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Innate Immune Response Induced in Gnotobiotic Piglets by a Mixed Culture of Commensal Bacteria

Roger B. Harvey, Kenneth J. Genovese, Haiqi He, and David J. Nisbet

U.S. Department of Agriculture, Agricultural Research Service, Food and Feed Safety Research Unit, 2881 F&B Road, College Station, TX, 77845 USA

Abstract

Our laboratory has developed a recombined porcine-derived mixed bacterial culture (RPCF) isolated from the ceca of a healthy, pathogen-free pig and have maintained it at steady state in a continuous-flow chemostat. The culture has been shown to protect neonatal and weaned pigs from infection and disease caused by *Salmonella* and *E. coli*. However, the mechanism of action of the protection from pathogens observed with the RPCF culture remains unclear. In the present study, 40 piglets were delivered by caesarian section and reared under gnotobiotic conditions. Piglets were either given RPCF within 1 hr after birth or were given sterile media. At times 0, 8, 24, 48, and 72 hr post-birth, piglets were euthanized and samples of spleen taken. Splenic cells from individual piglets were isolated and cultured with or without concanavalin A (conA). Splenic cells from RPCF-treated piglets had increased levels of IL-1 β , IFN- γ , IL-18, and IL-10 at 8 hr after birth compared to control piglets as measured by porcine cytokine ELISA. The increased levels of cytokines produced by RPCF-treated piglet splenocytes then declined over time, returning to levels observed in control pigs, or in some instances, below control levels. These results suggest that RPCF may act as a modulator for certain aspects of innate immune development.

Introduction

Our laboratory has developed a recombined, porcine-derived continuous-flow culture of mixed commensal bacteria (Gram-positive obligate anaerobes), designated as RPCF. RPCF has eliminated multiple strains of *Escherichia coli* and *Salmonella* from chemostats during *in vitro* challenge (1). When piglets are orally dosed with RPCF within 24 h of birth, the treated piglets have reduced colonization, shedding, and disease from *E. coli* and *Salmonella* following laboratory challenge with virulent strains of these organisms (2,3). Furthermore, under field trial conditions, treatment of piglets at birth decreased mortality and medication costs associated with enterotoxigenic strains of *E. coli* (4). The mechanism of protection is unknown, but the authors speculate that it could be exclusion of pathogens by competition for attachment sites, receptors, or nutrition; production of bactericidal compounds such as bacteriocins; modulation and enhancement of immune function; or all of the above. It is a known fact that commensal bacteria play an important role in the development of innate immune responses. The objective of the present study was to determine if RPCF could affect innate immune response in gnotobiotic piglets.

Materials and Methods

Forty piglets were delivered by caesarian section and reared under gnotobiotic conditions. Piglets were either given RPCF within 1 h after birth or were given sterile media. At times 0, 8, 24, 48, and 72 h post-birth, 4 piglets from each group (total of 8 per time frame) were euthanized and samples of spleen taken. Splenic cells from RPCF-treated piglets were isolated and cultured with or without concanavalin A (ConA) and analyzed by porcine cytokine ELISA for concentrations of IL-1 β , IFN- γ , IL-4, IL-10, and IL-18.

Results and Conclusions

Splenic cells from RPCF-treated piglets had increased levels of IL-1 β , IFN- γ , IL-10, and IL-18 at 8 h after birth compared to control piglets. The increased levels of cytokines produced by RPCF-treated splenocytes then declined over time, returning to levels observed in control pigs, or in some instances, below control levels. We conclude that RPCF can affect immune function in neonates and speculate that early innate responses observed herein may positively impact acquired immune responses later.

Discussion

These results suggest that RPCF may act as a modulator for certain aspects of innate immune function. If so,

RPCF could be used to “prime the pump” of the immune system which could induce earlier development of the innate system in neonates. In neonatal mammals, it appears that the immune system becomes “tolerant” of the commensal flora as it is exposed to them, eventually leading to a “non-response” to these bacterial species (5,6,7,8). Although there is a response to these organisms, this response is not detrimental to the host, keeping the normal flora relegated to their normal niche within or on the host. In this way, the host does not continue to respond to the normal flora in a costly way, saving a robust response for pathogens the host encounters. Failure of the immune system to tolerate the commensal flora can lead to inflammatory disease in the host (5). Indeed, in swine, *Lactobacillus* species have been shown to enhance the immune response of pigs to an *E. coli* challenge, inducing T cell differentiation and enhanced cytokine expression (9). IL-1 β , IFN- γ and IL-18 are considered pro-inflammatory cytokines, aiding in the production of an immune response to non-self antigens. These cytokines would also be considered to be indicators of an innate immune response, the first line of defense of the host, leading to the production of the acquired immune response. It is unclear, however, if the production of these cytokines and the subsequent induction of the innate immune response are responsible for the protective effects of the RPCF culture, or if this response is part of the tolerance process. The induction of IL-10 production would indicate an attempt to modulate the response to the microflora, and perhaps an indication of the beginning of the tolerance process. Further studies would need to be specifically targeted to the tolerance process to flesh out the response, perhaps covering longer periods of time after birth.

References

1. Harvey RB, Droleskey RE, Hume ME, et al. 2002. In vitro inhibition of *Salmonella enterica* serovars Choleraesuis and Typhimurium, *Escherichia coli* F-18, and *Escherichia coli* O157:H7 by a porcine continuous-flow competitive exclusion culture. *Curr. Microbiol.* 45:226-229.
2. Genovese KJ, Anderson RC, Harvey RB, and Nisbet DJ. 2000. Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic *Escherichia coli* infection in nursery-raised neonatal pigs. *Can. J. Vet. Res.* 64:204-207.
3. Genovese KJ, Harvey RB, Anderson RC, and Nisbet DF. 2001. Protection of suckling neonatal pigs against an enterotoxigenic *Escherichia coli* expressing 987p fimbriae by the administration of a bacterial competitive exclusion culture. *Microb. Ecol. Health Dis.* 13:223-228.
4. Harvey RB, Anderson RC, Genovese KJ, et al. 2005. Use of competitive exclusion to control enterotoxigenic strains of *Escherichia coli* in weaned pigs. *J. Anim. Sci.* 83(E Suppl.):E44-E47.
5. Pamer EG. 2007. Immune responses to commensal and environmental microbes. *Nature Immunology.* 8:1173-1178.
6. Burkey TE, Skjolaas KA, Minton JE. 2009. Porcine mucosal immunity of the gastrointestinal tract. *J. Anim. Sci.* 87:1493-1501.
7. Bailey M, Haverson K, Inman C, et al. 2005. The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effector function. *Proc. Nutr. Soc.* 64:451-457.
8. Collier-Hyams LS, Neish AS. 2005. Innate immune relationship between commensal flora and the mammalian intestinal epithelium. *Cell. Mol. Life Sci.* 62:1339-1348.
9. Wang A, Yu H, Gao X, et al. 2009. Influence of *Lactobacillus fermentum* I5007 on the intestinal and systemic immune responses of healthy and *E. coli* challenged pigs. *Antonie van Leeuwenhoek.* 96:89-98.

Meat Juice Multiserology – Developing a protein microarray for simultaneously detecting antibodies against zoonoses and production diseases in pigs

Meemken, D.*⁽¹⁾, Hahne, S.⁽²⁾, Baechlein, C.⁽³⁾, Engemann, C.⁽⁴⁾, Gabert, J.⁽⁴⁾, Sander, S.⁽⁴⁾, Lichter, D.⁽⁵⁾, Klein, G.⁽¹⁾, Blaha, T.⁽²⁾

⁽¹⁾Institute for Food Quality and Food Safety, University of Veterinary Medicine, Hannover, Bischofsholer Damm 15, D-30173 Hannover, Germany

⁽²⁾Field Station for Epidemiology, University of Veterinary Medicine Hannover, Buescheler Str. 9, D-49456 Bakum, Germany

⁽³⁾Institute of Virology, University of Veterinary Medicine Hannover, Buenteweg 17, D-30559 Hannover, Germany

⁽⁴⁾QIAGEN Leipzig GmbH, Leipzig, Deutscher Platz 5b, D-04103 Leipzig, Germany

⁽⁵⁾Alere Technologies GmbH, Jena, Loebstedter Str. 103, D-07749 Jena, Germany

*corresponding author: diana.meemken@tiho-hannover.de

Abstract

In the European Union serological and/or bacteriological monitoring results on zoonoses are to be taken into account for the risk assessment of slaughter pig herds in the framework of the risk-based meat inspection. Furthermore, the European food safety strategy pursues the additional goal to increase herd health and animal welfare. To meet the two goals identifying and controlling the mainly latent zoonoses as well as production diseases, the challenge was to develop and validate a cost-efficient diagnostic tool for simultaneously testing meat juice samples or blood serum samples for antibodies against various pathogens. The following antigens were chosen for detecting the corresponding antibodies by means of a protein microarray: a) zoonotic pathogens (*Salmonella* spp., *Toxoplasma gondii*, *Trichinella spiralis*, *Yersinia enterocolitica*, hepatitis E virus) and b) production disease causing pathogens (influenza A virus, *Mycoplasma hyopneumoniae*, PRRSV, *Actinobacillus pleuropneumoniae*). After validation by reference to the single-ELISA test results seven out of the nine chosen pathogens could be serologically detected by the developed microarray with test accuracy values between 0.71 and 1.

Introduction

The new European food safety concept for products of animal origin pursues three main and equally important goals: optimizing food safety, animal health as well as animal welfare by means of process control along the entire food chain (Anonymous, 2002). The need for this legislative enhancement is on the one hand due to the fact, that the traditional ante- and post-mortem meat inspection methods (inspecting, palpating and incising) alone are not able to control subclinical or asymptomatic zoonotic diseases (Hathaway and Richards, 1993 and EFSA, 2011). On the other hand there is a growing social demand for food from healthy herds with a high level of animal welfare quality. The Regulation (EC) No. 853/2004 defines as minimum nine criteria for the so-called food chain information (Anonymous, 2004).

Most parts of the food chain information are already available, because they have to be documented for other purposes at the farm or the abattoir or they are easy to attain. But other parts of the food chain information are not answerable without additional diagnostic tests. Although national serological salmonella monitoring systems are established in some European countries like Denmark, The Netherlands and Germany, information about other important zoonotic pathogens in pigs is missing. In the „Scientific opinion on the public health hazards to be covered by inspection of meat“ from 2011 the European Food Safety Authority (EFSA) ranked the following zoonotic pathogens as the most important public health hazards due to the consumption of pork: *Salmonella* spp., *Trichinella* spp., *Yersinia enterocolitica* and *Toxoplasma gondii*. The EFSA underlines the importance of serological monitoring programmes for subclinical zoonoses in pigs (EFSA, 2011).

The concept of the meat juice multi-serology (Meemken and Blaha, 2011) is to use meat juice samples from the salmonella monitoring programmes and to extend the analysis to other zoonotic and animal health relevant pathogens.

Material and Methods

For developing a swine specific microarray various recombinant or native antigens were acquired with relevance for zoonoses or for production diseases. Except for the hepatitis E virus antigen, which was provided by the Institute of Virology, University of Veterinary Medicine Hannover, Germany, all other zoonotic antigens were provided by QIAGEN Leipzig, Germany, who applies the same antigens in their commercial single-ELISA tests. The antigens with relevance for production dis-

eases were partly provided by third party or were self-made and, thus, have not been used so far in commercial single-ELISA tests. Assisted by Alere Technologies, Jena, Germany a “swine-specific protein microarray” was produced. The chosen antigens were spotted in different concentrations on a glass platform located at the bottom of a microarray tube (Fig. 1). On the surface of the glass platform which has a size of 4 mm² up to 200 antigens can be fixed.



Fig. 1: Microarray tube (3x1x0.8cm)

Positive and negative controls as well as antigens of the following zoonotic and production disease were spotted on the microarray: *Salmonella* spp., *Toxoplasma gondii*, *Trichinella spiralis*, *Yersinia enterocolitica*, hepatitis E virus, influenza A Virus, *Mycoplasma hyopneumoniae*, Porcine reproductive and respiratory syndrome disease (PRRSV) and *Actinobacillus pleuropneumoniae* (*App*). The concentration of specific antibodies in a sample is visualized by the coloration intensity of the spots after processing and is calculable via a computer software between zero (no specific antibody in the sample) and one (high concentration of antibodies in the sample). During the validation phase different dilutions of meat juice samples and blood serum samples, different test substrates and test conjugates as well as different concentrations of the chosen antigens were tested. The results of the microarrays were compared to the respective single-ELISA test results. For each spot, i.e. antigen in a specific concentration, a cut-off was determined for optimal sensitivity, specificity and test accuracy via a Receiver Operating Characteristic curve analysis (ROC curve analysis).

Results

The test duration of a microarray test procedure for establishing nine serological results accounts for 1.5 h and is comparable to the test duration of any of the corresponding single-ELISA tests. If meat juice samples are used as specimen, a preparation of the meat juice sample is required, i.e. that meat juice samples are to be diluted tenfold lesser than blood serum samples. Due to this preparation the test procedure for both specimens are equal. After validation of the microarray and determination of cut-off values per spot, specificity, sensitivity and test accuracy could be calculated for each antigen used in the microarray. The highest level of test accuracy values are measured for *Trichinella* spp. (1), *Toxoplasma gondii* (0.99), hepatitis E virus (0.95), *Yersinia enterocolitica* (0.94), *Salmonella* spp. (0.92), PRRSV (0.91) and influenza A virus (0.71) with values between 0.7 and 1. Lowest test accuracy values were measured for those spots using the self-made antigens of *Actinobacillus pleuropneumoniae* (0.61) and *Mycoplasma hyopneumoniae* (0.54) with values between 0.5 and 0.6.

In Fig. 2 a completely processed microarray is shown as example.

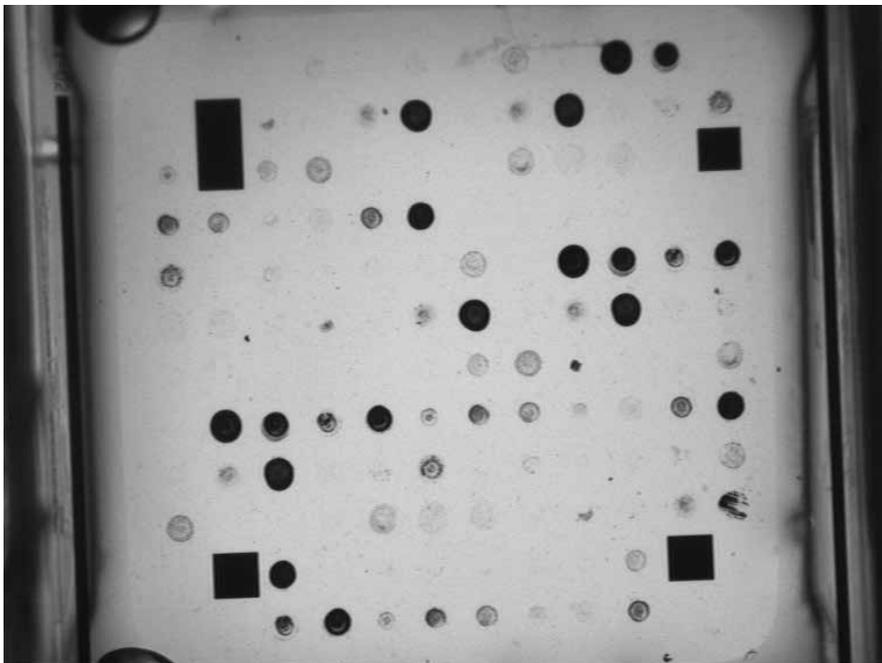


Fig. 2: Processed microarray test results with 2000x magnification

Discussion

The major challenges during the development of a swine specific protein microarray for simultaneously detecting antibodies against zoonoses and production diseases were a) acquiring efficient antigens for the antibody detection, b) preparing meat juice samples and blood serum samples suitable for testing via microarray, and c) applying the same conjugates and substrates as well as the same time sequences for each step of the procedure for nine different antigens due to the simultaneous approach in one reaction tube.

Regarding the suitability of the acquired antigens, all such antigens used in single-ELISA tests (*Trichinella* spp., *Toxoplasma gondii*, hepatitis E virus, *Yersinia enterocolitica*, *Salmonella* spp.) showed the highest sensitivity and specificity. This was due to the prior implemented standardisation of the single-ELISA tests regarding substrates, conjugates and procedures by the manufacturer. The self-made antigens of *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* showed the lowest sensitivity and specificity and are to be revised in further research projects.

Although most ELISA tests for production diseases are licensed for blood serum only, it could be shown by Meemken and Blaha (2011) that a tenfold lesser dilution of the meat juice sample compared to blood serum leads to comparable serological results for production diseases. This finding is in accordance with Nielsen et al. (1998) and Molina et al. (2008), who investigated a tenfold lesser *Salmonella* spp. antibody concentration and PRRSV antibody concentration in meat juice compared to the corresponding blood sera.

Measuring antibodies against zoonotic and animal disease pathogens in meat juice or blood serum samples and designing serological herd profiles is a meaningful tool for risk-categorizations of pig herds for the risk-based meat inspection and could be useful as basis for herd health management initiatives. By developing miniaturized and simultaneous test systems like the developed protein microarray the cost effectiveness can be considerably increased.

Furthermore, identifying herd specific serological differences on a continuous basis will help to understand the underlying management factors determining the infectious status of pig herds especially the latent infections and will become a valuable benchmarking tool for targeted intervention measures to improve food safety and herd health.

To design a serology based herd profile at least 60 meat juice samples per herd (to detect at least 5% intra-herd seroprevalence) should be taken at slaughter. If a serological monitoring programme like the German or the Danish salmonella monitoring programme is established, the already taken meat juice samples can easily be used for detecting antibodies against additional pathogens, too.

The composition of the test targets can be adapted to actual needs such as emerging risks, and special herd health threats.

Conclusion

The developed protein microarray is a valuable diagnostic tool to analyze antibodies against different zoonotic and pig disease pathogens at once in a meat juice or blood serum sample. Used in the framework of a serological monitoring program the resulting serological herd profiles can be used by farmers and veterinarians for targeted improvement measures as well as by food business operators and official veterinarians for risk-based decisions. The logistics of an existing meat juice or blood serum based salmonella monitoring programme could be utilised for the multi-serology concept to increase the feasibility and cost-effectiveness. If included in a surveillance system by continuously taking random samples per herd meat juice multi-serology via protein microarray could become a powerful diagnostic tool for improving food safety and pig herd health.

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References

ANONYMOUS, 2002, Regulation (EC) No 178/2002 of the European parliament and of the council laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, O. J., L 31.

ANONYMOUS, 2004, Regulation (EC) No 853/2004 of the European parliament and of the council laying down specific hygiene rules for food of animal origin, O. J., L 139.

EFSA, 2011, Scientific Opinion on the public health hazards to be covered by inspection of meat (swine), EFSA J., 9: 2351.

HATHAWAY S.C. and RICHARDS M.S., 1993, Determination of performance attributes of post-mortem meat inspection procedures, Prev. Vet. Med., 16: 119-131.

MEEMKEN D., BLAHA T., 2011, "Meat Juice Multi-Serology" - a tool for the continuous improvement of herd health and food safety in the framework of the risk-based meat inspection of slaughter pigs, J. Food Saf. Food Qual. 62: 192-199.

Approaches to reduce antibiotic resistance in the pork supply chain.

Heres, L^{*1}, Düsseldorf, S. ^{2,3}, Oorburg, D.^{1†}, and Urlings, B.A.P ^{1,4}.

¹VION Food Group, Boseind 10, 5281 RM, Boxtel, The Netherlands,

²University Bonn,

³Erzeuger Gemeinschaft Süd Ost Bayern,

⁴ Wageningen University.

* corresponding author: lourens.heres@vionfood.com; †presenting author.

Abstract

Occasionally, use of antibiotics is necessary to treat diseased animals. Prudent use is however necessary, as antibiotic usage can evoke the selection and propagation of antibiotic resistant bacteria. Antibiotic resistance in pigs is primarily a occupational risk for those who are in contact with pigs. Secondly, contamination of the environment and fresh food may expose the general population to resistant bacteria. Strict hygiene at slaughter (including a zero tolerance on fecal contamination) can prevent the contamination of food with resistant bacteria.

Recent experience in The Netherlands shows that obligatory reporting of antibiotic usage and accompanying benchmarking, can result in a marked reduction. Also a ban on use of critical antibiotics (fluoroquinolones and cephalosporins) appeared to be feasible. We hypothesize that good animal health and optimal biosecurity are crucial to further reduce the occurrence and propagation of antibiotic resistance. To support the farmers, veterinarians, and other advisors in these areas, we assumed that the collection of data on health parameters in the slaughterhouse has an added value. Pathological findings is “classical” information about the health of the slaughtered animals. It was studied whether serological results from blood collected in the slaughterhouse, can be complementary information

We show that differences in Salmonella-, Mycobacterium avium- and Toxoplasma- status can be used as a derivative of internal and external biosecurity at the farm. Serology on slaughterhouse blood for pathogens of the lung disease complex (e.g. PRRSV and *Mycoplasma hyopneumoniae*) provides additional information, which can support the animal health management.

It is concluded that future challenges lay in exchange of easily accessible information collected in slaughterhouses, development of management alternatives based on this information, and development of additional serological methods.

Introduction

Antibiotics have influenced the therapeutic possibilities in human and veterinary medicine drastically after their discovery in the 1940s. Occasionally, use of antibiotics is necessary to treat diseased animals. In animal populations where the transmission of infection is very likely, it can also be used to mitigate the severity of disease in not yet clinically ill animals. The question is however in which situations this is good veterinary practice. Prudent use is necessary, as antibiotic usage can evoke the selection and propagation of antibiotic resistant (ABR) bacteria.

Antibiotic resistance in pigs is primarily a occupational risk for those who are in contact with pigs. This is for example seen in LA-MRSA, where those people who are in contact with live pigs are most likely to be colonized (GILBERT, 2012), however the pathogenicity of LA-MRSA is questioned (HEALTH COUNCIL NL, 2011). Secondary spread of ABR bacteria may contaminate the environment and fresh foods, and may therewith expose the general population to resistant bacteria.

Which sources finally attribute most to human exposure (environment-to-human, human-to-human, animal-to-human, or food-to-human) is marginally known. Moreover, the presence of resistant bacteria in live animals does not necessarily mean that this will lead to contamination of meat, and afterwards consumers. Strict hygiene at slaughter can prevent the contamination of pork with Salmonella (VAN HOEK, 2012) and accordingly with resistant bacteria that are present in the intestinal tract of pigs.

Independent of the actual size of the contribution of antibiotic usage in animal production on the antibiotic resistance in men, there is societal and political pressure to reduce antibiotic usage in the animal production chains. Therefore the animal supply chain should prudently use antibiotic, and try to reduce the number of treatments and refrain from critical antibiotics.

Recent experience in The Netherlands shows that obligatory reporting of antibiotic usage and accompanying benchmarking, resulted in a quick reduction of its usage (MEVIUS, 2012). Also a ban on use of critical antibiotics (fluoroquinolones and third and fourth generation cephalosporins) was implemented.

We hypothesize that good animal health and optimal biosecurity are crucial to reduce the occurrence and propagation of antibiotic resistant microorganisms. When animals are healthy, there is no need to treat them with antibiotics. And when antibiotic resistance occurs, and good hygienic barriers are present, further transmission of resistant micro-organisms will be prevented. One of the building blocks that was investigated is the idea that the information generated from collection of data in the slaughterhouse has an added value to support farmers, veterinarians, and other advisors in these areas. Pathological findings is “classical” form of data collected at the slaughterhouse that contains information about the health of the slaughtered animals. It was studied whether serological results from blood collected in the slaughterhouse, contains complementary information for this purpose.

First serological data may be used as generic hygiene/biosecurity parameter. For this purpose serological data that is collected for other purposes may be used. In many countries serology in slaughterhouses (either on meat juice or on serum) is collected for Salmonella serology. To control Salmonella the herd status of pig herds is assessed by serology in several countries, like Denmark, Germany and The Netherlands. Recently, other serological analyses were added to this slaughterhouse serological screening. In risk based meat inspection systems Mycobacterium avium- and Toxoplasma- serology are used to verify the hygiene status of the farm supplying to the slaughterhouse. Mycobacterium avium serology is performed to eliminate the need of incising the lymph nodes during meat inspection (HILLER, 2012). Toxoplasma is assessed as this is a relevant zoonotic agent, ingested with raw meat from pigs and cattle too (JONES, 2009, OPSTEEGH, 2012). We assessed whether serology for these zoonoses provides additional information about the level of biosecurity control.

The use of serology on slaughterhouse blood can be extended. An important reason for usage of antibiotics in pig production are lung diseases. Other reasons are intestinal problems and streptococcal infections around weaning. The major lung pathogens in today's pig husbandry are Mycoplasma hyopneumoniae (Mhyo), Actinobacillus pleuropneumoniae serotype 2 (APP 2), swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus type 2 (PCV2). We therefore assumed that serology for pathogens of the porcine respiratory disease complex (PRDC) provides additional information, which can support the animal health management.

Material and Methods

Based on literature we made an assessment on the linkage between serology for Salmonella, Mycobacterium avium and Toxoplasma for biosecurity verification.

Secondly, the potential of slaughterhouse blood as predictor of pig herd health status, as reflected in technical performance of the herd and the usage of antimicrobial drugs, was explored. Twenty pig herds located in Southeast Bavaria were included in a study and followed during 4 fattening rounds. Blood samples at slaughter were randomly collected from a random delivery per round and submitted for ELISA serology. The blood was tested for antibodies of Mhyo, APP 2, SIV, PRRSV, and PCV2. Data on growth, feed usage and antibiotic usage were collected per farm. Statistical analyses were done to quantify the relation between serological response and technical performance (growth and feed efficiency) and antibiotic usage (for more details see: DÜSSELDORF, 2013).

Results

For the spread of Salmonella in pig herds biosecurity is of significant importance (BERENDS, 1999). From our assessment of the infection routes of Salmonella can be distinguished in external and internal routes; externally with piglet or gilts, rodents, birds etc. and internal transmission – from department to department by mixing pigs or carried by working personal, and failing cleaning and disinfection. Where biosecurity is well implemented, transmission of Salmonella will be prevented and less Salmonella positive serology will be found. *Mycobacterium avium* can be introduced by birds and contaminated water. Toxoplasma can be introduced by rodents or cats. Therefore positive serology may indicate that these introductory routes are

not in control. Pathogens that cause disease may use the same introductory or transmission routes, and ABR bacteria may use them as well.

Preliminary analyses showed that meaningful differences were found in serological statuses between the herds for the different lung pathogens. Analyses indicated that PRRSV was the most influential pathogen affecting technical herd performance. APP2, PCV2 and SIV significantly influenced the presence of pneumonia in pig herds, and APP2 was relevant to increasing clinical PRDC symptoms. The percentage of positive samples for the 5 parameters varied between zero and 85%. The proportion of positive samples was highly correlated with the percentage of pigs with symptoms of PRDC, the percentage of pneumonia per herd, the average daily growth rate (ADGR), and the average feed conversion rate (AFCR). (DÜSSELDORF, 2013).

Discussion

The assessment that was made indicates that the serology for zoonotic pathogens like Salmonella, MAA and Toxoplasma may predict the level of biosecurity. Without additional costs the serological results can be used to assess the biosecurity status and compare farms in a bench mark. .

The high correlation between the serological results for the lung pathogens and the herd technical performance indicates that the analysis of blood sampled at slaughter may aid in making better decisions on pig herd health management. Monitoring the five pathogens from the PRDC may enable an improvement in pig herd health, an increase in technical performance, and the promotion of the reduction of the usage of antibiotics in pig herds. Positive results have been reported for programs where blood is collected with regular intervals for serology on pathogens of the PRDC (e.g. Respig®, see: GEURTS, 2011). As the correlations with performance and positive serology at blood collected in the slaughterhouse are so good, the blood collection of these kind of strategies may as well be done in the slaughterhouse, were blood sampling can be done much easier and with less stress for the animal.

The promising thing in the serology in slaughterhouse blood is that results indicate that farmers who can improve their farm health will most likely improve their performance, and therewith increase their profitability. This may be an incentive for farmers to request for serology on slaughterhouse blood.

The serological screening is only an assessment. The information is getting value when the farmers, veterinarians and other advisors that have a professional role in advising the farmer use this information appropriately. Research is planned how farmers can use these data to set goal for further improvement of herd health and reduction of antibiotic usage.

Serological monitoring will only be a part of the solution. The serological tools described are the most easily available. For intestinal diseases additional tools have to be investigated.

Conclusion

It is concluded that future opportunities lay in exchange of easily accessible information collected in slaughterhouses, development of management alternatives based on this information, and development and application of additional serological methods.

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References

BERENDS, B. R., et al. Identification and quantification of risk factors in animal management and transport regarding *Salmonella* spp. in pigs. International journal of food microbiology, 1996, 30.1: 37-53.

DÜSSELDORF, S. 2013. Concept of key performance indicators controlling consumer oriented quality and herd health management in a Bavarian pork chain. Thesis. University of Bonn.

GEURTS, V.N.A.M, et al. The ResPig program as a tool for identifying risk factors affecting technical performance and post-mortem results at the slaughterhouse on Dutch pig farms and for restrictive antibiotic use. Maastricht, Safepork 2011.

GILBERT, Maarten J., et al. Livestock-associated MRSA ST398 carriage in pig slaughterhouse workers related to quantitative environmental exposure. *Occupational and environmental medicine*, 2012, 69.7: 472-478.

HEALTH COUNCIL NL (2011). Antibiotics in food animal production and resistant bacteria in humans. The Hague: Health Council of the Netherlands, 2011; publication no. 2011/16. <http://www.gezondheidsraad.nl/sites/default/files/201116.pdf>.

HILLER A, et al. (2012) Implementation of risk based meat inspection of fattening pigs without incision in a slaughterhouse in Lower Saxony. *Archiv für Lebensmittelhygiene* 63: 4, 107-114

JONES, Jeffrey L., et al. Risk factors for *Toxoplasma gondii* infection in the United States. *Clinical infectious diseases*, 2009, 49.6: 878-884.

MEVIUS, D. J., et al. (2012) Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN).

OPSTEEGH M, et al.(2011) A quantitative microbial risk assessment for meatborne *Toxoplasma gondii* infection in The Netherlands. *Int J Food Microbiol.* 150(2-3):103-14.

VAN HOEK, Angela HAM, et al. A quantitative approach towards a better understanding of the dynamics of *Salmonella* spp. in a pork slaughter-line. *International journal of food microbiology*, 2012, 153.1: 45-52.

Galactomannans for the control of *Salmonella* infection in fattening pigs

Mainar-Jaime, R.C.^{(1),(2)*}, Andrés, S.⁽²⁾, Auseré, M.⁽³⁾, Vico, J.P.⁽⁴⁾

⁽¹⁾Dpto. Patología Animal. Facultad de Veterinaria. Universidad de Zaragoza. Zaragoza, Spain.

⁽²⁾Centro de Investigación y Tecnología Agroalimentaria de Aragón. Zaragoza, Spain.

⁽³⁾Agropienso Sociedad Cooperativa. Huesca, Spain.

⁽⁴⁾Facultad de Ciencias Agropecuarias. Universidad Católica de Córdoba, Argentina.

*corresponding author: rcmainar@unizar.es

Abstract

The use of plant-derived products with antimicrobial activity appears as an alternative for the control of pig salmonellosis. The results of 3 field trials aimed at assessing the efficacy of different concentrations of a galactomannan (Salmosan®) in the control of *Salmonella* infection in fattening pigs are presented. It was found that the addition of ≥ 2 kg/T of Salmosan® to the regular diet of pigs during the entire fattening period was able to decrease significantly the prevalence of *Salmonella* infection and shedding at slaughter. This galactomannan could be considered a good complementary tool along with good hygiene and biosecurity for the control of this infection in the pig farm.

Introduction

Among the main zoonotic diseases transmitted by pigs and pork products in developed countries one of the most important is salmonellosis. In the USA this infection was considered the main bacterial foodborne illness in 2011 (1), while in the European Union (EU) it was ranked as the second after campylobacteriosis (2). After years of control programmes against poultry salmonellosis, pigs are now becoming a major source of infection for humans in the EU (2).

Salmonella spp. are characterized by a great environmental and antimicrobial resistance. In addition, detection of asymptotically *Salmonella*-infected pigs is not straightforward and vaccines are not usually used against zoonotic *Salmonella*. Thus, high standards on biosecurity and hygiene along the entire food chain are of utmost importance for the control of this infection (3).

The use of some non-digestible oligosaccharides has been proposed as a new alternative for the control of salmonellosis in weaning and postweaning pigs. These are usually plant or yeast-derived products that have shown some antimicrobial effects either through modulation of beneficial microbiota, thus favoring competitive exclusion, bacteriocin production, etc., or through the enhancement of the intestinal defense system by some immunomodulatory action. Of particular interest are the mannan-oligosaccharides due to their ability to bind to mannose-specific lectin of gram-negative pathogens that express Type-1 fimbriae, thus blocking the adhesion of these bacteria and excreting them from the intestine. However, when these products have been tested results have been inconsistent (4-6).

In this study we show the results of 3 field trials aimed at assessing the effect that the addition to the diet of fattening pigs of different concentrations of a galactomannan obtained from carob bean gum (*Ceratonia silicua*) had on the prevalence and seroprevalence of *Salmonella* spp.

Material and methods

Three different doses (0.5, 2 and 3 kg per Ton of feed) of a galactomannan (Salmosan®, ITPSA, Barcelona, Spain) were used during the entire period of fattening in 3 field trials carried out in a small commercial fattening unit previously identified as positive for *Salmonella*. This product had shown promising results against salmonellosis in previous trials on weaning pigs (7). The fattening unit was divided in 8 pens (12-14 tagged pigs/pen). Four of these pens were randomly chosen as treatment pens and pigs from these pens composed the treatment group (T). Pigs from the other 4 pens formed the control group (C). The farmer was unaware of the pen allocation.

All pigs were given in-feed colistin (120 ppm) for 2 weeks after arriving. Fifteen days after colistin treatment, serum (about 50 pigs/group) and fecal samples (20-25 pigs/group) were collected to confirm the absence of significant differences in

group seroprevalence and fecal prevalence. At 60 and 90 days of the fattening period serum and fecal samples were collected again from a similar number of pigs. One week before slaughter serum was collected from a minimum of 40 pigs in each group (except in the first trial), and mesenteric lymph nodes (MLN) and feces (except in the first trial) were collected at slaughter. The ISO 6579:2002 and the Herdcheck® Swine *Salmonella* ELISA (IDEXX Lab., cutoff %OD \geq 40%) were used for microbiological and serological analyses, respectively.

Univariable chi-squared analysis was used to assess the presence of differences in shedding, seroprevalence and prevalence between C and T at 60 and 90 days and at slaughter. A random-effects logistic regression (re-LG) with pen as the random factor and trial as the confounding factor was performed to measure the overall effect of the different doses of Salmosan® on the prevalence of infection and shedding at slaughter.

Results

Bacteriological results

Microbiological results for the C and T in the 3 trials are presented in Table 1. No significant differences were observed at any time when the lowest dose (0.5 kg/T) was used, but at higher doses (2 and 3 kg/T) the prevalences of infection and of shedding at slaughter were significantly lower for T.

Doses of 2 or 3 kg/T of Salmosan® supplemented during the entire fattening period decreased significantly the prevalence of infection after taking into account pig allocation to pens and adjusting by trial (Table 2). A dose-response trend was observed, with a higher reduction when a higher dose was used. According to results in Table 2, the efficacy of Salmosan® could reach from 90% (2kg/T) to 99% (3 kg/T) when compared to the control group. Similar results were observed for *Salmonella* shedding.

Seroprevalence

No differences were observed between C and T after colistin treatment in any of the trials. When the highest dose (3 kg/T) was used, a significant lower seroprevalence was observed in T compared to C after 60 days of fattening. No differences at any time were observed for the other two trials (Table 3).

Discussion

The use of antibiotics as growth promoters or with a preventative use is officially forbidden in the EU. Thus, the control of enteric bacterial infections must be based on strict hygiene and biosecurity measures and the use of vaccines when available. No vaccines are commonly used for the control of salmonellosis in fattening pigs in the EU so far. Thus, different products, such as mannan-oligosaccharides, with antimicrobial properties are being considered as potential alternative for the control of this infection. Mannan-oligosaccharides can be obtained from a wide variety of natural sources and their diverse biological composition and processing make their overall antimicrobial effect variable (4, 6).

We tried different doses of a galactomannan obtained from carob bean gum (Salmosan®) added to the regular diet of pigs for the entire period of fattening to assess its effect on *Salmonella* shedding, prevalence and seroprevalence. The results indicated that the addition of \geq 2 kg/T of Salmosan® was able to significantly decrease the prevalence of *Salmonella* infection and shedding at slaughter. In trial 2 this was supported by a significant lower number of seroconverting pigs (cutoff %OD $>$ 40) before slaughter. In trial 3, however, despite a very large number of control pigs were infected and shed *Salmonella* at slaughter, the proportion of seroconverting pigs was very low in both groups. This finding could be explained if *Salmonella* infection occurred mostly at the end of the fattening period, during the transport to the slaughter or at lairage (9), as seroconversion is seen around 2 weeks after infection (8). It could also be explained by a delayed onset of seroconversion that may happen with certain *Salmonella* strains (10).

The apparent and unexpected difference in seroprevalence between C and T in trial 3 (0% vs. 7.8%; $p=0.12$, respectively) was most likely due to the intrinsic variability of the ELISA used, as three animals from C had ODs higher than 36% but lower than 40%, and thus were deemed test negative.

Conclusion

In view of the bacteriological results, Salmosan® seems to be effective against *Salmonella* infection in fattening pigs. Its efficacy would be very high when doses \geq 2 kg/T are used for the entire period of fattening, and may be useful even if infection occurs during the transport to the slaughterhouse or at lairage. Overall, this galactomannan could be considered

a complementary tool along with good hygiene and biosecurity for the control of this infection in the pig farm. Further investigation is warranted to confirm these findings.

Table 1. Microbiological results (ISO 6579:2002) for fecal samples after 60 (60d) and 90 (90d) days in the fattening unit and for mesenteric lymph nodes (MLN) and fecal samples (Fecal) at slaughter.

		Fattening unit						Slaughter					
		60d			90 d			MLN			Fecal		
		N	No. + (%)	<i>p</i>	N	No. + (%)	<i>p</i>	N	No. + (%)	<i>p</i>	N	No. + (%)	<i>p</i>
T1	C	19	4 (21)	0.41	45	2 (4.2)	0.99	42	23 (54.8)	0.25			
	T	28	3 (10)		44	1 (2.3)		49	21 (42.9)				
T2	C	25	3 (12)	0.60	25	2 (8)	0.48	47	37 (78.7)	<0.01	45	26 (57.8)	<0.01
	T	25	1 (4)		25	0 (0)		39	0 (0)		39	1 (2.6)	
T3	C	26	7 (26.9)	0.15	28	0 (0)	1	49	20 (40.8)	<0.01	49	34 (69.4)	<0.01
	T	23	2 (8.7)		27	1 (3.7)		51	3 (5.9)		51	6 (11.8)	

T1: Trial 1 (0.5 kg/T); T2: Trial 2 (3 kg/T); T3: Trial 3 (2 kg/T). C: control group; T: treatment group.

Table 2. Results of the random-effect logistic regression on the effect of Salmosan[®] on *Salmonella* infection and shedding at slaughter.*

Factor	Logistic regression parameters					
	Infection (MLN)			Shedding (Fecal)		
	<i>P</i>	Odds Ratio (OR)	95%CI (OR)	<i>P</i>	Odds Ratio (OR)	95%CI (OR)
Doses						
0 kg /T feed		1			1	
0.5 kg /T feed	0.55	0.73	0.26, 2.07	-	-	-
2 kg /T feed	<0.01	0.09	0.04, 0.17	<0.01	0.06	0.02, 0.13
3 kg /T feed	<0.01	0.008	0.001, 0.07	<0.01	0.018	0.001, 0.23
Trial ^a						
1 (April 2009)		1		-	-	-
2 (May 2010)	0.15	3.1	0.64, 15.6		1	
3 (Feb. 2013)	0.47	0.68	0.25, 1.91	0.64	1.51	0.25, 8.86

*Pen as random factor and trial as confounding factor. No fecal samples collected in Trial 1 (0.5 kg/t.)

Table 3. Serological results (Herdcheck® *Salmonella* ELISA, cutoff %OD ≥40) for the three trials after 60 (60d) and 90 (90d) days in the fattening unit and one week previous slaughter (Slaughter).

		60 d			90d			Slaughter		
		N	No. + (%)	<i>p</i>	N	No. + (%)	<i>p</i>	N	No. + (%)	<i>p</i>
T1	C	55	3 (5.5)	0.98	55	4 (7.3)	0.46	-	-	-
	T	56	3 (5.4)		53	6 (11.3)		-	-	-
T2	C	55	18 (32.7)	<0.01	53	13 (24.5)	0.06	40	15 (37.5)	<0.01
	T	56	6 (10.7)		55	6 (10.9)		40	4 (10)	
T3	C	51	1 (1.9)	1	51	0 (0)	0.12	49	0 (0)	0.12
	T	51	2 (3.9)		50	3 (6)		51	4 (7.8)	

T1: Trial 1 (0.5 kg/T); T2: Trial 2 (3 kg/T); T3: Trial 3 (2 kg/T). C: control group; T: treatment group.

Acknowledgements

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References

- CENTERS FOR DISEASE CONTROL (CDC). 2011. Estimates of Foodborne Illness in the United States In: National Center for Emerging and Zoonotic Infectious Diseases. <http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html>: CDC 2012: 2.
- EFSA-ECDC. 2013. The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2011. *EFSA J.*, 11(4):3196.
- DE BUSSEER EV, DE ZUTTER L, DEWULF J, HOUF K, MAES D. 2013. *Salmonella* control in live pigs and at slaughter. *Vet J.*, 196:20-7.
- GALLOIS M, ROTHKÖTTER HJ, BAILEY M, STOKES CR, OSWALD IP. 2009. Natural alternatives to in-feed antibiotics in pig production: can immunomodulators play a role? *Animal*, 3:1644-61
- GAGGIA F, MATTARELLI P, BIAVATI B. 2010. Probiotics and prebiotics in animal feeding for safe food production. *Int J Food Microbiol*, 141: S15-S28.
- BECKER PM AND GALLETTI S. 2008. Food and feed components for gut health-promoting adhesion of *E. coli* and *Salmonella enterica*. *J. Sc. Food Agric.*, 88: 2026-35
- BADÍA R, ZHANG B, LIZARDO R, MARTÍNEZ P, BADIOLA I, BRUFAU J. 2009. The influence of carob bean gum on *Salmonella* prevalence of piglets experimentally infected with *S. Typhimurium*. XI Int Symp Digestive Physiol Pigs, May 20th-22nd, Montbrió, Spain.
- NIELSEN B, BAGGESEN D, BAGER F, HAUGEGAARD J, LIND P. 1995. The serological response to *Salmonella* serovars typhimurium and infantis in experimentally infected pigs. The time course followed with an indirect anti-LPS ELISA and bacteriological examinations. *Vet Microbiol* 47: 205-218.
- BOUGHTON C, EGAN J, KELLY G, MARKEY B, LEONARD N. 2007. Rapid infection of pigs following exposure to environments contaminated with different levels of *Salmonella typhimurium*. *Foodborne Pathog Dis.*, 4:33-40.
- VAN PARYS A, BOYENA F, LEYMANA B, VERBRUGGHEA E, MAESB D, HAESBROUCKA F, PASMANS F. 2013. Induction of seroconversion and persistence of *Salmonella Typhimurium* in pigs are strain dependent. *Comp. Immun. Microbiol. Inf. Dis.* May 10. doi:pii:S0147-9571(13)00023-4.

Swine zoonosis risk assessment with new herd seroprofiling assays from QIAGEN

Schroeder, C*, Sander C., Kanitz S., Engemann C.

QIAGEN Leipzig GmbH, Leipzig, Deutscher Platz 5b, D-04103 Leipzig, Germany

*corresponding author: carsten.schroeder@qiagen.com

Abstract

QIAGEN Leipzig developed the *pigtype*[®] product line of ELISA tests for screening for swine zoonoses. This product line now includes ELISA for detection of salmonella-, *Yersinia*-, *Trichinella*-, and *Toxoplasma*-antibodies in swine. These *pigtype* assays are validated for serum and meat juice samples and are officially approved by the German Friedrich-Loeffler-Institut. In order to follow the seroprofiling concept, the *pigtype* ELISA reagents and assay protocols are standardized. This product concept allows combining serological salmonella monitoring with serological testing for other zoonosis.

Introduction

In 2003, the European Parliament issued Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents¹. The purpose of this directive is to ensure that zoonoses are properly monitored, and that food-borne outbreaks receive proper epidemiological investigation. The collection of this information enables the evaluation of relevant trends and sources, which therefore permits the development of effective strategies to improve the herd health level and to contribute to consumer protection.

Since 2007 Germany has implemented an official state controlled program for salmonella reduction in pork. This program is managed by the QS (Quality Scheme for Food) organization based on their salmonella Monitoring and Reduction Program for pork production². This program is based on serological monitoring of pigs for antibodies to salmonella and classification according to its risk of salmonella into one of three categories³. One of three tests kits officially listed for this program is the SALMOTYPE[®] Pig Screen ELISA, which is now available as *pigtype* Salmonella Ab ELISA from QIAGEN Leipzig.

In order to allow serological monitoring for other infections, transmissible from pork to man, QIAGEN Leipzig developed ELISA test kits for detection of antibodies to *Trichinella*, *Toxoplasma*, and *Yersinia* in serum and meat juice samples. Those assays are fully validated and approved by Friedrich-Loeffler-Institute in Germany and harmonized, regarding reagents and protocols.

The purpose of this study was to evaluate the performance of new swine zoonosis assays in service and research laboratories based on well characterized samples. Furthermore, prevalence data for different zoonoses were obtained.

Material and Methods

Sets of 8 serum samples per zoonosis parameter were sent to 6 laboratories in Germany and Austria. The samples (each vial 50 µl) were shipped frozen to the participants and storage instructions requested the samples to be stored at -20°C until analysis.

The analysis of the samples was performed blinded. The samples were identifiable by sample ID numbers (#1 – #8) with the prefix SM (= salmonella), TOX (= *Toxoplasma*), TR (= *Trichinella*), and YS (= *Yersinia*), respectively.

In Table 1, the samples of each test panel are listed, supplemented with information to sample origin and status. The status of field sera refers to results determined during validation of the respective *pigtype* ELISA kit. Samples were sourced from field studies, the School of Veterinary Medicine of the Leipzig University, and the Federal Institute of Risk Assessment in Germany.

The participant laboratories were requested to test the samples in two runs (e.g., on separate days) according to manufacturer's instructions.

Two data points per sample and laboratory were generated, 12 data points per sample, 96 data points per assay, and 384 data points for the inter-lab trial samples sent.

Table 1. Description of the test panel samples for salmonella (= SM), *Toxoplasma* (= TOX), *Trichinella* (= TR), and *Yersinia* (= YS).

Sample ID	Origin		
SM-1	Salmonella field serum swine: weak positive	TOX-1	<i>Toxoplasma gondii</i> field serum wild boar: positive
SM-2	Salmonella field serum swine: strong positive	TOX-2	<i>Toxoplasma gondii</i> field serum wild boar: weak positive
SM-3	Field serum swine: negative	TOX-3	<i>Toxoplasma gondii</i> field serum sheep: positive
SM-4	Salmonella field serum swine: positive	TOX-4	Field serum swine: negative
SM-5	Salmonella field serum swine: strong positive	TOX-5	Field serum swine: negative
SM-6	Salmonella field serum swine: weak positive	TOX-6	Field serum swine: negative
SM-7	Field serum swine: negative	TOX-7	Field serum swine: negative
SM-8	Serum of a <i>Salmonella Typhimurium</i> vaccinated 84-day-old swine (21 d.p.i.; 3rd vaccination): positive	TOX-8	<i>Toxoplasma gondii</i> field serum wild boar: weak positive
TR-1	<i>Trichinella</i> field serum wild boar: positive	YS-1	<i>Yersinia</i> field serum swine: positive
TR-2	Field serum swine: negative	YS-2	Field serum swine: negative
TR-3	Field serum swine: negative	YS-3	Serum of a <i>Yersinia enterocolitica</i> experimentally infected 54-day-old swine (26 d.p.i.): strong positive
TR-4	<i>Trichinella</i> field serum wild boar: strong positive	YS-4	Serum of a <i>Yersinia enterocolitica</i> experimentally infected 41-day-old swine (13 d.p.i.): weak positive
TR-5	Serum of a <i>Trichinella spiralis</i> experimentally infected swine (20 w.p.i.): positive	YS-5	Field serum swine: negative
TR-6	Field serum swine: negative	YS-6	Serum of a <i>Yersinia enterocolitica</i> experimentally infected 51-day-old swine (23 d.p.i.): positive
TR-7	<i>Trichinella</i> field serum wild boar: positive	YS-7	<i>Yersinia</i> field serum swine: weak positive
TR-8	Field serum swine: negative	YS-8	Serum of a <i>Yersinia enterocolitica</i> experimentally infected 58-day-old swine (30 d.p.i.): strong positive

In addition, one participating laboratory tested serum from 32 fattening pigs, 20 organic pigs, and 25 wild boar samples from their sample collection.

QIAGEN Leipzig *pigtype* ELISA test kits were sent to each of the participants of the inter-lab trial. All laboratories received ELISA test kits with the same batch number (Table 2).

Table 2. ELISA test kits and associated batch number.

Test kit name	Batch No.
<i>pigtype</i> Salmonella Ab	11-11.2SM
<i>pigtype</i> Toxoplasma Ab	12.02.1TOX
<i>pigtype</i> Trichinella Ab	12-03.1TR
<i>pigtype</i> Yersinia Ab	12-01.1YS

The *pigtype* Salmonella Ab is an updated version of the well-known SALMOTYPE PigScreen ELISA. The assay detects antibodies to salmonella serotypes of group B, C, D, and E (Kauffmann-White-scheme) in porcine serum, plasma, and meat juice samples.

pigtype Yersinia Ab is an ELISA for detection of antibodies to pathogen *Yersinia* strains in porcine serum, plasma, and meat juice samples.

pigtype Toxoplasma Ab and *pigtype* Trichinella Ab are multi-species ELISA test kits for detection of antibodies to *Toxoplasma gondii* and *Trichinella* spp., respectively. The kits are capable of detecting their respective pathogens in porcine serum, plasma, and meat juice samples. Furthermore, the kits are capable of detecting their respective pathogens in serum

and plasma samples of different mammalian species such as fox, goat, sheep, cattle, horse, dog, and/or cat.

All the ELISA products have interchangeable sample dilution buffer, wash buffer, substrate-, and stop-solutions. They use the same test procedure steps, with a 60 minute sample incubation, 30 minute conjugate incubation, and 10 minute substrate incubation. All steps are performed at room temperature. The test procedures are flexible with the option of a 60 minute incubation period at room temperature or an overnight incubation at 2–8°C (ON protocol).

Results

All laboratories scored the correct results for negative and positive samples using the *pigtype* Salmonella Ab (Figure 1). The total CV for positive samples of the test panel, using *pigtype* Salmonella Ab, is 12.7%. Using *pigtype* Toxoplasma Ab, all laboratories scored the correct results. The total CV for the positive samples of the test panel, using *pigtype* Toxoplasma Ab is 13.5% (Figure 2).

Figure 1. *pigtype* Salmonella Ab box plot results.

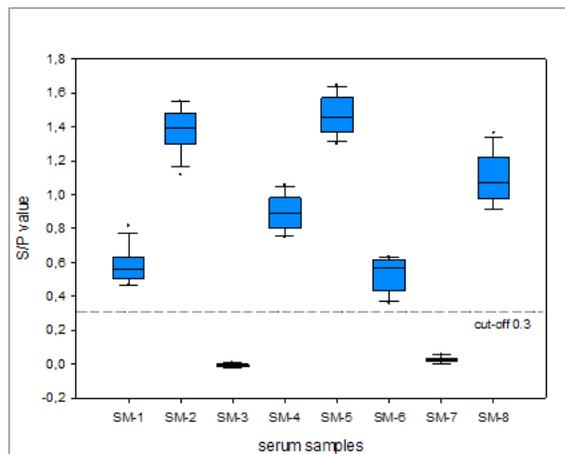
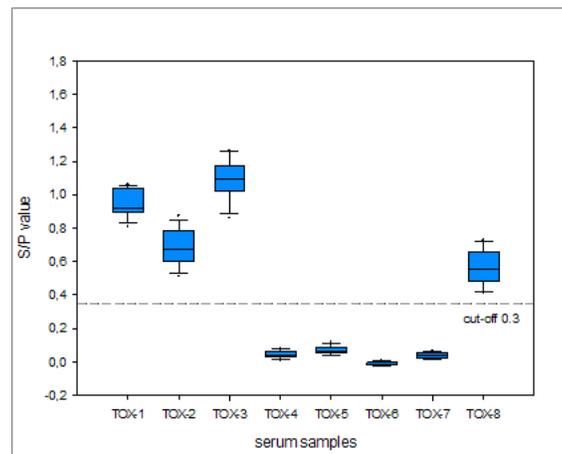


Figure 2. *pigtype* Toxoplasma Ab box plot results.



All laboratories scored the correct results using *pigtype* Trichinella Ab. The total CV for the positive samples of the test panel, using *pigtype* Trichinella Ab is 8.8% (Figure 3). Using *pigtype* Yersinia Ab, all laboratories scored the correct results. The total coefficient of variation is 8.8% for the positive samples of the test panel in the *pigtype* Yersinia Ab (Figure 4).

Figure 3. *pigtype* Trichinella Ab box plot results

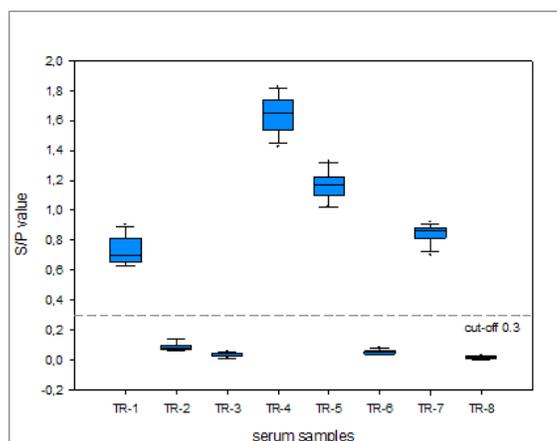
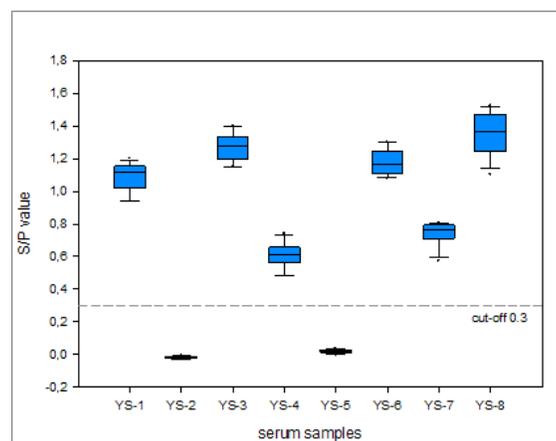
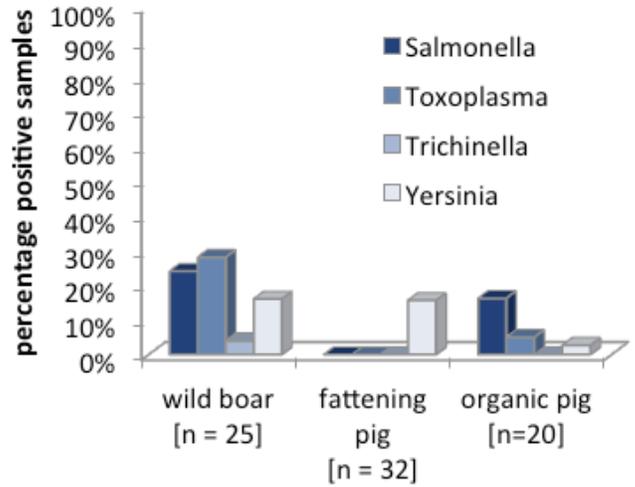


Figure 4. *pigtype* Yersinia Ab box plot results



As part of this study a limited number of field samples were tested. In fattening pigs a low percentage of *Yersinia* antibody positives were detected, while in organic pigs more salmonella and *Toxoplasma* antibody positives were found. The highest percentage of salmonella, *Toxoplasma*, and *Yersinia* positives was detected in wild boars. 4% of tested wild boar sera had antibodies specific to *Trichinella* (Figure 5).

Figure 5. Prevalence of antibodies to salmonella, *Toxoplasma*, *Trichinella*, and *Yersinia* in wild boar, fattening pigs, and organic pigs.



All of the participants were comfortable with sample dilution, quantity of reagents, and duration of the test. Two laboratories performed the ELISA manually and 4 laboratories semi-automated (no fully automated testing). There was little inter-lab variation with each sent ELISA. None of the laboratories reported any problems in the procedure of the tests.

Conclusions

In this study, there was a $\geq 99\%$ agreement of the test panel results for the participating laboratories. Our data suggest the suitability of the *pigtype* assays for an easy-to-use and cost-efficient serological monitoring for zoonotic diseases in swine herds. This could be an effective tool to use, under the European Directive on zoonoses, and bring improvements to herd risk assessment and risk oriented meat control.

References

- 1.) **DIRECTIVE 2003/99/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC Directive 2003/99/EC**
<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:325:0031:0040:EN:PDF>
- 2.) **Guideline Salmonella Monitoring and Reduction Program for Pork Production, www.q-s.de/dc_salmonella_programme_pig_lab.html**
- 3.) **Blaha T. (2004), Up-to-date information from the German QS salmonella monitoring and reduction programme. Dtsch Tierarztl Wochenschr. Aug; 111(8):324-326.**

The True Seroprevalence of Enteropathogenic *Yersinia* in Pigs, a Hayesian Approach

Vilar, M.J.*⁽¹⁾, Ranta, J.⁽²⁾, Virtanen, S.⁽¹⁾, Laukkanen-Ninios, R.⁽¹⁾, Korkeala, H.⁽¹⁾

⁽¹⁾ Department of Food Hygiene and Environmental Health Faculty of Veterinary Medicine. University of Helsinki. P.O. Box 66 (Agnes Sjöbergin katu 2) 00014 Helsinki University

⁽²⁾ Research and Laboratory Department. Risk Assessment Research Unit EVIRA. Finnish Food Safety Authority Mustialankatu 3 00790 Helsinki

*corresponding author: maria.vilar@helsinki.fi

Abstract

Bayesian inference was used to estimate the true seroprevalence of enteropathogenic *Yersinia* in pigs in Finland. Sensitivity and specificity of the diagnostic test were also estimated. One-hundred-seventy-two pigs of different ages were sampled and analysed for antibodies against enteropathogenic *Yersinia* outer proteins by a commercially ELISA test. Posterior probability estimates for sensitivity and specificity of the ELISA were 69.9% and 84.3%, respectively. The posterior probability of the true seroprevalence of enteropathogenic *Yersinia* was 78.6% for all pigs. There was an age tendency with the highest seroprevalence values in fattening pigs and in sows.

Introduction

Yersiniosis is a food-borne disease in humans, mainly caused by *Yersinia enterocolitica* (EFSA and ECDC, European Centre for Disease Prevention and Control 2012). *Y. enterocolitica* infections have been associated with the consumption of pork products (Tauxe and others 1987; Fredriksson-Ahomaa and others 2006; Rosner and others 2010). Healthy pigs are often asymptomatic carriers of *Yersinia*; and are considered the major reservoir of this zoonotic agent (Fredriksson-Ahomaa and others 2001; Fredriksson-Ahomaa and others 2006).

Serum samples can be analysed for antibodies against *Yersinia* by different serological test (Nielsen and others 1996; von Altröck and others 2006). In general, serology is a diagnostic tool that can be used for monitoring *Yersinia*, and it is cheaper and less time-consuming than the bacteriological methods (Fredriksson-Ahomaa and others 2011).

Detection of antibodies could provide a good estimation of the prevalence of enteropathogenic *Yersinia* in pigs at farms, when taking into account the sensitivity and specificity of the diagnostic tests used. The true seroprevalence can be estimated from an apparent seroprevalence by using the Bayesian inference which allows the incorporation of prior information in addition to the data. As well, the Bayesian approach provides a reliable estimate of the sensitivity and specificity when there is no gold standard test. The objective of the present study was to estimate the true seroprevalence of *Yersinia* in pigs using a Bayesian approach based on a cross-sectional sampling, and to estimate the diagnostic test sensitivity and specificity. The results showed that the sensitivity and specificity of the ELISA test were lower than previously reported by the manufacturer; and, the posterior probability estimate of the true seroprevalence was 78.6% (95%PI 61.5 – 90.5), for all pigs.

Material and Methods

In this study, serum samples were collected and analysed for occurrence of antibodies against enteropathogenic *Yersinia*. Individual serum samples from weaning pigs (20 to up to 50 kg), fattening (50 kg or more), and sows were collected on the farms, as previously described by Virtanen et al. (Virtanen and others 2012). The serum samples were tested for the presence of *Yersinia* antibodies by using a commercially available ELISA kit (Pigtype Yopscreen, Labor Diagnostik, Leipzig, Germany).

The definitions of apparent seroprevalence (A_p), true seroprevalence (T_p), sensitivity (S_e) and specificity (S_p) were considered as defined by Thrusfield (Thrusfield 2007). Independent beta prior distributions were used to take into account the uncertainty in the true seroprevalence, sensitivity, and specificity (Hanson and others 2003). The number of seropositive pigs (x) is conditional on the true seroprevalence, thus the model was: $x \sim \text{binomial}(A_p, n)$, where $A_p = T_p * S_e + (1 - T_p)(1 - S_p)$.

Prior beta distributions for the true seroprevalence were constructed based on a systematic review of the literature. Average pooled results of the apparent prevalence and its 95% confidence interval for each age group were calculated. Finally, they were used as inputs in the software Betabuster (downloaded from <http://www.epi.ucdavis.edu/diagnostictests/betabuster>).

html).

Information provided by the ELISA test's manufacturer validation report was used to estimate the prior distributions for sensitivity and specificity of the serological analyses. The sensitivity was estimated assuming a binomial model and uniform prior, where x out of n infected animals tested positive, thus $\beta(x+1, n-x+1)$. The specificity was similarly estimated.

Models were constructed in WinBUGS 1.4.3. Inferences were based in 50000 iterations after a burn-in of 1000 iterations for convergence. Results from the marginal posterior distributions are summarized as the median and their probability intervals (PI).

Results

The estimated sensitivity of the ELISA was 69.9% (95% PI 61.9 - 77.7), and the specificity was 84.3% (95% PI 51.9 - 99.2). The posterior probability estimates of the true seroprevalence in each age group of the Finnish pig population are presented in the Figure.

A sensitivity analysis was conducted using different beta prior distributions for each model, and no significant differences were found between the posterior estimates, F-value ($p > 0.05$).

Discussion

The commercial ELISA has been used to determine the seroprevalence of *Yersinia* in pigs (von Altrock and others 2006; Fredriksson-Ahomaa and others 2009; von Altrock and others 2011; Virtanen and others 2012) without questioning the accuracy characteristics reported by the manufacturer. The estimations obtained indicated that the commercial ELISA had lower sensitivity and specificity than previously reported by the manufacturer.

Several studies have reported apparent seroprevalence of *Yersinia* in pigs; however, differences in sensitivity and specificity between diagnostic methods result in dif-

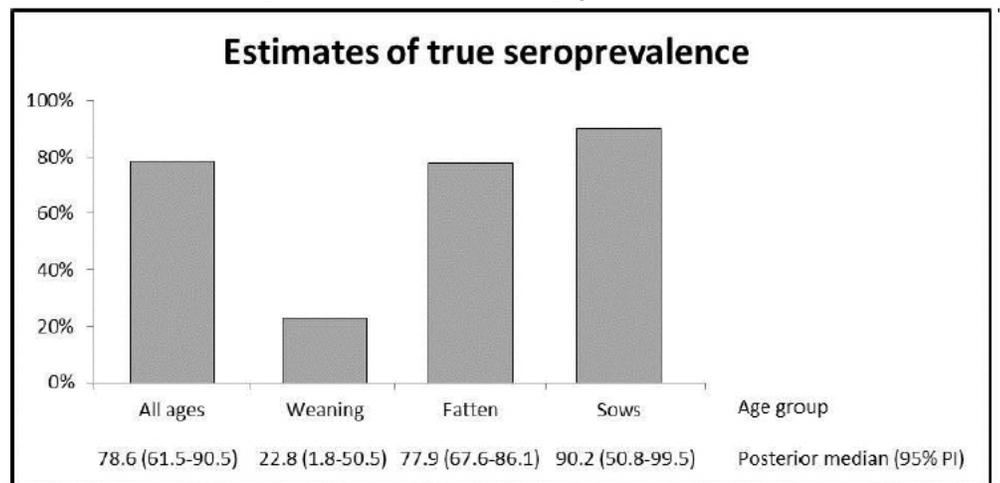
ferent true seroprevalence estimations. The true seroprevalence estimated in this study was significantly higher than the commonly reported apparent seroprevalence. The differences might be explained because those studies were based on a frequentist approach without taking into account the prior information nor the uncertainty of the sensitivity and specificity of the diagnostic test used. Therefore, the true seroprevalence estimated in the present study is not comparable directly.

Fattening pigs showed a true seroprevalence of 77.9%, value between the range of previously reported apparent seroprevalence of 2.5% (Nesbakken et al., 2007) to 82.1% (Virtanen et al., 2012). The use of two ELISA tests was reported to be used, which might explain the wide range of seroprevalence. The two test were the anti-LPS ELISA for specifically detection of *Y. enterocolitica* O:3 (Nesbakken and others 2006; Nesbakken and others 2007) and the same ELISA kit that we used that is against antibodies of enteropathogenic *Yersinia* (von Altrock and others 2006; von Altrock and others 2011; Virtanen and others 2012).

The lowest value of the true seroprevalence was found in weaning pigs, as they might be still protected by the maternal antibodies against *Yersinia*. Significant differences in seroprevalence between groups were observed, showing that the true seroprevalence increased with age, as previously reported by Vilar (Vilar and others 2013).

Conclusion

The Bayesian approach provided reliable information on the seroprevalence of enteropathogenic *Yersinia* in pigs, and also useful information of the ELISA diagnostic test commonly used to detect antibodies against *Yersinia*.



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References

1. EFSA E. F. S. A. & ECDC, EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL. (2012) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. The EFSA Journal 10, 2597
2. FREDRIKSSON-AHOMAA M., HALLANVUO S., KORTE T., SIITONEN A. & KORKEALA H. (2001) Correspondence of genotypes of sporadic *Yersinia enterocolitica* bioserotype 4/O : 3 strains from human and porcine sources. *Epidemiology and Infection* 127, 37-47
3. FREDRIKSSON-AHOMAA M., NESBAKKEN T., SKURMIK M., THISTED LAMBERTZ S., DICKSON J. S., HOORFAR J. & KORKEALA H. (2011) *Yersinia enterocolitica* in pork. In *Rapid Detection, Characterization, and Enumeration of Foodborne Pathogens*. Ed J. Hoorfar. Washington, DC, ASM Press. pp 195-297
4. FREDRIKSSON-AHOMAA M., STOLLE A., SIITONEN A. & KORKEALA H. (2006) Sporadic human *Yersinia enterocolitica* infections caused by bioserotype 4/O : 3 originate mainly from pigs. *Journal of Medical Microbiology* 55, 747-749
5. FREDRIKSSON-AHOMAA M., WACHECK S., KOENIG M., STOLLE A. & STEPHAN R. (2009) Prevalence of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in wild boars in Switzerland. *International Journal of Food Microbiology* 135, 199-202
6. GREINER M. & GARNER I. (2000) Epidemiologic issues in the validation of veterinary diagnostic tests. *Preventive Veterinary Medicine* 45, 3-22

Persistence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in pig herds over a two year period.

Van der Wolf, P.^{1*}, Meijerink, M.¹, Broens, E.², Graat, E.³, Köck, R.⁴, Friedrich, A.⁵

¹ Pig Health Department, Animal Health Service (AHS), Deventer, The Netherlands.

² Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

³ Quantitative Veterinary Epidemiology Group, Wageningen Institute of Animal Sciences, Wageningen University, Wageningen, The Netherlands.

⁴ Institute of Hygiene, University Hospital Münster, Münster, Germany

⁵ Medical Microbiology, University Medical Center Groningen, Groningen, The Netherlands

* Pig Health Department, Animal Health Service (AHS), Deventer, The Netherlands, PO Box 9, 7400 AA Deventer, The Netherlands, phone: +31-570-660378, e-mail p.vd.wolf@gddeventer.com

Aim of this study was to determine if a known MRSA contamination within a pig herd could persist for a period of two years.

Material and Methods:

16 pig herds with a known MRSA contamination were asked to participate in this study. Per herd, five dust swabs (Sodibox®) were collected every two months for a period of two years. Samples were tested for presence of MRSA by culture and two PCR's for confirmation of MRSA. Isolates were typed by spa- en MLVA-typing.

Results:

These 16 herds were sampled 177 times, varying from 4 to 13 times per herd. 72.9% of all samplings had at least one positive sample. Per herd this ranged from 38% to 100%. A total of 882 dust swabs were collected, of which 32.8% were positive, varying from 12% to 58% per herd. 284 isolates were spa-typed, yielding 17 different spa-types, mostly t011 and t108, with a minimum of 1 and a maximum of 6 spa-types per herd for the entire period and with a maximum of 3 per sampling. MLVA-typing did not lead to much more information, only in one herd there were two different types of spa-type t011: t011-398 (common) and t011-555 (only once). In 12 herds there was a spa-type which was found from beginning to the end of the test period. In 4 herds the same spa-type was found, and still prominent, as was found in the initial sampling in 2008. In many herds there were incidental findings of rare spa-types. Detailed results can be found in table 1.

Conclusions:

Overall a dynamic picture of contamination emerges, with herds being contaminated with a single type for a long time, dominant spa-types being replaced by another type and occasional findings of rare types. Both within herd reduction of contamination and prevention of new introductions will be necessary to control MRSA in pig herds.

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Table 1. Results of the longitudinal study on MRSA in swine herds, per sampling 5 dust swabs were taken. Presented are the spa-types and number of samples positive per herd per round and totals.

Herd	spa-type	Round													Total round 1-13	
		2008	1	2	3	4	5	6	7	8	9	10	11	12		13
1	t108	6			1		3	1	2	1	4	3	1	1	1	18
1	total	6	0	0	1	0	3	1	2	1	4	3	1	1	1	18
2	t108	14	1		1	3				2	4				3	14
2	total	14	1	0	1	3	-*	-	-	2	4	-	-	-	3	14
3	t108	2	2		1											3
3	t127	9	2	1	2	1										6
3	total	11	4	1	3	1	-	-	-	-	-	-	-	-	-	9
4	t011	8	3	1		1		1			2				4	12
4	total	8	3	1	0	1	0	1	0	0	2	0	0	-	4	12
5	t011	11	5				1		2	1		1	2	1		13
5	t3423											1				1
5	t6320					1										1
5	nt			1												1
5	total	11	5	1	0	1	1	0	2	1	-	2	2	1	0	16
6	t011	10			2	1	1	1	1			1		1		8
6	t108		2		1	2	1	1	1	1	1				4	14
6	t1606				1											1
6	total	10	2	0	4	3	2	2	2	1	1	1	0	1	4	23
7	t011		5	2	3	4	2	3	1	1					4	25
7	t1451							1								1
7	total	-	5	2	3	4	2	4	1	1	-	-	-	-	4	26
8	t011		3		3	1	1				2	2		2	1	15
8	t108						1	1			1					3
8	total	-	3	0	3	1	2	1	0	0	3	2	0	2	1	18
9	t011		1			1										2
9	t034				1	1										2
9	t108		2		1					1					3	7
9	t2330		1													1
9	total	-	4	0	2	2	0	0	0	1	0	0	0	0	3	12
10	t011							1							3	4
10	t108		5			2										7
10	t1457														1	1
10	total	-	5	0	0	2	0	1	0	0	-	-	-	-	4	12
11	t011		2	3	3	2	1	3	1		2	1	1	2		21
11	t034						2						1			3
11	t1184				1							1				2
11	t1456											2				2
11	t6320					2	2									4
11	t7621								4							4

Herd	spa- type	Round													Total round 1-13	
		2008	1	2	3	4	5	6	7	8	9	10	11	12		13
11	total	-	2	3	4	4	5	3	5	0	2	4	2	2	0	36
12	t108	9													1	1
12	t899	1	1	1	2		1		1	2			1		1	10
12	t1457	1														
12	total	11	1	1	2	0	1	0	1	2	0	0	1	0	2	11
13	t011	7			1		1									2
13	t899			3	1		2		4	2	2				2	16
13	total	7	0	3	2	-	3	0	4	2	2	-	-	-	2	18
14	t011													1		1
14	t108	10		1		3			3	1	3	2	2	2	2	19
14	nt		1					2								3
14	total	10	1	1	0	3	-	2	3	1	3	2	2	3	2	23
15	t011	7	5	1	2	2	3	5	3	2	2	1	2		2	30
15	t1451										1					1
15	t2346	4			1	1	1									3
15	total	11	5	1	3	3	4	5	3	2	3	1	2	0	2	34
16	t002	5			1	1				1						3
16	t2164		1													1
16	t899													2		2
16	nt			1												1
16	total	5	1	1	1	1	0	0	0	1	0	0	0	-	2	7
Total		208	42	15	29	29	23	20	23	15	24	15	10	10	34	289

* not done

nt: not typed

Exposure assessment of extended-spectrum beta-lactamases/AmpC beta-lactamases-producing in meat in Denmark

Carmo, L.P.^{*(1,2)}, Nielsen, L.R.⁽²⁾, da Costa, P.M.⁽¹⁾, Alban, L.^(2,3)

⁽¹⁾ Institute of Biomedical Sciences Abel Salazar, University of Porto. Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal.

⁽²⁾ University of Copenhagen. Grønnegårdsvej 8, 1870 Frederiksberg, Denmark.

⁽³⁾ Danish Agriculture and Food Council. Axelborg, Axeltorv 3, 1609 København V, Denmark.

*corresponding author: lpedro20@hotmail.com

Abstract

Extended-Spectrum Beta-Lactamases (ESBL) and AmpC Beta-Lactamases (AmpC) are of great concern because of their ability to cause antimicrobial resistance in *Enterobacteriaceae* hampering the effect of treatment with beta-lactam antibiotics. The main objective of this study was to assess the relative importance of different types of meat for the exposure of consumers to ESBL/AmpC and their potential relevance for human cases in Denmark.

This was assessed by weighting the prevalence of each genotype of ESBL/AmpC-producing *E. coli* (ESBL/AmpC-PEC) in imported and nationally produced broiler meat, pork and beef with the meat consumption patterns in Denmark. Data originated from the Danish surveillance programme for antibiotic use and antibiotic resistance (DANMAP) for 2009 to 2011. Data about human ESBL cases in 2011 were also collected to assess a possible genotype overlap. Uncertainty was assessed by inspecting beta distributions of the genotypes in each type of meat.

Broiler meat represented the largest part of the estimated ESBL/AmpC contaminated pool of meat (83.8%) compared to pork (12.5%) and beef (3.7%). CMY-2 was the genotype with the highest relative importance for human exposure (58.3%). However, it is rarely found in humans in Denmark.

In general the overlap between ESBL/AmpC genotypes in meat and those found in human *E. coli* infections isolates was limited. CTX-M-1 had a relative importance of 28.8% for human exposure through meat. The prevalence of CTX-M-1 in humans was 7.3% of *E. coli* urinary tract infections and 8.0% of *E. coli* bloodstream infections. Hence, the genotype CTX-M-1 was considered the most relevant genotype found in meat when referring to human exposure. This suggests that meat might constitute a less important source of ESBL/AmpC exposure of humans in Denmark than previously thought. Nonetheless, more detailed surveillance data are required to determine the contribution of meat compared to other sources, such as pets and hospitals.

Introduction

Extended-Spectrum Beta-Lactamases (ESBL) were defined by the EFSA Panel on Biological Hazards (BIOHAZ) as plasmid-encoded enzymes found in the bacterial family *Enterobacteriaceae*. ESBL confer resistance to a variety of beta-lactam antibiotics, including penicillins, 2nd, 3rd and 4th generation cephalosporins and monobactams (EFSA, 2011). The BIOHAZ panel also stated that AmpC Beta-Lactamases (AmpC) are intrinsic cephalosporinases found on the chromosomal DNA of many Gram-negative bacteria. However, the number of AmpC enzymes that are plasmid-borne, such as CMY-2, is increasing (EFSA, 2011).

Economically and socially, the burden of antimicrobial resistance is quite significant (De Kraker et al., 2011). The World Health Organization (WHO) calculated 25,000 deaths each year in the European Union (EU) due to infections with antibiotic resistant bacteria (WHO, 2011a). The increase of ESBL/AmpC is particularly worrying due to their resistance to 3rd and 4th generation cephalosporins, which have been considered critically important to human medicine (WHO, 2011b). Recently, a possible link has been made to food and food-producing animals (EFSA, 2011; SAGAM, 2009), stressing the importance of scrutinizing the role of food animal products to human ESBL/AmpC-producing bacteria (ESBL/AmpC-PB) infections.

Denmark, as the other Nordic countries, has always been considered having a low animal prevalence of anti-microbial resistance, due to the conscious use and restrictive measures related to antibiotics. The “Yellow card” initiative (Alban et al., 2013) and the cephalosporins ban for use in pigs (Agersø & Aarestrup, 2013) are examples of the precautionary Danish policy related to antimicrobial use in animal production.

In 2009, the first Danish surveillance study in meat and live animals with enriched culture method was performed, nowadays described as the preferred method for ESBL/AmpC producers isolation (EFSA, 2011), followed by genetic background investigation (Agersø et al., 2012). Since then, this protocol has been repeated yearly, revealing an increasing prevalence in Danish broiler meat from 3% in 2009 (data not published) to 44% in 2011 (DANMAP, 2011). In the same period, imported broiler meat had prevalences between 36% and 50% (DANMAP, 2009, 2010, 2011).

The aim of this project was to determine the relative importance of the various ESBL/AmpC-producing *E. coli* (ESBL/AmpC-PEC) genotypes in different meat types with respect to exposure of Danish meat consumers. Moreover, the genotype overlap between isolates found in meat and human cases was evaluated to assess the relative importance of meat as a source of ESBL/AmpC-PEC infections in humans.

Material and Methods

Data about the fresh and frozen meat available for consumption in 2009, 2010 and 2011 were collected and divided into six categories: imported and domestically produced broiler meat, pork and beef (DTU, 2011). Furthermore, the prevalence of the different genotypes found in each category of meat was obtained (DANMAP, 2009, 2010, 2011). In the DANMAP programme, detection of ESBL/AmpC-PEC is conducted using a selective culture method with antimicrobials. This method has a very high sensitivity and is qualitative, hence resulting in positive or negative outcome.

To calculate the relative importance of each type of meat and of each genotype for human exposure, the prevalence of ESBL/AmpC genotypes was weighted by the amount of meat available for consumption: Weighted prevalence (WP) = Prevalence*Meat available for consumption. All the WPs were added to have an ESBL/AmpC Total Pool. Finally, the WP of each genotype was divided by the ESBL/AmpC Total Pool: Relative importance (RI) = WP/ (ESBL/AmpC Total Pool).

Due to the low frequency of positive samples for pork and beef and the low frequency of testing in general, a pool of the 3 years’ sampling was used. However, the described procedure was also applied to each year separately. The genotype group “Others” included TEM-52 and unknown (i.e. where it was impossible to determine a specific genotype) in beef; unknown genotypes in pork; and TEM-20, TEM-52, up-regulated AmpC and unknown genotypes in broiler meat.

Using the software @Risk 4.5 Palisade Corporation®, beta distributions were created to assess the 95% credibility interval (CI) for each the genotype and hereby estimate how high the true prevalence could be given the available data. The beta distributions were made through a simulation using 1,000,000 iterations. The distributions were defined by (s+1, n-s+1), where s represents the number of positive samples for each genotype in each type of meat from 2009 to 2011, and n represents the total number of samples tested for ESBL/AmpC-PEC in each type of meat within the same 3-year period.

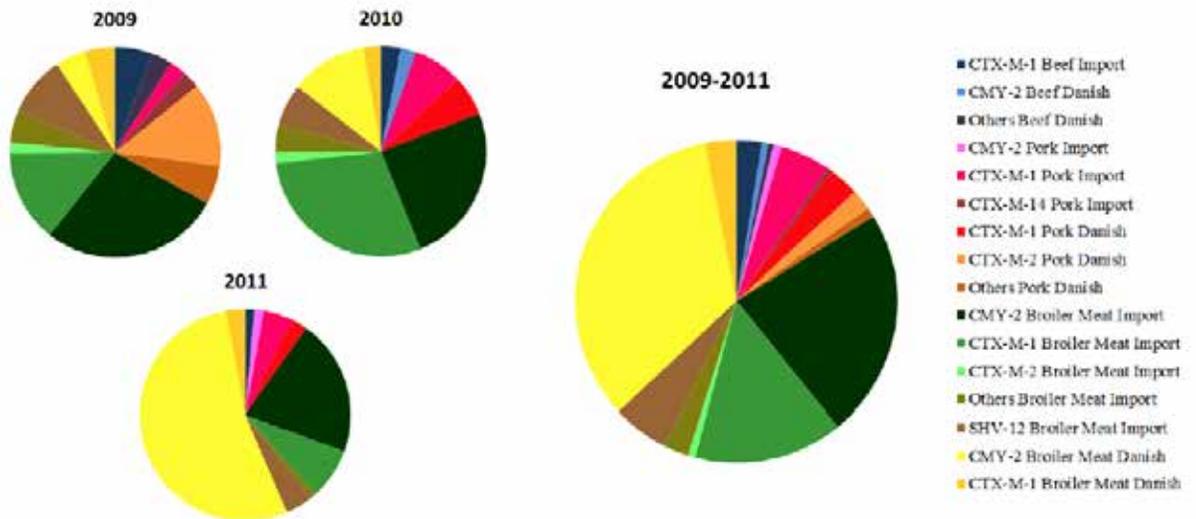
Results

During the period 2009 to 2011, broiler meat contributed the most to the total human exposure to ESBL/AmpC-PEC representing 83.8% of the total exposure. Danish broiler meat contributed with 37.0% and imported broiler meat with 46.8%. Danish and imported pork constituted 6.2% and 6.3% of the ESBL/AmpC positive meat available for consumption, respectively. Beef had minor relevance representing 1.2% in beef of Danish origin and 2.5% of imported origin.

The relevance of each genotype varied between the 3 years (Figure 1). Most important was the increase in the

role CMY-2 in Danish broiler meat from 4.6% in 2009 to 11.8% in 2010, increasing to 53.5% of the total exposure in 2011. Moreover, CMY-2 constituted the genotype that meat consumers were most exposed to (58.3% across the 3-year period). CTX-M-1 was the most frequently isolated genotype in pork and beef at 8.2% and 2.5% of the total exposure, respectively. Including broiler meat, CTX-M-1 represented 28.8% of the total ESBL/AmpC positive meat in the Danish market from 2009 to 2011. Overall, Danish meat presented a lower relative importance and imported meat contributed to 55.7% of the human exposure.

Figure 1 – Relative importance of each type of meat for human exposure considering the genotypes found in DANMAP surveillance from 2009 to 2011. The beef genotypes are represented in blue colours, while pork is in pink, red and orange, and broiler meat is in green, brown and yellow colours.



CTX-M-15 was the genotype most commonly found in human cases. It was found on rare occasions in Danish pigs and cattle at slaughter. However, it has not been detected in meat samples so far. This ESBL genotype was estimated to be present in Danish pork and beef below prevalences of 0.7% and 1.0%, respectively, given the available data. The prevalence of CTX-M-1 was 2.0% in imported pork (DANMAP, 2009, 2010, 2011), but this estimate is uncertain due to the low number of samples. The estimated upper limit of prevalence for this genotype was 3.8% in imported pork.

Discussion

A definitive cause-effect association cannot be established through our approach. The limited genotype overlap between the meat and human reservoirs indicates that meat might play a minor role for human cases of ESBL/AmpC-PEC.

Considering that cephalosporins have not been used in poultry in Denmark for more than 10 years it is possible that the high prevalences detected in Danish broiler meat might be caused by practices upstream in the production pyramid. This hypothesis has also arisen in Sweden (Börjesson et al., 2013; SVARM, 2010). Danish and Swedish broiler parents come from the same Swedish breeding stock, which in turn is supplied by a Scottish grand-parent breeding company where cephalosporins were used as a prophylactic measure until recently. Cross-contamination through the environment, humans or animals, as well as off-label use of cephalosporins, should also be considered and investigated. Moreover, it should be studied whether the animal feed is an important source of ESBL/AmpC-PEC. However, the fact that CMY-2 and CTX-M-1 were also the genotypes detected in Swedish broiler meat (SVARM, 2010) and both of them being the only ESBL/AmpC genes persistently detected in Danish broiler meat from 2009 to 2011, supports the hypothesis of a common source upstream in the production pyramid. Co-resistance patterns may have facilitated the spread and maintenance of ESBL/AmpC-PEC (Börjesson et al., 2013).

The discontinuation of the use of cephalosporins in pigs is the most likely explanation for the reduction on the

ESBL/AmpC-PEC prevalence seen in these animals in 2011 (Agersø & Aarestrup, 2013). Other factors could also have contributed to this diminution, i.e. the “Yellow card” initiative, adopted almost at the same time and that initially led to a 25% decrease in use of antimicrobials in livestock (Aarestrup, 2012; Alban et al., 2013).

In humans, the so-called “pandemic CTX-M-15” genotype dominated both in blood and urine ESBL-PEC isolates, with 68.0% and 59.3%, respectively (DANMAP, 2011). CTX-M-1 was detected in 7.3% of urine and 8.0% of blood isolates (DANMAP, 2011). However, it should be noted that, particularly for human ESBL-PEC septicaemia, the number of cases on which these results are based is quite small (n=25). Consequently, it is possible that the true ESBL genotype distribution is slightly diverse from what was found and some genotypes causing infections may have not been detected. As CTX-M-1, CTX-M-14 was detected both in human infections and meat.

ESBL/AmpC genes found in humans and meat overlapped to a limited extent. It should be highlighted, however, that although some genotypes were not found in meat they were found in live animals, indicating their possible presence in meat. This is the case of CTX-M-15, which was detected in very low prevalences in Danish pigs and in Danish cattle.

This was one of the main motives to assess the uncertainty. This investigation is especially interesting to apply to genotypes that were found in live animals and not in meat. The upper 95% CI expected prevalence for ESBL/AmpC genes not detected in meat (that is the case of CTX-M-15), considering the DANMAP sampling within the 3-year period, was 0.7% for Danish pork, 0.9% for imported pork, 1.0% for Danish beef, 1.4% for imported beef, 0.9% for Danish broiler meat and 0.7% for imported broiler meat. Such low prevalences points against a role of meat for the CTX-M-15 PEC infections in humans. Nonetheless, very little is known about the duration of carriage, dominance in the human gut microflora and different ESBL/AmpC-PEC’s ability to survive in the human gut environments.

It is of great relevance to highlight that CMY-2 was detected in human infections, but it was not possible to calculate its prevalence in humans. Nevertheless, the positive number of samples was low (data not published). Considering the possible emergence of CMY-2 *E. coli* and its relevance for human exposure through meat, it is important that its prevalence is calculated in the upcoming years.

Meat might not have such a high impact for human ESBL/AmpC-PEC infections as firstly thought. However, this does not exclude the importance of animal production, and even meat, for ESBL/AmpC dissemination, so a precautionary approach should probably be taken.

In Denmark, there are limited additional options to control ESBL/AmpC occurrence through the reduction in use of cephalosporins in livestock. It is known that other antimicrobials can co-select ESBL/AmpC genes. Therefore, measures to promote the reduction of antimicrobial use in general, such as the “Yellow card” scheme, may have some influence. However, downstream the production chain, other options can be explored. In first place, some studies have documented the existence of some risk factors for ESBL/AmpC growing in animals’ gut (Persoons et al., 2010). The impact of probiotics in the control of this type of bacteria should also be studied. Secondly, cross contamination during slaughter should be inspected and, if relevant, hygiene should be improved. Other option to consider is to implement decontamination after slaughter. Nonetheless, the role of other sources should be assessed, including other foodborne sources, pets and hospitals, so further studies are needed. ESBL/AmpC-PB is a very complex subject and their emergence, ecology and dynamics is nowadays poorly understood. Furthermore, very little is known about the impact of control strategies, which hampers prioritisation of measures to prevent the emergence and dissemination of ESBL/AmpC-PB. This is a critical point that must urgently be further investigated. Finally, we suggest that data collection, in human, food and animal reservoirs, become harmonized across the EU. This would not only allow comparison of data between countries, but also facilitate studying risk factors and suggest optimal control strategies.

Conclusion

The genotype overlap between the two reservoirs – humans and meat from poultry, pigs and cattle – is low,

suggesting that meat might not have such a relevant role for ESBL/AmpC-PEC human infections in Denmark in 2009-2011 as previously thought. Poultry meat was most relevant for human ESBL/AmpC-PEC exposure followed by pork and beef, and Danish meat had a lower relative importance than imported meat. CTX-M-1 and CTX-M-14 producing *E. coli* were found in both humans and animals, but the occurrence of these two genotypes in humans was not very frequent. “Pandemic” CTX-M-15 was not found in meat samples, but it should not be ruled out that it can occur in meat, because it has previously been detected in live animals although at a low prevalence. Future studies aiming to obtain a better understanding of the issue of ESBL/AmpC-PEC is required, unveiling the factors that lead to ESBL/AmpC emergence and explaining the interface between several sources and humans to clarify the increasing occurrence of human cases.

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References

To have access to a full reference list, please contact the first author.

#1447 Visual-only meat inspection of pigs fattened outdoors: a food safety risk?

Tongue, S.C.^{*},⁽¹⁾, Oliva-Abascal, R.⁽¹⁾, Evans, J.⁽¹⁾, McCrone, I.S.⁽²⁾, Webster, C.⁽¹⁾, Tucker, A.W.⁽²⁾, Gomes, C.⁽¹⁾

⁽¹⁾ Epidemiology Research Unit, SRUC (Scotland's Rural College), Inverness, UK.

⁽²⁾ Department of Veterinary Medicine, University of Cambridge, Cambridge, UK.

* *corresponding author*: sue.tongue@sruc.ac.uk

Abstract

The primary purpose of meat inspection is to contribute to the production of safe food for human consumption. The aim of this study was to investigate whether the implementation of modern, predominantly visual, inspection systems for carcasses from fattening pigs reared outdoors since weaning, in Great Britain (GB), would alter the risk to food safety. A combination of sources was used to inform a formal, mostly qualitative, risk assessment, based on Codex Alimentarius Commission (CAC) guidelines. These sources included: a retrospective analysis of meat inspection data; a field trial of visual inspection of pigs from non-controlled housing conditions; previous work, scientific literature and publically available information. It was concluded that the public and animal health risks associated with endocarditis changed from negligible to non-negligible (i.e. very low) and the risk of microbial contamination of carcasses may be reduced.

Introduction

In order to determine whether a carcass is fit for human consumption, traditional methods of meat inspection use observation, palpation and incision with a focus on the detection of gross lesions. These methods may not be suitable for detecting some of today's important food-borne microbial pathogens and they may contribute to cross-contamination of carcasses. Derogations from European Union (EU) regulations have enabled carcasses of fattening pigs to be inspected by visual-only methods, provided that certain requirements are met, and the pigs have been reared under controlled housing conditions, in integrated production systems, from weaning to slaughter (Anon. 2004). However, uptake of modified inspection programmes has been low with only three EU member states reporting implementation (Alban *et al.* 2011). One of the reasons for low uptake within the British pig industry is that slaughterhouses accept a mixture of indoor and outdoor reared pigs throughout the day; the latter are from non-controlled housing conditions and still need to be inspected by traditional means. In previous work by Hill *et al.* (2013), it was determined that the overall risk, from all hazards to public health, was negligible for all pigs if a visual-only inspection method was used. Despite this, there was still a concern that the implementation of such inspection systems may not be appropriate for carcasses from fattening pigs reared, since weaning, in outdoor management systems, due to increased variability in the epidemiology, occurrence and control of diseases on these units. But is this the case?

The aim of this study was to investigate whether the implementation of modern, predominantly visual, inspection systems for carcasses from fattening pigs reared outdoors since weaning in Great Britain (GB) would alter the risk to food safety. This was achieved by addressing the following four questions: firstly, does the prevalence of conditions observed at traditional meat inspection vary between fattening pigs reared from weaning to slaughter, under controlled housing conditions, in integrated production systems, and those reared in outdoor management systems? Secondly, does the frequency of conditions observed in carcasses of fattening pigs, reared from weaning to slaughter in outdoor management systems, vary when inspected using visual-only inspection compared to traditional methods? Thirdly, does the level of microbial contamination of these carcasses vary when inspected using visual-only inspection compared to traditional methods? And, finally, will the risks from hazards to public health change if visual-only inspection for fattening pigs from non-controlled housing conditions was implemented?

Material and Methods

To answer the first question, a year's worth of ante-mortem and post-mortem carcass inspection plus post-mortem offal inspection data for fattening pigs slaughtered at the study abattoir were acquired. These were combined with information about the management systems from which the pigs originated i.e. indoor or outdoor systems. The frequency (prevalence) of each condition in each batch and the proportion of the batches affected with a condition were calculated and then compared (confidence intervals and Z-test). Effects of batch size and season were investigated by comparing odds ratios (Mantel-Haenszel). The batches in which the conditions were found were also compared for the two finishing systems (mean prevalence - t-test).

To answer the second and third questions, a field study was implemented over five separate weeks of work in the study abattoir during the period from November 2011 until April 2012. Carcasses from fattening pigs, which were reared from weaning to slaughter in outdoor management systems, were inspected using both post-mortem inspection methods. The

number of carcasses affected by each condition was recorded at a batch level. The baseline of type, frequency and distribution of conditions detected by both post-mortem inspection methods was established and then compared (normal distribution - paired t-test and Pearson's correlation test; season effect - linear models; non-normal distribution - categorised as 'absence' or 'presence' for each batch and McNemar test). Bonferroni corrections were used to adjust the threshold level for statistical significance in all relevant statistical analyses, due to the number of analyses performed in the comparisons.

Sponge swabs were used to collect samples for microbiological investigation from a subset of the above carcasses, after visual-only inspection and after traditional inspection, using a systematic random sampling strategy. The sampling methodology followed that described in Regulation 2073/2005. Samples were not intentionally taken from the same carcass after both inspection methods. Samples were processed in the laboratory 24 hours after collection in the abattoir. Total aerobic plate count, *Enterobacteriaceae* count, and *Salmonella* spp. isolation were carried out using the following methods:

1. Total aerobic plate count - BS EN ISO 4833:2003
2. *Enterobacteriaceae* count - BS EN ISO 21528-2:2004
3. *Salmonella*: Isolation of *Salmonella* - BS EN ISO 6579:2002

Yersinia spp. isolation was carried out using the following methods:

4. British Standards Institution (2003b) - BS EN ISO 10273:2003
5. Health Protection Agency (2011c). Detection of *Yersinia* species. Microbiology Services. Food, Water & Environmental Microbiology Standard Method F33: Issue 1.0.

The mean of the microbial counts with their 95% confidence interval (C.I.) were calculated for both inspection methods and then compared. For total aerobic plate count the estimates calculated were the mean of the logs, compared by student t-test. For the *Enterobacteriaceae* count the estimates were the mean of the values. Due to a large number of zeros, this variable was categorised as presence/absence and compared with a chi-squared test. Samples with more than zero *Enterobacteriaceae* plate count were transformed to \log^{10} and the means compared between the inspection methods (student t-test). Linear models were also applied. Other variables included in the models were inspection method, week, date and line position. The variables were selected to enter in the multivariable model if $P < 0.15$. This approach was also used for the binary variable: presence/absence of *Yersinia* spp. Bonferroni corrections were used to adjust the threshold level for statistical significance in all relevant statistical analyses, due to the number of analyses performed in the comparisons. Bonferroni corrections were used, as above.

To address the final question - will the risks from hazards to public health change if visual-only inspection for fattening pigs from non-controlled housing conditions was implemented - the outcomes from the investigations outlined above were used with previous work, scientific literature, and publically available information to inform the mostly qualitative risk assessment, using a modified CAC approach (CAC 1999). Hazards were identified then characterised; the characteristics of each organism when ingested by humans were described. Occupational hazards were not considered. In addition, the general characteristics of the hazard in pigs were described. For exposure assessment, the contribution of pork meat to the total public health exposure to organisms that are associated with the identified hazards was explored. The risk that exists when outdoor pigs are inspected by traditional methods was then characterised by integration of hazard identification, hazard characterisation and exposure assessment to obtain a risk estimate. This was then compared to the risk that exists when outdoor pigs are inspected by a visual-only method. In addition to the food safety aspects (public health), animal health and welfare were considered.

Results

The analysis of the historic traditional inspection records included data from more than 1.2 million pigs from approximately 7,400 batches (the groups that they are submitted to the abattoir in). These pigs came from both indoor and outdoor rearing and fattening systems, with the latter accounting for approximately a quarter of the batches. Batch size and seasonality had no influence on the associations found. The prevalence of conditions detected on traditional inspection of pigs submitted to slaughter from the two different fattening systems were similar. Statistically significant findings were as follows:

- a higher percentage of indoor batches were recorded as affected with 'tail bite', 'lameness', 'oedema', and 'pericarditis' than outdoor batches;
- a higher percentage of outdoor batches were recorded as affected with 'hair contamination' and 'milk spot' than indoor batches;

- the mean prevalence of ‘milk spot’ was higher, in the batches in which the condition was present, in those batches from outdoor systems, compared to indoor systems;
- the mean prevalence of ‘kidney pathology’, in the batches in which the condition was present, was slightly higher for indoor pigs than for outdoor pigs; whereas for ‘hepatitis’, ‘peritonitis’, ‘pneumonia’ and ‘other pathology’, the reverse was the case.

In the field study, more than 11,000 carcasses of fattening pigs from non-controlled housing conditions from 62 batches and 12 farms were inspected. No effect of season and farm of origin were found. There were statistically significant differences in the frequencies found by the two inspection methods for six of the categories of conditions. However, the biological differences were very small. Hair contamination of carcasses was recorded at higher frequencies with the visual method of inspection than with traditional inspection. The recorded frequencies were higher with the traditional method of inspection for milk spot, renal pathology, enteritis, pluck pathology and faecal contamination, than with visual only inspection.

For total aerobic plate count, *Enterobacteriaceae* count and *Salmonella* spp. isolation, 800 swabs were taken (400 after the traditional inspection point and 400 after the visual-only inspection point). For the *Yersinia* spp. isolation a slightly different subset of carcasses was sampled. In the whole study 759 swabs were tested for *Yersinia*: 379 after traditional inspection and 380 after visual-only inspection. No *Salmonella* spp. were isolated from any sample in the study. In addition, no statistical difference was found in the proportion of carcasses contaminated with *Yersinia* spp. after the two inspection methods. Although there was no evidence for a difference in the general bacterial contamination of carcasses after the two inspection methods, for the carcasses where *Enterobacteriaceae* were present there was some evidence that the level of contamination of carcasses was lower after visual-only inspection compared to traditional inspection.

Of the five public health hazards that we identified (endocarditis [*Streptococcus* spp., including *S. suis*, & *E. rhusiopathiae*]; granulomatous lesions [*Mycobacterium* spp., *Rhodococcus equi*]; *Salmonella* spp., *Yersinia* spp., and the hygiene process indicators [total aerobic plate count and *Enterobacteriaceae* count], only two have a revised risk on a change in inspection method; the risk associated with endocarditis (inflammation of the internal lining of the heart), changed from negligible to non-negligible i.e. very low; and, it is possible that the risk of microbial contamination of carcasses with *Enterobacteriaceae* is reduced. Only two animal health hazards were identified and assessed (endocarditis and granulomatous lesions). Again, endocarditis has a revised risk on a change in inspection method from negligible to non-negligible i.e. very low.

Discussion

Most of the differences found in the historic data analysis were predictable from knowledge of the housing, management and fattening systems used and their relationship with the diseases, or circumstances from which the conditions arise.

The field trial outcomes on observation and recording of conditions using the two different inspection methods can be explained by the positioning of the visual-only inspection point on the line, the truly ‘hands-off’ nature of the visual-only inspection and the lack of incisions into offal with this method.

The lack of isolation of *Salmonella* spp. was slightly unexpected and further work is planned to determine if this was truly the case. The hygiene processes on the line were observed to be good and this is reflected in the outcomes from the hygiene process indicators. Nevertheless there was still some evidence for a reduction in the contamination of carcasses by changing the *post-mortem* inspection method to a visual-only system where handling of carcasses by official personnel was minimised. It is possible that a change in the inspection method from traditional to visual would lead to reduced microbial contamination of carcasses in any abattoir with a level of contamination as low as, or higher than, the study premises. If the level of contamination of carcasses is reduced by a change in inspection method, then it could be hypothesised that the potential for cross-contamination would also be reduced; however, we cannot draw that as a conclusion from our study.

Despite the revised risk classification for public and animal health attributable to endocarditis for visual- only inspection compared to traditional inspection for outdoor pigs, the fact still remains (Hill *et al.*, 2013) that outdoor pigs from non-controlled housing conditions present at least the same, if not less of a risk than indoor pigs from controlled housing conditions.

Conclusion

The main conclusion is that the public and animal health risks associated with endocarditis would change from negligible to non-negligible (i.e. very low), if visual-only inspection for carcasses from fattening pigs reared outdoors since weaning in Great Britain (GB) (non-controlled housing conditions) was implemented, and the risk of microbial contamination of carcasses may be reduced. However, despite this change, these risks would be expected to be no greater than those that are expected if visual-only inspection were to be implemented for fattening pigs from controlled housing conditions.

Acknowledgements

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References

ANONYMOUS, 2004, Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption., Off. J. Eur. Comm., L155/206

HILL A., BROUWER A., DONALDSON N., LAMBTON S., BUNCIC S., GRIFFITHS I., 2013, A risk and benefit assessment for visual-only meat inspection of indoor and outdoor pigs in the United Kingdom., Food Control, 30, 255-264 doi:10.1016/j.foodcont.2012.04.031

CODEX ALIMENTARIUS COMMISSION (1999) Principles and guidelines for the conduct of microbiological risk assessment, CAC/GL-30

<http://www.who.int/foodsafety/publications/micro/cac1999/en/>

Evaluation of penicillin G residues by kidney inhibition swab tests in sow body fluids and tissues following intramuscular injection

Shelver, W.L.^{*(1)}, Lupton, S.J.⁽¹⁾, Newman, D.J.⁽²⁾, Larsen, S.⁽³⁾, Smith, D.J.⁽¹⁾

⁽¹⁾USDA-ARS, Biosciences Research Laboratory, Fargo, USA

⁽²⁾Department of Animal Sciences, North Dakota State University, Fargo, USA

⁽³⁾National Pork Board, Des Moines, USA

*Corresponding author: Weilin.Shelver@ars.usda.gov

Abstract

In 2011, the USDA-Food Safety and Inspection Service (FSIS) changed the method used for screening swine tissues for antimicrobial residues from the Fast Antimicrobial Screen Test to the Kidney Inhibition Swab (KIS™). Here, we describe the use of KIS™ test for the detection of penicillin G residues in kidney, liver, plasma, urine, and skeletal muscle of heavy sows following the administration of a 5x label dose of penicillin G procaine. Such off-label use is legal in the United States under the Animal Medicinal Drug Use Clarification Act (AMDUCA) when label routes or doses are ineffective at treating disease and is commonly used to treat bacterial infections in heavy sows. Heavy sows (n=126; 228 ± 30.1 kg) were administered intramuscular (IM) doses of penicillin G (33,000 U/kg bw) for 3 consecutive days using 3 different administration patterns. Within treatment, six sows each were slaughtered after 5, 10, 15, 20, 25, 32, or 39 withdrawal days. The IM administration pattern had no discernible effect on penicillin G depletion from kidney, skeletal muscle, serum, urine or liver. Residues were depleted most rapidly from liver and skeletal muscle and more slowly from kidney and urine. While kidney residues were a poor predictor of penicillin G residues in skeletal muscle, kidney was the most sensitive target tissue for detecting penicillin G residues, with two positive results even after a 39-day withdrawal period. The most suitable ante-mortem matrix to replace FSIS on-site tests using kidney was urine. Serum, another ante-mortem matrix predicted muscle residue well albeit showing more positives than muscle. These data support a 15-day withdrawal period suggested by Food Animal Residue Avoidance Databank for extra-label penicillin G treated heavy sows with the caveat that kidney tissues be excluded from human consumption.

Introduction

Penicillin G is active against a variety of Gram-positive pathogens affecting livestock production and is indicated for treatment of a number of bacterial diseases in a variety of animal species including erysipelas in swine (NADA 065-010). For most food animals, the typical route of penicillin G administration is by IM injection at daily dose of 6,600 IU/kg with treatment not to exceed 4 consecutive days. Under label conditions, approved pre-slaughter withdrawal periods are 7 days for swine with zero tolerance for penicillin G residues in tissues. However, sows are commonly treated with higher doses which is allowed under a veterinarian's supervision when labeled doses are ineffective. Under AMDUCA, the veterinarian prescribing the off-label use must recommend an appropriate pre-slaughter withdrawal period to ensure that drug residues remaining in edible tissues deplete to safe levels (21 CFR Part 530).

There are very few data which describe the depletion of penicillin G residues under off-label conditions. The studies available indicate increased doses increase the elimination time. Apley et al. (2009) conducted a residue depletion study in sows using a single 5x label dose of 33,000 IU/kg administered by intramuscular injection or with a needle free device. Intramuscular injection in the "hip" produced flip-flop kinetics (KuKanich et al., 2005; Riviere 2011) in which the terminal elimination rate is controlled by the rate of absorption rather than by the rate of elimination. Between-animal plasma half-lives were highly variable. After an 8-day withdrawal period two of five hogs had a quantifiable residue. From these data, they recommended of a 28 day withdrawal period based on a 95% confidence interval that 99% of treated animals would have no detectable residue in the kidney.

The US-FSIS changed the method for screening swine tissues for antimicrobial residues from the Fast Antimicrobial Screen Test to the KIS™ test in September of 2011 (FSIS notice 45-11). An increase in the detection of penicillin residues in sow tissues subsequent to the adoption of the new screening assay may be attributed to the survey method change. This paper describes the detection of penicillin G procaine residues with the CHARM-KIS test in kidney, liver, plasma, urine, and skeletal muscles of heavy sows after an extra-label penicillin-G procaine administration. Sows were treated IM with 33,000 IU/kg bw for 3 consecutive days and were slaughtered with withdrawal periods extending to 39 days post-treatment.

Material and Methods

Chemicals and Supplies. Penicillin G procaine (300,000 U/mL; Norocillin; Norbrook Pharmaceuticals, Lenexa, KS) injection solution was purchased from Ivesco, LLC (Iowa Falls, Iowa). Penicillin G procaine monohydrate reference standard was purchased from U. S. Pharmacopeia, Rockville, MD. Kidney inhibition swabs, neutralization tablets, penicillin G controls,

and heating blocks were obtained from Charm Laboratories (Lawrence, MA). Driploss containers were purchased from the Danish Meat Research Institute, Taastrup, Denmark.

Animal Housing and Treatment Assignment. A study protocol was approved by the North Dakota State University Institutional Animal Care and Use Committee prior to the initiation of the live-phase of the study. Heavy sows were purchased from the North Dakota Pig Cooperative (Larimore, ND) and delivered to the North Dakota State University Animal Research Center (Fargo, ND) and acclimatized for at least 14 days. Animals were randomly assigned to one of three treatments and were each provided unique identification numbers by ear tag. For treatment 1, sows received injections (10 mL on each side) in a single location for 3 days at the same location. For treatment 2, sows received consecutive injections (10 mL on each side) for 3 consecutive days at locations separated by approximately 2 inch intervals. For Treatment 3, sows received 20 mL in the one side with overflow injections occurring on the other side of the neck. Injections occurring on consecutive days were separated by approximately 2-3 inches.

Sows were slaughtered with 5, 10, 15, 20, 25, 32, or 39 day withdrawal periods relative to the last off-label dosing day. Positive control sows (n = 2 per withdrawal period) were treated with the label dose (6,600 U/kg bw) of penicillin G via IM administration for 3 consecutive days and were euthanized after a 7-day withdrawal period, consistent with the product label; or a 15-day withdrawal period. Negative control sows (n = 2) were dosed IM with sterile saline (1 mL per 45.5 kg) for 3 consecutive days and were slaughtered 5 days after the last saline injection.

On-Site Analysis of Kidneys. Kidney samples were screened for penicillin residues on the kill floor using the Charm-KIS microbial inhibition test according to FSIS procedures (FSIS CLG-ADD 3.01, 2011); the manufacturer's recommended incubation time, without the automatic shut-off option, was followed. All samples were determined in duplicate by separate operators and each result scored independently by both operators.

Sample Collection and Treatment. At collection, skeletal muscle and additional kidney were collected using a 3-cm diameter tissue coring device driven by a cordless drill and trimmed to approximately 3 x 3 cm. Samples were placed into Driploss containers and were frozen at -80 °C until analysis. Samples were thawed at room temperature for 1 hr and the tissue juices were collected from the drip tubes after centrifuging at 1,200 x g for 10 min. The collected tissue juices were used to saturate Charm-KIS swabs. Microbial inhibition tests for tissues were then conducted as described by the FSIS (2011) for determination of the presence of penicillin G. Liver required the addition of an equal volume of water followed by boiling (1 min) and centrifugation at 14,000 x g prior to swab. Urine or serum aliquots (500 µL) were combined with a single Charm-KIS neutralization tablet and vortexed; particulates were allowed to settle for 1 min. Serum/urine supernatant was adsorbed for 10 seconds with a cotton swab, after which, the CHARM-KIS microbial inhibition assay was performed.

Assay Sensitivity Determination. Control serum, urine, skeletal muscle juice, and kidney juice were prepared as previously described and tissue matrix aliquots were spiked with 0, 10, 20, 30, 40, and 50 ppb of penicillin G procaine. Liver juice was spiked with 0, 10, 25, 50, 75, and 100 ppb of penicillin G procaine. Fortification of each matrix was repeated on three separate days.

Results and Discussion

Typical color indication for positive, negative, and "caution" KIS results are shown in Figure 1. Sensitivity for penicillin residues in kidney juice, muscle juice, urine, and serum was 20, 30, 20, and 30 ppb respectively and can be lot dependent. When dilution factor is accounted for, liver juice sensitivity was 100 ppb.

Negative control sows that received normal saline and which were slaughtered on withdraw day 5 had negative readings for all matrices tested. The positive control sows, which received the label dose and were slaughtered at withdrawal day 7 showed positive Charm-KIS results for all tissues. For withdrawal day 15, one out of two positive control animals tested positive using Charm-KIS in kidney and urine samples but the rest of matrices returned negative results. As seen in Figure 2 there is no difference in the various treatments indicating the differences from the various injection patterns are not discernibly different. The variability is probably due to the variability in absorption since penicillin G procaine would demonstrate flip-flop kinetics. Apley's (2009) estimate of a 28 day withdrawal period is at least reasonable and our data suggest it may be

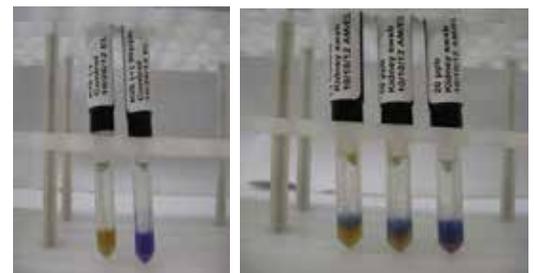


Figure 1. Typical results returned from the Charm-KIS rapid screening assay, from left to right, KIS negative control, KIS positive control, negative, caution, and positive results from kidney swabs. A caution results is considered to be negative by FSIS.

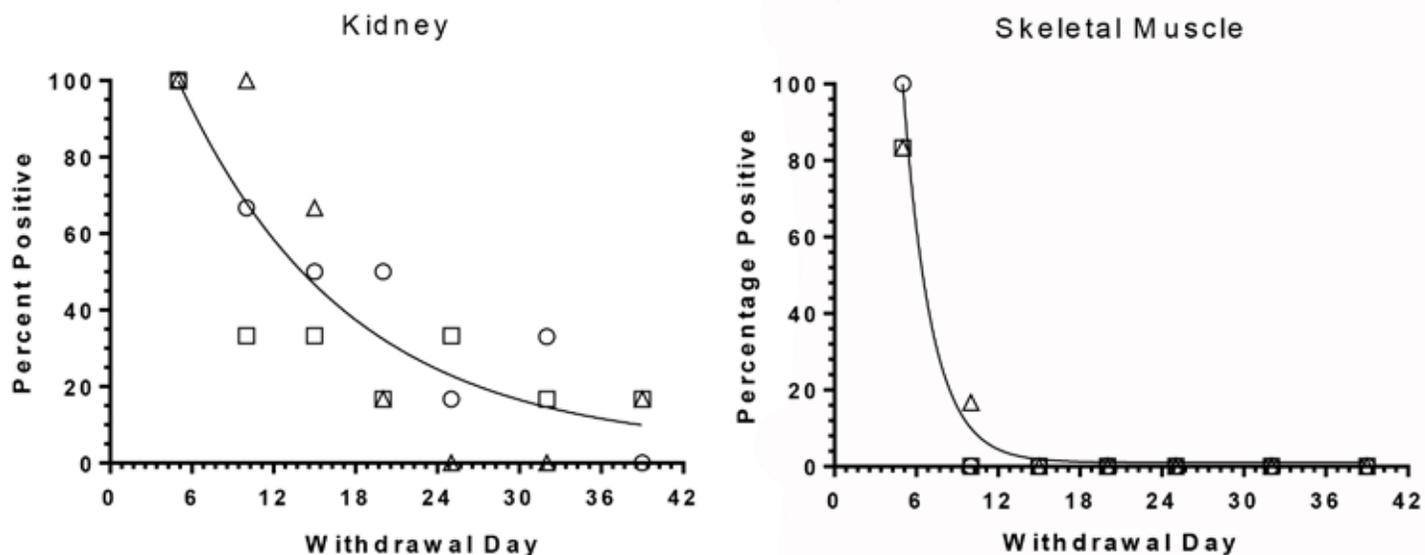


Figure 2. Depletion of Penicillin G residues in kidneys (left panel) and muscle (right panel) of heavy sows as detected by the Charm-KIS rapid screening assay. Line is a first order depletion fit of combined data from Treatments; false positive results (where there is no measureable residues from LC-MS/MS, data not shown) were removed from the skeletal muscle data prior to plotting.

longer before kidney levels become non-detectable as we observed positives even at day 39. Slow penicillin depletion could be attributed to absorption from the injection sites where residues remain and the fact that penicillin concentrated in the kidney where it is excreted by an active process. From sow penicillin results reported by Korsrud et al (1998), penicillin concentrations in kidney were approximately 40-70 times greater than residues in corresponding muscle samples. The Charm-KIS assay returned smaller numbers of muscle positives than in kidneys for the same animal and time point, in agreement with Korsrud et al's findings. Charm-KIS assays of liver returned fewer positive results than the assays of other tissues, even at day 5 (Figure 3) possibly caused by the much poorer assay sensitivity with liver compared to other matrices.

Applicability of using the Charm-KIS for pre-slaughter screening of treated animals was tested with assays of serum and urine. No false-positive Charm-KIS assay results were returned for either matrices (LC-MSMS data not shown). The urine was a much better surrogate matrix for detecting potential violative penicillin G residues in kidney (Figure 3) than was serum. Serum results clearly demonstrate that serum is an inadequate surrogate for kidney because serum is more rapidly cleared of penicillin than kidney.

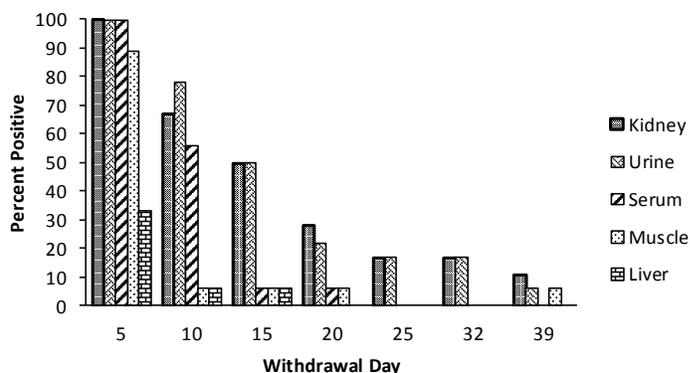


Figure 3. Comparison of Charm-KIS test results in kidneys, urine, serum, muscle, and liver of off-label treated sows as a function of withdrawal day.

Conclusion

Based on kidney results a prolonged withdrawal period is needed prior to culling sows that have been previously treated with high doses of penicillin G procaine. The labeled dose can also return positive kidney penicillin tests beyond the prescribed withdrawal period. Urine can serve as an ante-mortem test reflecting the levels which will be observed in the kidney.

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References

- APLEY M., GOETZEE H., GEHRING R., KARRIKER L., 2009. Pharmacokinetics and tissue residues of procaine penicillin G in sows after administration of 33,000 IU/kg intramuscularly and by needle-free injection in the hip. National Pork Board Research Report NPB #07-234.
- Food Safety and Inspection Service, Swab test for antimicrobial drug detection, CLG-ADD 3.01. Effective 05/29/2011.
- Food Safety and Inspection Service Notice 44-11, Using the kidney inhibition swab (KIS™) test to detect antimicrobial drug residues in swine in selected establishments - Phase III.
- Food Safety and Inspection Service Notice 43-12, Using the kidney inhibition swab (KIS™) test to detect antimicrobial drug residues in all livestock in slaughter establishments – phase IV. 6/26/12.
- KUKANICH B., GEHRING R., WEBB A. I., CRAIGMILL A. L., RIVIERE J. E, 2005. Effect of formulation and route of administration on tissue residues and withdrawal times. *JAVMA*. 227, 1574-1577.
- KORSRUD G. O., SALISBURY C. D. C., RHODES C. S., PAPICH M. G., YATES W. D. G., et al.1998, Depletion of penicillin G residues in tissues, plasma, and injection sites of market pigs injected intramuscularly with procaine penicillin G. *Food Additives Contam.* 15, 421-426.

Depletion of penicillin G residues in sows after intramuscular injection

Smith, D.J.^{*(1)}, Lupton, S.J.⁽¹⁾, Shelver, W.L.⁽¹⁾, Newman, D.J.⁽²⁾, and Larsen, S.⁽³⁾

⁽¹⁾USDA ARS Biosciences Research Laboratory, Fargo, ND, USA

⁽²⁾North Dakota State University, Department of Animal Science, Fargo, ND, USA

⁽³⁾National Pork Board, Des Moines, Iowa, USA

*corresponding author: david.j.smith@ars.usda.gov

Abstract

A penicillin G procaine residue depletion study was conducted in heavy sows to estimate the pre-slaughter withdrawal periods necessary to clear penicillin from kidney and muscle. Heavy sows (n=126) were treated with penicillin G procaine at a 5x dose (33,000 IU/kg) for 3 consecutive days by intramuscular (IM) injection using 3 separate patterns of drug administration. Treatments differed by pattern and volume of penicillin G procaine administration. Sets of 6 animals per treatment were each slaughtered with 5, 10, 15, 20, 25, 32, and 39 day withdrawal periods; skeletal muscle and kidney were collected for penicillin G analysis by LC-MS/MS. Penicillin residues in skeletal muscle averaged 23.5 ± 10.5 ng/g at withdrawal day 5 for all treatments, but averaged $3,760 \pm 1,930$ ppb in kidney. By 15 days of withdrawal, skeletal muscle penicillin G residues were quantifiable in only one of 18 (5.5%) treated hogs (3.4 ng/g) but were easily detected in kidneys of 50% of the treated hogs, with kidney residues in all hogs averaging 119 ± 199 ng/g (mean includes 8 non-detects counted at $\frac{1}{2}$ the limit of detection). Using an action limit of 25 ng/g and a ln-linear depletion model, the withdrawal period required for penicillin depletion in muscle was 13 days, whereas a 52-day withdrawal period was required for kidney. The FARAD recommended withdrawal period of 15 days for hogs treated with extra-label doses of penicillin is adequate for skeletal muscle, but is inadequate for kidney. Slaughter of penicillin treated hogs after a 15-day withdrawal period, with kidney discard into inedible offal would ensure the human food safety of skeletal muscle.

Introduction

Penicillin in its various forms has been used by the swine industry for decades; during this period penicillin's use has not typically been associated with violative drug residues. Since 2011, however, detections of penicillin residues in sow tissues by the Food Safety and Inspection Service (FSIS) have increased substantially resulting in a number of carcass condemnations and the risk that swine producers may be 'blackballed' from packing plants. A major change in the regulatory framework for penicillin detection has been FSIS's employment of the new Charm-KIS test kit in place of the "FAST" swab test for screening animal tissues for penicillin residues (FSIS, 2011a). Currently the US FDA has not established a tolerance set for allowable penicillin residues in edible tissues of pork.

Sows are typically treated with about 5 times the label penicillin G procaine dose that is used for growing swine. Producers, with the advice and consent of a consulting veterinarian, can legally use this dose in an "off-label" manner provided a proper pre-slaughter withdrawal time is selected. Unfortunately, few data exist which describe the depletion of penicillin residues from sow tissues under such use conditions. Apley et al. (2009) conducted a residue depletion trial in which heavy sows were dosed with a single 5x penicillin G procaine dose, but pre-slaughter withdrawal periods selected (2, 4, 6, and 8 days) were too short to properly model penicillin G residue depletion. For example rates of penicillin detection after an 8-d withdrawal period were 60, 80, and 100% in kidney, muscle, and injection sites, respectively; in the same tissues, frequencies of quantifiable residues were 40, 40, and 100% respectively. Because penicillin G procaine depletion rates are poorly defined, a residue depletion study was conducted to provide withdrawal period estimates for sows treated with extra-label doses of penicillin G procaine.

Material and Methods

The North Dakota State University (NDSU) Animal Care and Use Committee approved a detailed protocol prior to the initiation of the study. Heavy sows (n = 160) were purchased from the North Dakota Pig Cooperative (Larimore, ND) and were group housed in concrete-floored pens with straw bedding. Animals were group fed a corn-soybean ration daily; pens were cleaned daily and water was available on an ad libitum basis. Animals that were lame, which had visible abscesses or other visible anomalies were not included in the study. Within the pool of healthy animals, sows were randomly allocated to pen and treatment (injection pattern) so that each pen contained 21 sows. Within treatment, sows were randomly allocated to a post treatment withdrawal slaughter day. Treatments, which differed in the injection volume and pattern, are not defined here because there was insufficient evidence to infer that treatment had a discernible effect on residue depletion.

Sows (n = 126) were weighed and penicillin G procaine (Norocillin; Norbrook Laboratories, Lenexa, KS; 33,000 U/kg BW) was administered via intramuscular injection through 3.8 cm, 16-gauge needles for three consecutive days. Sows were killed after 5, 10, 15, 20, 25, 32, or 39 day withdrawal periods relative to the last dosing day. Kidney and skeletal muscle (mid portion

of the longissimus dorsi) were collected. Samples were placed on dry-ice and subsequently stored at -80 °C until analysis.

Tissues were processed by homogenization on dry ice to prevent thaw and degradation of penicillin residues. Kidney and muscle samples were extracted using the FSIS method CLG-BLAC.02 with some modifications (FSIS, 2011b). Blank (negative control) and fortified samples (spiked with 25 ng/g penicillin G procaine) were extracted with each sample set in duplicate. Trial samples were extracted in duplicate by withdrawal day. Before analysis, a deuterated internal standard (Penicillin G-d7; Sigma Aldrich, St Louis, MO) was added to each sample extract at an end concentration of 100 ng/mL. Blank sample extracts were utilized to prepare matrix matched standard curves containing 2 to 500 ng/mL of penicillin G. A fresh standard curve was made for each sample set and samples and curves were analyzed within 24 hours of preparation. Apley et al's. (2009) analytical method was modified slightly for penicillin analysis using a Waters (Milford, MA) Ultra High Performance Liquid Chromatograph coupled to a tandem quadrupole mass spectrometer. The detection method was modified to monitor additional fragment ions for Penicillin G to improve confirmation and quantification of residues. Reported data are not corrected for recovery (US FDA CVM, 2006).

Estimations of withdrawal period were completed for kidney and skeletal muscle tissues using FDA and CVMP guidelines (FDA, 2006; CVMP, 1995) with the following criteria.

- For a given time point to be included in the analysis, at least three animals had to have returned residues above the method limit of quantification (US FDA CVM, 2006)
- Nominal values that were below the limit of quantification, but above the limit of detection, were used if there were at least three points at the withdrawal period above the limit of quantification
- Values falling below the limit of detection were included at ½ the method limit of detection and were included in withdrawal period calculations (CVMP, 1995)

For kidney calculations, data obtained from withdrawal days 10, 15, 20, and 25 were used in the analysis. Data from day 32 were not employed even though there were 3 animals with penicillin residues above the LOQ because the 32 day data did not continue the linear trend with respect to days 20 and 25. For skeletal muscle, only one animal out of 18 had residues above the LOQ at the 15-d withdrawal period; 3 animals had residues above the LOD, but below the LOQ. Because a minimum of 3 days of data are required to determine the terminal, linear elimination period, the 15-d withdrawal period data were used for estimation of a withdrawal period, even though these data did not strictly comply with FDA guidelines. Excel 7.0 was used in conjunction with the tables of Owen (1962) to calculate the critical values or the non-central t-distribution used in FDA calculations. Withdrawal periods were estimated using the FSIS action level for penicillin G of 25 ppb (FSIS, 2013).

Results

Injection pattern had no discernible effect on residue depletion, so data were pooled across treatment. The depletion of ln-transformed penicillin G residues from kidneys of heavy sows are presented in Figure 1 as geometric means. Penicillin residues were measured in kidneys of all animals 5 days after the final treatment, but by 10 days 6 of 18 animals had residues below the assay LOD (1.8 ng/g). By 20 days of withdrawal, 5 of 18 animals had residues above the assay LOQ (6.1 ng/g) with 3 of these animals having kidney residues above 100 ng/g and one animal with penicillin residues greater than 300 ng/g. Penicillin residues (22.7 and 17.0 ng/g) were present in kidneys of two hogs after a 39-d withdrawal period. The estimated withdrawal period to ensure penicillin G depletion to a 25 ng/g action level in 99% of animals with 95% confidence was 52 days.

In contrast to kidney tissues, Penicillin G residues depleted quickly from skeletal muscle (Figure 2). Residues in skeletal muscle at 5 days of withdrawal averaged only 23.5 ± 10.5 ng/g and depleted rapidly thereafter. By the 15th day of withdrawal, only 1 sow had skeletal muscle residues greater than the assay LOQ (2.4 ng/g) with 4 other sows having residues greater than the method LOD (0.7 ng/g). Thus, the estimated withdrawal period for skeletal muscle was calculated to be 13 days (Figure 2).

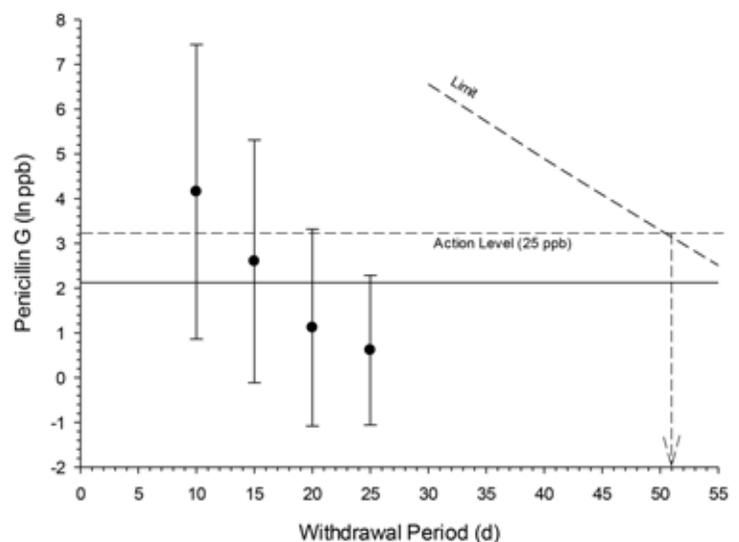


Figure 1. Penicillin G depletion from kidney of heavy sows. Geometric means of ln-transformed residues are shown. Hatched lines are the action level of 25 ng/g, the calculated statistical tolerance limit, and corresponding time (d) to for penicillin levels in 99% of animals to fall to the action level with 95% confidence.

Discussion

A withdrawal period was estimated for kidney tissues using the log-linear approach promulgated by the US-FDA CVM (2006) with modifications suggested by the CVMP (1995). In making a withdrawal period estimation, the essential assumptions of equal variance and normal distributions (Shapiro-Wilk) of data were violated ($P < 0.001$), thus the estimated withdrawal period presented here was admittedly calculated with data that did not conform to statistical ideals. As discussed by Concordet and Toutain (1997a) and documented by Sanquer et al. (2006) these assumptions are, at best, difficult, and are sometimes impossible to meet. Non parametric approaches to withdrawal period calculations proffered by Sanquer et al (2006) and Concordet and Toutain (1997b) were not attempted on this data set.

While the time required for kidney residues to deplete to FSIS action levels (52 d) far exceeded the FARAD estimated withdrawal time of 15 days for extra-label penicillin use, 15 d was more than sufficient for penicillin residues to deplete from skeletal muscle.

Conclusion

The use of penicillin G procaine as an economical and effective therapeutic drug for use in heavy sows will likely require the guarantee that kidneys from treated animals be included as offal not fit for human consumption.

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References

- APLEY M., COETZEE H., GEHRING R., KARRIKER L., 2009. Pharmacokinetics and tissue residues of procaine penicillin G in sows after administration of 33,000 IU/kg intramuscularly and by needle-free injection in the hip. Pork Safety Research Report. NPB #07-234.
- CONCORDET D., TOUTAIN P. L., 1997a. The withdrawal time estimation of veterinary drugs revisited. *J. Vet. Pharmacol. Therap.* 20:380-386.
- CONCORDET D., TOUTAIN P. L., 1997b. The withdrawal time estimation of veterinary drugs: A non-parametric approach. *J. Vet. Pharmacol. Therap.* 20:374-379.
- CVMP. 1995. Note for guidance: Approach towards harmonisation of withdrawal periods. European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit.
- Food Safety and Inspection Service. 2011a. Swab test for antimicrobial drug detection, CLG-ADD 3.01. Effective 05/29/2011.
- Food Safety and Inspection Service. 2011b. Screening and Confirmation of β -Lactam Antibiotics by HPLC-MS/MS, CLG-BLAC.02. Effective 07/02/2011.
- Food Safety and Inspection Service. Screening and Confirmation of Animal Drug Residues by UHPLC-MS-MS. GLC-MRM1.02. Effective 02/22/2013.
- OWEN D. B., 1962. Handbook of Statistical Tables. Addison-Wesley Publishing Co. Reading, MA. USA.
- SANQUER A., WACKOWIEZ G., HAVRILECK B., 2006. Critical review on the withdrawal period calculation for injection site residues. *J. Vet. Pharmacol. Therap.* 29:355-364.
- US FDA CVM. 2006. Guidance for Industry, #3. General principles for evaluating the safety of compounds used in food-producing animals. US Dept. Health and Human Services. <http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052180.pdf>

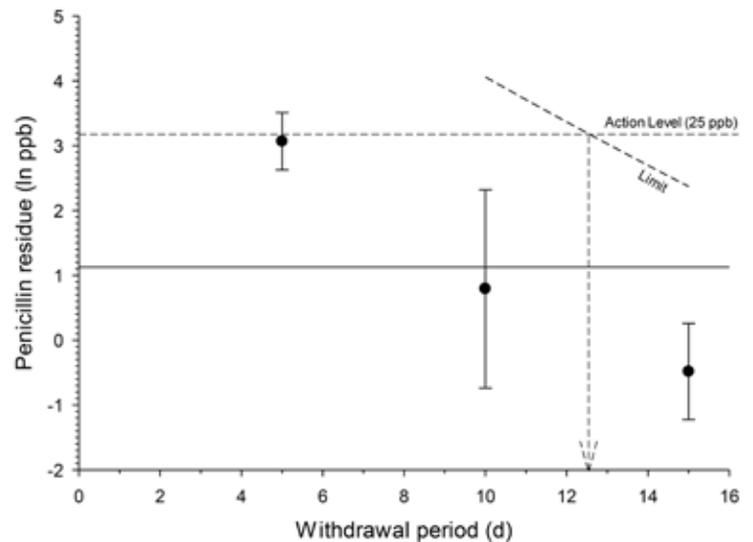


Figure 2. Penicillin G depletion from skeletal muscle of heavy sows. Geometric means of ln-transformed residues are shown. Hatched lines are the 25 ng/g action level, the calculated action level limit, and corresponding time (d) to for penicillin levels in 99% of animals to fall to the action limit with 95% confidence.

TLR4 Single Nucleotide Polymorphisms (SNPs) Associated with *Salmonella* Shedding in Pigs

Jalusa Deon Kich^{1,2*}, Jolita Janutenaite Uthe^{1,3}, Magda Vieira Benavides⁴, Maurício Egídio Cantão², Ricardo Zanella⁵, Christopher Keith Tuggle³ and Shawn Michelle Dunkin Bearson¹

¹ USDA/ARS/National Animal Disease Center, 1920 Dayton Ave, Ames, IA, USA; ² Embrapa Swine and Poultry, Br 153, Km 110, Concórdia, SC, Brazil; ³ Department of Animal Science, Iowa State University, 2255 Kildee Hall, Ames, IA, USA; ⁴ Embrapa LabEx USA/USDA, Beltsville Agriculture Research Center, Beltsville, MD, USA; ⁵ Embrapa Swine and Poultry BJT/CNPq, Concórdia, SC, Brazil

*Corresponding author: jalusa.kich@embrapa.br

Abstract

Toll-like receptor 4 (TLR4) is a key factor in the innate immune recognition of lipopolysaccharide (LPS) from Gram-negative bacteria. Previous studies from our group identified differences in the expression profile of *TLR4* and genes affected by the *TLR4* signaling pathway among pigs that shed varying levels of *Salmonella*, a Gram-negative bacterium. Therefore, genetic variation in this gene may be involved with the host's immune response to bacterial infections. The current study screened for single nucleotide polymorphisms (SNPs) in the *TLR4* gene and tested their association with *Salmonella* fecal shedding. Pigs (n=117) were intranasally challenged at 7 weeks of age with 1×10^9 CFU of *S. Typhimurium* χ 4232 and were classified as low or persistent *Salmonella* shedders based on the levels of *Salmonella* being excreted in fecal material. *Salmonella* fecal shedding was determined by quantitative bacteriology on days 2, 7, 14, and 20/21 post exposure, and the cumulative levels of *Salmonella* were calculated to identify the low (n=20) and persistent (n=20) *Salmonella* shedder pigs. From those 40 animals, the *TLR4* region was sequenced, and 18 single nucleotide polymorphisms (SNPs) in *TLR4* were identified. Twelve SNPs have been previously described and six are novel SNPs of which five are in the 5' untranslated region and one is in intron 2. Single marker association test identified 13 SNPs associated with the qualitative trait of *Salmonella* fecal shedding, and seven of those SNPs were also associated with a quantitative measurement of fecal shedding ($P < 0.05$). Using a stepwise regression process, a haplotype composed of SNPs *rs80787918* and *rs80907449* ($P \leq 4.0 \times 10^{-3}$) spanning a region of 4.9Kb was identified, thereby providing additional information of the influence of those SNPs on *Salmonella* fecal shedding in pigs.

Keywords: *TLR4*, single nucleotide polymorphisms (SNPs), *Salmonella*, swine

Introduction

Salmonella is a widespread foodborne pathogen with the ability to adapt to different environments. Swine (*Sus scrofa*) are an important reservoir of *Salmonella* because colonization and shedding of this bacterium, imposing elevated risks to public and animal health. Thus, diverse intervention strategies are needed to control the transmission of *Salmonella* from pork to humans and to the environment.

In bacterial infections, the severity of infection is impacted by the pathogenicity of the microorganism and its interaction with the host immune defense system (Zanella et al., 2011). Toll-like receptor 4 (TLR4) is a well-characterized gram-negative bacterial lipopolysaccharide (LPS) recognition receptor and a host inflammatory response activator well conserved among animal species (Noreen et al., 2012, Yang et al. 2012). Schröder and Schumann (2005) suggested that mutations in the *TLR4* regions involved with pathogen recognition and transduction signaling may affect host susceptibility to infection. Polymorphisms in the *TLR4* gene have been associated with different infectious diseases in humans, cattle, chicken and pigs (Noreen et al., 2012, Kataria et al. 2011, Leveque et al. 2003, Yang et al., 2012).

In swine, *TLR4* is located on *Sus scrofa* 1 (SSC1) V10.2 (289,776,058 bp to 289,785,087 bp). Thomas et al. (2006) identified the genomic structure of porcine *TLR4*, and Shinkai et al. (2006) described the distribution of SNPs for five *TLRs* in pigs. Specifically for *TLR4*, 13 SNPs were widely distributed in 11 pig breeds, and of those, seven were non-synonymous. Thirty four SNPs were identified in *TLR4* using pigs representing European commercial breeds and some traditional breeds (n=259), and of these, 17 SNPs were located in the non-coding region and 17 SNPs were found in the coding region (Palermo et al., 2009). Furthermore, polymorphisms in the *TLR4* gene have been identified as potential genetic markers for disease susceptibility in pigs (Uenishi & Shinkay, 2009). Our group had reported up-regulation of *TLR4* and its target genes in pigs challenged with *Salmonella enterica* serovar Typhimurium (Huang et al., 2011). Therefore, to determine if *TLR4* is a possible candidate gene associated with *Salmonella* shedding, we first sequenced the *TLR4* gene for SNPs identification and tested their associations with *Salmonella* shedding status.

Material and Methods

Briefly, 117 pigs were intranasally challenged at 7 weeks of age with 1×10^9 CFU of *S. Typhimurium* χ 4232 as previously described (Huang et al., 2011, Uthe et al., 2009). At days 2, 7, 14 and 20/21 post-inoculation (pi), *Salmonella* fecal shedding was quantified using a standard bacteriological test previously described (Uthe et al., 2009). Forty pigs were chosen based on their fecal culture status; quantitative classification of the phenotype was scored based on cumulative *Salmonella* fecal shedding. Genomic DNA was extracted from blood samples and purified as previously described (Uthe et al., 2009). Nine sets of primers were designed to cover all exons (n=3) of *TLR4* (SSC1: 289,775,345bp - 289,786,312bp V. 10.2). PCR products were sequenced and polymorphisms were identified using Phred/Phrap/Consed/PolyPhred software. A single and multiple marker association tests were conducted within PLINK (V1.07) and R statistical programs (Purcell et al., 2007).

Results

For the quantitative measurement of *Salmonella* shedding cumulative measurements were taken within days 2, 7, 14 and 20/21 pi (Figure 1). Sequencing analysis of those forty (n=40) animals identified 18 SNPs; twelve were previously described in the literature and/or annotated in GenBank and six are novel SNPs (Table 1).

Of the 18 SNPs, thirteen (n=13) SNPs were associated ($P \leq 0.05$) with *Salmonella* shedding as a qualitative phenotype using a Chi-squared test; of those 13 SNPs, seven were also associated with *Salmonella* shedding as a quantitative phenotype using a Wald Statistical test (Table 1).

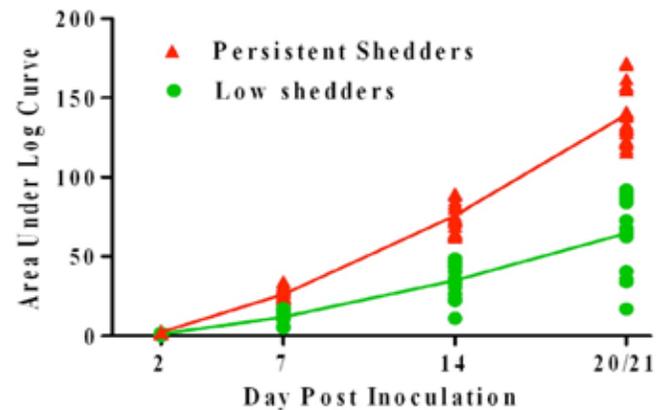


Figure 1: Area under the log curve illustrating the log of cumulative colony forming units (CFU). Quantitative bacteriology of *Salmonella* shedding in swine fecal samples was performed at day 2, 7, 14 and 20/21 days post-challenge with *S. Typhimurium*, and CFU were determined.

Table 1. Identified SNPs and position in the *TLR4* gene of *Salmonella* low and persistent shedder pigs.

SNP	Location in Sus Scrofa genome (bp)	GenBank accession number	Single Marker Association (P-value)	
			qualitative	quantitative
SNP1#	SSC1:289,774,983	No	0.033	0.064
SNP2#	SSC1:289,775,046	No	0.033	0.064
SNP3#	SSC1:289,775,081	No	0.033	0.064
SNP4#	SSC1:289,775,543	No	0.133	0.244
SNP5	SSC1:289,775,665	rs80830544	0.363	0.550
SNP6#	SSC1:289,775,979	No	0.025	0.056
SNP7	SSC1:289,780,226	rs80881287	0.004	0.029
SNP8**	SSC1:289,780,292	rs80787918	0.002	0.013
SNP9#	SSC1:289,782,761	No	0.001	0.025
SNP10	SSC1:289,782,834	rs80923358	0.003	0.037
SNP11	SSC1:289,782,933	rs80951861	0.003	0.037
SNP12*	SSC1:289,783,127	rs80811682	0.007	0.054
SNP13*	SSC1:289,783,342	(Shinkai et al., 2006)	0.285	0.514
SNP14	SSC1:289,783,476	rs80981701	0.064	0.105
SNP15*	SSC1:289,783,478	rs80955017	0.034	0.046
SNP16*	SSC1:289,783,543	rs80894552	0.176	0.231
SNP17	SSC1:289,784,913	rs80834103	0.025	0.056
SNP18**	SSC1:289,785,250	rs80907449	0.002	0.011

*non-synonymous SNPs; **haplotype components; # novel SNPs

Using a haplotype construction and the backward-elimination process, the most significant haplotype for both measurements of *Salmonella* shedding, qualitative ($P \leq 7.9 \times 10^{-4}$) and quantitative ($P \leq 4.0 \times 10^{-3}$) comprised a region of 4.9Kb composed of SNPS, *rs80787918* (SNP8) and *rs80907449* (SNP18) ($r^2=0.902$) located at SSC1:289,780,292 bp and SSC1:289,785,250 bp, respectively (**Table 2**).

Discussion

Four SNPs, *SNP12*, *SNP13*, *SNP15* and *SNP16*, located on exon three of *TLR4* gene are non-synonymous mutations and they are positioned between *SNP8* and *SNP18*. When the additive effects of those markers were tested within the haplotype constructed with markers *SNP8* and *SNP18*, it was not observed any improvement in the association test. The significance of those results was possibly penalized by the addition of markers into the association test, due to the limited number of samples.

The haplotype CC of *SNP8* and *SNP18* was identified in higher frequency in persistent shedding pigs (67.5%) compared to low shedding pigs (30%); furthermore, the frequency of haplotype TT in low shedding pigs (65%) was greater when compared to persistent shedding pigs (32.5%). No animals from the persistent shedding group were identified with the haplotype TC or CT, while it was observed in low frequency in the low shedding group (2.5%). Together, these results suggest that the region located between *SNP8* and *SNP18*, more specifically on exon 3, is possibly harboring the causative mutation for *Salmonella* colonization and shedding variation in swine.

Conclusion

The results from this study support the concept that *TLR4* is an important modulator associated with the porcine response to *Salmonella* infection in swine.

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References

- Huang, T.H., Uthe, J.J., Bearson, S.M., Demirkale, C.Y., Nettleton, D., Knetter, S., Christian, C., Ramer-Tait, A.E., Wannemuehler, M.J., Tuggle, C.K., 2011. Distinct peripheral blood RNA responses to *Salmonella* in pigs differing in *Salmonella* shedding levels: intersection in IFNG, TLR and miRNA pathways. *PLoS ONE*. 6, e28786.
- Kataria, R.S., Tait, R.G. Jr., Kumar, D., Ortega, M.A., Rodriguez, J., Reecy, J.M., 2011. Association of toll-like receptor four single nucleotide polymorphisms with incidence of infectious bovine keratoconjunctivitis (IBK) in cattle. *Immunogenetics*. 63, 115-119.
- Leveque, G., Forgetta, V., Morroll, S., Smith, A.L., Bumstead, N., Barrow, P., Loredano-Osti, J.C., Morgan, K., Malo, D., 2003. Allelic variation in *TLR4* is linked to susceptibility to *Salmonella enterica* serovar Typhimurium infection in chickens. *Infect. Immun.* 71, 1116-1124.
- Noreen, M., Shah, M.A., Mall, S.M., Choudhary, S., Hussain, T., Ahmed I., Jalil, S.F., Raza, M.I., 2012. *TLR4* polymorphisms and diseases susceptibility. *Inflamm. Res.* 61, 177-188.
- Palermo, S., Capra, E., Torremorell, M., Dolzan, M., Davoli, R., Haley, C.S., Giuffra E., 2009. Toll-like receptor 4 genetic diversity among pig populations. *Anim. Genet.* 40, 289-299.
- Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J., Sklar, P., de Bakker, P.I., Daly, M.J., Sham, P.C. 2007 PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *Am. J. Hum. Gen.* 81, 559-575.
- Schröder, N.W.J., Schumann R.R., 2005. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infect. Dis.* 5, 156-164.
- Shinkai, H., Tanaka, M., Morozumi, T., Eguchi-Ogawa, T., Okumura, N., Muneta, Y., Awata, T., Uenishi, H., 2006. Biased distribution of single nucleotide polymorphisms (SNPs) in porcine Toll-like receptor 1 (*TLR1*), *TLR2*, *TLR4*, *TLR5* and *TLR6* genes. *Immunogenetics*. 58, 324-330.
- Thomas, A.V., Broers, A.D., Vandegaart, H.F., Desmecht, D.J., 2006. Genomic structure, promoter analysis and expression of the porcine (*Sus scrofa*) *TLR4* gene. *Mol. Immunol.* 43, 653-659.

Table 2. Haplotypes frequency (SNPs *rs80787918* and *rs80907449*) and associations with qualitative and quantitative phenotypes of *Salmonella* shedding.

Haplotype	Haplotype frequency		Qualitative (P Val.)	Quantitative (P Val.)
	Persistent Shedders	Low Shedders		
CC	0.675	0.3	0.00079	0.004201
TC	0	0.025	0.3143	0.1054
CT	0	0.025	0.3143	0.1445
TT	0.325	0.65	0.00334	0.02912

Uenishi, H, Shinkay, H., 2009. Porcine Toll-like receptors: The front line of pathogen monitoring and possible implications for disease resistance. *Dev. Comp. Immunol.* 33, 353-361.

Uthe, J.J, Qu, L., Couture, O., Bearson, S.M., O'Connor, A.M., McKean, J.D., Torres, Y.R., Dekkers, J.C., Nettleton, D., Tuggle, C.K., 2011. Use of bioinformatics SNP predictions in differentially expressed genes to find SNPs associated with *Salmonella* colonization in swine. *J. Anim. Breed. Genet.* 128, 354-365.

Uthe, J.J., Wang, Y., Qu, L., Nettleton, D., Tuggle, C.K., Bearson, S.M., 2009. Correlating blood immune parameters and CCT7 genetic variant with the shedding of *Salmonella enterica* serovar Typhimurium in swine. *Vet. Microbiol.* 135, 384-388.

Yang, X.Q., Murani E., Ponsuksili, S., Wimmers, K., 2012. Association of TLR4 polymorphism with cytokine expression level and pulmonary lesion in pigs. *Mol. Biol. Reports.* 39, 7003-7009.

Zanella, R., Settles, M.L., McKay, S.D., Schnabel, R., Taylor, J., Whitlock, R.H., Schukken Y., Van Kessel, J.S., Smith, J.M., Neibergs, H.L., 2011. Identification of loci associated with tolerance to Johne's disease in Holstein cattle. *Anim. Genet.* 42, 28-38.

Assessment of the impact of omitting palpation of the lungs and the liver at meat inspection

Alban, L.*, Pacheco, G., Kruse, A.B., Petersen, J.V.

Danish Agriculture & Food Council, Copenhagen, Denmark

*corresponding author Lis Alban: lia@lf.dk

Abstract

Meat inspection of finisher pigs is gradually moving from traditional inspection involving palpation and incision to a more visual inspection. However, what do we miss if we no longer palpate and incise? This is addressed in this paper which focuses specifically on palpation of the lungs and the liver. A risk assessment following international guidelines was undertaken. The assessment shows that omission of these routine palpations on finisher pigs from controlled housing (i.e. herds with high biosecurity) will have no significant impact on food safety. The reasoning for reaching these conclusions is presented in the following.

Introduction

The main aim of meat inspection is to ensure safe and savoury meat. However, much has changed since the birth of modern meat inspection 100 years ago. Today, *Campylobacter*, *Salmonella* and *Yersinia* fill up the human statistics for zoonotic infections. And it is well-known that meat inspection in itself can do only little to mitigate the risk associated with such agents, because they do not result in macroscopic lesions in affected carcasses or plucks.

In fact, palpation and incision result in cross-contamination of the carcasses. In line, it has been suggested to omit unnecessary palpation and incisions to limit the cross-contamination with *Salmonella*.

However, what would we risk if we stopped the routine palpations and incisions? Two earlier risk assessments - following international guidelines - concluded that omitting routine palpation of the mandibular and mesenteric lymph nodes and discontinuing the routine opening of the hearts did not carry any increase in the food safety risks (Alban et al., 2008; Alban et al., 2010).

Next, we have looked at the impact of omitting palpation of the lungs, the liver and their lymph nodes.

We looked at food safety as well as the impact on animal health (including the ability to identify notifiable diseases) and welfare of the pigs. Only the food safety results are described in this paper. For the other aspects, the reader is kindly referred to the full risk assessment which can be found on the following link: http://www.lf.dk/~media/lf/Aktuelt/Publikationer/Svinekod/Risk%20assessment_lungs%20liver%202013%2002%2028.ashx

Material and Methods

The work followed OIE guidelines for risk assessment. This implies that the following five steps were undertaken: Hazard identification, release assessment, exposure assessment, and consequence assessment. Finally, risk estimation was made based on an integration of the four previous steps.

Data consisted of a comparison study involving 3,000 plucks, own collection of slaughterhouse samples (N=104) sent for microbiological investigation, slaughterhouse statistics, literature and expert opinion.

Results

Hazard identification: embolic pneumonia and liver abscesses were identified as the two most important lesions that might escape detection if routine palpation is omitted. This was based on a literature review and discussions with experts in the field.

Release assessment: Two studies were conducted to assess the proportion of plucks with embolic pneumonia that might escape detection, if visual inspection is applied instead of traditional inspection. The first was a comparison study including 3,000 plucks (Table 1). The second consisted of an evaluation of 104 plucks with embolic pneumonia collected during meat inspection (Table 2). The comparison study showed that one out of three cases found in traditional inspection was missed by visual inspection (Table 1). Likewise, the evaluation study showed that around one out of five cases would be missed if visual inspection was used compared to traditional inspection.

Based on these data as well as slaughterhouse data, it was estimated that between 1,080 and 1,800 cases might be missed in a year, if visual only was applied. This figure is probably a worst case scenario because the visual inspection that formed part of the present risk assessment was conducted under suboptimal conditions: the chain was not set up to allow an easy visual inspection prior to the traditional inspection.

Exposure assessment: The distribution of the agents found in the lungs with embolic pneumonia is shown in Table 3. It is noted that *Staph. aureus* and *E. coli* were found predominantly. Only two livers were found with abscesses. These were large and easy recognizable. Here, *Staph. aureus* were found.

The human exposure risk related to the hazards identified in embolic pneumonia was assessed as negligible since lungs are not considered edible tissue in Denmark. The human exposure risk related to meat from pigs with embolic pneumonia that escaped detection seems low, because the bacteria are normally not present in the muscle tissue – and if present it will be in low numbers.

Moreover, the low numbers of abscesses present in the carcass associated with pyaemia are most likely found during cutting. It was also shown that although presence of pyemia is a risk factor for abscesses in the carcass (RR=4.4, P<0.001) it was less than 1% of the abscesses that were found in pigs with embolic pneumonia (Table 4).

Livers for human consumption are handled individually which will make it easy to identify abscesses. Therefore, the exposure risk was assessed as low.

The exposure risk for pets was assessed to be negligible, because lungs destined for pet food are heat-treated. Exposure will only take place in case raw lungs are fed directly to pets, which is thought to occur only infrequently. Likewise, for fur animals, the exposure is very low because most animal by-products are heat-treated prior to being fed to fur animals in Denmark.

Consequence assessment: None of the agents involved in the development of embolic pneumonia or liver abscesses in pigs had a significant foodborne zoonotic potential. This is supported by the fact that these hazards do not show up in the human statistics, where *Salmonella*, *Yersinia*, *Toxoplasma* and *Trichinella* have been identified by the European Food Safety Authority as the pig/pork-relevant hazards (EFSA, 2011).

Risk estimation: The prevalence of embolic pneumonia is low; however, because of the substantial size of the Danish pig production a non-negligible number of cases might be overlooked if visual inspection replaces traditional inspection. The food safety impact of this is very low, because lungs are not considered edible tissue and because the agents involved in

Table 1. Association between visual and traditional inspection of plucks for embolic pneumonia, Denmark (N=3000 finisher pigs)

	Number of plucks found with lesions			Assessment of association		
	Traditional inspection	Sum	Visual/Traditional*	Kappa	P-value	
Visual inspection	+	-				
+	2	7	9	3.0	0.33	0.0339
-	1	2990	2991			
Sum	3	2997	3000			

*: Prevalence of cases found at visual versus traditional inspection

Table 2. Summary statistics on the ability to detect by visual inspection the 104 plucks with lesions indicative of embolic pneumonia, found during meat inspection of finisher pigs from controlled housing herds, October-November 2012, Denmark

	Yes	Maybe	No
Lesion visually detectable?	65 (62.5%)	19 (18.3%)	20 (19.2%)

Table 3. Distribution^(*) of agents found in 104 lungs with embolic pneumonia and 98 associated lymph nodes. The lungs and lymph nodes were found during meat inspection of finisher pigs from controlled housing herds, October-November 2012, Denmark

Agents/growth	Lung samples		Lymph node samples	
	Number	Percentage	Number	Percentage
<i>Staphylococcus aureus</i>	82	78.8	69	70.4
<i>Escherichia coli</i>	61	58.7	40	40.8
<i>Proteus spp.</i>	6	5.8	1	1.0
<i>Pasteurella multocida</i>	4	3.8	4	4.1
<i>Bacillus cereus</i>	20	19.2	11	11.2
<i>Pseudomonas spp</i>	6	5.8	7	7.1
Growth	96	92.3	79	80.6
No Growth	8	7.7	19	19.4
Total	104	-	98	-

*: More than one agent could be found in a sample. Therefore, the percentages do not add to 100.

these lesions have a limited foodborne impact.

The prevalence of liver abscesses is very low. The agent involved has a limited food-borne impact, and cases will be identified during the unique handling of the organ if destined for human consumption.

Discussion

Visual inspection does not necessarily result in a lower number of plucks being suspected of embolic pneumonia. This was also seen of other lesions in the pluck (results not shown here but in the risk assessment report. Registration intensity merely depends upon what we want the meat inspectors to record.

Consumers might expect the abattoir to do whatever is possible to detect pigs with pyaemia from an aesthetic point of view. Therefore, in case of doubt plucks should be palpated – or sent to the rework area for extended examination.

Conclusion

The assessment showed that routine palpation of the liver and lungs is an unnecessary part of meat inspection in finisher pigs, if there are no visual indications of infection or other data pointing to disease.

Epilogue

The EU Commission has recently (May 2013) amended the EU Meat inspection regulation 854(2004). The changes - which will come into force in June 2014 - open up for visual inspection as the standard for inspection of pigs unless ante mortem, post mortem, any other finding on the individual or food chain information or geographical data indicate otherwise.

The next step is to investigate how the results of this risk assessment might be used in practice by the abattoirs in the modernisation process of meat inspection. Here, simultaneous inspection by one inspector of plucks hanging over the intestines might be of interest – if judged feasible. Communication with important trade partners prior to implementation of such changes is required to ensure recognition of equivalence.

Continued discussion about meat inspection is needed to ensure the most food safety for the resources spent. Next work will focus on an evaluation of the decision code “accepted for deboning” – does this code result in value for money?

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References

Alban, L., Vilstrup, C., Steenberg, B., Jensen, H.E., Aalbæk, B., Thune-Stephensen, Jensen, S., 2008c. Assessment of the risk for humans associated with Supply Chain Meat Inspection – The Danish way. Danish Veterinary and Food Administration /DMA Available online: http://www.lf.dk/Aktuelt/Publikationer/~media/lf/Aktuelt/Publikationer/Svinekod/Modernisation%20of%20Meat%20Inspection_DK.ashx 54 pp.

Alban, L., Steenberg, B., Petersen, J.V., Jensen, S., 2010. Is palpation of the intestinal lymph nodes a necessary part of meat inspection of finisher pigs? Landbrug og Fødevarer Available online:

<http://www.lf.dk/Aktuelt/Publikationer/~media/lf/Aktuelt/Publikationer/Svinekod/UKRisikovurdering%20kroeslymfeknuder%20koedkontrol.ashx> 27pp.

EFSA, 2011. Scientific Opinion on the public health hazards to be covered by inspection of meat (swine). EFSA Journal 2011;9(10):2351. [198 pp.] doi:10.2903/j.efsa.2011.2351. Available online: <http://www.efsa.europa.eu/en/efsajournal/doc/2351.pdf>

Table 4. Association between pyaemia and abscesses found in finisher pigs from Danish Crown Horsens, October-December 2012

Pyaemia	Abscesses*		Sum	Assessment of association
	+	-		
+	97	620	717	Relative Risk = 4.4
-	37.195	1.172.660	1.209.855	P<0.0001
Sum	37.292	1.173.280	1.210.572	Pos. pred. value= 0.14

*: Defined as abscess on the carcass including legs and toes except the head and abdomen

Risk-based Surveillance of Antimicrobial Residues – Identification of Potential Risk Factors

Alban, L.*, Pacheco, G., Petersen, J.V.

Danish Agriculture & Food Council, Copenhagen, Denmark

*corresponding author Lis Alban :lia@lf.dk

Abstract

Today in Denmark, residue surveillance is conducted at random. However, if the surveillance is targeted to high-risk finisher pig herds, then the number of samples can be reduced whereby resources can be saved without jeopardizing public health. An analysis of Danish data covering 2.5 years has shown that finisher pig herds with a very high prevalence of chronic pleuritis have a higher risk of residue findings in the surveillance programme. If this is incorporated into the own control, then cost savings of 25% can be obtained. However, as shown in the Danish data there are other reasons for the presence of residues such as miscommunication and insufficient marking of treated animals. Such cases should be prevented through information campaigns addressed to the farmers and their advisors.

Introduction

Around 20,000 samples are analysed each year for presence of antibacterial residues in Danish slaughter pigs, and between zero and five samples are positive above the maximum residue level (MRL). The intention was to develop a risk-based surveillance programme involving fewer samples while ensuring equal safety. Therefore, risk factors were searched for.

Material and Methods

Data were obtained from the Danish slaughterhouse database covering the period from July 2010 to December 2012. Residues were suspected in 17 cases. In nine of these, the farmer called in to prevent the pigs from being slaughtered. Hence, eight cases were found through the surveillance programme, and two of these were above MRL (Table 1).

For these eight cases, the number of pigs slaughtered and the number in which each of the following lesions were found were included in a statistical analysis: chronic pleuritis, tail bite, chronic pericarditis, chronic pneumonia, chronic peritonitis, osteomyelitis, abscess in hindquarters, abscess in leg/toe and abscess in forequarters. Next, data from all herds delivering pigs for slaughter to the same abattoir were included covering a 3-month period prior to the residue finding.

The prevalence (calculated as y/n) of chronic pleuritis in each of the eight case herds was compared with the prevalence in all the herds that delivered pigs to the same slaughterhouse in the same period. This was done by use of logistic regression model where the response was y/n and the explanatory variable was whether the finding was part of the surveillance programme or not. Overdispersion was taken into account by use of the P-scale option in PROC GENMOD in the software SAS® Version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Among the nine lesions investigated, only chronic pleuritis was associated with presence of residues (Table 2). The subsequent detailed analysis revealed that the prevalence of chronic pleuritis was 1.7 times higher in the eight case herds compared to all other herds (Table 3). In two cases, the prevalence was significant higher ($p \leq 0.05$), and in one case substantially higher, but only borderline significant ($p = 0.1$). In the remaining cases, the prevalence did not differ from the other herds delivering pigs to the abattoir (Table 3). It is noted in Table 3 that the average of chronic pleuritis measured over 3 months varied between the abattoirs and over the year; minimum 16.3% and maximum 25.3%. In a quarter of the herds (24.7%) the prevalence of chronic pleuritis was above 40% - corresponding to two times the rounded average of chronic pleuritis.

Table 1. List of 17 finisher pig herds included in a study of antimicrobial residues, July 2010 to December 2012, Denmark, sorted by identification number

Case No.	Found* in surveillance	Finding	Explanation for mistake
1	Yes	Doxycyclin above MRL	Inadequate use of water-medication mixer
2	Yes	Penicillin G below MRL	Inadequate marking of treated animal
3	Yes	Oxytetracyclin below MRL	Pig with residue was poorly growing and this might have caused a slower elimination of the drug from the body
4	Yes	Penicillin G above MRL	Inadequate marking of treated pigs

5	Yes	Not found in confirmatory test	Information unavailable
6	Yes	Penicillin G below MRL	Interview with farmer did not reveal reason for finding
7	Yes	Tylathromycin	Information unavailable
8	No	Penicillin G below MRL	Interview with farmer did not reveal reason for finding
9	No	n.a.	Lack of internal communication - Farmer called slaughterhouse in time and treated pigs were driven back
10	No	Penicillin and dihydro-streptomycin Suspected	Lack of internal communication and inadequate marking of treated pigs - Farmer called slaughterhouse too late
11	No	n.a.	Farmer called slaughterhouse in time and treated pigs were driven back
12	No	n.a.	Farmer called driver in time to avoid deliverance of treated pigs. Problem arose because of lack of communication in combination with defect medication mixer
13	No	Lincomycin, Ethacylin, Denagard suspected	Farmer called slaughterhouse too late
14	No	Pigs tested negative for the drugs of interest	Farmer called slaughterhouse too late. All 170 pigs tested
15	No	n.a.	Farmer called slaughterhouse in time and treated pigs were driven back
16	No	n.a.	Farmer called slaughterhouse in time and pigs killed before slaughter
17	No	Two pigs known to be treated with injectable antibiotics	Inexperienced driver took wrong pigs to slaughter. Farmer called slaughterhouse when aware of problem, but it was too late

*: Consisting of both official samples and own check samples.

n.a.: Not applicable

Table 2. Average prevalence of nine selected lesions in each of the eight pig herds where residues of antimicrobials were found through the Danish residue surveillance, July 2010 - August 2012

Code/Lesion	Prevalence ^a of lesions in eight herds								Average ^b of company
	H1	H2	H3	H4	H5	H6	H7	H8	
Chronic pleuritis	22.86	48.10	8.05	21.68	69.16	32.49	47.34	52.33	18.76
Tail bite	0.14	0	0.13	0	0.93	0.08	0	0.39	0.17
Chronic pericarditis	0.22	0	3.05	4.21	13.08	0	0.18	0	2.01
Chronic pneumonia	0.72	0.77	0.50	0.97	0.93	0.37	0	0	0.53
Chronic peritonitis	0.86	0.19	0.79	0	1.87	0.37	0.12	2.52	0.78
Osteomyelitis	0.22	0	0.26	0.32	0.93	0.29	0.18	0.39	0.31
Abscess hindquarters	0.50	0.26	1.19	0.65	2.80	1.80	0.67	1.55	0.98
Abscess leg/toe	0.79	0.26	1.29	3.56	0	0.82	0.79	1.79	1.07
Abscess forequarters	0.14	0.13	0.50	0.97	0	0.33	1.03	0.39	0.48

^a: Figures in bold indicate a prevalence higher than the slaughterhouse company average.

^b: Based on data from the last quarter of 2012 for the entire slaughterhouse company.

Table 3. Comparison of prevalence of chronic pleuritis in each of eight case herds with the average prevalence in the abattoir during a 3-month period prior to the finding of residues

Case No.	# of herds	Prevalence of chronic pleuritis in finisher pigs			Prevalence ratio ^c	P-value (log. regr.)
		Average of abattoir ^b (%)	90% C.I. ^a (%)	Case herd y/n (%)		
1	2274	23.9	2.0-59.5	318 / 1391 (22.9)	0.96	0.87
2	2128	20.5	1.4-53.3	745 / 1549 (48.1)	2.35	0.04
3	1297	25.3	3.1-62.5	243 / 3020 (8.1)	0.32	0.39
4	1356	24.2	3.4-67.3	67 / 309 (21.7)	0.90	0.94
5	1354	23.7	3.7-66.3	74 / 107 (69.2)	2.92	0.38
6	2250	21.1	1.4-54.0	794 / 2444 (32.5)	1.54	0.33
7	2167	22.6	1.6-55.3	782 / 1652 (47.3)	2.09	0.05
8	1042	16.3	2.2-61.9	270 / 516 (52.3)	3.21	0.10
All herds^d	13868	22.3	2.0-60.0	(37.6)	1.69	n.a.

^a: 90% percentile interval of the observed prevalence in the herds.

^b: Average prevalence of chronic pleuritis on the abattoir for that specific time period.

^c: ratio between the prevalence of chronic pleuritis in the case herd versus all other herds that delivered pigs to the same abattoir in the same period.

^d: one herd could contribute with more deliverances of pigs, because for each case herd all herds that delivered to the same abattoir in the same period were included.

Discussion

The results of this study indicate that chronic pleuritis might be a risk factor for use in surveillance. This is confirmed by Dutch data, which show a three times higher risk for such herds. In the Netherlands, risk-based surveillance for antimicrobial residues has been implemented in the pig abattoirs (Jelsma et al., 2006).

However, if a high prevalence of chronic pleuritis in a herd is used as the sole criterion for inclusion into the programme, only one fourth of the population will be covered in the surveillance. And this might result in some farmers losing the incentive to comply with the withdrawal period. Moreover, because there are other reasons for finding residues than a high prevalence of chronic pleuritis – as noted in Table 1 – it would make sense to include samples taken at random as well. Moreover, herds with prior history of non-compliance with withdrawal periods should also be included in the programme. For the group consisting of risk-based samples, a 50% reduction can be introduced because sampling is taking place in a compartment with a risk that is at least two times higher than in the remaining group. This implies that 25% of the costs related to taking samples can be saved without jeopardizing public health, because it will lead to more or less the same number of positive samples as in the current programme.

It is important to evaluate the results of the programme separately for the different subgroups - random and risk-based - in order not to discourage slaughterhouse companies or governments from introducing risk-based programmes which will result in a higher number of positive samples due to the fact that sampling is taking place in herds with a higher risk.

Other risk factors - reported in the cases where the farmers called in or where follow-up visits were conducted after findings in the surveillance programme - were inadequate marking of treated animals, lack of communication, and incorrect use of medication dispensers. And these latter reasons cannot be used as risk factors for a monitoring or surveillance programme. Instead, these are dealt with through information campaigns.

Conclusion

The prevalence of antibacterial residues in Danish finisher pigs is negligible; only eight cases were found over a 2.5-year time period, and among these only two were above the MRL. This makes it of interest to extend the use of risk-based principles in the surveillance programme. Resources could be spent more effectively, if sampling takes place in high-risk sub-populations, because such pigs have a higher probability of harbouring residues of antimicrobial origin. Hence, the number of positives might remain unchanged or increase – reflecting an equivalent level of safety – even when the sample size is reduced.

Our study shows that in two of the eight cases, the prevalence of chronic pleuritis was significantly higher ($p \leq 0.05$) compared to all the other herds that delivered pigs to the same abattoir in the same time period, and in one herd it was borderline significant ($p = 0.1$). Hence, pigs from herds with a high prevalence of chronic pleuritis might be associated with a higher risk of residues compared to pigs from other herds.

Information campaigns targeted pig farmers should be aimed for to prevent presence of residues in meat at time of slaughter.

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References

Jelsma A, Lesuis R, Ronteltap E. 2006. Final report on the data analysis from the 'Visual Inspection pilot'. Dutch Food and Consumer Product Safety Authority. 36 pp.

Possible impact of the “yellow card” antimicrobial scheme on meat inspection lesions in Danish finisher pigs

Alban*, L., Dahl, J., Andreasen, M., Petersen, J.V., Sandberg, M.
Danish Agriculture & Food Council, Copenhagen, Denmark
*corresponding author Lis Alban: lia@lf.dk

Abstract

In 2010, the “yellow card scheme” adopted by the Danish Veterinary and Food Administration imposed restrictions on pig farmers who used more antimicrobials than twice the average. We studied the impact on antimicrobial consumption and vaccine use based on data from the monitoring programme Vetstat covering the time period January 2010 and July 2011. The decrease in antimicrobial consumption was pronounced for all age groups treated for either gastro-intestinal or respiratory disease. Data from meat inspection of finisher pigs from before and after introduction of the scheme were compared (N=1.7 million finisher pigs). Nine lesions of chronic nature and infectious origin and the code “condemned” were selected. Logistic regression models with year and week as explanatory variables were used to identify whether the prevalence of a lesion changed from 2010 to 2011. The most common lesion seen was chronic pleuritis (~23%) while the other lesions occurred less-commonly (<1%). For osteomyelitis, pleuritis, chronic arthritis and condemnation, no differences were observed between the 2 years. The prevalence of chronic peritonitis (OR=1.5), umbilical hernia (OR=1.2) and chronic enteritis (OR=1.2) were statistically higher in 2011 compared to 2010, whereas it was lower for tail bite infection (OR=0.6), chronic pericarditis (OR=0.6), and chronic pneumonia (OR=0.7) ($P<0.001$). Our results indicate that marked reduction in use of antimicrobials is associated with a short-term increase in the prevalence of specific lesions found during meat inspection and higher coverage of vaccines against respiratory diseases might impact the prevalence of chronic pneumonia positively. Other factors that impact on pig health were not included in the study. Moreover, effect of productivity was not evaluated.

Introduction

Discussion about prudent use of antimicrobials in animal production is on-going. There is concern about the development of bacteria that are resistant to the antimicrobials commonly used to cure infections in both humans and livestock. However, it might be speculated that animal health might deteriorate if diseased animals are not treated, hereby jeopardizing animal welfare.

In 2010, the “yellow card scheme” adopted by the Danish Veterinary and Food Administration imposed restrictions on pig farmers who used more antimicrobials than twice the average. Such an approach might have negative implications for the pig health. We therefore decided to study the potential impact on pig health. This paper shows the main part of the results – more details can be found in Alban et al. (2013).

Material and Methods

We made use of the antimicrobial consumption and vaccine use data from the monitoring programme Vetstat, covering all treatments between January 2010 and July 2011. A linear regression using the ordinary least square regression method was fitted to the antimicrobial use data by use of Microsoft Excel[®] and the R^2 was calculated. Based on the linear regression, the relative decrease (%) in consumption was calculated for a 12-month period. A similar exercise was made for the vaccine data.

Data from meat inspection of finisher pigs from before and after introduction of the scheme were compared. A total of 1.7 million finisher pigs originating from 2,765 pig farms were included in the analyses. These pigs were all slaughtered on one large Danish abattoir. These data covered the first 9 weeks in 2010 and 2011, respectively. Nine lesions of chronic nature and infectious origin and the code “condemned” were selected.

Logistic regression models with year and week as explanatory variables were used to identify whether the prevalence of a lesion changed from 2010 to 2011. Possible clustering due to correlation within herds and between weekly shipments of animals originating from the same herd was taken into account in the models. The software programme SAS was used for this part of the analysis.

In datasets that are very large, statistical significance is easily obtained. Moreover, we had 10 different models, which would make it easier to obtain statistical significance by chance. Therefore, we applied a significance level of 1% in the univariable models and 0.1% in the final model instead of the customary 5%. Moreover the Odds Ratio – as a measure of biological importance – had to be <0.9 or >1.1.

Results

The decrease in antimicrobial consumption was pronounced for all age groups treated for either gastro-intestinal or respiratory disease. Vaccine use increased in general: PCV2-related infections (+31%), gastro-intestinal disease (27%), and respiratory infections (21%). Figure 1 shows the decline in the use of antimicrobials for the gastro-intestinal diseases, whereas figure 2 shows the increase in the use of vaccines: PCV2-related infections: +31%, gastro-intestinal disease: +27%, respiratory infections: +21%.

The most common lesion seen was chronic pleuritis (~23%) while other lesions occurred less-commonly (<1%). For osteomyelitis, pleuritis, chronic arthritis and condemnation, no differences were observed between the 2 years. Chronic peritonitis (OR=1.5), umbilical hernia (OR=1.2) and chronic enteritis (OR=1.2) were more prevalent in 2011 compared to 2010, whereas tail bite infection (OR=0.6), chronic pericarditis (OR=0.6), and chronic pneumonia (OR=0.7) occurred less-frequent ($P<0.001$) (Table 1).

Discussion

The introduction of the Yellow Card scheme led to a substantial decrease in the consumption of antimicrobials in pigs in Denmark. This enabled a national evaluation of the impact on the health of pigs. Meat inspection data can be seen as indicators of health although there are inherent weaknesses in such data. These weaknesses were minimised by choosing data from one abattoir only. These data were considered the best available at the time for the purpose of our study. Later, more refined analyses including data describing productivity can be made.

The current permit limits for antimicrobial use in the yellow card scheme were set by the Danish Veterinary and Food Administration based on an intention to focus on the herds with the highest use. Naturally, when the consumption decreases among the farmers with the highest use, a permit limit defined as twice the average consumption will decrease. When considering the future permit limits, a thorough assessment should be conducted to find appropriate permit limits, which will not be leading to under-treatment and thereby poor animal health and welfare.

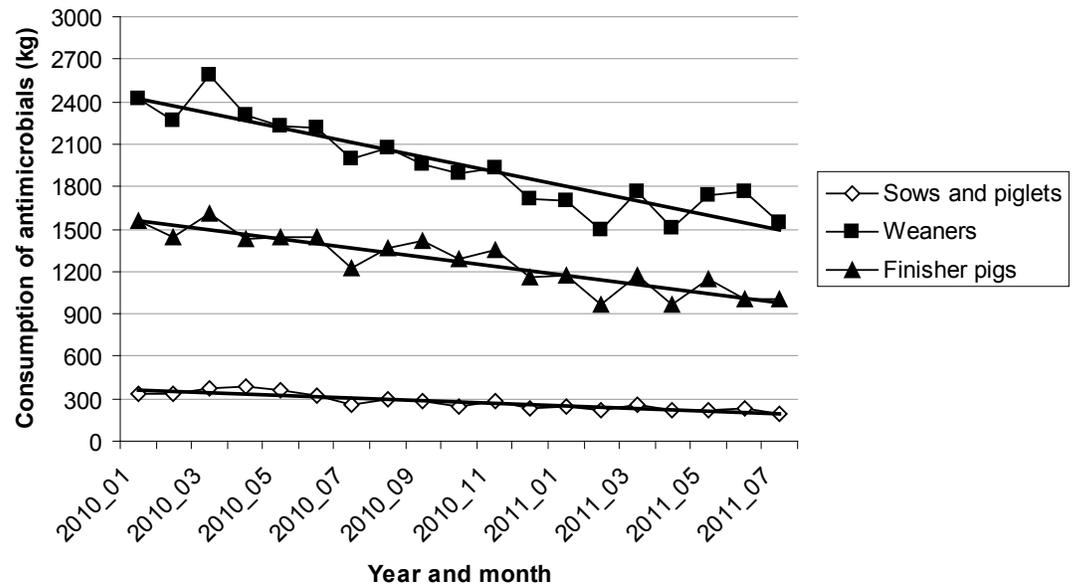


Fig. 1. Development in monthly consumption and linear trend of antimicrobials used in Danish pigs for treatment of gastro-intestinal disease, divided according to age group, January 2010 to July 2011. Source: Vetstat, the Danish Veterinary and Food Administration.

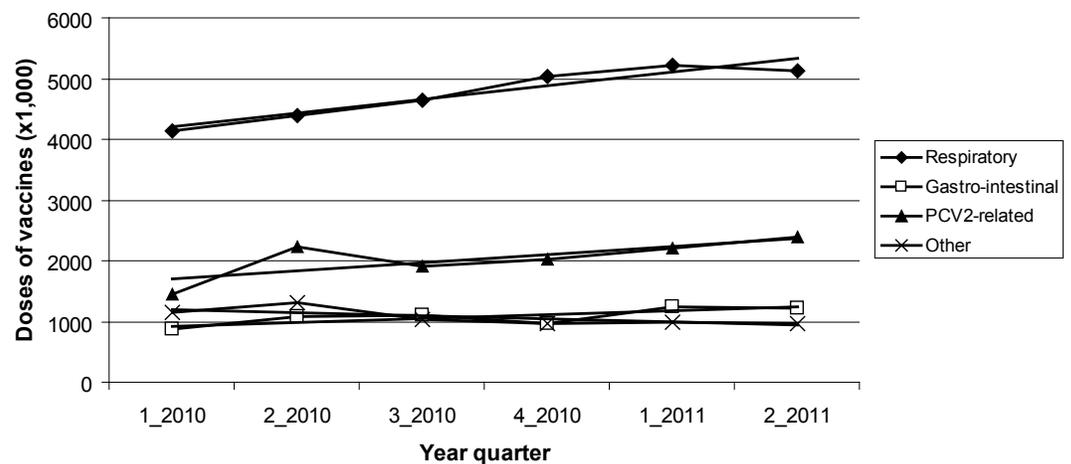


Fig. 2. Development in number of sold vaccines doses and linear trend, divided according to indication, used in Danish pigs between first quarter of 2010 and second quarter of 2011. Source: Vetstat, the Danish Veterinary and Food Administration.

The amounts of antimicrobials used per pig are already low in Denmark compared to other countries with a similar pig production (European Medicines Agency, 2011). The existing low use has an effect on the total release of resistant strains of zoonotic bacteria into environment as such, which can be demonstrated by the relatively low prevalence of resistance seen in zoonotic bacteria of human and animal origin (DANMAP, 2011). As long as the existing hygienic measures are kept in place, both in primary production and at slaughterhouse level – the food safety risk related to antimicrobial resistant strains of zoonotic bacteria on meat will remain low. Therefore, a further decrease in use of antimicrobials in production animals in Denmark might have limited impact on food safety.

Moreover, strict measures in one country's animal production have a limited impact on the general release of resistant strains, when animals, humans and meat are moved freely between European Member states as well as in and out of the European Union. Initiatives regarding prudent use of antimicrobials in humans and livestock on a European or global level are therefore welcomed.

A system like the yellow card might not as easily be introduced in other pig-producing countries due to the unique structure of the Danish pig production where almost all producers and slaughterhouses are member of the same organisation and most producers operate with high biosecurity measures (SPF-system).

Table 1. Results of analyses of effect of year in 10 logistic regression models^a describing association between year and each of 10 specific lesions found at meat inspection of 1.7 million Danish finisher pigs at one large abattoir during the first nine weeks of the year 2010 and 2011, respectively..

Model with lesion as dependent variable:	<u>Frequency of lesion in 2011 compared to 2010</u>						
	Random effects			Fixed effects			
	Covariance subject	Parameter estimate	Standard Error	Parameter estimate	Odds Ratio	Standard Error	P-value (Wald's)
Chronic peritonitis ^b	Herd	0.2619	0.0238	0.4332	1.54	0.0313	<0.0001
Umbilical hernia	Herd	0.6340	0.0397	0.2058	1.23	0.0314	<0.0001
	Week	1.1032	0.0135				
Chronic enteritis	Herd	0.1534	0.0183	0.1645	1.18	0.0313	<0.0001
	Week	1.3872	0.0166				
Condemnation ^b	Herd	0.4813	0.0371	0.1184	1.13	0.0382	0.0019
Osteomyelitis ^c	n.a.	n.a.	n.a.	0.0867	1.09	0.0263	0.001
Chronic arthritis	Herd	0.5229	0.0387	-0.0236	0.98	0.0373	0.526
	Week	1.0329	0.0124				
Chronic pleuritis	Herd	1.3188	0.0415	-0.0439	0.96	0.0104	<0.0001
	Week	5.4904	0.0684				
Chronic pneumonia	Herd	0.6746	0.0402	-0.3413	0.71	0.0344	<0.0001
	Week	1.08	0.0129				
Chronic pericarditis ^b	Herd	0.7885	0.0585	-0.5233	0.59	0.0484	<0.0001
Tail bite infection ^b	Herd	0.9499	0.5343	-0.6030	0.55	0.0354	<0.0001

^a: The dependent variable consisted of the number of finisher pigs (y) in which a specific lesion was recorded at meat inspection divided by the number of pigs (n) sent for slaughter from that producer in a week. Week was kept in all models as a categorical variable

^b: The model specifying both herd and week as random effects did not converge, therefore a reduced model was run with only herd as a random effect

^c: The model specifying herd as a random effect did not converge, therefore a reduced model was run without any random effects

n.a.: Non applicable

Conclusion

Our results indicate that marked reduction in use of antimicrobials is associated with a short-term increase in the prevalence of specific lesions found during meat inspection, and higher coverage of vaccines against respiratory diseases might impact the prevalence of chronic pneumonia positively.

References

Alban, L., Dahl, J., Andreasen, M., Petersen, J.V., Sandberg, M., 2013. Possible impact of the “yellow card” antimicrobial scheme on meat inspection lesions in Danish finisher pigs. *Prev. Vet. Med.*, 108, 334-341.

European Medicines Agency, 2011. Trends in the sales of veterinary antimicrobial agents in nine European countries (2005-2009). EMA/238630/2011. 77 pp. http://www.ema.europa.eu/docs/en_GB/document_library/Report/2011/09/WC500112309.pdf

DANMAP, 2010. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. National Food Institute, Technical University of Denmark. http://www.danmap.org/Downloads/~media/Projekt%20sites/Danmap/DANMAP%20reports/Danmap_2011.ashx

Prevalence of *Salmonella*, *Toxoplasma*, *Yersinia*, Hepatitis E and Porcine Reproductive and Respiratory Syndrome virus in UK pigs at slaughter

Cheney, T.*¹ and Powell, L.F.¹ (on behalf of Defra and its Agencies, FSA, BPEX, PHE and the *Toxoplasma* Reference Unit)
¹ Epidemiology, Surveillance and Risk Group, Animal Health and Veterinary Laboratories Agency - Weybridge, Woodham Lane, New Haw, Addlestone, Surrey, KT15 3NB, UK

*Corresponding author: Tanya Cheney, AHVLA - Weybridge, New Haw, Surrey, KT15 3NB, UK. E-mail: tanya.cheney@ahvla.gsi.gov.uk; fax: +44 (0)1932 359429

Abstract

In 2006/7 the prevalence of *Salmonella* in UK slaughter pigs was found to be amongst the highest in Europe. In advance of the anticipated EU-wide National Control Programme for *Salmonella* in pigs, a UK-wide monitoring programme was set up by Defra and funded in collaboration with key Government and industry partners. The monitoring programme presented a cost-effective opportunity to concurrently collect data on other potentially zoonotic pathogens for which prevalence data is currently limited, including *Toxoplasma*, *Yersinia*, Hepatitis E virus (HEV) and Porcine Reproductive and Respiratory Syndrome virus (PRRSv). Antimicrobial resistance testing of *Campylobacter coli* isolates and testing for ESBL *E. coli* was also undertaken (details not reported here). This study will meet urgent evidence needs and improve our knowledge regarding potential risks to public health in the pig meat supply chain. Between January and April 2013, 600 slaughter pigs from 14 abattoirs throughout the UK were sampled. Sampling was weighted according to abattoir throughput. Sampling dates and pig carcasses were randomly allocated utilising the abattoir daily throughput as a sampling frame. Samples collected from each pig include a post-stun rectal swab (*Salmonella*), post-bleed serum samples (*Toxoplasma*, HEV and PRRSv), the caecum (*Salmonella*) and tonsils (*Yersinia* and PRRSv) at the evisceration point, and pre-chill carcass swabs (*Salmonella* and *Yersinia*). *Salmonella* isolation (ISO 6579), serotyping and phage typing, and the isolation of *Yersinia*, were undertaken at AHVLA. Plasma samples were tested by ELISA for antibodies to *Toxoplasma* (at the *Toxoplasma* Reference Unit), HEV (at Public Health England (PHE)) and PRRSv (at AHVLA), and also tested for HEV RNA (at PHE). Tonsil material from PRRSv-seropositive pigs was tested by PCR and used for virus sequencing (at AHVLA).

Introduction

Foodborne diseases are estimated to cost the UK nearly £1.5 billion per year (FSA, 2011). *Salmonella* is the second most common foodborne zoonosis, with 9,455 laboratory-confirmed cases in the UK in 2011 (EFSA, 2013). Control of *Salmonella* in the EU has thus far focused predominantly on poultry, with *Salmonella* National Control Programmes (NCPs) having been implemented in the various chicken and turkey sectors from 2007 (Defra, 2011). The success of the NCPs in poultry has resulted in a progressive reduction in the number of cases in people. However, the proportion of human cases attributed to pork and pork products appears to be rising.

In 2006/7, an EU baseline survey was conducted to assess the prevalence of *Salmonella* in pigs at slaughter (Commission Decision 2006/668). Levels in the UK were above the EU average; 40.4% (259/641) of pigs had evidence of current infection and/ or carcass contamination, with *Salmonella* isolated from 21.8% of lymph node samples, 15.1% of carcass swabs and 21.9% of caecal samples. A NCP for *Salmonella* in pigs is expected to commence in 2015 and reduction targets based on the EU mean prevalence are likely to be challenging for the UK pig industry. Since the last survey, efforts continued to reduce the prevalence of *Salmonella* in pigs. Therefore, a new monitoring programme to reassess the prevalence of infection was proposed. This presented an opportunity to simultaneously gather national-level prevalence data on other organisms to address additional knowledge gaps.

In a recent EFSA Opinion on pig meat inspection, *Toxoplasma gondii* was identified as one of the most significant foodborne public health hazards (EFSA, 2011). There are an estimated 350,000 toxoplasmosis cases in the UK each year, of which 10-20% are symptomatic (ACMSF, 2012). Despite pig meat being considered a high risk food for transmission, a recent report from the Advisory Committee on the Microbiological Safety of Food (ACMSF) noted that there is virtually no data concerning the prevalence in UK livestock (ACMSF, 2012).

In the EFSA Opinion, *Yersinia* was also deemed very important (EFSA, 2011). Yersiniosis was the fourth most frequently reported zoonosis in the EU in 2011 and infections are often acquired from raw or undercooked pig meat (EFSA, 2013). Consequently, EU

Member States have been advised to gather prevalence data for pigs at slaughter at regular intervals (EFSA, 2009).

The number of indigenous hepatitis E virus (HEV) infections in people in England have increased substantially recently. Pigs have been hypothesised to play a role due to the high prevalence of anti-HEV antibodies identified in previous surveys and the close phylogenetic relationship between HEV strains infecting humans and pigs. Pork products are unlikely to become laden with virus by faecal contamination, so it was pertinent to assess the quantity of pigs that are viraemic at slaughter from which products subsequently entering the food chain may include viraemic blood, such as muscle.

Porcine Reproductive and Respiratory Syndrome virus (PRRSv) can have a major economic impact on pig production. Infection can cause direct costs from loss of production, increased mortality and reproductive failure, and indirect costs associated with treatment, disease control, pig disposal costs and disrupted breeding programmes. Consequently, PRRSv has been targeted for control by the British Pig Executive (BPEX, 2011). Minimal data is presently available on seroprevalence in the UK, so the monitoring programme provided an ideal opportunity to determine how many pigs were potentially infectious at slaughter and to augment current data on PRRSv diversity.

A UK-wide multi-agency monitoring programme was launched to address these various evidence needs, involving collaboration between the Department for Environment, Food and Rural Affairs (Defra), the Department of Agriculture and Rural Development (DARD), the Food Standards Agency (FSA), the British Pig Executive (BPEX), Public Health England (PHE), Public Health Wales (PHW), the Veterinary Medicines Directorate (VMD) and the Animal Health and Veterinary Laboratories Agency (AHVLA). The study design was consistent, as far as possible, with the technical specifications of the EU baseline survey of 2006/7 to enable comparisons.

Methods

Sample size: The target sample size for the EU baseline survey was 600 pigs based on an estimated prevalence of 50% with an accuracy of 4% and 95% confidence (Commission Decision 2006/668). Utilising the same sample size would enable detection of a 20% overall reduction in *Salmonella* prevalence or a 40% reduction in carcass contamination (with 80% power and 95% confidence). An additional 10% (60 pig carcasses) were included as a contingency.

Participants: The target population was all slaughtered pigs (finishers and cull sows and boars) in the UK. The following animals were excluded: any carcass that was totally condemned; animals with a live weight <50kg; animals that had undergone emergency slaughter; and animals kept in the UK <3 months before slaughter. The largest capacity slaughterhouses in Great Britain (GB) and Northern Ireland (NI) were used, which together represented over 80% of all pigs slaughtered in the UK. The number of pigs sampled at each slaughterhouse was proportional to the annual throughput.

Sampling schedule: Samples were scheduled for collection between January and April 2013. The date of sampling and the specific pig carcass to sample on each occasion were randomly assigned by AHVLA utilizing the daily throughput as a sampling frame for the latter.

Sample and data collection: Samples were collected by trained staff at the FSA and DARD. Samples collected from each pig comprised of one post-stun rectal swab, two post-bleed EDTA whole blood samples, the caecum, heart, tongue and tonsils at the evisceration point, and two pre-chill carcass swabs. Samples were transported by courier (at 2-8°C) as soon as possible after collection. A standardised questionnaire was also completed at the time of sampling to gather epidemiological data, including information concerning the pig (e.g. age, weight) and abattoir processes (e.g. slaughter speed, scalding temperature).

Laboratory analysis: Bacteriological examination was carried out within 24 hours of receipt at the lab and 96 hours of sample collection.

Salmonella: Caecal contents, carcass swabs and rectal swabs were tested for *Salmonella* at AHVLA following the method in ISO6579 annex D, with pre-enrichment in Buffered Peptone Water, subculture to Modified Semi-Solid Rappaport-Vassiliadis medium then plating to Xylose-Lysine-Desoxycholate Agar and Novobiocin Brilliant Green Agar. Suspect colonies were confirmed serologically or biochemically. Positive isolates were serotyped according to the White-Kaufmann-Le Minor scheme and Typhimurium isolates were phage typed at the AHVLA national reference laboratory.

Yersinia: Carcass swabs and tonsils were tested for *Yersinia* at AHVLA via the cold enrichment method; samples were

stored at 2-8°C and inoculated onto *Yersinia* selective agar at weekly intervals for 3 successive weeks. Speciation was via colony morphology and API 20E biochemical strips.

PRRSv: EDTA plasma samples were tested for PRRSv antibodies by ELISA at AHVLA. Tonsil material from seropositive pigs was tested by PCR and a subset of viruses was sequenced.

Toxoplasma: EDTA plasma samples were tested for *Toxoplasma* antibodies using the Sabin-Feldman Dye Test at PHW. Heart and tongue tissue from seropositive pigs were stored for future investigations.

HEV: EDTA plasma samples were also tested for HEV antibodies by ELISA at PHE. In addition, HEV RNA levels were quantified using quantitative Taqman or hemi-nested PCR as appropriate (Garson *et al.*, 2012; Jothikumar *et al.*, 2006). Samples with sufficient viral loads were subject to sequence analysis in the ORF2 region.

Data collection and analysis: Data were registered on a survey specific MS Access database. Data cleaning and statistical analyses will be conducted in Stata 12.0. The overall prevalence of each organism will be calculated accounting for clustering of pigs within farms. These will be compared with previous abattoir surveys to investigate recent trends and the data for *Salmonella* will help develop the NCP in pigs. Agreement between sample types will be examined via kappa tests. Variation in regional prevalence will also be investigated. Co-infections will be explored. The questionnaire data will be used to investigate associations with the various microorganisms via multivariable logistic regressions models.

Results

The prevalence results from this unique multi-agency, multi-funded project should be available later this year. By combining numerous infectious agents into one monitoring programme, the project will be able to efficiently and simultaneously address urgent evidence needs and improve knowledge of numerous potential risks to public health in the pig meat supply chain. The survey has attracted considerable interest to date and the results are likely to be extremely useful to both the industry and public health bodies. The project has also enabled the strengthening of the collaborative ties between veterinary and public health organisations and the industry in the UK, which will prove beneficial for future research and surveillance and the implementation of the pig NCP.

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References

- ACMSF, 2012. Risk profile in relation to *Toxoplasma* in the food chain. London: FSA, pp.1-70
- FSA, 2011. Foodborne Disease Strategy 2010-15 v1.0. London: FSA, pp.1-24
- BPEX, 2011. 20:20 Pig Health and Welfare strategy. Kenilworth: BPEX, pp.1-19
- Defra, 2011. Zoonoses Report UK 2010. London: Defra, pp.1-83
- EFSA, 2007. Report of the Task Force of Zoonoses Data Collection including a proposal for a harmonized monitoring scheme of antimicrobial resistance in *Salmonella* in fowl (*Gallus gallus*), turkeys, and pigs and *Campylobacter jejuni* and *C. coli* in broilers. EFSA Journal. 96, pp.1-46.
- EFSA, 2009. Technical specifications for harmonised national surveys of *Yersinia enterocolitica* in slaughter pigs on request of EFSA. EFSA Journal. 7(11), pp. 1374.
- EFSA, 2011. Scientific Opinion on the public health hazards to be covered by inspection of meat (swine). EFSA Journal. 9(10), pp. 2351.
- EFSA, 2013. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011. EFSA Journal. 11(4), pp. 3129.
- Garson, J.A., Ferns, R.B., Grant, P.R., Ijaz, S., Natouli, E., Szypulska, R., Tedder, R.S., 2012. Minor groove binder modification of widely used TaqMan probe for hepatitis E virus reduces risk of false negative real-time PCR results. Journal of Virological Methods. 186 (1-2), 157-160.
- Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. Journal of Virological Methods. 131, 65-71.

Dynamic of *Campylobacter* infection within pig farms from sows to fattening pigs

Leblanc-Maridor M. ^(1,2), Denis M. ^(3,4), Chidaine B. ^(3,4), Belloc C. ^(1,2)

¹INRA, UMR1300 Biology, Epidemiology and Risk Analysis in animal health, F-44307 Nantes, France

²LUNAM Université, Oniris, Nantes-Atlantic College of veterinary medicine and food sciences and engineering, UMR Bio-EpAR, BP 40706, F-44307 Nantes, France

³Anses, Unité Hygiène et Qualité des Produits Avicoles et Porcins, BP 53, 22440 Ploufragan, France

⁴Université Européenne de Bretagne, France

*Corresponding author : mily.leblanc-maridor@oniris-nantes.fr

Abstract

This work aimed at (i) describing *Campylobacter* excretion by conventional pigs in field conditions from sows to fattening pigs, and (ii) assessing the role of the environment as a source of pig contamination. Five sows and six piglets per sow were individually followed in two farrow-to-finish farms. Faecal shedding, contamination of pens (empty or with animals), feed and water were monitored from birth to finishing for pigs and during one production cycle for sows. *Campylobacter*, mainly *C. coli*, was highly prevalent for sows and their piglets. All the sows excreted *Campylobacter*. Piglets became infected early: 84 and 86% of them excreted *Campylobacter* three to five days after their birth. The prevalences increased progressively through the rearing period with some young pigs becoming punctually negative. The amount of *Campylobacter* in faeces was highly variable between pigs (from 0 to 10⁹ CFU/g of faeces) and between sampling times for a given pig, with sometimes no detection. Samples of the pigs' environment during the down period were always negative and became positive when pigs were housed in the pens. Nevertheless, no correlation was established between the excretion level of the pigs and the contamination level of their environment. Some samples of feed, initially free of *Campylobacter*, became positive due to contamination by faecal material. Finally, our study underlines the role of the sows as a *Campylobacter* contamination source for their piglets.

Introduction

Campylobacter, a major cause of food-borne human infection, is commonly carried in the intestinal tract of a wide range of animals, including livestock animals, without causing clinical signs (EFSA, 2007). Pigs are known to be frequently infected by *Campylobacter* with prevalence between 50% and 100% and excretion levels ranging from 10² to 10⁷ Colony Forming Units (CFU) of *Campylobacter* per gram of faeces (Alter *et al.*, 2005). For the implementation of control measures in farms, dynamics of *Campylobacter* excretion and sources of contamination have to be known. Previous studies describe that piglets became infected by their mothers within the first weeks of life (Hume *et al.*, 2002). Other sources could be suspected among which the environment (Alter *et al.*, 2005). This work aimed at (i) describing *Campylobacter* excretion (presence/absence, main species and quantity) by conventional pigs in field conditions along one production cycle from sows to fattening pigs and (ii) assessing the role of the environment as a potential source of pig contamination.

Material and Methods

Farms and animals individual follow up

This study was carried out in two farrow-to-finish farms. In each farm, five sows from the same batch have been followed along one production cycle (before farrowing and until the slaughter of their offspring). After farrowing, 6 piglets per sow have been individually followed from their first week of life until their departure to the slaughterhouse. Sows were monitored weekly 2 weeks before farrowing. Piglets and sows were followed weekly from the first week to 5 weeks of age then growing pigs were monitored every three weeks until slaughter whereas the sows have been sampled at the 8th, 11th and 17th weeks after farrowing.

Samples and analysis

Fresh faecal samples from sows and young pigs were collected individually and 10 g of each were diluted to 1:10 in Preston broth for detection and enumeration. For the environment, surface swabs (floors, walls, structures) were collected from each pen and from the floors and walls around each pen (i) in empty rooms after cleaning/disinfection (during the down period) and (ii) in presence of the animals. Surfaces swabbed were measured to detect and quantify the number of *Campylobacter* per square meter. Swabs were humidified with 45ml Preston broth. Enumeration for all the samples was done

using a 10-fold serial dilution on Karmali plates.

Presence/absence of *Campylobacter* was checked in feed and water. Feed samples were collected before the distribution to the animals (in bags or silos) and in presence of pigs (into the trough of each studied pens). Water samples were collected upstream to the distribution network and at the faucet in each unit (service, gestation, farrowing, post-weaning, fattening).

Species of isolates were identified by real-time PCR (Leblanc-Maridor *et al.*, 2011).

For the statistical analysis, the correlation between the contamination level of the environment and the excretion level of *Campylobacter* by the animals was estimated.

Results

For one farm (Farm I), all the faecal samples from sows were positive for *Campylobacter* whatever the sampling times while in the second farm (Farm II), no *Campylobacter* were detected in the faecal samples of one sow at three different sampling times. Level of *Campylobacter* excretion varied from less than 10^2 to 10^7 CFU of per gram of faeces, and varied between sows at one sampling time and also for a given animal at different sampling times.

At 3 to 5 days after birth (first faecal sample), 84% and 86% of the piglets (respectively Farm I and Farm II) excreted *Campylobacter* (Figure 1). These prevalences increased progressively through the rearing period except at three weeks of age where only 60 % of the piglets excreted *Campylobacter*. Afterwards, prevalences were above 90 % with some pigs becoming punctually negative.

% of *Campylobacter* excreting pigs

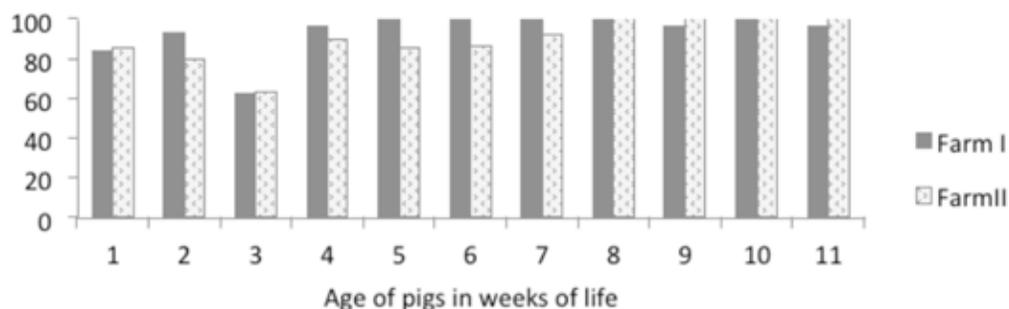


Figure 1: Evolution of the percentage of young pigs excreting *Campylobacter* in their faeces

The *Campylobacter* faecal excretion of young pigs was on average 10^5 to 10^7 CFU of *Campylobacter* per gram of faeces with a high variability in the quantities between two different samples for a given animal or between pigs at the same sampling time (from 0 to 10^9 CFU/g) (Figure 2). A decrease of the quantity of *Campylobacter* excreted by piglets was observed between the 4th and the 5th weeks of age for both farms after treatment with tylosine given to the piglets at the entrance into the post-weaning unit.

No *Campylobacter* was ever found in the environmental samples taken after the cleaning-disinfection process (without animals) as well as in the feed samples taken before distribution to the animals or in the water. When the animals were in the pens, feed samples and environmental samples were positive with a high variability of the contamination level (from less than 100 to 10^9 CFU/square meter). Concerning the environmental samples (walls or floors), the number of positive samples increased with the age of the pigs. There is no significant correlation between the level of *Campylobacter* excretion by pigs and the contamination level of the environment, whatever the samples (walls or floors of the pens and food) ($P > 0.05$).

Faecal excretion of *Campylobacter* by pigs log (CFU/g faeces)

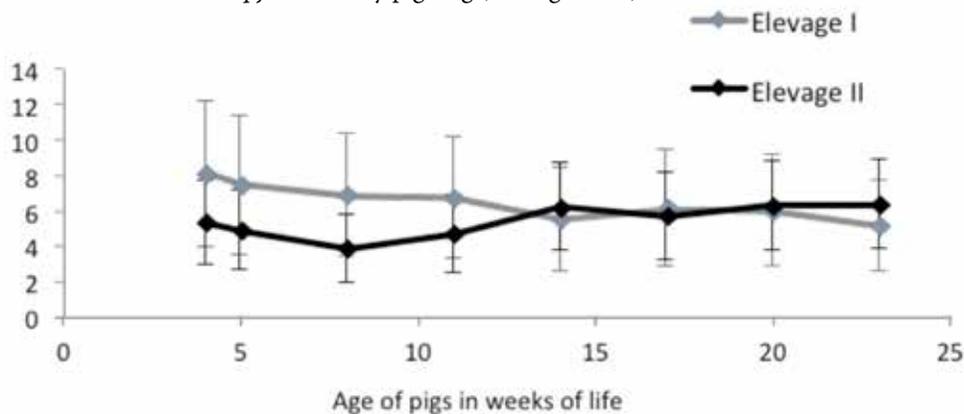


Figure 2: Faecal excretion of *Campylobacter* by young pigs

All *Campylobacter* isolates from faecal samples of sows and pigs, environmental samples and feed were identified as *C. coli*.

Discussion

The objective of this study was to describe the dynamics of *Campylobacter* excretion in a conventional farrow-to-finish farm and to assess the role of the environment as a potential source of contamination. The originality of this study was the individual follow-up of the same animals all along a production. Indeed, in the other studies, the comparisons of prevalence or excretion levels of *Campylobacter* between pigs at various stages have been done on different animals, at one sampling time or on a grouped way (Alter *et al.*, 2005; Weijtens *et al.*, 1993; Weijtens *et al.*, 1999).

In this survey, the high prevalence observed for sows throughout a production cycle is close to that found in the literature. Nevertheless, this result has to be taken with caution considering the low number of individuals sampled (5 sows per farm). Regarding the piglets, 84 and 86% of them are contaminated at the first sampling time, 3 to 4 days after their birth. This early contamination of piglets was similar to the results previously described (Weijtens *et al.*, 1997; Weijtens *et al.*, 1999).

In our study, the early contamination of the piglets seems to be due to the contact with their mothers as the environment was negative during the down period (after the cleaning and disinfection process) as well as feed and water of the farrowing unit. Moreover, sows excrete high quantities of *Campylobacter* which could promote the contamination of the piglets. The role of the sows as the first contamination source of *Campylobacter* for their piglets was previously described (Weijtens *et al.* 1999; Hume *et al.*, 2002; Alter *et al.* 2005).

Besides, the progressive increase of the prevalence of young pigs' infection is in accordance with the results of Weijtens *et al.* (1997). However, like Alter *et al.* (2005) we did not observe a decrease of the percentage of carrier pigs during the fattening period as observed by Weijtens *et al.* (1993). To our knowledge, the punctual decrease of prevalence (60% of the piglets) observed in both farms at the third week of life has not been reported previously. The origin of this decrease is difficult to explain and not linked to bias of bacteriological method as the positive sows samples have been treated at the same time.

For sows and for piglets, the amount of *Campylobacter* in faeces was highly variable (from 0 to 10⁹ CFU/g). After the 5th week of life, all young pigs became shedders until the end of the study with a level of excretion similar to those found in previous studies, except for one animal. Nevertheless, similar to previous findings (Weijtens *et al.*, 1999), variations in the average colony count of *Campylobacter* in the faeces between both animals and samples from a given animal were observed in our trial. *Campylobacter* could not be detected in one animal at one time point whereas high counts were observed in faeces from the same pigs at previous and following sampling times. This situation was previously described in an experimental trial (Leblanc-Maridor *et al.*, 2008). These observations suggest an intermittent excretion of *Campylobacter* or elimination followed by re-contamination of pigs by *Campylobacter* (Weijtens *et al.*, 1999).

No significant correlation has been found in our study between the contamination level of the environment and the excretion level of the pigs. However, the environment can play a role as source of indirect contamination for pigs, especially due to pig having frequent oral contacts with their environment. In this work, the resistance of *Campylobacter* in the environment in a pig farm seems limited as shown by a high number of negative environmental samples in the presence of the animals on dirty soil. In the same way, no positive environmental samples have been observed after the down period showing that good measures of hygiene between two batches (cleaning, disinfection, down period) allow the elimination of *Campylobacter* in the concerned rooms. The epidemiological role of the environment in the infection dynamics and the excretion pattern of *Campylobacter* by pigs might be limited if this environment is not constantly recontaminated by pigs faeces. Weijtens *et al.* (2000) underlined in a study that *Campylobacter* infection in pig farm can be reduced even eliminated through the implementation of strict hygienic measures associated with a repopulation with *Campylobacter* non-carriers pigs. Among the feed samples taken into the troughs in presence of animals, few were positive due probably to a contamination *via* the faecal material (the presence of faeces into the trough has been sometimes observed or strongly suspected). These results highlight that feed could play a role in indirect transmission of *Campylobacter* between pigs.

Conclusion

Our study confirmed the high prevalence of *Campylobacter* infection for sows and pigs in conventional farms all along a production cycle. The early contamination of the piglets could be mostly due to the contact with their mothers. Even if no significant correlation has been shown between the environment contamination and the excretion levels of *Campylobacter* by the animals, the environment appeared frequently contaminated notably by faecal material (walls, floor and structures of the pen/room, feed in the troughs) and is probably an element of *Campylobacter* transmission between animals.

References

- Alter, T., Gaull, F., Kasimir, S., Gurtler, M., Mielke, H., Linnebur, M., Fehlhaber, K., 2005, Prevalences and transmission routes of *Campylobacter* spp. strains within multiple pig farms. *Vet. Microbiol.* 108, 251-261.
- EFSA 2007. The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006, Journal, T.E., ed. (EFSA), p. 130.
- Hume, M.E., Droleskey, R.E., Sheffield, C.L., Harvey, R.B., 2002, *Campylobacter coli* pulsed field gel electrophoresis genotypic diversity among sows and piglets in a farrowing barn. *Curr. Microbiol.* 45, 128-132.
- Leblanc Maridor, M., Denis, M., Lalande, F., Beaurepaire, B., Cariolet, R., Fravallo, P., Federighi, M., Seegers, H., Belloc, C., 2008, Experimental infection of specific pathogen-free pigs with *Campylobacter*: excretion in faeces and transmission to non-inoculated pigs. *Vet. Microbiol.* 131, 309-317.
- Leblanc-Maridor, M., Beaudeau, F., Seegers, H., Denis, M., Belloc, C., 2011, Rapid identification and quantification of *Campylobacter coli* and *Campylobacter jejuni* by real-time PCR in pure cultures and in complex samples. *BMC Microbiol.* 11, 113.
- Weijtens, M.J., Bijker, P.G., Van der Plas, J., Urlings, H.A., Biesheuvel, M.H., 1993, Prevalence of *Campylobacter* in pigs during fattening; an epidemiological study. *Vet. Q* 15, 138-143.
- Weijtens, M.J., van der Plas, J., Bijker, P.G., Urlings, H.A., Koster, D., van Logtestijn, J.G., Huis in't Veld, J.H., 1997, The transmission of *Campylobacter* in piggeries; an epidemiological study. *J. Appl. Microbiol.* 83, 693-698.
- Weijtens, M.J.B.M., Reinders, R.D., Urlings, H.A.P., Van der Plas, J., 1999, *Campylobacter* infections in fattening pigs; excretion pattern and genetic diversity. *J. Appl. Microbiol.* 86, 63-70.
- Weijtens, M.J.B.M., Urlings, H.A.P., Van der Plas, J., 2000, Establishing a *Campylobacter*-free pig population through a top-down approach. *Letters in Appl. Microbiol.* 30, 479-484.

Porcine Hepatitis E

P. Buholzer¹, S. Wacheck², R. Fries³, H. Irsigler³, K. Reisp⁴, F. Schmoll⁴

¹ Prionics AG, Schlieren, Switzerland

² Chair of Food Safety, Faculty of Veterinary Medicine, Ludwig-Maximilians University, Munich, Germany

³ Freie Universität Berlin, Faculty of Veterinary Medicine, Institute of Meat Hygiene and Technology, Berlin, Germany

⁴ AGES, Institute for Veterinary Disease Control, Mödling, Austria

Hepatitis E virus (HEV) is a zoonotic agent that can be transmitted from pigs to humans via consumption of pork and products derived of it. Recently, the European Food Safety Authority (EFSA) has published a scientific opinion urging for measures to prevent Hepatitis E virus (HEV) from entering the food chain.

Unfortunately, there is only limited knowledge about the world-wide prevalence of HEV in pig herds and consequently on the potential risk for consumers.

Various studies demonstrated high levels of HEV in the pig production units. Antibody prevalence of up to 60% is commonly found in pig herds. However, also antibody-negative herds are identified, even in high prevalence regions. These findings are of great interest; it is crucial to learn more about these negative herds and about the differences between non-infected and infected herds. Identification of differences in holding conditions, management, sourcing of piglets etc. will help to identify risk factors for HEV infection in pig herds as well as for the introduction of the virus into herds. Strategies and measurements to reduce or eliminate HEV in pig herds can then be implemented and consequently the risk of introducing HEV into the food chain reduced.

The implementation of an appropriate risk based surveillance system for HEV in the pre-harvest sector would be an effective and economic way to collect information on the herd infectious status and to continuously reduce the prevalence of HEV infection among livestock. Risk based surveillance systems help reduce the risk of introducing zoonotic diseases into the food chain and increase consumer health protection.

The high prevalence of HEV found in many countries shows the importance of surveillance and control of this important zoonotic pathogen. For this purpose it is of high importance to use adequate diagnostic tools, such as the PrioCHECK[®] HEV Ab porcine. The PrioCHECK[®] HEV Ab porcine is, due to the use of antigens of both, genotype 1 and 3, capable to detect antibodies against all HEV genotypes.

Austrian model approach to assess quality of post-mortem feedback-information systems in pigs

Wanda, S.*⁽¹⁾, Schleicher, C.⁽²⁾, Scheriau, S.⁽²⁾, Koefer, J.⁽¹⁾

⁽¹⁾ *Institute of Veterinary Public Health, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria*

⁽²⁾ *Division of Data, Statistics and Risk Assessment, Austrian Agency for Health and Food Safety, Graz, Austria*

*corresponding author : Sabine.Wanda@vetmeduni.ac.at

Abstract

A novel quality assurance approach was tested for its applicability to assess data validity and meat inspection performance by means of modeling and training of official meat inspectors (OMIs). General linear mixed models (GLMM) were used to estimate the variance in 20 selected lesions assessed by 12 official meat inspectors for 247.507 pigs. The pigs originated from 72 conventional farms and were slaughtered at one abattoir in Austria in the period January 2008 to June 2011. The heterogeneity in the health state of the pigs, variation due to the farms of origin, piglet producer, quarter and medication was considered in the statistical model. Based on the model meat inspection showed hardly any variation for post-mortem findings such as pericarditis, arthritis and milkspots, whereas meat inspection of blood aspiration, scalding water lungs, skin lesions and hepatitis can be deemed as not sufficiently standardized. Training of OMIs resulted in better detection rates of blood aspiration and pleurisy, but not in the detection of skin lesions related to slaughter technology and animal welfare. Grading of pneumonia has to be further improved in future training sessions. An improved data record system was established based on the outcome of the model and training of OMIs. In future research the benefit of the new code system and training effect to standardize meat inspection will be assessed in form of statistical re-evaluations.

Introduction

In order to revise meat inspection towards the introduction of a risk-based approach, information feedback systems have been established throughout Europe, legally required by European Commission regulations (EC) 854/2004 and (EC) 2074/2005 to grant safety along the production chain. It became mandatory for official meat inspectors (OMI) to record slaughter lesion data, and provide information about the predominant diseases affecting finishing pigs from the slaughter house to the pig producer and farm veterinarian, thus assist in monitoring disease in national herds. Although the variability in scoring lesions among OMIs is believed to be a major contributor to variability in lesion prevalence among slaughter prevalence in monitoring programs, hardly anyone has questioned the quality of data recorded in such databanks for reporting.

In Austria a novel quality assurance (QA) approach was evaluated for its applicability to assess data validity and meat inspection performance by means of modeling and training of OMIs. As described in Schleicher et al. (2013) statistical models were fitted to estimate the probabilities of a positive finding.

Furthermore, the amount of variation among these probabilities that contribute to the OMIs was determined by calculating variance partitioning coefficients (VPCs). Primary aim was to assess those lesions with the highest variability among OMIs for further training and to assess practically at the slaughter plant the feasibility of the model as training base.

Material and Methods

Study population

The population under study comprised all conventional pigs from 72 farms located in the province of Styria, Austria, that were slaughtered in the period January 2008 to June 2011. Farms included 21 (29.2%) fattening farms and 51 (70.8%) farms with “farrow to finish units”. Herd size ranged from 70 to 2025 finishing pigs. All farms participating in the study had no “all in/all out” management. Each farm sent batches of finishing pigs to slaughter according to growth performance. Additionally, only farms which sent more than 400 pigs to slaughter in the study period were included in the study. A total of 247.507 pigs were examined.

Meat inspection and Data recording

The study was conducted at one slaughter plant located in the province of Styria, Austria, slaughtering approximately 2000 – 2300 pigs per week. Finishing pigs were sent to slaughter with about 115 – 120 kg (253.5 – 264.6 lb). The abattoir killed about 115 -120 pigs per hour and used carbon dioxide stunning followed by conventional sticking with the animals lying on the side. At the slaughterhouse post-mortem data were recorded by a total of 12 official meat inspectors (OMI) with 11-

19 years of experience in meat inspection. Each veterinarian was registered under a certain “vetcode” in the system of the slaughterhouse. Along the pluck- and carcass line the veterinarian was able to select post-mortem findings on a touch screen out of 55 pre-defined parameters. In the study focus was laid on 20 lesions according to their frequency and animal health significance from the abattoir company’s database. The parameters were assessed for their validity, thus quality in post-mortem feedback-information systems.

Statistical analysis

General linear mixed models (GLMM) were used to estimate the variability due to the OMIs for each of the 20 selected lesions as described in Schleicher et al. (2013). The heterogeneity in the health state of the pigs, the variation due to farms of origin, piglet producer, quarter and medication (PCV2 vaccination, deworming, scabies treatment) were considered in the statistical models. For each lesion the amount of dispersion among OMIs and the farm of origin were quantified in the model using Variance Partitioning Coefficients (VPCs). Special care was taken that a balanced sample was given for the models. Each of the 12 OMIs examined more than 6.000 plucks and more than 7.000 carcasses. The latter ensured that each OMI inspected plucks and carcasses of at least 51 out of 72 farms, and that pigs originating from one farm were examined by at least 7 OMIs. The models were implemented in R (version 2.14.2) using the package lme4.

Training of veterinarians in meat inspection

Eleven of the twelve OMIs participated in the practical training. Each of the OMIs had to examine 12 preselected carcasses and plucks within 12 minutes. They were asked to record lesions out of a list of 14 pre-defined pathological abnormalities, namely: mild, moderate and severe forms of pneumonia, pleuritis visceralis, blood aspiration, scalding water lungs, pericarditis, milkspots, perihepatitis, hepatitis, skin lesion related to a) slaughter technology b) infectious agent and c) animal welfare, scabies, pleuritis parietalis and arthritis. Precise definitions and guidelines were given beforehand. Additionally, the OMIs were asked to record multiple lesions, not only the most predominant one. Subsequently, a photo documentation of each lesion was completed.

Results

In the study period January 2008 to June 2011 247.507 pigs were examined by 12 OMIs. In total about 70% of the plucks and approximately 40-50% of the carcasses were recorded with lesions. A detailed list on slaughter statistics is given in Table 1.

The abattoir company’s database was screened and finally 20 lesions, namely 18 pathological abnormalities and 2 findings related to slaughter technology chosen for further evaluation according to their relative frequency (Table 2) and animal health significance.

Table 1. Number of pigs [%] slaughtered in the study period and number [%] of plucks, carcasses and number of pigs (carcass + pluck) recorded without lesion.

criteria	2008	2009	2010	2011*
	number [%]			
Pigs total	56.760 [22.9]	68.013 [27.5]	74.890 [30.3]	47.842 [19.3]
Pigs (fattening ¹)	18.348 [7.4]	21.713 [8.8]	26.673 [10.8]	16.045 [6.5]
Pigs (farrow ²)	38.412 [15.5]	46.300 [18.7]	48.217 [19.5]	31.797 [12.8]
Plucks (P) no lesion	16.089 [28.3]	20.267 [29.8]	23.541 [31.4]	13.770 [28.8]
Carcasses (C) no lesion	27.191 [47.9]	33.318 [49]	42.822 [57.2]	28.271 [59.1]
C + P no lesion	8.984 [15.8]	11.672 [17.2]	15.759 [21]	1.397 9.6]

¹originating from fattening farms ²originating from farms with farrow to finish units

*6months

Range of relative frequencies in lesions was found highest for blood aspiration, followed by scalding water, hepatitis, bursitis and skin lesion. The most frequent lesion was bursitis in carcasses (13.8%) and pneumonia (+/+/+/++) in plucks (30.4%). However, a simple descriptive analysis will not allow a distinction between lesion variability due to farm/herd management and/or OMI performance. Therefore, a statistical analysis (GLMM) was conducted to estimate the influence of OMI and farm on each of the 20 lesions and to determine the level of standardization and homogeneity in meat inspection as an indicator for data quality. The results of the models include fixed effects (influence of quarterly time effect, farm type and piglet producer). Largest variance among OMIs (VPC estimates) was given for the finding skin lesion (3.9-20.8%), followed by blood aspiration (8.2-19.8%) and hepatitis (2.9-18.9%). A negligible amount of variation was determined concerning pericarditis (0 – 0.1%), peritonitis (0-0.6%) and arthritis (0-0.4%). Particularly large variation on farm level was shown concerning milkspots. Subsequent analysis of the raw data revealed that the pigs from 2 of the considered piglet producers showed a noticeably larger risk for severe milkspots (>3). Training of the OMIs improved the detection rate of blood aspiration (90.9-100%) and pleuritis visceralis (63.6-90.9%). However, hardly any improvement was given for the detection of skin lesions. Localization and severity of the skin lesions affected highly the detection rate of the OMIs (18.2-100%). In addition, a certain disagreement in recording multiple lesions was noted between OMIs. The

performance among meat inspectors to assess different forms of pneumonia were found acceptable, but need to be further improved. Lowest variability was given for pneumonia +++, which was found consistent with the model.

Discussion

Meat inspection might be considered standardized and homogeneous if the probability of a specific post-mortem finding is independent of the OMI carrying out the examination. Emphasis was laid to establish a model fitted for real work conditions. In contrast to trial designs no repeated measurements or reference standards were available. Focus of the work was not the estimation of the rater's sensitivity and specificity, but rather the analysis of the variation of the probabilities of a finding between different OMIs. It was considered essential in the model to take the variation between farms and seasonal effects into account to reduce the influence of the heterogeneity in the health state of pigs. Otherwise, the model fit might be poor, in particular for infrequent findings with low relative frequency (e.g. scabies). Findings for which there were different levels of gradation to choose from (i.e. pneumonia) typically exhibited larger variation among OMIs. In general good cooperation between trainer and OMIs was given. OMIs, who participated in the study, voluntarily gave feedback on how to improve the abattoirs' data recording system.

Conclusion

Inspection of pigs at slaughter has been widely used in epidemiological studies of risk factors associated with raised prevalence of lesions. However, the prevalence of lesions recorded in databanks must be based on valid data and reflect a certain consistency in data recording of OMIs to establish a functional post-mortem feedback information system. The statistical model (GLMM) was found an essential and helpful tool to estimate on the one hand the amount of variation in post-mortem findings that can be accredited to the OMI and on the other hand as a training base for OMIs. As a consequence of the statistical analysis and training of OMIs an improved code record system will be established in the abattoir. Focus was laid to assess fewer findings, but with high animal health significance in the future. Precise guidelines and definitions on each lesion were provided for the OMIs in cooperation with the veterinary section of the local government. Frequent training sessions and a re-evaluation of the OMIs' performance might be considered in the future to standardize meat inspection on the long term.

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References

EUROPEAN COMMISSION REGULATIONS (EC) 854/2004 of the European parliament and of the council of 29 April 2004 laying down specific rules for the organization of official controls on products of animal origin intended for human consumption

EUROPEAN COMMISSION REGULATIONS (EC) 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organization of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004

SCHLEICHER, C., SCHERIAU, S., KOPACKA, I., WANDA, S., HOFRICHTER, J., KOEFER, J., 2013, Analysis of the variation in meat inspection of pigs using variance partitioning. Preventive Veterinary Medicine, <http://dx.doi.org/10.1016/j.prevetmed.2013.05.018>

Table 2. Overview on the total relative frequency (r.f., %) assessed by 12 OMIs and range of r.f. (min.—max.) for each OMI for 20 selected lesions over the full study period (01/08 – 06/11).

Carcass		%		Pluck	
finding	r.f. (%)	min.-max.	finding	r.f. (%)	min.-max.
bursitis	13,8	4.9-27.1	pneumonia +	13,8	3.2-26.8
arthritis	0,6	0.2-1.4	pneumonia ++	11,9	4.8-23.5
scabies	0,5	0-6,3	pneumonia +++	4,7	1.5-16.2
skin lesion	8,6	0-19,8	pleuritis visc.	14,1	4.9-22.2
pleuritis +	7,3	3.6-13.2	blood aspiration	17,7	1.2-41.5
pleuritis ++	8,3	3,9-12	scalding water	12,9	2.1-35.2
abscess	1,5	0.3-3.0	lung adhesion carcass	0,5	0.1-0.9
peritonitis	0,1	0-0,5	pericarditis	3,1	2.5-3.9
			milk spots ≤3	2,5	1.0-3.9
			milk spots >3	4,7	2.0-10.1
			hepatitis	8,3	0.9-29.1
			perihepatitis	2,7	0.9-6.1

Data on distribution and characterization of *Listeria monocytogenes* strains in a pork slaughter and cutting plant in Quebec support an earlier surveillance in the meat production chain.

Fravallo, P.^{1,2,3*}, Larivière-Gauthier, G.^{1,2,3}, Kerouanton, A.⁵, Quessy, S.^{1,2,3}, Fournaise, S.⁴, Bekal, S.⁶ and Letellier, A.^{1,2,3}.

1 NSERC, Industrial Research Chair in Meat Safety, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

2 CRIPA, Centre de recherche en infectiologie porcine et avicole, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

3 GRESA, Groupe de recherche et enseignement en salubrité alimentaire Faculté de médecine vétérinaire, Université de Montréal

4 Olymel S.E.C.

5 Anses, The French agency for Food, Environmental and Occupational Health Safety-Ploufragan (France)

6 LSPQ Laboratoire de santé publique du Québec -Sainte-Anne-de-Bellevue (Québec).

*corresponding author : philippe.fravallo@umontreal.ca

Listeria monocytogenes is a major public health concern. It has been associated with highly mediated outbreaks. It was the case in 2008, when an important episode (23 deaths) in Canada showed the need to reinforce the policies to better control *Listeria* in ready-to-eat products. Improved surveillance in the production plants environment increased the detection levels and represents a challenge for both industries and control agencies. In Canada, there is currently no surveillance policy of this microorganism in the steps preceding ready-to-eat production. The distribution, diversity and characterisation of this microorganism in the slaughter and cutting environments are not well documented either. The aim of the present study is to provide such data, starting with a plant in Quebec. Moreover, recent studies suggested that the ability to produce biofilm and some virulence attributes (presence of SNP in InlA) are linked with “environmental” strains compared to virulent ones. Such characterization will be presented. The plant was sampled at three occasions, after the cleaning and disinfecting procedures, during a two-year period. A total of 874 samples were collected. *Listeria* detection followed the Health Canada standard method, geno-serogrouping was obtained by PCR and isolates were compared by PFGE after *Apa*1 and *Asc*1 restriction. Four serogroups (mainly I/IIb) were found, and 6 out of 21 pulsotypes were regularly or sporadically associated to human clinical strains profiles. We observed an increase in occurrence of *Listeria monocytogenes* following the processing steps (χ^2 p<0.05). On the contrary, the diversity strongly and repeatedly decrease from step to step : 96.1% of the strains recovered from the cutting room presented the same profile. The “resident strains” have not been represented in clinical cases surveillance. Biofilm formation ability of strains alone could not explain strain transitions. Our results indicate that better knowledge of *Listeria monocytogenes* before food processing could be helpful to optimise *Listeria monocytogenes* control under a risk analyse approach.

Introduction :

Listeria monocytogenes is the etiologic agent of listeriosis. The frequently deadly disease has been recognized as a food-borne disease since 1984. Then, numerous outbreaks were identified, associated with consumption of heavily contaminated “ready to eat” products (RTE). Canadian regulation (Health Canada Agency, 2011) requires from RTE food industry, including meat and delicatessen production, the control of *L. monocytogenes* both from products and from surfaces in the plant. The introduction of the bacteria in plants, via raw products, is scarcely documented in Canada. Nevertheless, a recent study confirmed raw meat as the main source of *L. monocytogenes* in the meat transformation industry (Choy *et al.*, 2012) and rare previous works, from the end of the previous century, suggested the presence of a single, largely distributed, clone from slaughter (Giovannacci *et al.*, 1999). The present study aimed to describe and follow the evolution of *L. monocytogenes* strains in a pig slaughter/cutting plant in Quebec. Strains were characterised and compared with provincial surveillances data from food and clinical.

Material and method:

Sampling: In the lairage, 60 pen floors were sampled (10 X 100cm²) just after emptying. One year later, in the same plant, during 3 successive sampling (one month separated) a total of 274 surface samples were done, after sanitation procedures. Samples consisted in swabbing of 900 cm² after gentle mechanical mobilisation (individual brushes) of the surfaces to be sampled. The slaughter (pre and post evisceration, chilling room) and the cutting zones (surfaces in contact and not with

meat) of the plant were sampled by a total of 92 sites, systematically swabbed per visit.

Detection: *L. monocytogenes* detection followed a procedure derived from MFHPB-30 standard (Health Canada): 2 enrichment steps, respectively 24h at 30°C and 48h at 37°C, in UVM-1 and Fraser broths. Each broth was isolated on chromogenic Aloa agar, incubated 24h at 37°C.

Identification: typical colonies (max. 2 per positive sample) were confirmed and geno-serogrouped by a multiplex PCR protocol (K rouanton *et al.*, 2010). PFGE profiles (2 enzymes *AscI* and *ApaI*) were obtained according to the CDC Pulsenet standardized protocol (Graves, 2001) for each isolate. Profile comparisons were conducted using BioNumerics® (Applied Maths) software. Strain pulsotype was determined and a new pulsotype was attributed when the profile similarities were strictly fewer than 100%, according to Dice correlation coefficient (1% band position tolerance). Diversity in the strain collections from the different zones of the plant was quantified by Simpson Index calculation.

Characterization: biofilm forming ability of strains was assessed by a biomass producing test: static incubation of strains, 24h in 1/20 TSB-Ye broth in round bottom micro-plates, and staining with crystal violet (Djordjevic *et al.*, 2002). Strains were compared to the collections established during *L. monocytogenes* surveillance, food associated and clinical origin, in Quebec from 2001.

Results:

L. monocytogenes detections from surfaces in the raw meat processing zone were not infrequent after sanitation procedures. Several geno-serogroup including those relevant in a public health perspective: groups IIB and IVB were found. In this study, the more frequent serogroups were different in the different zones: lairage (IIA), slaughter (IIC) and cutting (IIB) (table 1).

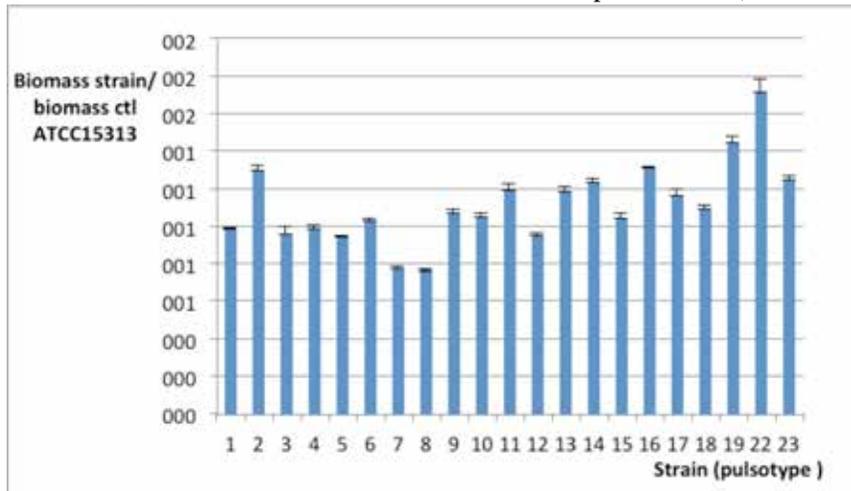
Table 1: synthesis of the identification of *L. monocytogenes* isolates from the lairage to the cutting zones.

Zone (Simpson index)	Geno-serogroup	# of isolates	% per zone	Pulsotypes		
Lairage (D=0.01)	IIA	8	73%	2, 3, 11, 12, 19, 22, 23		
	IIB	1	9%	7		
	IVB	2	18%	4, 6		
				Sampling 1	Sampling 2	Sampling 3
Slaughter (D=0,19)	IIA	1	8%	18	-	-
	IIB	5	38%	1, 8, 10	5, 9	-
	IIC	7	54%	16	16	16, 17
Cutting (D=0,69)	IIA	8	9%	11, 13, 15	11, 13, 14	
	IIB	74	86%	1, 5	1, 5	1, 5
	IIC	3	4%	17	16	16
	IVB	1	1%			6

The increase of the value of Simpson index well described that the diversity (number of different pulsotypes per zone) was high in lairage and low in cutting zone (despite it was easier to detect *L. monocytogenes* from surfaces in cutting zone) (Table 1). This suggests a selection, a sorting, of strains between lairage and slaughter and moreover between slaughter and cutting zones (surfaces sampled concomitantly). In slaughter, the pulsotype 16 strains were predominant and detected at each visit. They became sporadic and were replaced by pulsotype 1 and 5 in the cutting zone. Those pulsotypes (1 and 5) were present but much less abundant in slaughter.

This transition could not be explained by a switch during chilling of the carcasses (as it

Fig.1: Comparison of biofilm ability of the *Listeria monocytogenes* strains, named according to their pulsotype. (Mean values standardized against ATCC15313; bar: standard deviation; n>15 from 2 independent trials)



could be frequently evocated) as *L monocytogenes* were seldomly detected in the chilling room in our conditions and presented the pulsotype 18, never shown anywhere else during the study.

Some pulsotype present in lairage appeared at some occasions in the cutting place (pulsotype 6 and 11).

Some difference in biofilm forming abilities was shown depending on strain pulsotypes. Pulsotypes 1 and 5 strains did not present particular properties in biofilm forming. Pulsotype 16, and strains 19 and 22, were the strains that shown the higher values in biomass forming.

Table 2: Distribution of the strains in surveillance of food chain/human cases (*Detected in food during outbreak investigations).

	Not previously detected	Previously detected during food surveillance		Previously detected in human cases	
		regularly	sporadically	regularly	sporadically
Pulsotype as determined in the present study	2; 3; 4; 8; 10; 11; 12; 13; 16; 18; 23		1; 5; 15; 17	14*	6;7; 9; 19*; 22

The strains (pulsotype 1 and 5), frequently detected in cutting plant, have never been associated with human cases, nor pulsotype 16 and 17 strains frequent in slaughter. But 6 out the 21 strains pulsotypes isolated were previously (regularly for pulsotype 14 or sporadically) detected in patients (Table 2).

Discussion:

In the conditions of this study, isolation of *L monocytogenes* from environment of the plant, after sanitation procedures, described an increasing in homogeneity in the strain collections, from slaughter to cutting zone. The homogeneity of the *L monocytogenes* population in the cutting room is in agreement with previous study Giovannacci *et al.*, 1999). Ability to form biofilm, as assessed in vitro, didn't appear to be the key to explain persistence.

But we described from lairage and slaughter that numerous strains are susceptible to enter the plant, and some can persist in the slaughter environment. It encourages reconsidering the idea that the primary production is a weak source of *L monocytogenes* in meat production (Farzan *et al.*, 2010 and Wesley *et al.*, 2008), two studies that didn't used chromogenic agar for strain isolation. Consideration of the "entering strains" is relevant (Boscher *et al.*, 2012) as in our study, some of them presented a profile related to clinical strains.

For industrial and public health considerations, better knowledge of the *L monocytogenes* present before the RTE step of meat production chain (identification of the persistent strains in plants and characterization of their virulence properties, e.g. integrity of the InlA gene sequence) should be particularly relevant to support efficient control measures.

Conclusion:

Many different *L monocytogenes* strains enter the meat production chain, some sorting exists in subsequent steps. Surveillance earlier in the primary production would permit to adapt the control measures for raw meat production and at the processing step (RTE level).

References:

K rouanton A, Marault M, Petit L, Grout J, Dao TT, Brisabois A. Evaluation of a multiplex PCR assay as an alternative method for *Listeria monocytogenes* serotyping. *J Microbiol Methods*. 2010 Feb;80(2):134-7.

Giovannacci I, Ragimbeau C, Queguiner S, Salvat G, Vendevre JL, Carlier V, Ermel G. *Listeria monocytogenes* in pork slaughtering and cutting plants. Use of RAPD, PFGE and PCR-REA for tracing and molecular epidemiology. *Int J Food Microbiol*. 1999 Dec 15;53(2-3):127-40

Choi YM, Park HJ, Jang HI, Kim SA, Imm JY, Hwang IG, Rhee MS. Changes in microbial contamination levels of porcine carcasses and fresh pork in slaughterhouses, processing lines, retail outlets, and local markets by commercial distribution. *Res Vet Sci*. 2012 (12)348-7.

Graves LM, Swaminathan, B. PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. *Int J Food Microbiol* 2001;65:55-62.

Djordjevic D, Wiedmann M, McLandsborough LA. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl Environ Microbiol.* 2002 Jun;68(6):2950-8.

Farzan A, Friendship RM, Cook A, Pollari F. Occurrence of *Salmonella*, *Campylobacter*, *Yersinia enterocolitica*, *Escherichia coli* O157 and *Listeria monocytogenes* in swine. *Zoonoses Public Health.* 2010 Sep;57(6):388-96.

Wesley IV, Larsen S, Hurd HS, McKean JD, Griffith R, Rivera F, Nannapaneni R, Cox M, Johnson M, Wagner D, de Martino M. Low prevalence of *Listeria monocytogenes* in cull sows and pork. *J Food Prot.* 2008 Mar;71(3):545-9.

Boscher E, Houard E, Denis M. Prevalence and distribution of *Listeria monocytogenes* serotypes and pulsotypes in sows and fattening pigs in farrow-to-finish farms (France, 2008). *J Food Prot.* 2012 May;75(5):889-95

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***Yersinia enterocolitica* prevalence, on fresh pork, poultry and beef meat at retail level, in France.**

Esnault E^{*}.^{1,2}, Labbé A.^{1,2}, Houdayer C.^{1,2}, Denis M.^{1,2}

⁽¹⁾ Anses Laboratory of Ploufragan/Plouzané , Unit of Health and Quality of Poultry And Pig Products, BP 53, 22440 Ploufragan, France.

⁽²⁾ European University of Brittany, France.

*corresponding author: emilie.esnault@anses.fr

Abstract

Y. enterocolitica is a zoonotic agent, and the third bacterial cause of human enteritis in Europe. The objective of this study was to assess consumer exposure to the pathogen *Y. enterocolitica* through meat consumption over a one-year period, in France. In this context, the prevalence of *Y. enterocolitica* was established on samples of fresh pork, beef and poultry collected at retail level in France. Of the 649 samples, 5.1% (34) were positive for *Y. enterocolitica*. No significant difference in prevalence between the categories of fresh meat was observed: the prevalence was 5.2 % for pork, 5.2% for beef and 5.9% for poultry meat. However, tongues of pork were highly contaminated by *Y. enterocolitica* (12.5%) compared to other type of meat.

Although the isolation methods of *Y. enterocolitica* was done by a method promoting the detection of pathogenic biotypes 1B, 2, 3, 4 and 5 (enrichment ITC and streaking on CIN), only strains carrying biotype 1A were isolated. Strains of biotype 1A are considered non-pathogenic for humans. However, recent studies indicate that biotype 1A strains isolated from clinical cases have in their genomes genes known to play a role during disease. The presence of the three virulent genes *inv*, *myfA* and *ystB* were tested; the gene *ystB* being strongly related to the clinical biotype 1A. The three genes were detected for some of our strains and 71% of our isolates carried the *ystB* gene. Thus, these strains can be potentially pathogenic for humans.

Introduction

Y. enterocolitica is an important food-borne enteropathogen, known to cause a wide variety of clinical symptoms ranging from mild gastroenteritis to invasive syndromes like terminal ileitis (Bottone, 1999). *Y. enterocolitica* is the third bacterial cause of human enteritis in Europe (EFSA and ecdc, 2012).

This pathogen is of particular concern for consumers' safety because it is able to growth in food stored at refrigeration temperatures without apparent signs of spoilage.

The species *Y. enterocolitica* is divided into six biotypes. The biotype 1A generally regarded as nonpathogenic and the pathogenic biotypes 1B, 2, 3, 4, 5 (Wauters *et al*, 1988). In France and most other countries worldwide, biotype 4 is the most prevalent biotype isolated from humans (69%), followed by biotype 2 (30%) and biotype 3 (Savin and Carniel, 2008). Genes involved in virulence have been characterized. Among them, the gene *inv* for invasive gene mediates cell invasion, the *myf*-gene encodes the production of fibrillae Myf and the *yst*-gene which encodes enterotoxin.

Y. enterocolitica has frequently been isolated from animals, food and environment (Falcao *et al.*, 2006). Pigs are considered the principal reservoir for human pathogenic strain of *Y. enterocolitica*. Pigs do not develop clinical signs, but they do carry *Y. enterocolitica* in their oral cavity, on tongues and tonsils, and in lymph nodes, and excrete this bacterium in their feces (Thibodeau *et al.*, 1999).

It is assumed that the main sources of infection in humans are pig and pork products (Bottone, 1999; Gousia *et al.*, 2011), however *Y. enterocolitica* have been found on other type of meat (Bonardi *et al.*, 2010).

A one year survey, done on pig tonsils at slaughterhouse in 2010-2011 by our laboratory, estimated that 13.7% of the pigs carried *Y. enterocolitica* and that 74.3% of the pig batches contained at least one positive pig (Fondrevez, 2012). Most of the strains were of biotype 4. In this context we investigate the occurrence of *Y. enterocolitica* in the major meat species, pork, beef and poultry at retail level in France. The isolated stains were then screened for the presence of virulence genes.

Material and Methods

Food samples

A total of 649 raw meat samples was collected at retail level in France during the year 2012. Samples were consisted in raw meat of pork (n=237), beef (n=210) and chicken (n= 202). All the samples were kept at 4°C during transport and during

storage before analysis. The analysis was done within 4 days after purchase.

Microbiological analysis

The presence of *Y. enterocolitica* in meat samples was assessed as follow: 25g of meat were diluted 1:10 in peptone salt broth (AES chemunex) and homogenized in stomacher for 90s. One ml of this suspension was then placed in a tube containing 9 ml irgasan–ticarcillin–potassium chlorate (ITC) broth (Bio-Rad, Marnes La Coquette, France).

The ITC enrichment broth was incubated for 48h at 25°C. Streaking was done on cefsulodin–irgasan–novobiocin (CIN) agar plates (*Yersinia* Selective Agar Base and *Yersinia* Selective Supplement, Oxoid, Basingstoke, UK).

After 24h at 30°C, we checked for the presence of typical colonies on CIN plates. We then streaked a maximum of four characteristic colonies per sample on plates containing the *Y. enterocolitica* chromogenic medium (YeCM) (prepared in the laboratory as described by Weagant, 2008), for the presumptive selection of *Y. enterocolitica* isolates carrying pathogenic biotypes (red “bull’s-eye” colonies) and of *Y. enterocolitica* isolates carrying the biotype 1A (bleu-purple colonies). Each isolate was then subcultured on Plate Count Agar (PCA) plates (AES, Bruz, France) and incubated at 30°C for 24h for biochemical assays. The ability of isolate to degrade urea was used to confirm that the isolates belong to *Yersinia*. Strains were stored in peptone glycerol broth, at –80°C.

DNA extraction and Real-Time PCR for detection of virulence genes

Real Time PCR was used to evaluate the presence of virulence genes *inv*, *myfA* and *ystB*. Strains were sub-cultured on PCA at 30°C for 24h. DNA was extracted from some colonies with QIAamp DNA mini kit (Qiagen, USA) following the manufacturer’s instructions. The PCRs were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California), in a final volume of 25µl with the Sybr® Green Jumpstart™ Taq ReadyMix™ (Sigma-Aldrich, Saint Louis, Missouri) at 1X. The genes *inv* (Rasmussen *et al.*, 1994), *myfA* (Kot and Trafny, 2004) and *ystB* were detected with specific primers as indicated in table 1. The final concentration of primers in the PCR reaction was 0.3 µM. The amplification conditions for each gene are detailed in Table 1.

Table 1: Primer sequences and PCR conditions for detection of virulence genes by Real-Time PCR

Genes	Primer sequence	First step	Cycle of amplification	Melt Curve	Size in bp	Expected Tm
<i>inv</i>	F-CTG TGG GGA GAG TGG GGA AGT TTG G R-GAA CTG CTT GAA TCC CTG AAA ACC G	94°C 2 min	34 cycles of Denaturation 94°C 60 sec Annealing 61°C 60 sec Extension 72°C 30 sec	56°C -> 95°C, increment of 0.5°C for 5 sec	570	87.5°C
<i>myfA</i>	F - CAG ATA CAC CTG CCT TCC ATC T R- CTC GAC ATA TTC CTC AAC ACG C	94°C 2 min	35 cycles of Denaturation 94°C 60 sec Annealing 58°C 90 sec Extension 72°C 90 sec	56°C -> 95°C, increment of 0.5°C for 5 sec	272	84.5°C
<i>ystB</i>	F-AAA GCG TGC GAT ACT CAG AC R-CAG CAT ACC TCA CAA CAC CA	95°C 5 min	34 cycles of Denaturation 94°C 30 sec Annealing 55°C 30 sec Extension 72°C 30 sec	56°C -> 95°C, increment of 0.5°C for 5 sec	68	79°C

Results

A total of 649 samples, including raw meat of pork, beef and chicken were obtained from different supermarkets in France. Of the 649 samples, 5.1% (32) were positive for *Y. enterocolitica*. Although the isolation methods of *Y. enterocolitica* was done by a method promoting the detection of pathogenic biotypes 1B, 2, 3, 4 and 5 (enrichment ITC and streaking on CIN), only strains carrying biotype 1A were isolated. The prevalence of this biotype in the different categories of meat was 5.2% for pork, 5.2% for beef and 5.9% for poultry meat (Table 2).

No significant difference in prevalence of *Y. enterocolitica* was observed between the three categories of fresh meat. Among pork meat sample, the tongues showed the highest prevalence (12.5%) followed by the minced meat (6.9%) (Table 3). The other pork meats like pork chop, fillet and roast were very little contaminated (2.1%). The occurrence of *Y. enterocolitica* in tongue was significantly higher than that of other pork meats (χ^2 ; $p=0.05$) but not of that of minced meat (χ^2 ; $p=0.42$).

Because recent studies indicate that biotype 1A strains may have in their genomes genes known to play a role during disease, the strain were screened for the presence of virulence genes *inv*, *myfA* and *ystB*. The incidence of virulence genes detected in the three categories of meat is presented in table 4.

Among the strains tested, only six lacked the virulence genes tested. The *inv*, *myfA* and *ystB* genes were detected with an incidence of 65%, 12% and 71%, respectively. The *inv*, and *ystB* virulent genes were detected in the various categories of meat. Only two isolates from pork and two isolates from chicken carried the gene *myfA*. This gene is not detected in isolates obtained from beef meat.

Discussion

In the present work, *Y. enterocolitica* were detected in the three main category of meat. The prevalence was about 5% to 6% for raw pork, poultry and beef meat. This prevalence was lower than that observed in Italy by Bonardi *et al.* (2010) which detected a contamination rate of 15.2% for raw pork meat and 32.5% for chicken meat. In our study, tongues of pork were highly contaminated by *Y. enterocolitica* (12.5%) compared to other type of meat. This is coherent with the carriage of *Y. enterocolitica* by pig for which *Y. enterocolitica* is particularly present in their oral cavity (Thibodeau *et al.*, 1999). The great majority of *Y. enterocolitica* isolates from food product belongs to biotype 1A (Bonardi *et al.*, 2010), which is in agreement with our results. Indeed, none *Y. enterocolitica* carrying enteropathogenic biotype were detected in the present study although the isolation methods used promote the detection of pathogenic biotypes. Like previous studies (Bonardi *et al.*, 2010; Falcao *et al.*, 2006; Grant *et al.*, 1998), the predominant genotype for virulent genes was *inv+* and *ystB+*. The presence of *ystB* is strongly related to the clinical biotype 1A (Grant *et al.*, 1998) and is found in 71% of our isolates. The presence of the three genes *ystB*, *inv* and *myfA* for some of our strains reveals that these strains can be potentially pathogenic for humans.

Conclusion

Our study indicates that consumption of meat from pork, beef or poultry presents a low risk of *Y. enterocolitica* for humans. Indeed, the prevalence of *Y. enterocolitica* is low and strains carried the biotype 1A considered as non-pathogenic for humans. However, some of these strains have several genes associated with pathogenicity especially the *ystB* gene often associated with clinical cases with this biotype. This risk should not be ignored.

Acknowledgements

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References

Bonardi, S., Paris, A., Bassi, L., Salmi, F., Bacci, C., Riboldi, E., Boni, E., D'Incau, M., Tagliabue, S., Brindani, F., 2010. Detection, semiquantitative enumeration, and antimicrobial susceptibility of *Yersinia enterocolitica* in pork and chicken meats in Italy. *J Food Prot* 73, 1785-1792.

Bottone, E.J., 1999. *Yersinia enterocolitica*: overview and epidemiologic correlates. *Microbes Infect* 1, 323-333.

Table 2 : *Y. enterocolitica* contamination rate of the three categories of meat

Categories of meat	Number of samples	Number of positive samples	% of positive samples
Pork	237	11	5.2
Beef	210	11	5.2
Poultry	202	12	5.9
Total	649	34	5.1

Table 3 : *Y. enterocolitica* contamination rate of the three types of pork meat

types of pork meat	Number of samples	Number of positive samples	% of positive samples
Tongue	24	3	12.5
Minced meat	72	5	6.9
Other pork meats	141	3	2.1
Total	237	11	5.2

Table 4: Distribution of virulence genes among the categories of meats

Categories of meat	n° of strains	Virulence genes					
		<i>inv</i>		<i>myfA</i>		<i>ystB</i>	
		number	%	number	%	number	%
Pork	11	6	55	2	18	7	64
Beef	11	8	72	0	0	10	91
Poultry	12	8	67	2	17	7	58
Total	34	22	65	4	12	24	71

- EFSA and ECDC, 2012. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010. EFSA Journal 2012 10, 442.
- Falcao, J.P., Falcao, D.P., Pitondo-Silva, A., Malaspina, A.C., Brocchi, M., 2006. Molecular typing and virulence markers of *Yersinia enterocolitica* strains from human, animal and food origins isolated between 1968 and 2000 in Brazil. J Med Microbiol 55, 1539-1548.
- Fondrevez, M. , 2012. *Yersinia enterocolitica* chez le porc: détection, caractérisation, et prévalence. Mémoire de Thèse, université de Rennes 1, France.
- Gousia, P., Economou, V., Sakkas, H., Leveidiotou, S., Papadopoulou, C., 2011. Antimicrobial resistance of major foodborne pathogens from major meat products. Foodborne Pathog Dis 8, 27-38.
- Grant, T., Bennett-Wood, V., Robins-Browne, R.M., 1998. Identification of virulence-associated characteristics in clinical isolates of *Yersinia enterocolitica* lacking classical virulence markers. Infect Immun 66, 1113-1120.
- Kot, B. and Trafny, E.A. (2004) The application of PCR to the identification of selected virulence markers of *Yersinia* genus. Pol J Vet Sci; 7: 27-31.
- Rasmussen HN, Rasmussen OF, Andersen JK, Olsen JE. 1994. Specific detection of pathogenic *Yersinia enterocolitica* by two-step PCR using hot-start and DMSO. Mol Cell Probes. 8:99-108.
- Savin, C., Carniel, E., 2008. Les diarrhées d'origine bactérienne : le cas de *Yersinia enterocolitica*. Revue Francophone des Laboratoires 400, 49-58.
- Thibodeau, V., Frost, E.H., Chenier, S., Quessy, S., 1999. Presence of *Yersinia enterocolitica* in tissues of orally-inoculated pigs and the tonsils and feces of pigs at slaughter. Can J Vet Res 63, 96-100.
- Wauters, G., Goosens, V., Janssens, M., Vandepitte, J., 1988. New Enrichment Method for Isolation of Pathogenic *Yersinia enterocolitica* Serogroup 0:3 from Pork. App. Env. Method. 54, 851-854.
- Weagant, S. D., 2008. A new chromogenic agar medium for detection of potentially virulent *Yersinia enterocolitica*. J. Microbiol. Met. 72, 185-90.

Prevalence and Antimicrobial Resistance of *Salmonella*, *E. coli*, and *Campylobacter* in Pigs from Swine Producing States in the United States

Abley, M.⁽¹⁾, Fedorka-Cray, P.J.^{(1)*}, Gebreyes, W.⁽²⁾, McKean, J.⁽³⁾, Davies, P.⁽⁴⁾, Thakur, S.⁽⁵⁾, Larsen, S.⁽⁶⁾

⁽¹⁾ USDA-ARS, BEAR, 950 College Station Rd., Athens, GA 30605

⁽²⁾ The Ohio State University, Columbus, OH

⁽³⁾ Iowa State University, Ames, IA

⁽⁴⁾ University of Minnesota, St. Paul, MN

⁽⁵⁾ North Carolina State University, Raleigh, NC

⁽⁶⁾ National Pork Board, Des Moines, IA

*corresponding author: paula.cray@ars.usda.gov

Abstract

The purpose of the study was to determine the prevalence and antimicrobial susceptibility of *Salmonella*, *Campylobacter* and generic *E. coli* (commensal bacteria which may harbor antimicrobial resistance genes) from swine feces collected over one year from the top three swine producing states (Iowa, North Carolina, and Minnesota), which represent 51% of the total pig crop in the U.S, plus Ohio. The prevalence of *Salmonella* (n=462/4426), *Campylobacter* (n=994/1184) and *E. coli* (n=833/845) at the sample level was 10.4%, 98.6% and 83.6%, respectively. Overall, the top three *Salmonella* serotypes were Typhimurium (42%), Derby (25%) and Adelaide (5%) while *C. coli* was the predominant *Campylobacter* species. *Salmonella* serotypes varied by barn within state and strain differences within serotypes by antibiogram and pulsotype were observed. In general, *Salmonella* were most often resistant to Tetracycline (76%), Sulfisoxazole (59%), and Streptomycin (55%); however, serotype variation occurred. *E. coli* was most often resistant to Tetracycline (89%) and the Sulfonamides (33%); *C. coli* were most often resistant to Tetracycline (87%), Erythromycin (43%) and Azithromycin (43%). Less than 5.8% of *E. coli* and 5.6% of *Salmonella* were resistant to Ceftriaxone, a clinically important antimicrobial. Seasonal variations by serotype and resistance were observed.

Introduction

Foodborne illness is a global problem and foodborne infections with *Salmonella* and *Campylobacter* continue to be problematic in the United States with 1.03 million and 0.8 million, respectively episodes of foodborne illnesses a year (1). Collectively, foodborne illness costs in the U.S. are projected to be \$152 billion per year (2). Although gastroenteritis associated with foodborne infections often resolves without treatment, the development of antimicrobial resistance to clinically important antimicrobials remains a significant concern when treatment is indicated (3). Antimicrobials are important for use in both human and food animal production.

Studies have been conducted to determine the prevalence and antimicrobial resistance of food borne pathogens in swine (4,5,6). The purpose of the study was to determine the prevalence and antimicrobial susceptibility of *Salmonella*, *Campylobacter* and generic *E. coli* (commensal bacteria which may harbor antimicrobial resistance genes) from swine feces collected over one year from the top three swine producing states (Iowa, North Carolina, and Minnesota), which represent 51% of the total pig crop in the U.S, plus Ohio.

Material and Methods

Using recent projections from USDA NASS (7), operations with more than 5,000 head accounted for 61.1% of the total hog and pig inventory (projected at approximately 68 million pigs) in 2008 with the ten largest states (Iowa, North Carolina, Minnesota, Illinois, Indiana, Nebraska, Missouri, Oklahoma, Ohio, and Kansas) accounting for over 85% of this inventory. Therefore, we started initial sampling in the top three states, Iowa, North Carolina, and Minnesota which represented 51% of the total swine inventory. Based on National Animal Health Monitoring System Swine 2006 projections of 7.2% *Salmonella* prevalence on-farm (8), the expected total number of *Salmonella* isolates recovered were projected to equal approximately 324 which exceeded our target number of 300 *Salmonella* isolates.

Up to 30 fresh fecal samples were collected per barn pen floor from a total of 148 barns across all states (n=4,426 samples collected from a majority of 1-2 pens/barn) (Table 1); collections were divided by season. Ohio was included to represent smaller operations and contributed 3 barns per quarter (12 barns total).

Salmonella, *E. coli* and *Campylobacter* were isolated using standard culture methods. Antimicrobial susceptibility testing was determined using broth microdilution (Sensititer, Trek Diagnostic System, ThermoFisher Scientific Inc., Cleveland, OH)

and a custom panel of 15 antimicrobial agents according to manufacturer's directions. Resistance was determined using CLSI interpretive standards (9,10).

Molecular subtyping was determined using pulsed field gel electrophoresis (11).

Results

The prevalence of *Salmonella* (n=462/4426), *Campylobacter* (n=994/1184) and *E. coli* (n=833/845) at the sample level was 10.4%, 98.6% and 83.6%, respectively. Overall, the top three *Salmonella* serotypes were Typhimurium (42%), Derby (25%) and Adelaide (5%). Serotypes by state are shown in Table 2.

Salmonella serotypes varied by barn within state and strain differences within serotypes by antibiogram and pulsotype were observed. In general, *Salmonella* were most often resistant to Tetracycline (76%), Sulfisoxazole (59%), and Streptomycin (55%); however, serotype variation occurred. Seasonal variations by serotype and resistance were observed.

C. coli was the predominant *Campylobacter* species which were most often resistant to Tetracycline (87%), Erythromycin (43%) and Azithromycin (43%). *E. coli* was most often resistant to Tetracycline (89%) and the Sulfonamides (33%). Less than 5.8% of *E. coli* and 5.6% of *Salmonella* were resistant to Ceftriaxone, a clinically important antimicrobial.

Discussion

The prevalence of *Salmonella* was 10.4% based on a recovery of 462 positive samples from 4,426 submitted samples. Of the 462 positive samples, 2 serotypes were recovered from 19 samples yielding 481 isolates. It is interesting to note the distribution of serotypes across states in Table 2. With the exception of the top two serotypes and Infantis and Anatum which are recovered from three states, no other serotype is recovered in more than two states.

It is also interesting to note that there is considerable within serotype diversity with respect to within barn recovery by state (data not shown). This may be attributed to the movement of pigs themselves which carry different bacterial flora or the transmission of bacteria by other means. Further diversity within serotype is also noted with respect to acquisition of resistance as some phenotypes exhibit more resistance (Typhimurium) while others tend to exhibit little or no resistance or resistance primarily to the historical antimicrobials (streptomycin, sulfas, tetracycline) (Derby) (data not shown). Although seasonal variation in both prevalence and resistance would not be unexpected, the effect on serotypes and subsequent impact on resistance should be monitored.

Campylobacter coli is not typically associated with human illness. However, since Erythromycin is a drug of choice in human medicine continued surveillance is warranted in addition to the emerging resistance to

Table 1. Number of barns sampled per site.

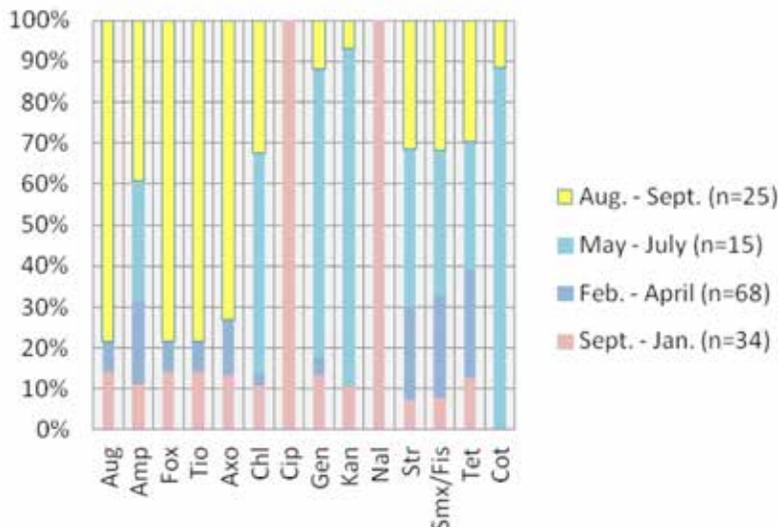
	Sept. - Jan.	Feb. - April	May - July	Aug. - Sept.	Total
A	12	12	12	12	48
B	12	10	14	12	48
C	5	2	4	4	15
D	6	10	10	11	37

Table 2. *Salmonella* serotypes by state

Serotypes	A n=181	B n=137	C n=21	D n=142	Total n=481
Typhimurium, I 4, 5, 12 : i : -, or Albert*	89	57	9	46	201
Derby	15	71	1	33	120
Adelaide	0	0	2	23	25
Worthington	19	0	0	2	21
Infantis	11	0	1	5	17
Mbandaka	15	0	0	0	15
Alachua	0	0	0	11	11
Anatum	1	0	3	4	8
Rissen*	8	0	0	0	8
Johannesburg	0	0	0	8	8

*serotyping was done by using the SMART molecular PCR developed in our laboratory which does not differentiate between Typhimurium, I 4,5,12 : i : -, or Albert ; Rissen was confirmed by PFGE

Figure 1. Seasonal variation in antimicrobial resistance in all *Salmonella* spp. from state D (n=142)



Ceftriaxone, the quinolone Nalidixic Acid, and fluoroquinolone Ciprofloxacin in *E. coli* and *Salmonella*.

Conclusion

Further characterization of persistent versus non-persistent bacterial strains including those that readily acquire resistance more than others may offer areas for development of mitigation strategies.

Acknowledgements

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References

1. Scallan E., Hoekstra R.M., Angulo F.J., Tauxe R.V., Widdowson M.A., Roy S.L., Jones J.L., and Griffin P.M. 2011. Foodborne illness acquired in the United States--major pathogens. *Emerging Infectious Diseases* 17: 7-15.
2. Scharff, R.L., Health related costs from food borne illness in the United States. Available at <http://www.producesafetyproject.org/admin/assets/files/Health-Related-Foodborne-Illness-Costs-Report.pdf-1.pdf> Accessed 18 June 2010
3. Lee, L. A., N. D. Puh, E. K. Maloney, N. H. Bean, and R. V. Tauxe. Increase in antimicrobial-resistant *Salmonella* infections in the United States, 1989-1990. *J. Infect Dis.* 170:128-134. 1994.
4. Haley, C.A., Dargatz, D.A., Bush, E.J., Erdman, M.M., and Fedorka-Cray, P.J. 2012. *Salmonella* prevalence and antimicrobial susceptibility from the National Animal Health Monitoring System swine 2000 and 2006 studies. *Journal of Food Protection*. 75(3):428-436.
5. Gebreyes, W.A., Thakur, S., Morrow, W.E. 2006. Comparison of prevalence, antimicrobial resistance, and occurrence of multi-drug-resistant *Salmonella* in antimicrobial-free and conventional pig production. *Journal of Food Protection*. 69(4):743-748.
6. Tadesse, D.A., Bahnson, P.B., Funk, J.A., Thjakus, S., Morrow, W.W., Wittum, T., Degraes, F., Rajala-Schultz, P., Gebreyes, W. 2011. Food borne Pathogens and Disease. Prevalence and antimicrobial resistance profiles of *Campylobacter* spp. isolated from conventional and antimicrobial-free swine production systems from different U.S. regions. *Food borne Pathogens and Disease*. 8(3):367-374.
7. USDA NASS. Overview of the U.S. Hog Industry. Available at <http://usda.mannlib.cornell.edu/usda/current/hogview/hogview-10-30-2009.pdf>. Accessed 28 July 2010.
8. USDA NAHMS Swine Info Sheet. Available at http://nahms.aphis.usda.gov/swine/swine2006/Swine2006_salmonella_infosheet.pdf. Accessed 28 July 2010.
9. CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-first Informational Supplement. CLSI Document M100-S21. Clinical and Laboratory Standards Institute 2013.
10. United States Department of Agriculture. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Animal Arm Annual Report. Washington DC. 2009. United States Food and Drug Administration. NARMS executive report. Rockville, MD 2009.
11. Ribot, E. M., M.A. Fair, R. Gautom. 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella* and *Shigella* for PulseNet. *Food borne Pathogens and Disease*. 3:59-67.

Development in with-in herd prevalence, between herd prevalence and carcass prevalence in Danish pigs and pork compared to number of attributable human cases from 1995 to 2012.

Dahl, J.

⁽¹⁾ Danish Agriculture and Food Council, Axeltorv 3, Copenhagen, Denmark

*corresponding author : jd@lf.dk

Abstract

The Danish Salmonella program for pigs and pork has been running for 20 years, and has resulted in a 90 % reduction of human cases attributed to Danish pork from 1993 to 2012 (Anonymous, 2013). The development in prevalence in breeding herds, sow herds, finisher herds and at carcass level was analysed as part of the ongoing preparations for the fifth Danish Salmonella plan for pigs and pork, which will be initiated from January 2014.

Analyses of the serological surveillance of the prevalence in breeding herds showed an increase in positive herds from 25 % in 1998 to 50 % in 2012. The within-herd prevalence in positive breeding herds fluctuated between 3 and 6% seropositive samples (cut-off 40 OD%) with no clear pattern.

Prevalence in sow herds could not be analysed directly, but two microbiological surveys in 1998 and 2008 showed an increase in positive sow herds.

The serological surveillance in finisher herds showed an initial decrease from 1995 to 1998. From 1998 the number of positive herds increased from 30 % to 65 %. Within herd prevalence in positive finisher herds fluctuated between 6 and 9 % (cut-off 40 OD%) with no clear pattern.

The carcass surveillance program was initiated in 2001. The carcass prevalence on slaughterhouses affiliated with the Danish Agriculture and Food Council dropped from 1.7 % in 2001 to 1.2 % in 2010. In 2010 the sampling area per carcass was increased from 300 cm² to 400 cm², which coincided with an increase in carcass prevalence to 1.5 % for 2011 and 2012. The relative and absolute prevalence of *Salmonella Typhimurium* dropped from 0.8 % in 2001 to 0.4 % in 2012, consistent with the development in human cases attributed to pork, whereas the prevalence of *Salmonella Derby* increased from 0.2 % to 0.4 %, making it more prevalent than *Typhimurium* in 2012. This increase has not been followed by an increase in the number of human cases of *Derby*, which remains low at around 5-10 cases each year.

Overall the results indicate a steady increase in the number of positive herds in primary production. But improved hygiene in the slaughter house kept the carcass prevalence low, and the number of human cases attributed to Danish pork has remained at a low level since 2000 (Anonymous, 2013).

Introduction

In the spring of 2013 the Danish Veterinary and Food Administration initiated an analysis of the results of the Danish Salmonella program for pigs and pork as part of the planning of the fifth Salmonella plan. The development in salmonella prevalence in breeding herds, sow herds, finisher herds and at carcass level was analysed using surveillance data from the ongoing program.

Material and Methods

Breeding and multiplying herds

Ten blood samples are collected each month from 4-7 months old gilts on all Danish breeding and multiplying herds. The blood samples are analysed using the Danish Mix-ELISA (Mousing, 1997).

The surveillance data were analysed in the period from 1996 to 2012. The cutoff for positive samples was OD% >40.

The data were analysed using a zero-inflated binomial model, as described by Stevenson (2005). The zero-inflated model can in one step handle overdispersion caused by excess zeros, due the existence of truly negative herds, and estimate the prevalence in positive herds. Herd effects were included by adding a herd effect as a random effect.

Sow herds

Salmonella surveillance in Danish sow herds is risk based, and the results cannot be used for analyses over time. Instead two bacteriological screenings of sow herds from 1998 (Anonymous, 1999) and 2008 (Anonymous, 2009) were used to evaluate the progress.

Finisher herds

Serological results from the meat-juice Mix-ELISA (Mousing, 1997) from 1995 to 2012 were analysed using the same statistical model as for breeding and multiplying herds (Stevenson, 2005).

The sampling frame was changed several times over the period. To standardise results over the years, only the first sample obtained per herd-month was used for the evaluation. The cut-off was OD% 40.

Carcass swabs

From 2001 to 2010 3*100 cm² per carcass was swabbed on 5 different, randomly chosen carcasses per day per slaughterhouse. The 5 swabs were analysed as one pooled sample. Individual carcass prevalences were estimated by dividing the pool prevalence by 3, following Sørensen (2007).

In 2011 swabbing was extended to 4*100 cm² per carcass to comply with EU-standards. Over the years, microbiological methodology has changed, and PCR-based methods have replaced standard microbiological methods.

Results

Breeding and multiplying herds

After an initial drop from 1996 to 1998, the proportion of positive herds has increased from 25 % in 1998 to 50 % in 2012. The within-herd prevalence has fluctuated over the years, with no clear trend (figure 1). 682 breeding and multiplying herds were active in the period. The number of herds active per year ranged from 208 to 354. The number of breeding and multiplying herds declined over the years, from 354 in 1999 to on average 220 herds per year from 2007 to 2012.

Sow herds

A bacteriological screening based on fecal samples in 1998 found at least one positive sample in 17 % of the sow herds. A screening in 2008 found at least one positive sample in 41 % of the sow herds.

In 1998 the most frequent serotype was *S. Typhimurium*. In 2008 *S. Typhimurium* was found in 29 % of the herds, but *Salmonella Derby* was now just as frequent as *S. Typhimurium*.

Finisher herds

Figure 2 represent results from all herds (from 16000 herds in 1995 to 5000 in 2012). After an initial drop from 1995 to 1998, the proportion of positive herds has doubled from 1998 to 2012 (figure 2). But the average prevalence in positive herds has been fairly constant over the period.

Carcass prevalence

Figure 3 shows the carcass prevalence from 2001 to 2012 from Danish slaughterhouses affiliated to the Danish Agriculture and Food Council (figure 3).

From 2001 to 2010 the prevalence of salmonella positive carcasses dropped from 1.7 % to 1.2 %. In 2011, the swabbed area was extended from 3*100 cm² to 4*400 cm². The apparent prevalence increased to 1.7% the first year.

Salmonella Typhimurium decreased from 0.8 % in 2001 to 0.4 % in 2012. In the same period *Salmonella Derby* increased, and exceeded the *S. Typhimurium* prevalence in 2012.

Figure 1. Development in proportion of positive breeding and multiplying herds and within-herd prevalence in positive herds from 1996 to 2012 in Denmark.

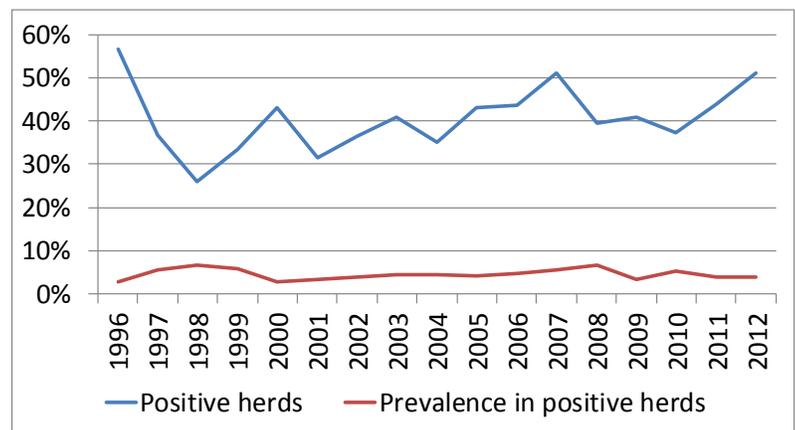
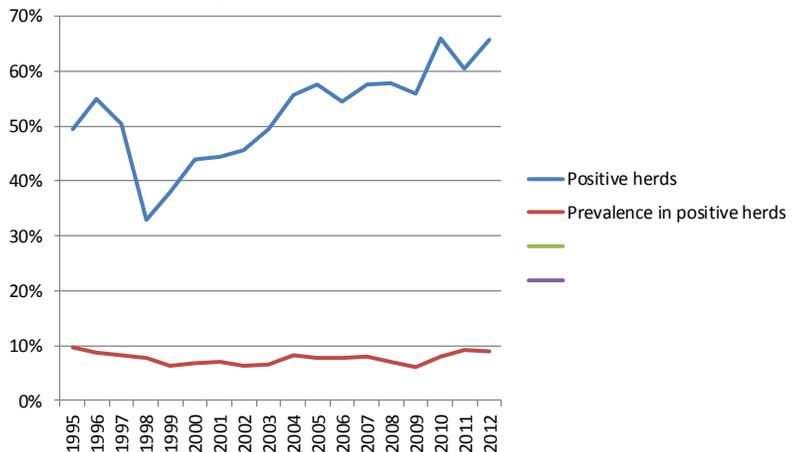


Figure 2. Development in the proportion of positive finisher herds and with-in herd prevalence from 1995 to 2012 in Denmark.



Discussion

The zero-inflated model showed a steady increase in the number of sero-positive breeding herds and finisher herds from 1998 to 2012.

A more detailed analysis of data from 1998 and 2012 showed that herd-size had increased considerably, and the proportion of specialized finisher herds had increased from 50 % in 1998 to 75 % 2012 (details not shown).

The increase in positive herds could not be explained by the increase in herd size, but part of the increase could be attributed to the increase in specialized finisher herds. Specialized finisher production had a higher proportion of positive herds in both years compared to integrated herds (sow herds with production of finishers).

Focus in the Danish salmonella program in primary production has been on reducing high prevalence herds, based on the idea, that buying gilts, weaners and growers from high prevalence herds was more problematic than buying from medium to low prevalence herds.

This allowed for a continued spread of infection from herd to herd.

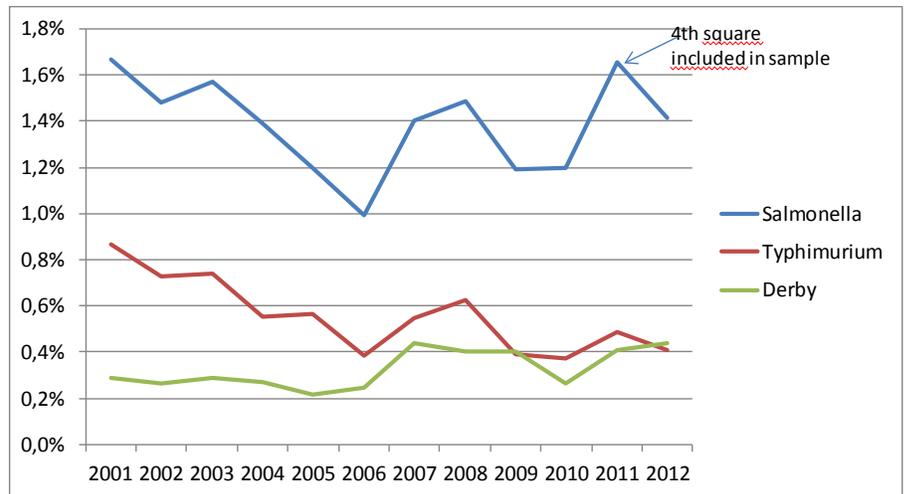
In 2010 the declaration system for breeding, multiplying herds and sow herds was changed to positive or negative status for each herd, making it easier for sow herds or finisher herds to get salmonella-free replacement animals.

The increase in herd prevalence has not resulted in an increase in carcass prevalence and in human cases. Baptista (2010) showed that the association between herd prevalence and carcass prevalence is positive, although not very strong for large slaughterhouses. The improvement in slaughterhouse technology and hygiene more than compensated for the increase in salmonella prevalence in primary production.

References

- Anonymous (1999). Annual Report on Zoonoses in Denmark 1999. http://www.food.dtu.dk/Publikationer/Foedevaresikkerhed/Annual_report_on_zoonoses.
- Anonymous (2009). Annual Report on Zoonoses in Denmark 2008. http://www.food.dtu.dk/Publikationer/Foedevaresikkerhed/Annual_report_on_zoonoses. P. 20.
- Anonymous (2013). Annual Report on Zoonoses in Denmark 2012. http://www.food.dtu.dk/Publikationer/Foedevaresikkerhed/Annual_report_on_zoonoses. P. 6.
- Baptista, F. M (2010) Factors influencing Salmonella carcass prevalence in Danish pig abattoirs. Preventive Veterinary Medicine 95 (2010) 231–238
- Mousing, J. (1997). Nation-wide Salmonella enterica surveillance and control in Danish slaughter swine herds. Prev Vet Med Feb;29(4):247-61.
- Stevenson, J. D. Analysis of presence/absence data when absence is uncertain (false zeroes) : An example for the Northern Flying Squirrel using SAS. Extension note 74, July 2005.
- Sørensen, L. L.(2006). Estimation of Salmonella prevalence on individual-level based upon pooled swab samples from swine carcasses. Vet Microbiol. 2006 Sep 18.

Figure 3. Carcass prevalence on Danish slaughterhouses affiliated to the Danish Agriculture and Food Council.



Case study: Tuberculation, serology and bacteriology of sows at a farrowing unit suspected of an infection with *Mycobacterium avium*.

Oorburg, D.^{1,5*}, Hiller, A.^{1,5}, Bakker, D.², Wisselink, H.², Buholzer, P.³, Cooremans, S.⁴, Heres, L.⁵, Urlings, H.A.P.^{1,5}

¹Wageningen University, Animal Sciences Group, Wageningen, the Netherlands

²Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands

³Prionics, Schlieren, Switzerland

⁴Lintjeshof, Nederweert, Netherlands

⁵VION Food Group, Boxtel, The Netherlands

* VION Food Group, P.O. Box 1, 5280 AA Boxtel e-mail Derk.Oorburg@vionfood.com

Abstract

Mycobacterium avium (MA) is considered a zoonotic hazard in pork. Herds delivered at slaughter showing gross lesions indicative of a mycobacterial infection, eg. specific abscesses in lymphoreticular tissue, were bacteriologically positive for MA.

A risk factor analysis revealing different possible sources of primary infection was carried out at farms supplying these pigs. Also the common farrowing farm supplying the piglets to these farms was taken into account as a possible source of infection.

Intradermal tuberculin testing, serology and tissue sampling was carried out on the sows and finishing pigs.

Positive results in tuberculation, serology and bacteriology of pigs and bacteriology of environmental samples are presented. Intradermal tuberculation below the tail is compared with the standard procedure of testing behind the ear (lege artis). This new method of tuberculation is easier to perform.

Using relevant slaughterhouse information can be an effective tool to improve the control of food safety hazards in the pork production chain.

Introduction

Mycobacterium avium (MA) is considered a zoonotic hazard in pork (1,2) and can cause severe illness in immunocompromised people and young children (3,4).

Known risk-factors for the infection of pigs with MA are feeding of contaminated peat, compost, sawdust/wood shavings and pests (5,6,7,8).

These infections can cause typical gross lesions eg. abscesses in lymphoreticular tissue (9). All slaughter pigs are subjected to post mortem meat inspection (PM) according to EU legislation (EU/854/2004) During PM specific attention has to be given to the presence of zoonotic agents such as mycobacterial infections. Traditionally the mandibular lymph nodes are cut and inspected. In risk based meat inspection MA serology is used (10,11,12).

Pig herds originating from different farms showed typical abscesses in the mesenteric lymph nodes, liver and spleen but sometimes also in the heart muscle, kidneys and lungs.

Risk factor analysis showed two possible routes of contamination; 1) contamination of the animal's drinking water and 2) contamination at the breeder farm because all piglets originated from the same breeder farm.

The objective of this study was to find out if an MA infection could be confirmed in the affected finishing pigs, on the finisher farms, on the breeder farm and in the sows. Additionally, this study also presents an evaluation of an alternative injection site for intradermal tuberculin testing.

Material and Methods

Breeder farm and finishing farms

The breeder farm, located in the South of the Netherlands, holds 3000 clinically healthy sows in group housing and produces approximately 85.000 piglets a year. The farm operates under an integrated farm assurance scheme (Dutch IKB) and has a high level of biosecurity control (eg. full site pest control by a third party, no outside access for the animals, only GMP+ certified feed is fed, all visitors must shower and dress in farm clothes before entering the farm etc.). The piglets are distributed around an age of 10 weeks to different fattening farms in Belgium.

The fattening farms are part of a cooperative group where all animals are fed from the same animal feed source. The different farms operate under an integrated farm assurance scheme (Belgian Codiplan plus) and have a high level of biosecurity control. The pigs are slaughtered at an age of 26 weeks in an abattoir in the Netherlands.

Environmental samples

On 2 finishing farms and the breeder farm environmental samples were collected. Drinking water, biofilm formation and debris in water and feed pipes, wood shavings and feed samples were collected.

Intradermal skin test

To screen the sow herd for a possible MA infection, 1000 newly arrived gilts, 1700 multiparous sows and 500 piglets aged 10 weeks or older were subjected to an intradermal tuberculin test at different visits within 2 months. The skin test was performed by administering 0.1 ml Avian Tuberculin PPD (25.000 I.U., ASG, Lelystad, The Netherlands) into the base of the ear using a McLintock pre-set syringe (Bar Knight, Glasgow, U.K). The reaction was read after 48 to 72 hours by inspecting the injection site for signs of swelling or redness.

To facilitate the skin testing procedure animals were restrained in individual pens making the injection site more accessible. To be able to assess if an alternative injection site would be effective we injected 900 animals both at the base of the ear as well as under the tail about 2 cm out of the median.

Blood sample and tissue sample collection at slaughter

Three consignments of sows (total n=66), all individually identified, were sampled at slaughter. During bleeding blood samples were collected in 10 ml test tubes for serum collection using a coagulation inducer. After coagulation samples were stored and shipped at 4°C to the laboratory. The laboratory carried out the MA-ELISA (PrioCHECK® M. avium Ab porcine).

A gross examination was carried out at PM giving special attention to both mandibular and mesenteric lymph nodes. Samples were collected and identified on the individual sow level to facilitate paired analysis.

Bacteriology and molecular detection by polymerase chain reaction

Samples were cultured as described by Komijn (1) and subsequently ZN positive colonies were further characterized at the species and subspecies level by real time polymerase chain reaction (PCR). Specific primers as described by Slana (13) for the detection of the specific insertion sequences IS1245, present in Mycobacterium avium subspecies avium (MAA) and Mycobacterium avium subspecies hominisuis (MAH) and IS901 present in MAA only, were used.

For the direct detection of MAA and/or MAH in tissue samples the same real time PCR was used as described above (13).

Results/discussion

Fattening pigs

In 15 deliveries from 4 different finishing farms lesions in different organs and lymph nodes were detected indicative of a mycobacterial infection. Prevalence of these abnormalities ranged from 0.5% up to 8.5% within a herd. Bacteriology was performed on affected tissue and revealed the presence of MAA (Table 1).

Sows

At the breeder farm the 500 piglets and the 1000 gilts tested negative in the tuberculin skin test. Of the 1700 sows 139 animals tested positive in the tuberculin skin test.

In 66 sows delivered to slaughter no gross pathological lesions were detected during PM inspection, even though 25 animals showed a positive skin test and 29 showed a positive result in serology (Table 2).

Comparison of ear and tail tuberculin skin testing.

In 900 animals tested both at the base of the ear and under the tail, 32 showed positive reactions in both tests, 4 only in the ear, 8 only under the tail and 856 tested negative (Table 3). This results in a very good agreement of both tests (Kappa= 0.835, CI

95% : 0.77-0.90 (14)) with good relative test characteristics for the alternative injection site compared to the base of the ear (SE=0.800, CI 95% : 0.68-0.92 SP=0.995, CI 95% : 0.989-0.999).

Environmental samples

Environmental samples on one of the finishing farms as well as on the breeder farm showed presence of generic Mycobacteria, but preliminary analysis could not confirm these to be MA.

Conclusion

For the purpose of screening large herds intradermal skin testing under the tail is an effective method compared to testing at the ear base. This facilitates rapid administration when the animals can only be restrained in a way where the head is presented away from the person carrying out the test.

No Mycobacterium avium could be recovered from the environmental samples or from the sows.

Results are indicative of an MA infection in the sows but this could not be proven conclusively at this stage. Further investigation is necessary.

If an infection in the sows can be demonstrated the sources of infection have to be identified to successfully control the spread of MAA within the farm.

This case study show that when information collected during post mortem inspection is fed back to the farm of origin, farmers and veterinarians can take corrective and preventive measures to control zoonotic infections and improve animal health.

References

1. Komijn, R.E., 1999, Prevalence of Mycobacterium avium in slaughter pigs in The Netherlands and comparison of IS1245 restriction fragment length polymorphism patterns of porcine and human isolates. Journal of Clinical Microbiology 1999, 37, 1254-1259.
2. Möbius, P., 2006, Comparative macrorestriction and RFLP analysis of Mycobacterium avium subsp. avium and Mycobacterium avium subsp. hominissuis isolates from man, pig, and cattle. Veterinary Microbiology 2006, 117, 284-291.
3. Biet F., 2005, Zoonotic aspects of Mycobacterium bovis and Mycobacterium avium-intracellulare complex (MAC). Vet Res. 2005 May-Jun;36(3):411-36.
4. Wagner D., 2004. Nontuberculous mycobacterial infections: a clinical review. Infection. 2004 Oct;32(5):257-70.
5. Matlova L., 2004. Impact of sawdust and wood shavings in bedding on pig tuberculous lesions in lymph nodes, and IS1245 RFLP analysis of Mycobacterium avium subsp. hominissuis of serotypes 6 and 8 isolated from pigs and environment. Vet Microbiol. 2004 Sep 8;102(3-4):227-36.
6. Matlova, L., 2005, Distribution of Mycobacterium avium complex isolates in tissue samples of pigs fed peat naturally contaminated with mycobacteria as a supplement. Journal of Clinical Microbiology 2005, 43, 1261-1268.
7. Trckova, M., 2005 Peat as a feed supplement for animals: a review. Veterinarni Medicina 2005, 50, 361-377
8. Fischer O, 2000, Findings of mycobacteria in insectivores and small rodents. Folia Microbiol (Praha). 2000;45(2):147-52.
9. Thoen, C.O., 2006. Tuberculosis. In Diseases of Swine, 9th ed.; Straw B.E., Zimmermann J.J., D'Allaire S., Taylor D.J.

Table 1. Results of Post Mortem meat inspection and bacteriology of finishing pigs suspected of an MA infection.

Finishing pigs					
Farm	Slaughtered	Detected at PM	Bacterial analysis	negative	positive
A	1300	53	25	9	16
B	1980	29	5	0	5
C	671	15	1*	0	1*
D	605	5	5	2	3

* pooled sample of affected tissues of 5 pigs.

Table 2. Results of tuberculin skin testing, PM inspection, serology and bacteriology of 66 sows from a farm suspected of an MA infection.

Sows			
Test	negative	positive	total
Tuberculin skin testing	41	25	66
Post mortem inspection	66	0	66
Serology	37	29	66
Bacteriology	inconclusive		66

Table 3. Results of tuberculin skin testing of animals both in the ear base and under the tail.

Test position		ear		total
		positive	negative	
under tail	positive	32	4	36
	negative	8	856	864
	total	40	860	900

Eds.; Blackwell Publishing: Iowa, USA,2006; pp. 807-822.

10. Wisselink, H.J.; 2010. Serodiagnosis of *Mycobacterium avium* infections in pigs. *Veterinary Microbiology* 2010, 142, 401-407.
11. Hiller A, (2012) Implementation of risk based meat inspection of fattening pigs without incision in a slaughterhouse in Lower Saxony. *Archiv für Lebensmittelhygiene* 63: 4, 107-114
12. Hiller A.. (2013). Prevalence of *Mycobacterium avium* in slaughter pigs based on serological monitoring results. *Int. J. Environmental Research and Public Health*. Accepted.
13. Slana I, 2010 Distribution of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis* in artificially infected pigs studied by culture and IS901 and IS1245 quantitative real time PCR. *Vet Microbiol.* Aug 26;144(3-4):437-43.
14. Landis, J. R., & Koch, G. G. (1977). The measurement of observer agreement for categorical data. *biometrics*, 159-174.

Origin of *Listeria monocytogenes* on meat products.

Oorburg, D.^{1,2*}, Dijkman, R.³, Heres, L.², Urlings, H.A.P.^{1,2}

¹Wageningen University, Animal Sciences Group, Wageningen, the Netherlands

²VION Food Group, Boxtel, The Netherlands

³Gezondheidsdienst voor dieren, Deventer, The Netherlands

* VION Food Group, P.O. Box 1, 5280 AA Boxtel e-mail Derk.Oorburg@vionfood.com

Abstract

Listeria monocytogenes is a relevant food safety hazard in ready to eat products. Inactivation during processing, prevention of recontamination and control of multiplication are the main instruments to secure the safety of meat products. Intensive microbiological monitoring of products and the production environment are valuable tools to assess the level of control in a meat processing plant. During the course of a year all isolates found during hygiene monitoring at a meat processing plant were stored at -70 degrees Celsius. A total of 94 *L. monocytogenes* isolates have been analyzed by pulsed-field gel electrophoresis (PFGE) and were divided into 30 different types.

Some types were observed both in samples taken from the environment and machinery as well as on products, indicating cross contamination of the final product. Two types could be identified at different moments during the year, indicating persistent contamination.

Processing equipment is playing an important role in *L. monocytogenes* contamination of meat products because it was contaminated before the start of processing.

Good hygienic practices, effective cleaning and disinfection and hygienic design of machinery are important to control *L. monocytogenes* on meat products. Genotyping of harvested *L. monocytogenes* isolates is a valuable tool to review the status of hygiene control in processing of meat products.

Introduction

Listeria monocytogenes is a relevant food safety hazard in ready-to-eat (RTE) products with potentially severe consequences (1,2,3). Preventing contamination of RTE products is therefore important. *L. monocytogenes* is an ubiquitous organism, also found in pigs and pork (4). Inactivation during pork processing, preventing recontamination and controlling the multiplication are therefore important points of an HACCP based quality system to assure the control of this hazard.

Food processing facilities can be contaminated with *L. monocytogenes* for long periods of time (5,6). Pulsed field gel electrophoresis (PFGE) has shown to be an effective tool to identify genetic relations of *L. monocytogenes* isolates recovered from food products and processing facilities (7,8). Genetic analysis shows some *L. monocytogenes* strains to be persistently present in a plant where other strains are not (5). Raw materials entering a meat product processing facility are a known source of environmental and product contamination (9,10). Complex processing machines, which are difficult to clean, are known to be the source of persistent contamination (8,11).

The objective of this study was to find the source of contamination of RTE products in a meat processing plant by means of PFGE typing and to determine its effectiveness for hygienic control at a processing plant accordingly.

Material and Methods

Description of the processing plant

The processing plant produces RTE products like fermented products (e.g. salami type), cooked products (e.g. bologna type) and oven-heated pork products (e.g. meatballs). Raw materials are cut, minced and mixed in a designated area for raw material processing. These processed raw materials are either fermented or heat treated and taken to an area with higher hygiene status ("high care area"). Here the finished products are sliced and/or packed to be dispatched. Employees from either area are not allowed to come in contact with each other or to enter the other area to minimise risk of cross contamination.

Sampling and isolates of *L. monocytogenes*

According to the processing plant's sampling plan as prescribed by their HACCP-based quality assurance system, RTE products are sampled on a weekly basis. Incoming raw materials as well as the production area (e.g. floor, drains) and processing equipment are sampled on a regular basis and sample collection was intensified after positive findings in the RTE

Distribution of PFGE pulsotypes in 94 isolates of different origin collected in a meat processing plant.

Origin	n	PFGE types	
<i>product</i>	29		
	RTE	25	8, 9(2), 11(13), 15, 19(4), 23, 26, 29, 30
	raw material	4	12, 13, 16, 17,
<i>environment</i>	65		
	area	27	1, 2, 5, 6, 7, 9(2), 11, 18, 19(9), 20, 21(3), 22, 24, 25, 27, 28
	machine	38	3, 4, 10, 11(28), 14, 15, 19(4), 22
Total	94		

Numbers in **(Bold)** indicate the frequency the PFGE types are found if found more than once.

Genetically identical PFGE pulsotypes in RTE products and in the processing area as well as the processing machines were found. This indicates cross contamination of RTE from the environment.

Only PFGE pulsotype 11 and 19 are considered persistent when using the definition of persistent strains as described by Keto-Timonen (6), (Fig. 2).

This distribution of the PFGE pulsotypes according to the origin (Table 1) combined with the occurrence in time (Fig. 2) suggests that PFGE pulsotype 11 was most likely originating from a persistent contamination of the processing machines. The machine was identified as a former unit used for altering the texture of cooked meat. After this machine was subjected to improved cleaning and disinfection procedures, including sterilizing the unit in an industrial steam cooker before each use, no *L. monocytogenes* could be recovered after intensive sampling.

PFGE pulsotype 19 appears to be persistently present both in the production environment and processing machines. Contamination of RTE could occur from either source further demonstrating the importance of good hygienic practices during food production.

The single PFGE pulsotypes from RTE and production area underline that also new introductions and contamination was occurring.

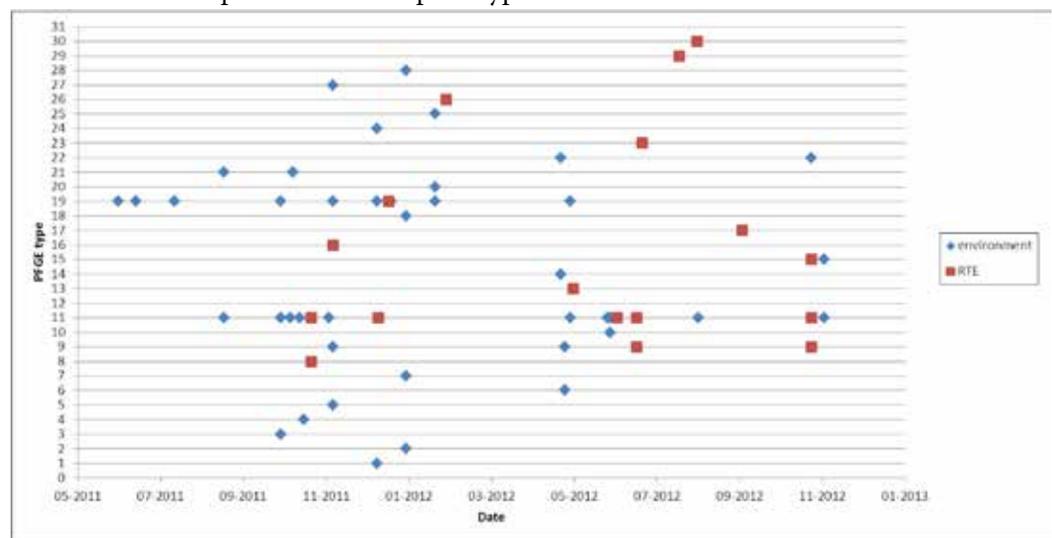
Conclusion

Using PFGE for genotyping of *L. monocytogenes* isolates is a valuable tool to review the status of hygiene control in a meat processing facility. It can reveal important contamination pathways and point out main sources of *L. monocytogenes* contamination which are specific for the processing facility. This provides valuable insights for changing hygienic operating procedures to improve food safety control.

References

1. EFSA, 2013. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011;EFSA Journal 2013,11(4):3129, 250 pp. doi:10.2903/j.efsa.2013.3129
2. CDC. 2012 Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet Surveillance Report for 2011 (Final Report). Atlanta, Georgia: U.S. Department of Health and Human Services, CDC. 2012

Fig 2. Distribution over time of different PFGE pulsotypes recovered from the environment and RTE products. When a PFGE pulsotype was recovered more than once on the same date and the same sample type, it is represented as a single point in the graph. An arrow is used to indicate a persistent PFGE pulsotype.



3. Mead PS, 2006. Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiol Infect.* Aug;134(4):744-51.
4. Autio, T (2000). *Listeria monocytogenes* contamination pattern in pig slaughterhouses. *Journal of Food Protection*®, 63(10), 1438-1442.
5. Lundén JM, 2003, Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. *J Food Prot.* Nov;66(11):2062-9.
6. Keto-Timonen, R (2007). An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. *Journal of Food Protection*®, 70(8), 1866-1873.
7. Autio, T (1999). Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Applied and Environmental Microbiology*, 65(1), 150-155.
8. Berzins, A., (2010). Contamination Patterns of *Listeria monocytogenes* in Cold-Smoked Pork Processing. *Journal of Food Protection*®, 73(11), 2103-2109.
9. Berrang ME, 2010. Colonization of a newly constructed commercial chicken further processing plant with *Listeria monocytogenes*. *J Food Prot.* Feb;73(2):286-91.
10. Hellstrom, S (2010). *Listeria monocytogenes* contamination in pork can originate from farms. *Journal of Food Protection*®, 73(4), 641-648.
11. Lunden, J. M., (2002). Transfer of persistent *Listeria monocytogenes* contamination between food-processing plants associated with a dicing machine. *Journal of Food Protection*®, 65(7), 1129-1133.
12. Anonymous.1996. *Microbiology of food and animal feeding stuffs; horizontal method for the detection and enumeration of listeria monocytogenes. Part 1: Detection method. ISO method 11290-1, 1st ed.* International Organisation for standardization, Genève, Switzerland.

Salmonella serovar distribution and risk factors associated with persistence of shedding in finishing pigs

Pires, A. F. A.⁽¹⁾, Funk, J.⁽¹⁾, Bolin, C.⁽²⁾

¹Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA.

²Diagnostic Center for Population and Animal Health, College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA.

* 1129 Farm Lane, B41 Food Safety Building and Toxicology Building, MSU, East Lansing, MI 48824, USA. Email: pire-sald@cvm.msu.edu; afapires11@gmail.com

Abstract

The objectives of this study were to describe the *Salmonella* serovar distribution and to identify risk factors associated with serovar persistence in finisher pigs. A longitudinal study was conducted in 18 cohorts of pigs. Fecal culture and serotyping were conducted using standard methods. Among the 446 *Salmonella* isolates (total 187 pigs), there were 18 distinct serovars. The six most common serovars were *S. Derby* (47.3%), *S. Agona* (27.4%), *S. Johannesburg* (10.5%), *S. Schwarzengrund* (2.7%), *S. Litchfield* (2.5%) and *S. Mbandaka* (2.2%). Survival analysis techniques, Kaplan-Meier methods and Log rank test were used to estimate the duration of *Salmonella* shedding in days and test differences in shedding by site, sex, serovar and nursery and environment *Salmonella* status. Cox proportional hazard models, including a shared frailty for cohort, were used to analyze the effect of the risk factors (sex, site, nursery status, environment status, and cold exposure) on duration of *Salmonella* shedding. Overall, the Kaplan-Meier median duration of fecal *Salmonella* shedding was 28 days, and the maximum 112 days. The median duration of shedding in *S. Derby* was 28 days and 14 days for *S. Agona* and *S. Johannesburg*. There was a significant difference of duration of shedding among sites, nursery and environmental *Salmonella* status. Site and nursery status were significantly associated with *Salmonella* shedding. These preliminary results suggest that duration *Salmonella* shedding might depend on farm site or cohort level risk factors.

Introduction

Salmonellosis remains a major foodborne disease threat to public health in the United States (CDC, 2010). *Salmonella* serovars have been recognized to have differential ability to cause illness in different species (e.g., host-adapted serovars such as *Choleraesuis* in swine, *Typhi* in humans and *Gallinarum* in poultry). There is further evidence that some serovars, although capable of zoonotic transmission, are more commonly associated with certain food animal species as compared to others (e.g., *Derby* in swine). Multiple *Salmonella* serovars can be isolated within an individual pig (Funk et al., 2000; Gebreyes et al., 2004), within groups of swine sampled at one point in time (Funk et al., 2001; Gebreyes et al., 2004; Rajic et al., 2005), within the same group of swine sampled at different ages/production stages (Funk et al., 2001); and across the same farms sampled over several years (Farzan et al., 2008). Risk factors for persistence of serovars in swine have predominantly focused on environmental contamination or transmission from sows to litters (Funk and Gebreyes, 2004). Dorr et al. (2009) described differential distribution of *Salmonella* genotypes by ecological niches, finding persistence of some genotypes more common in the environment, while other genotypes were more commonly isolated from swine. A few genotypes were identified in both swine and environmental samples. These data suggest differential abilities, at least by genotype, for persistence in different swine associated ecological niches (Dorr et al., 2009). The objectives of this study were: (1) describe *Salmonella* serovar distribution in finishing swine; (2) compare persistence of *Salmonella* by serovar; (3) identify risk factors associated with *Salmonella* persistence.

Material and Methods

The samples were collected from a longitudinal study of growing pigs in a multi-site farrow-to-finish production system (3 finishing sites with 6 cohorts each, 18 cohorts total) located in the Midwestern, United States (Pires et al., 2012). Fifty individual pig fecal samples per cohort were collected and cultured every 2 weeks for 16 weeks (8 collections). Fecal samples were processed and cultured using standard methods described previously (Pires et al., 2012). Identification of serotypes was conducted according to Kauffmann-White scheme and was conducted by MSU Diagnostic Center for Population and Animal Health and NVSL.

Risk factors investigated in this study were: sex (male/female), site, exposure to cold, nursery status and environment *Salmonella* status (Pires et al., 2012). The cold exposure was defined as ordinal variable, number times that the pig was exposed to temperatures below the lower critical temperature of the thermal neutral zone (TNZ) in lag times 12h, 24h, 72h, 1 week and 1 month prior the sampling.

Statistical Analysis:

The relative proportion of serovars-specific to the type of sample was described. To determine measures of serovar persistence on farm, time to event data (days of shedding) was calculated. For individual pigs, the duration of shedding for each new infection was defined as the interval between the sampling date of the first *Salmonella*-positive culture and the sampling date of the last positive culture for an individual pig; 7 days were added to the shedding duration to account for shedding during the half the sampling interval after the last positive culture with the same serovar.

The duration of *Salmonella* shedding (days) was analyzed using Stata (version 11.0; StataCorp LP College Station, TX) using parametric Kaplan-Meier survival graphs and semi-parametric Cox proportional hazard models, handling ties with Breslow method. Kaplan-Meier graphs were used to visually assess the survival curves (by risk factor groups and the four major serovars). Testing the differences in survivor functions across groups (site, sex, nursery and environment *Salmonella* status) was evaluated using the Log-Rank test. Cox proportional hazard models, including a shared frailty (random effect to account within-cohort correlation among pigs) for cohort, were used to analyze the effect of the risk factors (sex, site, nursery status, environment status, and cold exposure) on duration of *Salmonella* shedding (non-specific to serovar, i.e., *Salmonella* spp, total 175 pigs). Results were considered statistically significant at p -value < 0.05. Model diagnostics for the Cox models were based on Cox-Snell and Schoenfeld residuals. The event of interest was *Salmonella* negative (clearance of *Salmonella*), thus survival time was interpreted as the time period (days) a pig shed *Salmonella*. The hazard ratio was calculated for significant variables and interpreted as the higher the hazard ratio, the higher probability for clearance of the infection (the lower the hazard ratio, the higher probability of *Salmonella* shedding).

Results

Among the 446 *Salmonella* isolates (187 pigs total), there were 18 distinct serovars. The six most common serovars were *S. Derby* (47.3%), *S. Agona* (27.4%), *S. Johannesburg* (10.5%), *S. Schwarzengrund* (2.7%), *S. Litchfield* (2.5%) and *S. Mbandaka* (2.2%) (Table 1). Estimation of shedding duration was determined by the 4 major serovars in the 151 pigs. Estimation of shedding duration was determined by site, sex and nursery and environment *Salmonella* status in 175 pigs. Overall, the Kaplan-Meier median duration of fecal *Salmonella* shedding was 28 days, and the maximum 112 days. The median duration of shedding in *S. Derby* was 28 days and 14 days for *S. Agona*, *S. Johannesburg* and *S. Schwarzengrund*. There was a significant difference of duration of shedding among sites, nursery and environment status but not among serovars or sex (p -value < 0.05).

The probability for clearance of *Salmonella* from cohorts with a high *Salmonella* nursery status (positive nursery pools greater than the mean) was 0.66 times lower (hazard risk = 0.65 ; 95% C.I. 0.44- 0.94) than pigs from cohorts with positive nursery pools equal to or lower than the mean. There was significant differences among sites; pigs from site C presented higher probability to clear the infection (shorter *Salmonella* shedding duration) compared to site A. However no difference was found between site A and B (hazard ratio = 0.99; 95% C.I. 0.68-1.47). Environment *Salmonella* status, gender and cold exposure (12h, 24h, 48h, 72h, 1 week and 1month) were not significant predictors of shedding duration in final multivariable models.

Discussion

A number of studies have described *Salmonella* prevalence and serovar distribution, mainly related to risk factor studies in finishing pigs (Funk et al., 2001; Gebreyes et al., 2004; Rajic et al., 2005); but a few have examined the duration of shedding and its association with risk factors. The four most common serovars found in this study have been reported in other studies in North America (Farzan et al., 2008; Funk et al., 2001; Gebreyes et al., 2004; Rajic et al., 2005). A significant difference was observed among sites despite belonging to the same production system and having an identical pig source, feed and overall management procedures. Variability in *Salmonella* prevalence among herds and within the same herd over time has been previously reported (Pires et al, 2013; Funk et al., 2001b; Rajic et al., 2005).

There was a significant influence of nursery status (cohorts with positive nursery pools greater than the mean) on duration of fecal shedding. Pigs entering the finisher from these cohorts were exposed to *Salmonella* in the nursery and may have been shedding at arrival to the finishing barn. It seems that previous infection during the nursery increases the duration of

shedding. Future analysis should be conducted to assess the serovar-specific of nursery on *Salmonella* shedding during the finishing phase.

In this preliminary analysis, cold exposure did not affect the duration of shedding, as opposed to the previous findings on *Salmonella* prevalence (Pires et al., 2013). The variable cold exposure was considered time-constant, future analysis should investigate if the effect varies along the finishing phase, since young pigs are more susceptible to cold stress. In addition, other thermal factors should be investigated such as exposure to heat index and to temperatures above the TNZ.

Conclusion

These results suggest that duration *Salmonella* shedding might depend on farm site or cohort level risk factors. These are preliminary findings. Identification of risk factors associated with duration of shedding may allow more targeted interventions to control *Salmonella* by evaluation of control measures not only for prevalence reduction, but also to decrease the duration of shedding once exposed.

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References

- CDC, 2010, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food - 10 states, 2009. Morbidity and Mortality Weekly Report 59, 418-422.
- Dorr, P.M., Tadesse, D.A., Zewde, B.M., Fry, P., Thakur, S., Gebreyes, W.A., 2009, Longitudinal study of *Salmonella* dispersion and the role of environmental contamination in commercial swine production systems. Appl. Environ. Microbiol. 75, 1478-1486.
- Farzan, A., Friendship, R.M., Dewey, C.E., Poppe, C., Funk, J., Muckle, C.A., 2008, A longitudinal study of the *Salmonella* status on Ontario swine farms within the time period 2001-2006. Foodborne Pathog. Dis. 5, 579-588.
- Funk, J., Gebreyes, W.A., 2004, Risk factors associated with *Salmonella* prevalence on swine farms. Journal of Swine Health and Production 12, 246-251.
- Funk, J.A., Davies, P.R., Nichols, M.A., 2000, The effect of fecal sample weight on detection of *Salmonella enterica* in swine feces. J. Vet. Diagn. Invest. 12, 412-418.

Table 1: *Salmonella* serovar distribution in a longitudinal study in finishing pigs.

Serovar	Individual fecal pigs (%) (n=446)	Nursery (%) (n=62)	Environment (%) (n=33)	TOTAL
S. Derby	211 (47.3)	23 (37.1)	3 (9.1)	237
S. Agona	122 (27.4)	28 (45.2)	4 (12.1)	154
S. Johannesburg	47 (10.5)	3 (4.8)	9 (27.3)	59
S. Schwarzengrund	12 (2.7)	1 (1.6)	0	13
S. Litchfield	11 (2.5)	1 (1.6)	0	12
S. Mbandaka	10 (2.2)	0	0	10
S. Infantis	6 (1.3)	1 (1.6)	0	7
S. Livingstone	5 (1.1)	0	0	5
S. Bovis-morbificans	4 (0.9)	0	0	4
S. Meleagridis	4 (0.9)	1 (1.6)	0	5
S. Montevideo	4 (0.9)	2 (3.2)	17 (51.5)	23
S. Worthington	3 (0.7)	0	0	3
S. Typhimurium	2 (0.4)	0	0	2
S. Barranquilla	1 (0.2)	1 (1.6)	0	2
S. Lexington	1 (0.2)	0	0	1
S. Orion	1 (0.2)	0	0	1
S. Paratyphi B, L-tartrate+	1 (0.2)	0	0	1
S. Rough O:b:l,w	1 (0.2)	0	0	1
S. Muenchen	0	1 (1.6)	0	1

- Funk, J.A., Davies, P.R., Nichols, M.A., 2001, Longitudinal study of *Salmonella enterica* in growing pigs reared in multiple-site swine production systems. *Vet. Microbiol.* 83, 45-60.
- Gebreyes, W.A., Davies, P.R., Turkson, P.K., Morrow, W.E.M., Funk, J.A., Altier, C., 2004, *Salmonella enterica* serovars from pigs on farms and after slaughter and validity of using bacteriologic data to define herd *Salmonella* status. *J. Food Prot.* 67, 691-697.
- Pires, A.F.A., Funk, J.A., Bolin, C., 2012, Longitudinal study of *Salmonella* shedding in naturally infected finishing pigs. *Epidemiol. Infect.* Published online on November 13th 2012; doi 10.1017/S0950268812002464.
- Pires, A.F.A., Funk, JA; Manuzon, R; Darr, M; Zhao, L. Longitudinal study to evaluate the association between thermal environment and *Salmonella* shedding in a Midwestern US swine farm. *Prev. Vet. Med.*(*in press*).
- Rajic, A., Keenlside, J., McFall, M.E., Deckert, A.E., Muckle, A.C., O'Connor, B.P., Manninen, K., Dewey, C.E., McEwen, S.A., 2005, Longitudinal study of *Salmonella* species in 90 Alberta swine finishing farms. *Vet. Microbiol.* 105, 47-56.

Review of the International Commission on Trichinellosis Workshop on Surveillance for *Trichinella*

Pyburn, D.*¹, Gamble, H.R.²

¹United States Department of Agriculture, Des Moines, Iowa, USA

² National Academy of Sciences, Washington, DC, USA

*corresponding author: David.G.Pyburn@aphis.usda.gov

Abstract

The International Commission on Trichinellosis (ICT) provides science-based advice on the subject of *Trichinella*. In May 2013, the ICT held a workshop with the goal of developing recommendations on surveillance for *Trichinella* that could contribute to classifying the public health risk of pork from defined compartments or regions. This workshop was initiated based on discussions at the national and international levels regarding best practices for assuring pork safety relative to *Trichinella*.

A proposed revision of the OIE Terrestrial Animal Health Code Chapter on *Trichinella* provides guidance for establishing negligible risk for *Trichinella* in compartments that are audited for specific controlled management practices. In November 2012, the Codex Alimentarius Committee on Food Hygiene requested that OIE consider an alternative approach for defining negligible risk, allowing surveillance to be used for verification in place of individual farm auditing. Some European Union countries have already been approved for derogation from individual carcass testing based on documenting a low level of risk through animal testing. These derogations are based on testing performed on pigs raised under controlled management conditions.

With the high quality of pig management in industrialized pork production systems, the availability of historical and contemporary testing data, and public health records demonstrating the rare occurrence of trichinellosis in many countries, population-based surveillance programs might be a viable alternative to individual farm audits for verifying negligible risk status.

Given the disparate nature of the various national and international standards for *Trichinella* control, and the lack of specific science-based guidance to document negligible risk through testing and other surveillance data, the recommendations provided by the ICT should form the basis for future decisions on establishing risk categories for pork relative to *Trichinella*.

Workshop Report

A total of 15 participants attended the workshop. Nine participants were elected members of the International Commission on Trichinellosis, including the current and immediate-past presidents. Other participants were selected based on their knowledge of the global pork industry, epidemiology of animal diseases, diagnostic methods, statistical sampling protocols to document prevalence, and analysis of risk for transmission of foodborne disease. The workshop attendees:

Lis Alban, Danish Bacon and Meat Council (Denmark)

Rafael Blasco (OECD-CRP representative)

Alvin Gajadhar, Canadian Food Inspection Agency (Canada) (co-organizer)

Ray Gamble, National Academy of Sciences (USA) (co-organizer)

Ian Gardner, University of Prince Edward Island (Canada)

Joke van der Giessen, National Institute of Public Health and the Environment (Netherlands)

Steve Hathaway, Ministry of Agriculture and Forestry (New Zealand)

Dolores Hill, U.S. Department of Agriculture (USA)

Gillian Mylrea, World Animal Health Organization (OIE)

Karsten Noeckler, Federal Institute for Risk Assessment (Germany)

Sarah Parker, Canadian Food Inspection Agency (Canada)

Edoardo Pozio, Istituto Superiore di Sanita (Italy)

David Pyburn, U.S. Department of Agriculture (USA)

Pascal Boireau, Agency for Food, Environmental and Occupational Health Safety (France)

Isabelle Vallee, Agency for Food, Environmental and Occupational Health Safety (France)

There were three objectives for this workshop: 1) to develop recommendations on the appropriate level of testing to assure safety of pork (relative to *Trichinella*) and to allow derogation from slaughter testing or further processing; 2) to develop recommendations for appropriate application of testing methods to meet objective 1; and 3) to define gaps in scientific knowledge that affect the best use of current surveillance tools. These objectives were designed to result in a series of recommendations which could be used by the World Organization for Animal Health and the FAO Codex Alimentarius Commission in developing international requirements for food safety and trade. These recommendations will assist in the revision and supplementation of comprehensive science based guidance from the International Commission on Trichinellosis, which is the definitive body on the subject of *Trichinella* and trichinellosis.

The format of the workshop was divided into sections in which presentations from various subject matter experts would provide appropriate background knowledge such that consensus recommendations could be reached. Specific points addressed in the presentations included:

- How to define the composition/characteristics of a population to be surveilled/tested?
- How should testing/surveillance be structured (risk-based) and how might this vary by country/region?
- What are the appropriate tests to be used (minimum performance characteristics)?
- What statistical sampling protocols could be used to achieve acceptable levels of risk?

Given the importance of the outcome relative to guidance in food safety and trade, substantial time was allowed for discussion and planning of how to proceed with formulation of final recommendations.

Following an initial review of the objectives of the workshop, presentations were made on the following subjects:

- Overview of roles and responsibilities and current regulations/guidelines, and future directions for protection of public health relative to *Trichinella* in pork.
- Current and proposed standards of the World Organization for Animal Health (OIE) for mitigating risk of *Trichinella* infection in pork production systems.
- Current and proposed requirements of the Codex Alimentarius for mitigating public health risk of trichinellosis.
- Epidemiology of *Trichinella* in controlled and non-controlled management systems
- Structure of pork production and marketing systems in developing countries as it pertains to new OIE and Codex regulations.
- Structure of pork production systems in industrialized countries; status and risk of trichinellosis.
- Defining populations of pigs that can be subjected to surveillance – animal ID, auditing, etc.
- Surveillance tools for establishing prevalence data.
- Performance characteristics of the artificial digestion test and practical applications.
- Performance characteristics of the ELISA including new data on ELISA performance (ICT collaborative studies).
- Design elements for structuring risk-based surveillance.
- Statistical sampling protocols for achieving acceptable levels of risk.

Discussion occurred during and after each presentation. Following the conclusion of all presentations, workshop participants discussed how to define acceptable levels of risk for controlled management systems and began to develop specific recommendations for surveillance program design and applications. The group also identified gaps in knowledge regarding testing systems that should be addressed.

This workshop resulted in a consensus on several key principles for new recommendations on methods for verifying the integrity of a compartment of negligible risk for *Trichinella* in swine. The workshop affirmed that swine that are not included in a negligible risk compartment as defined in the new OIE Animal Health Code Chapter on *Trichinella* should follow current ICT guidelines on control. The workshop developed the following general definitions:

Negligible risk herd – Swine that are managed under conditions of biosecurity (as defined by the ICT in other recommendations) that minimize exposure to *Trichinella*. Recognition of negligible risk for a herd is dependent on all animals being raised under these principles.

Negligible risk compartment – A compartment consisting of negligible risk herds in a management system.

Importantly, the group concluded that the status of a negligible risk compartment may be verified through an assurance program for maintenance of conditions of biosecurity. Assurance may include, but not be limited to, a program of regular audits and/or swine testing/surveillance data. It was also concluded that, where auditing is used to assure maintenance of conditions of biosecurity, there is adequate scientific justification that testing is not required within the negligible risk compartment, once established. When surveillance is used to assure maintenance of conditions of biosecurity, the design prevalence for swine testing should be determined based on the intended purpose and swine testing should be performed using suitably standardized and validated digestion or serological methods.

Specific points where we identified gaps in scientific knowledge included the following: specific and detailed knowledge of the performance characteristics of digestion and ELISA tests; clarification of processes necessary to consider an audit valid; requirements for audit frequency; and, definition of competency standards for the auditors.

This workshop addressed the assessment of best practices in the establishment and maintenance of compartments, regions, and countries where the achievement of a negligible health risk of trichinellosis can be documented. By providing guidance on implementation of scientifically defensible surveillance systems, the outcomes of this workshop will inform and guide national and international regulatory authorities (OIE/Codex), which in turn will result in 1) a reduction in the cost of assuring pork safety, 2) alleviating complex barriers in international trade, and 3) assure the consumer of a *Trichinella*-free food chain.

The Impact of Pig Health on Public Health: Quantitative Data for Risk Assessments

DeClercq, A.^{1*}, Hurd, H.S.¹, Ramirez, A.¹, Frana, T.¹

¹Iowa State University Veterinary Diagnostic and Production Animal Medicine Department, Ames, IA USA

Abstract

Management practices can play an important role in affecting animal health. Sub-clinically ill animals entering harvest can have pleural adhesions known as peel-outs. The focus of this study was to examine how these pleural adhesions can be associated with *Salmonella* and respiratory pathogens, as well as to get a national estimate of peel-outs. The results suggest pleural adhesions are not significantly associated with *Salmonella* or respiratory bacterial contamination.

Introduction

With increased scrutiny being placed on management practices such as housing and antibiotic usage, it has become more important than ever to study how changes in these practices can affect animal health. While clinically ill animals will not pass ante-mortem inspection, it is possible that animals with subclinical illness or lesions could pass inspection and be harvested. These animals could in turn be more likely to harbor pathogens that could cause food-borne illness, such as *Salmonella*.

One possible source of contamination is what is referred to as a peel-out, or a pleural adhesion which does not allow for complete removal of the viscera. As a result, extra trimming is required. A previous study found that approximately 7% or 1 in 15 carcasses had some degree of pleural adhesions, and carcasses with peel-outs were 90% more likely to be contaminated with *Salmonella*.¹

To our knowledge, this is the only study conducted on peel-outs, and the findings were isolated to one plant. No studies have been conducted examining which pathogens are associated with peel-outs. The hypothesis is that *Streptococcus suis*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and *Actinobacillus suis* may be possible causes of peel-outs, as these pathogens are associated with pleuritis and respiratory illness.

The objectives of this study are threefold: 1) estimate the prevalence of peel-outs across the country, 2) determine what common respiratory pig pathogens are more likely to be associated with peel-outs, and 3) determine if peel-outs are associated with an increase in food-borne pathogens (specifically *Salmonella*).

Materials and Methods

Sample Collection

Six different slaughtering facilities were selected from different geographical areas. Two sets of samples were collected: lung samples immediately after evisceration and pleural swabs after the final trimming and before the final carcass wash. One person identified the cases and controls, and labeled with numbered tags or food-grade markers, depending on the individual plant's preference. Selected carcasses were separated by at least 10-15 non-selected carcasses. Cases and controls were selected in random order to blind the person doing the pleural swabs. On a separate sheet it was recorded if the carcass was a case or control, allowing for blinding during bacteriological analysis. The total number of carcasses, both cases and controls, were counted to determine peel-out frequency.

A piece of lung measuring approximately 1-2 inches in diameter was taken for the lung collection. Scissors were dipped in alcohol or 180° water (depending on plant preference) and changed frequently. Disposable gloves were worn throughout and also changed frequently. However, because the lung surface was seared before analysis, it was not necessary to change gloves and scissors after each sample was collected.

For the pleural swab collection, 18 oz Whirl-Pak bags with Speci-Sponges were used. The sponges were hydrated with 10 ml of buffered peptone water. After the final trimming and before the final carcass wash, both sides of the carcass were swabbed. Gloves were changed after each swab to prevent cross contamination. Both sets of samples were put on ice until analysis could be performed.

Bacteriological Analysis

Samples were submitted for bacteriological isolation at the Iowa State University College of Veterinary Medicine Veterinary Diagnostic Laboratory in Ames, IA. Lung and pleural swabs were initially set up on 5% sheep blood agar and incubated

aerobically with 10% CO₂, as well as incubated anaerobically. Additionally, samples were streaked onto 4% bovine blood agar and Tergitol 7 and incubated aerobically without CO₂. A Staph nurse colony was added to the sheep blood agar plate and 4% bovine blood agar plate. Plates were examined once a day for three two days. Typical *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Streptococcus suis*, and *Actinobacillus suis* isolates were identified with biochemical testing, gram stain, and matrix-assisted laser desorption time of flight mass spectrometry. Additional bacterial populations were identified if they had significant growth.

For *Salmonella* isolation, 100 ml of buffered peptone water (BPW) was homogenized in the Whirl-Pak bag with Speci-Sponge and incubated for 18hrs at 35°C). Subsequently 0.1 ml of BPW was transferred to 10 ml of Rappaport-Vassiliadis (RV) broth and incubated for 18 hours at 42°C.

Aliquots (10µl) of RV broth were streaked onto XLT4 and Brilliant Green with Novobiocin agars. Suspect colonies were confirmed as *Salmonella* with biochemical analysis (lysine-iron agar, motility-indole-lysine agar) and slide agglutination with polyvalent anti-O sera.

Statistical Analysis

Data was analyzed using SAS® 9.2 using two logistic regression models: a univariate model testing for *Salmonella* contamination as a result of peel-outs, and a multivariate model testing for peel-outs as a result of bacterial contamination. Three different replicates were run: Trial 1, Trial 2, and both trials put together. Frequency counts and percentages were given for *Salmonella* and bacterial contamination for both case and control carcasses (Table 1). The odds ratios, beta estimates, standard

¹Table 1. Frequency counts and percentages for *Salmonella* and bacterial contamination

	Trial 1		Trial 2**		Total	
	Lesioned carcass	Non-lesioned carcass	Lesioned carcass	Non-lesioned carcass	Lesioned carcass	Non-lesioned carcass
<i>Salmonella</i>	17 (5.28%)	13 (4.05%)	13 (6.50%)	8 (4.08%)	30 (5.75%)	21 (4.06%)
<i>Streptococcus suis</i>	71 (22.54%)	59 (19.09%)	52 (25%)	55 (27.23%)	123 (23.52%)	114 (22.31%)
<i>Pasteurella multocida</i>	32 (10.16%)	42 (13.60%)	14 (6.73%)	13 (6.44%)	46 (8.80%)	55 (10.76%)
<i>Actinobacillus pleuropneumoniae</i>	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>Haemophilus parasuis</i>	0 (0%)	1 (0.32%)	0 (0%)	0 (0%)	0 (0%)	1 (0.20%)
<i>Actinobacillus suis</i>	0 (0%)	1 (0.32%)	0 (0%)	0 (0%)	0 (0%)	1 (0.20%)

Table 2. Statistical analysis for *Salmonella* contamination

Salmonella contamination							
Explanatory variable	Response	β	SE	OR	95% CI	Chi-square	p-value
	Lesions	0: non-lesioned carcasses 1: lesioned carcasses	referent				
Trial 1		0.2780	0.3772	1.321	(0.631-2.766)	0.5434	p=0.4610
Trial 2**		0.4908	0.4611	1.634	(0.662-4.033)	1.1333	p=0.2871
Total		0.3647	0.2915	1.440	(0.813-2.550)	1.5651	p=0.2109

Table 3. Statistical analysis for bacterial contamination*

Peel-outs							
Explanatory variable	Response	β	SE	OR	95% CI	Chi-square	p-value
	<i>Streptococcus suis</i>	0: negative 1: positive	referent				
Trial 1		0.2329	0.1990	1.262	(0.855-1.864)	1.3706	p=0.2417
Trial 2**		0.0418	0.2301	1.043	(0.664-1.637)	0.0329	p=0.8560
Total		0.1520	0.1502	1.164	(0.867-1.563)	1.0245	p=0.3114
<i>Pasteurella multocida</i>	0: negative 1: positive	referent					
Trial 1		-0.3545	0.2510	0.701	(0.429-1.147)	1.9960	p=0.1577
Trial 2**		-0.0374	0.4067	0.963	(0.434-2.138)	0.0085	p=0.9267
Total		-0.2643	0.2122	0.768	(0.507-1.164)	1.5521	p=0.2128

¹*Inadequate data for statistical analysis of *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and *Actinobacillus suis*

**Data collection still in progress

errors, 95% confidence intervals, chi-square and p-values were also recorded for each model (Table 2 & 3). All results were pooled together.

Results

For the first sampling period, 643 carcasses were tested for *Salmonella* (322 cases and 321 controls) and 624 carcasses were tested for respiratory pathogens (315 cases and 309 controls.) For the second sampling period, 396 carcasses were tested for *Salmonella* (200 cases and 196 controls) and 410 carcasses were tested for respiratory pathogens (208 cases and 202 controls.) (The second sampling period is still ongoing.) The total number of carcasses tested for *Salmonella* to date is 1,039 (522 cases and 517 controls) and for respiratory pathogens is 1,035 (523 cases and 511 controls.) (Some samples had to be discarded due to either misclassification, or the carcasses being railed off before the pleural swab collection.) The prevalence of peel-outs varied significantly between plants, with a range of 2.64%-28.39% for the first sampling period (average 8.31%). The prevalence of peel-outs for the second sampling period varied from 4.08%-10.96% (average 7.82%). Overall, the prevalence of peel-outs varied from 2.64%-28.39%, with an average of 8.11%.

Discussion

Based on previous research, it was hypothesized that carcasses exhibiting peel-outs would be more likely to be contaminated with *Salmonella*, and that common respiratory pathogens would cause peel-outs. However, the data gathered does not support these hypotheses. While this study did have a significantly larger sample size than the previous study, we were only able to spend one day at each plant for each sampling period. Because bacteria counts and peel-out prevalence could vary from day to day, it would be beneficial if sampling was taken over multiple days. Also, it is possible that there are other respiratory pathogens that are more associated with peel-outs. Additional microbial testing could help answer this research question.

Conclusions

The data suggests that *Salmonella* contamination and respiratory pathogens are not highly associated with pleural adhesions as previously thought. Because there is limited information in this area, further research is needed.

Acknowledgments

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References

¹Hurd, H.S., Yaeger MJ, Brudvig JM, Taylor DD, Wang B. 2012. Lesion Severity at Processing Used as a Predictor of *Salmonella* Contamination of Swine Carcasses. *American Journal of Veterinary Research*. 73:1 pp 91-97.

Efficacy of a *Salmonella* Typhimurium and *Salmonella* Choleraesuis experimental combination vaccine against *S. Typhimurium* challenge in growing pigs.

Jordan, D.*, Kaiser, T., Cline, G.

Boehringer Ingelheim Vetmedica Inc., St. Joseph MO, USA

Salmonella Typhimurium (ST) and Choleraesuis (SC) are primary pathogens in swine. The objective of this study was to evaluate the efficacy of an experimental vaccine containing two avirulent live cultures (ALC) of ST and SC administered in pigs to aid in the prevention of salmonellosis caused by a *Salmonella* Typhimurium (ST) challenge. Pigs (n=48) were randomly assigned to one of two treatment groups: 1) placebo or 2) vaccinated. Pigs were administered either placebo or vaccine at 16-17 days of age through the drinking water with individual cup waterers. At four weeks post-vaccination, placebo-treated and vaccinated pigs were commingled and intranasally challenged with >10 logs of a virulent ST. Clinical observations and fecal shedding of ST were assessed daily for ten days post-challenge. Surviving pigs were then euthanized, and intestines were scored for pathological lesions consistent with *Salmonella* infection. Pigs were considered affected with salmonellosis if the cecum, spiral colon, mesenteric or ileocecal lymph nodes had a non-zero macroscopic lesion score or if the pig died due to challenge. Prevented fraction (PF) analysis was utilized to evaluate prevention of salmonellosis, and mitigated fraction (MF) analysis was used to evaluate reduction of diarrhea and ST shedding. Vaccination reduced salmonellosis with a PF of 53.3% (95% CI; 7.7, 80.7). Vaccination reduced the duration of diarrhea post-challenge with a MF of 75% (95% CI; 47.8, 94.7). Although all pigs shed ST post-challenge, vaccination reduced the duration of shedding with a MF of 45% (95% CI; 20.8, 72.2). In addition, body weight was measured to assess the impact of challenge on weight gain. Vaccination resulted in 2.42 kg heavier pig during the post-challenge period (P=0.0006). The data supports vaccination with a single dose of this ALC vaccine containing *Salmonella* Typhimurium and *Salmonella* Choleraesuis administered through the drinking water aids in the prevention of salmonellosis.

Introduction

Salmonella enterica serovar Typhimurium (ST) and *Salmonella* Choleraesuis (SC) are identified as primary pathogens in swine. ST is a primary cause of enteritis and subclinical production losses in growing or finishing swine and contributes to environmental and carcass contamination. SC is a primary cause of septicemia, pneumonia and/or enterocolitis in growing pigs. The objective of this vaccination-challenge study was to evaluate the efficacy of an experimental combination vaccine composed of two avirulent live bacterial cultures (ALC) of ST and SC administered once to two-week-old pigs via the drinking water as an aid in the prevention of salmonellosis caused by a virulent *Salmonella* Typhimurium challenge.

Materials and Methods

Sixty conventional, two-week old pigs originated from a herd without a history of clinical salmonellosis and without historical use of *Salmonella* vaccines were utilized in this study. Pigs were sourced from twelve litters and randomly assigned to each treatment group accordingly: one pig to the strict control group, and two pigs for each of the two treatment group (placebo and vaccine). A description of the treatment groups is shown in Table 1. Baseline fecal samples and blood samples were collected for *Salmonella* culture and *Salmonella* serology (tested by IDEXX *Salmonella* ELISA). Fecal samples were required to be negative by enrichment culture for pigs to be included in the study. Weights were collected on D-1, D27 and D38. Pigs were individually offered the ST/SC vaccine or placebo in 60mL of drinking water per pig at 16-17 days of age (D0). During the vaccination phase each of the three groups were housed in separate rooms to avoid unintentional exposure to vaccine that may be shed in the feces by the vaccinated pigs. The twelve strict control pigs were included as cohorts to the study animals in order to monitor biosecurity and were not included in statistical comparisons. The strict control animals were necropsied on D27 of the study to evaluate overall health. Bacterial culture of the organs determined no prior *Salmonella* colonization. Prior to challenge, the remaining pigs were re-penned and comingled so that vaccinated and placebo-treated pigs were both represented within rooms.

On D28, the vaccinated and placebo treated pigs were challenged intranasally with 2mL of a virulent ST (>10¹⁰ CFU per dose)(1). For ten days, pigs were observed daily for clinical signs of salmonellosis. Feces were scored for the presence, severity, and duration of diarrhea; duration was assessed by the number of days elapsed from the first to the last positive observation. Necropsies were conducted ten days post-challenge (D38) or earlier in the event of severe disease and mortality. Blood was collected for serology and fecal samples were collected throughout the study to monitor shedding via direct and modified enrichment cultures (3). A pig was considered to be shedding ST if a fecal sample was positive for non-vaccine serogroup B *Salmonella* post-challenge. Direct culturing was performed on fecal samples post-challenge and organs at necrop-

sy with an approximate sensitivity of 150 colony forming units per gram (cfu/gram). Dilutions of fecal material were done for quantitative shedding. To determine an average cfu/gram of feces for the group, enrichment positive cultures were assigned the values of “1” and the lower limit of detection for direct cultures was determined to be 150 cfu/gram. Body weights were collected prior to vaccination, prior to challenge and at the scheduled necropsy; average daily gain (ADG) was calculated. A case definition was defined a priori to incorporate the major end point manifestations of salmonellosis: enterocolitis, lymphadenopathy and death.

Preventive fractions with 95% CI were calculated as the complement of the risk ratio (StatXact 8.0). The 95% CI for the mitigated fractions were estimated using bootstrapping methods, stratifying by pen (SAS 9.2). All hypothesis testing was conducted using an alpha level of 0.05 (SAS 9.2) and Least Square Means (LSM) are reported. Fisher’s exact test was utilized when appropriate to compare differences between treatment groups.

Table 1: Study design table

Group	N	Treatment Day 0 (PO')	Challenge Day 28 (IN')	Necropsy	Parameters evaluated
Placebo	24	Media	> 10 ¹⁰ cfu/dose	D 38	Clinical signs, average daily weight gain, diarrhea, fecal shedding, organ colonization, mortality, seroconversion, organ pathology
Vaccinate	24	ST/SC	> 10 ¹⁰ cfu/dose	D 38	
Strict Control	12	None	n/a	D 27	Fecal shedding, organ colonization, seroconversion, organ pathology

* PO=per os, IN=intranasal

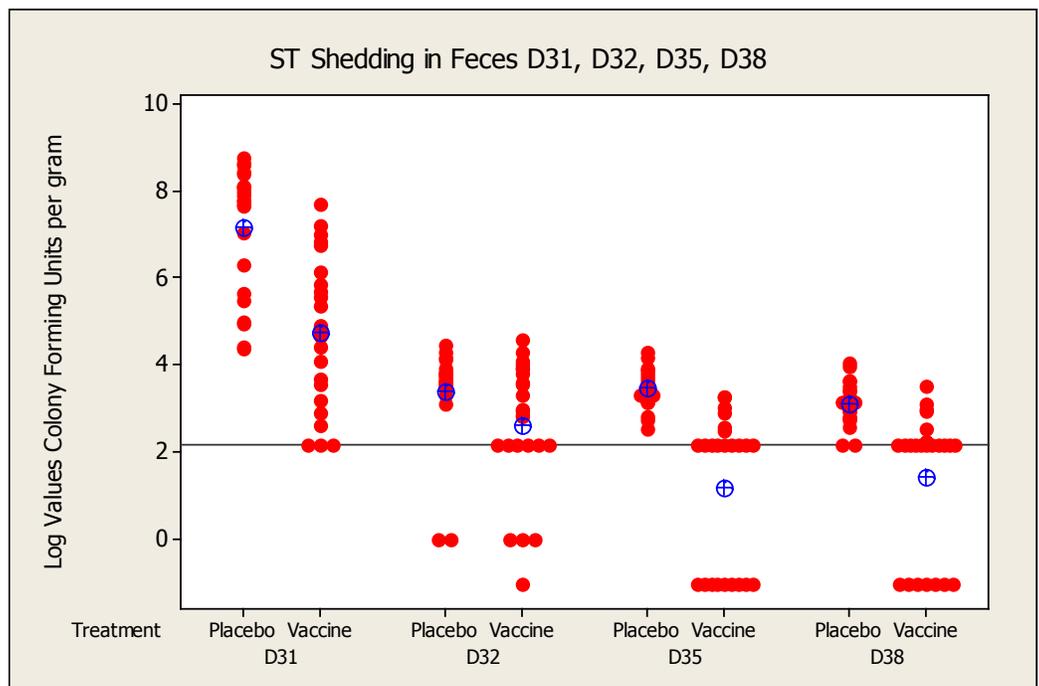
Results

All pigs in the strict control group were negative for any indication of colonization by *Salmonella*. Four pigs in the placebo treated group either died or were euthanized due to resultant disease post-challenge. No vaccinated pigs succumbed to severe clinical disease due to the ST challenge. Fifty percent of vaccinated pigs did not develop diarrhea at all, and 21% of the vaccinated pigs exhibited diarrhea for a single day only. Significantly more placebo treated pigs (18 of 24) exhibited diarrhea for two or more consecutive days post challenge than did vaccinated pigs (7 of 24) (Fisher’s Exact test $p=0.0117$). The vaccinated group demonstrated a significant reduction in the duration of diarrhea ($p<0.0001$) from 8.05 days in the placebo group to 2.54 days in the vaccinated group (Mitigated Fraction of 75% (95% CI; 47.8, 94.7)).

Following vaccination and prior to challenge, no difference in ADG between the placebo and the vaccinated group was observed (ADG=0.43 kg in both groups). However, the vaccinated pigs exhibited significantly greater ADG (0.37 kg) compared to placebo treated pigs (0.15 kg) during the post-challenge period ($p=0.0006$), resulting in, on average, a 2.42 kg heavier vaccinated pig during the ten-day post-challenge period ($p=0.0006$).

Blood was collected for serological testing for antibodies against *Salmonella* using the IDEXX *Salmonella* ELISA

Figure 1: Bacterial counts of ST shedding in feces, reported as Log cfu/gram of feces over time. The line represents the detection limit for direct quantification at 150 cfu/gram. Values at “0” represent those samples were positive only by enrichment culture. Points below the zero line represent samples that were negative by direct and enrichment culture for *Salmonella*. Mean values are denoted by circled crosses.



prior to vaccination, challenge and necropsy. One pig in each of the placebo and strict control group was positive for *Salmonella* antibodies. On the day of challenge, all placebo and strict control pigs were negative for *Salmonella* antibodies and three of the twenty-four pigs (13%) were positive in the vaccinated group. At the time of necropsy, all vaccinates had seroconverted with *Salmonella* antibodies and twelve of the twenty (60%) remaining placebo pigs had seroconverted.

When considering the parameters included in the case study definition, 15 out of 24 placebo pigs (63%) met the case definition where as only 7 of the 24 vaccinated pigs (29%) met the case definition. The reduction of disease due to ST in the vaccinated pigs was statistically significant (LSM difference $p=0.0023$, Preventive Fraction of 53.3% (95% CI; 7.7, 80.7)).

Post-challenge fecal samples were enumerated on D31, D32, D35 and D38 of the study. On the other days they were determined to be positive by direct or enrichment culture, or negative. As plotted in Figure 1 the vaccinated pigs had lower numbers of ST shed in the feces on each of the four days quantified. On D31 of the study, the amount of ST shed in the vaccinated pigs compared to the placebo treated pigs was reduced. As time continues the number of negative pigs in the vaccinate group increases while all of the remaining placebo pigs continue shedding through d38.

All placebo treated pigs shed the ST challenge strain for up to ten days (Table 2). Conversely, the vaccinated pigs had reduced shedding after D 31 on all but one day of the post-challenge period when compared to the placebo pigs. Vaccination reduced the duration of shedding of the ST challenge with a Mitigated Fraction of 45% (95% CI; 20.8, 72.2).

Discussion

Post-challenge, four placebo treated pigs succumbed to challenge while all vaccinated pigs were protected from mortality. At necropsy, the placebo treated pigs had more frequent and more severe lesions suggestive of ST than the vaccinated pigs. Placebo treated pigs demonstrated significantly increased frequency and duration of diarrhea compared to the vaccinated pigs. Furthermore, there was a reduction in the amount of ST shed in the feces on three of the four days in which numbers of *Salmonella* shed was quantified. An important outcome to note is that even after a very high ST challenge, the incidence of fecal shedding by vaccinated pigs dropped to 71% within ten days while all of the placebo pigs continued to shed *Salmonella* at levels detectable by direct culture. If an infectious dose of *Salmonella* is considered to be at least 1000 cfu/gram (2), the incidence of shedding in the vaccinates after ten days drops to 8% for pigs shedding over 1000 cfu/gram, on average, while the placebo treated pigs had an incidence of 60%. Reduced shedding by vaccinates could reduce pathogen transmission, the number of subclinical salmonellosis cases, and improve pig performance. Additionally, a reduction in colonization and shedding may reduce *Salmonella* risk during lairage.

Conclusion

Overall the vaccine significantly reduced clinical signs associated with salmonellosis including diarrhea and ST shedding. Furthermore, the post-challenge weight gain was significantly improved in vaccinated pigs. This data is supportive that a single dose of this lyophilized avirulent live vaccine containing *Salmonella* Typhimurium and *Salmonella* Choleraesuis administered in drinking water aids in the prevention of salmonellosis.

References

1. Curtiss, R., III, S.B.Porter, M.Munson, S.A.Tinge, J.O.Hassan, C.Gentry-Weeks, and S.M.Kelly. 1991. Nonrecombinant and recombinant avirulent *Salmonella* live vaccines for poultry, p. 169–198. In L. C. Blankenship, J. S. Bailey, N. A. Cox, N. J. Stern, and R. J. Meinersmann (ed.), Colonization control of human bacterial enteropathogens in poultry. Academic Press, New York, N.Y.
2. Loynachan, A.T., and D.L.Harris. 2005. Dose determination for acute Salmonella infection in pigs. Appl Environ Microbiol. 71(5):2753-5.
3. Rostagno, M.H., J.K.Gailey, H.S.Hurd, J.D.Mckean, R.C.Leite. 2005. Culture methods differ on the isolation of *Salmonella enterica* serotypes from naturally contaminated swine fecal samples. J Vet Diagn Invest 17:80–83.

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Table 2: Percentage of pigs with ST detected in feces by enrichment or direct culture

Group	D29	D30	D31	D32	D33
Placebo	79	100	100	100	100
Vaccinates	46	88	100	96	71
Group	D34	D35	D36	D37	D38
Placebo	100	100	100	100	100
Vaccinates	75	63	58	79	71

Genetic diversity of ST5 *Staphylococcus aureus* isolated from swine veterinarians in the USA

Jisun Sun, Leticia Linhares, My Yang, Srinand Srivatsan, Peter Davies*

Department of Veterinary Population Medicine, University of Minnesota, 385 AnSci/Vet Med Bldg., 1985 Fitch Ave., Saint Paul, MN, USA.

*Corresponding author: davie001@umn.edu

Abstract

The term livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) has been synonymous with sequence type ST398 since the identification of this lineage of MRSA in Holland. Subsequent research indicates broader genetic diversity of MRSA strains in swine, with MRSA variants belonging to MLST sequence type ST9, and ST5 also being reported in studies of swine in various countries. Unlike ST398, considered a novel sequence type, the ST5 lineage has long been associated with human MRSA infections, and particularly the USA100 group (defined by *sma1* PFGE).

We investigated the prevalence of *S. aureus*, and associated health events, in swine veterinarians in US and evaluate the genetic diversity of isolates using *spa* typing, MLST and PFGE. The ST398 and ST5 were the most prevalent *spa* types and 60 (18%) of 325 MSSA isolates and 6 (19%) of 32 MRSA isolates detected were *spa* type t002 (ST5). *Sma1* PFGE analysis of 23 isolates indicated that t002 isolates from the swine veterinarians were not clonal. Given previous reports of t002-ST5 MRSA in North American pigs, our findings of diverse ST5 *spa* types as well as genetic diversity within *spa* type t002, suggest that *S. aureus* variants of the ST5 lineage may be livestock associated. These organisms need to be more broadly characterized to understand their potential implications for human health.

Introduction

Livestock associated *Staphylococcus aureus* (LA-MRSA) has been deemed an emerging zoonotic pathogen in food animals, especially in pigs (Voss et al. 2005). Among early studies, the first and most predominant LA-MRSA type was sequence type (ST) 398 in Europe and North America while ST 9 variants appear to be predominant in Asia (Lewis et al. 2008; Smith et al. 2009; Cui et al. 2009; Asai et al. 2012). Various genetic studies are revealed more genetic diversity in LA-MRSA using molecular epidemiologic characteristics. There is considerable evidence for pig-to-human transmission of ST398 in occupationally exposed people (Garcia-Graells et al. 2012).

Recently, ST5 MRSA, well known as most globally disseminated hospital associated MRSA lineages, has been identified from pigs, retail meat and farm workers in North America (Khanna et al. 2008; Frana et al. 2013). The occurrence of ST5 variants in swine is arguably more concerning than ST398 MRSA, which to date have had minimal public health impact despite high prevalence of exposure in people with livestock contact.

The aim to this study was to compare ST5 MRSA and MSSA strains from US swine veterinarians using pulse-field gel electrophoresis (PFGE). The isolates were obtained as part of a longitudinal study of *S. aureus* nasal colonization in swine veterinarians. We postulated that a high degree of clonality among these isolates would suggest recent emergence of this lineage in the livestock sector, whereas greater diversity would suggest longer term association of ST5 with swine, or exposures from other sources.

Materials and methods

In an ongoing longitudinal study, nasal swabs are being collected from 67 US swine veterinarians. Participants mail self-collected nasal swabs monthly to the laboratory, along with information on recent animal exposure and health events. The most prevalent MLST types of *S. aureus* isolated in the first 7 months of the study (July 2012 – January 2013) were ST398, ST5 and ST9, with ST398 and ST5 the most common among MRSA isolates. The veterinarians reside in 15 major swine producing states of the USA and typically interact with diverse swine populations in their work.

Pulsed-field gel electrophoresis (PFGE)

To evaluate the diversity of ST 5, the 23 ST5 *S. aureus* isolates (3 MRSA and 20 MSSA isolates) determined to be *spa* type t002 (the predominant *spa* type among ST5 isolates) PFGE was performed using the *sma1* restriction enzyme (McDougal

et al. 2003). Visualized images were analyzed using BioNumerics software (Version 5.0) and PFGE types were defined using a similarity coefficient of 85% and USA 100 to USA 800 strains were included as references.

Results

Overall in first 7 months of the longitudinal study, 60 (18.5%) of 325 MSSA isolates and 6 (18.8%) of 32 MRSA isolates detected were spa type t002 (ST5). Other ST5 MSSA spa types found included t045 (14), t062 (6), t570 (3) and t2049 (1). We purposively selected 23 of the t002_ST5 isolates to attempt to maximize diversity of isolates among months and participants. For participants from whom t002-ST5 was isolated on more than one occasion, we sampled the first and last isolates available.

PFGE analysis showed that the 23 t002-ST5 isolates from swine veterinarians were not uniform, and therefore unlikely to share a recent clonal relationship. Fifteen distinct pulsotypes were seen, and eight isolates were classified as USA100, a common pulsotype in human clinical infections. Among participants with two MSSA isolates analyzed, PFGE patterns from one were exactly identical, while isolates from another individual participant showed only 85% similarity. For one participant with multiple MRSA isolates, the PFGE patterns were identical although there was a 6-month interval between the sampling events.

Discussion

Recently, ST5 has been reported backyard-raised pigs in Michigan (Gordoncillo et al. 2012) and from retail pork or pork farms in U.S (Molla et al. 2012) and even pigs and pig farmers in Canada (Khanna et al. 2008). In a pilot study of *S. aureus* colonization of pigs on 2 farms in Minnesota (unpublished), we also found the predominant spa types to be t034 (ST398), and t002 (ST5), comprising 37% and 29% of isolates respectively (all MSSA). Originally ST 5 is a common hospital associated MRSA type, mostly related to nosocomial infections. Although the isolates we analyzed were from human nasal swabs, our ongoing studies (unpublished) and a recent study from Holland (Verkade et al. 2013) suggest that most *S. aureus* isolates from swine veterinarians originate from occupational exposures. The diversity of PFGE types among these isolates, together with increasing reports of ST5 *S. aureus* from pigs in diverse locations suggest that this lineage may have a long association with swine rather than reflect a recent event of interspecies transmission. Further evaluation of human and swine ST5 *S. aureus*, particularly with respect to the presence of virulence determinants, is required to evaluate the potential occupational health risk associated with these organisms.

Conclusion

The presence of diversity in ST5 *S. aureus* isolates from swine veterinarians on PFGE suggests that ST5 MSSA and MRSA **have colonized this population** having a long association with pigs.

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References

- Asai, T., Hiki, M., Baba, K., Usui, M., Ishihara, K., Tamura, Y., 2012. Presence of Staphylococcus Aureus ST398 and ST9 in Swine in Japan. Jpn. J. Infect. Dis. 65, 551-552.
- Cui, S., Li, J., Hu, C., Jin, S., Li, F., Guo, Y., Ran, L., Ma, Y., 2009. Isolation and Characterization of Methicillin-Resistant Staphylococcus Aureus from Swine and Workers in China. J. Antimicrob. Chemother. 64, 680-683.
- Frana, T.S., Beahm, A.R., Hanson, B.M., Kinyon, J.M., Layman, L.L., Karriker, L.A., Ramirez, A., Smith, T.C., 2013. Isolation and Characterization of Methicillin-Resistant Staphylococcus Aureus from Pork Farms and Visiting Veterinary Students. PLoS One 8, e53738.
- Garcia-Graells, C., Antoine, J., Larsen, J., Catry, B., Skov, R., Denis, O., 2012. Livestock Veterinarians at High Risk of Acquiring Methicillin-Resistant Staphylococcus Aureus ST398. Epidemiol. Infect. 140, 383-389.
- Gordoncillo, M.J., Abdujamilova, N., Perri, M., Donabedian, S., Zervos, M., Bartlett, P., 2012. Detection of Methicillin-Resistant Staphylococcus Aureus (MRSA) in Backyard Pigs and their Owners, Michigan, USA. Zoonoses Public.

Health. 59, 212-216.

- Khanna, T., Friendship, R., Dewey, C., Weese, J.S., 2008. Methicillin Resistant Staphylococcus Aureus Colonization in Pigs and Pig Farmers. *Vet. Microbiol.* 128, 298-303.
- Lewis, H.C., Molbak, K., Reese, C., Aarestrup, F.M., Selchau, M., Sorum, M., Skov, R.L., 2008. Pigs as Source of Methicillin-Resistant Staphylococcus Aureus CC398 Infections in Humans, Denmark. *Emerg. Infect. Dis.* 14, 1383-1389.
- McDougal, L.K., Steward, C.D., Killgore, G.E., Chaitram, J.M., McAllister, S.K., Tenover, F.C., 2003. Pulsed-Field Gel Electrophoresis Typing of Oxacillin-Resistant Staphylococcus Aureus Isolates from the United States: Establishing a National Database. *J. Clin. Microbiol.* 41, 5113-5120.
- Molla, B., Byrne, M., Abley, M., Mathews, J., Jackson, C.R., Fedorka-Cray, P., Sreevatsan, S., Wang, P., Gebreyes, W.A., 2012. Epidemiology and Genotypic Characteristics of Methicillin-Resistant Staphylococcus Aureus Strains of Porcine Origin. *J. Clin. Microbiol.* 50, 3687-3693.
- Smith, T.C., Male, M.J., Harper, A.L., Kroeger, J.S., Tinkler, G.P., Moritz, E.D., Capuano, A.W., Herwaldt, L.A., Diekema, D.J., 2009. Methicillin-Resistant Staphylococcus Aureus (MRSA) Strain ST398 is Present in Midwestern U.S. Swine and Swine Workers. *PLoS One* 4, e4258.
- Verkade, E., van Benthem, B., den Bergh, M.K., van Cleef, B., van Rijen, M., Bosch, T., Kluytmans, J., 2013. Dynamics and Determinants of Staphylococcus Aureus Carriage in Livestock Veterinarians: A Prospective Cohort Study. *Clin. Infect. Dis.* 57, e11-7.
- Voss, A., Loeffen, F., Bakker, J., Klaassen, C., Wulf, M., 2005. Methicillin-Resistant Staphylococcus Aureus in Pig Farming. *Emerg. Infect. Dis.* 11, 1965-1966.

Longitudinal investigation of *Salmonella* spp. from farm to fork in the pig industry in Reunion Island

Tessier C *,^(1,2) Atiana, L.^(2,3), Cardinale, E.^(2,3), Denis, M.^(4,5)

⁽¹⁾ *Coopérative des producteurs de porcs de la Réunion, Saint Pierre, Réunion*

⁽²⁾ *Centre de coopération internationale en recherche agronomique pour le développement, Sainte Clotilde, Réunion*

⁽³⁾ *Centre de Recherche et de Veille dans l'Océan Indien, Saint Clotilde, Réunion*

⁽⁴⁾ *Anses Laboratory of Ploufragan/Plouzané, Unit of Health and Quality of Poultry And Pig Products, BP 53, 22440 Ploufragan, France.*

⁽⁵⁾ *European University of Brittany, France.*

*corresponding author : ctessier@cppr.fr

Abstract

Salmonella is, after *Campylobacter*, the most reported zoonotic pathogen in Europe. Pork meat is frequently incriminated in salmonellosis. However, asymptomatic carrying of *Salmonella* by pigs makes its detection difficult. Despite its tropical situation, pig industry of Reunion Island is also concerned by this public health burden.

Our epidemiological study aims to identify the pathways of contamination along the pig chain industry. Three *Salmonella* positive farms were followed from the farrowing to the pork cuts. At farm level, *Salmonella* excretion was detected by individual samples of feces at birth, farrowing, post-weaning and fattening. During slaughtering, samples were taken from pigs entering and from carcasses after polishing, splitting and chilling. Caeca and pork cuts were also collected. Environmental samples of water, food and environment at farm and at different stages before slaughtering and cutting of the day were taken.

Among the 900 analysed samples, 229 isolates were collected and divided into 42 pulsotypes and 12 serotypes. *S. monovariant* 4,[5],12:i:-, *S. Rissen*, *S. Typhimurium* and *S. Livingstone* were predominant. Three different infection profiles were observed. At the nursing stage, piglets were *Salmonella* free. Infection occurred either after post-weaning (2/3) or during the fattening period (1/3). Truck and lairage are cross-contamination stages (100% are positive before pig loading). On the contrary, slaughterhouse is a decreasing contamination stage (equipment and pork cuts are *Salmonella* free). Farm surroundings play a major role in the reinfection of pigs like other animal productions.

This original investigation, made for the first time until now, give an accurate photography of *Salmonella* spreading along the chain.

Introduction

Salmonella is one of the main zoonotic pathogens in Europe. Pork meat is frequently incriminated in salmonellosis. Despite being highly studied in poultry production, epidemiological data are missing concerning *Salmonella* contamination of pork meat in Reunion Island. Limiting *Salmonella* contamination is dependent on identifying the source and ways of dissemination of *Salmonella* transmission at each stage during production and during slaughtering.

The aim of this study was to identify the pathways of contamination all along the chain industry of Reunion Island, from the farm to pork cuts.

Material and Methods

Three farrow-to-finish farms, previously detected *Salmonella* positive, were selected for this project. Farms were selected from different geographical locations in Reunion Island and with different environmental characteristics and different general husbandry practices. They were monitored from farrowing to the pork cuts. Three sows and 5 piglets by sow were selected from one batch per farm.

At farm level, *Salmonella* excretion was detected by individual samples of feces, after farrowing and throughout each growing stage. At slaughterhouse level, gauze swabs from pigs entering and from carcasses after polishing, splitting and chilling, caecal contents and pork cuts were sampled. Environmental samples were collected at both farm and slaughterhouse levels.

They were collected at each visit in farms and all along the slaughtering and cutting process at the beginning of the day, before the slaughter of the followed pig batch.

Environmental and feces samples were pre-enriched in buffered peptone water (BPW) in 1:10 sample/broth ratio. Disinfectant neutralizer was added in BPW broth (1:10) for samples collected after cleaning and disinfection. Environmental swabs and socks were pre-enriched with 150 and 300 ml of BPW respectively. After incubation at 37°C for 18h ± 2h, one ml and 3

drops of BPW broth were inoculated respectively to 10 ml of Müller–Kauffmann Tetrathionate-Novobiocin broth (MKTn) and on Modified Semisolid Rappaport Vassiliadis agar (MSRV) plate. Both were incubated respectively at 37°C for 24 h and 41.5°C for 24–48h. Samples of feed were analysed as described previously. However, selective enrichment was done with MKTn and Rappaport Vassiliadis Broth (RVB), incubated respectively at 37°C and 41.5°C for 24h. A loopful of selective enrichment in MKTn or RVB was then streaked on Xylose-Lysine-Desoxycholate Agar (XLD) and Rambach Agar. MSRV plates with characteristic halo of migration were streaked onto XLD and Rambach agar. After 24h at 37°C, four typical colonies per sample (one per way if possible) were biochemically confirmed as *Salmonella* and serotyped according to the Kauffman-White scheme.

Results

Amongst the 900 analyzed samples, 229 isolates have been collected. They were divided into 42 *Xba*I pulsotypes and 12 serotypes: monovariant of Typhimurium 4,[5],12:i:- (72), Rissen (60), Typhimurium (32), Livingstone (25), Weltevreden (12), Derby (10), London (8), Senftenberg (3), Newport (3), Bredeney (1), Blockley (1) and Give (1). Some serotypes were predominant in some farms: 4,[5],12:i:- in farm A and B, Rissen in farm B and Typhimurium / Livingstone in farm C. Distribution of pulsotypes in farm B from farrowing to pork cuts is illustrated in Figure 1.

Water, feed, cockroach and rodents samples return all negative.

Although floor of maternity and corridor were positive for *Salmonella*, neither sows nor piglets excreted *Salmonella* until the end of nursing (2/3).

Piglets started to excrete *Salmonella* a few weeks after the beginning of post-weaning. Pulsotypes were similar to the ones found in the room after cleaning and disinfection, and in corridor before the beginning of post-weaning stage (2/3).

Afterwards, until the end of pig fattening, pulsotypes were identical to the ones previously found during post-weaning or in farm environment (including other animal productions). For one of the 3 farms, pigs were excreting *Salmonella* at a late fattening stage whereas, in the other ones, the number of contaminated pigs decreased during the fattening period

Loading bay (2/3), truck (3/3) and lairage room at slaughterhouse (3/3) were contaminated with new pulsotypes. Before slaughtering and cutting, equipment and tools were negative except knives (1/3). However, this pulsotype was not detected in any other sample. At slaughterhouse, just prior to slaughter, pigs were carrying both previously detected pulsotypes and new ones. Some pulsotypes were detected from carcasses after polishing (3/14 for farm B) and after splitting (2/14 for farm B and 2/15 for farm C). However, all samples collected after splitting steps were negative.

Discussion

All samples of water, feed, rodents and cockroaches were *Salmonella* negative. However, *Salmonella* has ever been isolated from same types of samples (Davies *et al.*, 1997; Letellier *et al.*, 1999) as well as in flies (Khalil *et al.*, 1994), all collected from *Salmonella* positive farms. These findings may suggest that they play a role in the spread of *Salmonella*.

None of the sows and none of the piglets were shedding *Salmonella* during nursing. Kranker *et al.* (2003) showed that, although some sows were seropositive during nursing, none piglets were *Salmonella* positive. This finding suggests two explanations. The first one could be that sows play a minor role in *Salmonella* contamination of pigs during growing. And the second one that passive maternal immunity of sows may have protected pre-weaned pigs from *Salmonella* contamination (Wales *et al.*, 2009). Moreover, maternal antibodies secreted in colostrum and milk could protect piglets against colonisation by different *Salmonella* serotypes (Funk *et al.*, 2001).

No piglets were excreted *Salmonella* before weaning and pulsotypes isolated from piglets and from residual infection were similar. These results confirm that horizontal transmission may be due to residual contaminations (Kranker *et al.*, 2003; Wales *et al.*, 2009).

Contamination of pens in farms studied after cleaning and disinfection is not surprising. Several studies have shown that cleaning and disinfecting pig house is recognized as a challenge (Magistrali *et al.*, 2008; Wales *et al.*, 2009). Moreover, *Salmonella* can persist outside animals for days to months, and is able to adapt himself to host but also to farm environment (Berends *et al.*, 1996).

Salmonella was isolated from cleaned truck suggesting that procedures for cleaning and disinfecting was not enough efficient to eliminate *Salmonella*. Same findings were already shown by (Swanenburg *et al.*, 2001). Lairage room was also positive before arrivals of pigs. Similar pulsotypes were isolated from truck and lairage room before loading and further on back of pigs, from carcasses after polishing and splitting and from caecal contents. This confirms that transport and lairage are important stages of cross-contamination (Magistrali *et al.*, 2008; Bolton *et al.*, 2013). No *Salmonella* was detected after splitting. This suggests that slaughter process reduce *Salmonella* contamination, notably after chilling. However, prevalence after this step could be underestimated, because a formation of an ice layer on the carcasses after chilling (Swanenburg *et al.*, 2001). Despite no pork meat were *Salmonella* positive, we cannot conclude that there is not a risk for consumer.

Conclusion

This investigation allows us to identify sources of *Salmonella* contamination at production and slaughterhouse stage. However, characterisation of all isolates per samples could confirm ways of contamination highlighted or to identify new ones. Indeed, individuals may be infected simultaneously with different serotypes (Funk *et al.*, 2001). MLVA on *S. Typhimurium* and *S. 4,[5],12:i:-* is in progress and will clarify the ways of contamination, indeed, the origin of some pulsotypes have not yet been identified.

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References

- Berends, B.R., Urlings, H.A.P., Snijders, J.M.A., Van Knapen, F., 1996, Identification and quantification of risk factors in animal management and transport regarding *Salmonella* spp. in pigs. *International Journal of Food Microbiology* 30, 37-53.
- Bolton, D.J., Ivory, C., McDowell, D., 2013, A study of *Salmonella* in pigs from birth to carcass: serotypes, genotypes, antibiotic resistance and virulence profiles. *Int J Food Microbiol* 160, 298-303.
- Davies, P.R., Morrow, W.E., Jones, F.T., Deen, J., Fedorka-Cray, P.J., Harris, I.T., 1997, Prevalence of *Salmonella* in finishing swine raised in different production systems in North Carolina, USA. *Epidemiol Infect* 119, 237-244.
- Funk, J.A., Davies, P.R., Nichols, M.A., 2001, Longitudinal study of *Salmonella enterica* in growing pigs reared in multiple-site swine production systems. *Vet Microbiol* 83, 45-60.
- Khalil, K., Lindblom, G.B., Mazhar, K., Kaijser, B., 1994, Flies and water as reservoirs for bacterial enteropathogens in urban and rural areas in and around Lahore, Pakistan. *Epidemiol Infect* 113, 435-444.
- Kranker, S., Alban, L., Boes, J., Dahl, J., 2003, Longitudinal Study of *Salmonella enterica* Serotype Typhimurium Infection in Three Danish Farrow-to-Finish Swine Herds. *Journal of Clinical Microbiology* 41, 2282-2288.
- Letellier, A., Messier, S., Quessy, S., 1999, Prevalence of *Salmonella* spp. and *Yersinia enterocolitica* in finishing swine at Canadian abattoirs. *J Food Prot* 62, 22-25.
- Magistrali, C., Dionisi, A.M., De Curtis, P., Cucco, L., Vischi, O., Scuota, S., Zicavo, A., Pezzotti, G., 2008, Contamination of *Salmonella* spp. in a pig finishing herd, from the arrival of the animals to the slaughterhouse. *Research in Veterinary Science* 85, 204-207.
- Swanenburg, M., van der Wolf, P.J., Urlings, H.A., Snijders, J.M., van Knapen, F., 2001, *Salmonella* in slaughter pigs: the effect of logistic slaughter procedures of pigs on the prevalence of *Salmonella* in pork. *Int J Food Microbiol* 70, 231-242.
- Wales, A.D., McLaren, I.M., Bedford, S., Carrique-Mas, J.J., Cook, A.J., Davies, R.H., 2009, Longitudinal survey of the occurrence of *Salmonella* in pigs and the environment of nucleus breeder and multiplier pig herds in England. *Vet Rec* 165, 648-657.

	After C&D	Entering	Farrowing +4days	Weaning	After C&D	+ 2 weeks	End	After C&D	+ 1 mo.	+ 1 mo.	+ 1 mo.	End
Sows	/	-	-	-								
Pigs	/	-	-	-	/	X06	X06		X06	X06 ; X42; X43	X20	X06
Room	-	-	-	X26	X06	X06	X06	X03	X05; X06	X05	X12; X20	X06
Corridor	X29	/	/	/	X06	/	/	-	/	/	/	X06
Loading bay	/	/	/	/	/	/	/	/	/	/	/	X11
Adjacent environment	/	/	X42	X26	/	X42	/	/	X04	X28	-	/
Other productions	/	/	X10	-	/	/	-	/	X04; X06	X07	-	/

	Loading	Lairage	Polishing	Evisceration	Splitting	Chilling	1 st cutting	2 nd cutting
Truck ±	<u>X45</u>							
Lairage room ±		<u>X45</u> ; <u>X52</u>						
Back of pigs *		X06 ; X15; <u>X45</u> ; <u>X52</u>						
Equipment ±			-	X53 (knife)	-	/	-	-
Carcasses *			X20; X33; <u>X45</u>		X44	-		
Caeca *				X06 ; X15; X33; X42; <u>X45</u>				
Pork cuts *							/	-

Figure 1: Distribution of *Salmonella* pulsotypes detected along the investigation in farm B from farrowing to pork cuts
C&D: Cleaning and disinfection. /: not done. -: negative sample. *: sampled after step indicated in the arrow. ±: sampled before step indicated in the arrow. Pulsotypes X03, X04, X05, X06, X07, X10, X12, X20 are S. 4,[5],12,;i:-; X15, X26, X28 and X29 are S. Typhimurium; X33 is S. Rissen; X42, X43 and X44 are S. Livingstone; X45 is S. Derby; X52 is S. London and X53 is S. Bredeney. X06 (in bold) is a predominant pulsotype. X45 and X52 (underlined) are pulsotypes identified from truck and lairage room and detected on carcasses.

Prevalence and risk indicators associated with *Salmonella* infection in farrow-to-finish farm in Reunion Island

Tessier C*, ^(1,2,3), Atiana, L. ^(2,3), Denis, M. ^(4,5), Cardinale, E. ^(2,3)

⁽¹⁾ *Coopérative des producteurs de porcs de la Réunion, 97410, Saint Pierre, Réunion*

⁽²⁾ *Centre de coopération internationale en recherche agronomique pour le développement, 97490, Sainte Clotilde, Réunion*

⁽³⁾ *Centre de Recherche et de Veille dans l'Océan Indien, Saint Clotilde, Réunion*

⁽⁴⁾ *Anses Laboratory of Ploufragan/Plouzané, Unit of Health and Quality of Poultry And Pig Products, BP 53, 22440 Ploufragan, France.*

⁽⁵⁾ *European University of Brittany, France.*

*corresponding author : ctessier@cpvr.fr

Abstract

Salmonella is the second cause of foodborne diseases in France and pork products are regularly incriminated. For the implementation of control measures in farms, it is necessary to better understand the risks of infection of pigs by *Salmonella* at farms. Our epidemiological study aims to determine *Salmonella* prevalence of fattening pigs in Reunion Island, oversea territory of France, and the main risk indicators for pig infection.

Fifty farms (farrow-to finish and multiplier) were randomly selected and visited 4 times: at slaughtered previous batch, after cleaning and disinfection, beginning and end of the fattening period for the studied batch. Pools of fresh faeces, gauze socks and gauze swabs were sampled to assess the bacteriological status of these pig farms. A questionnaire was submitted to farmers and analysed for the further identification of risk indicators.

Thirty-nine farms (78%_{IC95%} [68.3-87.6]) were tested positive for *Salmonella*. High prevalence of *Salmonella* may be related to poor decontamination. S. 4,[5],12:i:-, S. Livingstone, S. Weltevreden, S. Derby, S. Rissen, S. Typhimurium and S. Newport were the prominent serovars.

Among the risk indicators identified, farm located in low altitude, with a low number of houses, where ceiling and natural service area were not cleaned and disinfected, and with ineffective pest control were identified as risk indicators of *Salmonella* shedding by fattening pigs.

Introduction

Salmonellosis is one of the most important foodborne disease in many country, up to 95000 confirmed human cases in 2011 was infected by *Salmonella* in Europe (EFSA, 2013). In industrialized countries, 15% of cases of salmonellosis have been associated to the consumption of pork products (Berends *et al.*, 1998). The pig industry of Reunion Island, tropical island located in Indian Ocean, is also concerned by this public health burden. Despite the asymptomatic carriage of *Salmonella* by pigs, it's necessary to understand the different ways of contamination of breeding pigs in order to limit the presence of *Salmonella* on carcasses at the slaughterhouse and to minimize the risk of transfer of *Salmonella* to humans. However, today, few data on *Salmonella* have been collected in pig in Reunion Island (Cardinale *et al.*, 2010). Our epidemiological study aims to determine *Salmonella* prevalence of fattening pigs and identify the main risk indicators for pig infection in Reunion Island.

Materials and methods

Farms : Our study was carried out from January 2011 to August 2012 and involved 50 pig farms: 42 farrow-to-finish farms and 8 multiplier farms. These farms were selected randomly in membership of pork producers' cooperative (CPVR) in Reunion Island. Farms with several different animal productions on the same site were included in this survey. The farm selection was also based on the owner's willingness to cooperate. The sample size was calculated on the basis of a target assumed-herd-prevalence of 40% with an accuracy of 5% and 95% confidence limits (Cardinale *et al.*, 2010).

Samples and data collection : Only one fattening pig batch (pigs born on the same week) was considered per farm. Each farm was visited four times.

The first visit was held at the end of the previous batch before slaughtering. The second visit occurred after cleaning and disinfection, just prior to new loading. The third and the fourth visits took place at the beginning and at the end (25 weeks of age) of the fattening period. Data concerning the farm and housing conditions, manure storage, drinking and feeding practices, pest control, cleaning and disinfection procedures, environmental circumstances as well as hygiene and health aspects were collected from a questionnaire administered by a single investigator to each farmer. At the first (previous batch), third (beginning of the fattening period) and fourth visits (end of the fattening period), samples consisted of 4 pools of 20 fresh fecal samples and of soil surface using a pair of gauze socks. During the second visit, after cleaning and disinfection, walls were sampled using gauze swabs and soil surface using gauze socks.

Salmonella isolation and identification : Samples were pre-enriched in buffered peptone water (BPW) in 1:10 sample/ broth ratio. Disinfectant neutralizer was added in BPW broth (1:10) for samples collected after cleaning and disinfection. Environmental swabs and socks were pre-enriched with 150 and 300 ml of BPW respectively. After incubation at 37°C for 18h ± 2h, 1 ml and 3 drops of BPW broth were inoculated respectively to 10 ml of Müller–Kauffmann Tetrathionate-Novobiocin broth (MKTn) and on Modified Semisolid Rappaport Vassiliadis agar (MSRV) plate. Both were incubated respectively at 37°C for 24 h and 41.5°C for 24-48h. A loopful of selective enrichment in MKTn was then streaked on Xylose-Lysine-Desoxycholate Agar (XLD) and Rambach Agar. MSRV plates with characteristic halo of migration were streaked onto XLD and Rambach agar. After 24h at 37°C, four typical colonies per sample (one per way if possible) were biochemically confirmed as *Salmonella* and serotyped according to the Kauffman-White scheme.

Statistical analysis : The epidemiological unit of the study was the batch. The batch followed in this study was the one that was transferred from post-weaning room to the fattening room during the third visit. The batch was declared infected if at least one sample tested positive. The outcome variable was thus dichotomous (infected batch versus non-infected batch). A bivariate analysis related *Salmonella* status of the batch to each of the explanatory variables. The variables associated with *Salmonella* status of the batch were selected. All bilateral relationships between the possible explanatory variables were checked. For relationships between variables evidencing strong structural collinearity, one of the two variables of interest (the most related to the outcome variable) was chosen.

Results

Salmonella shedding : Among the 50 farms, 39 (78%_{IC95%} [68.3-87.6]) were tested positive for *Salmonella* at the end of the fattening period. The bacterium was detected in 74%, 66% and 82% of the samples collected during the first, the second and the third visit. Among the eleven different *Salmonella* serotypes identified, the predominant serotypes were S. 4,[5],12,i:- (28% of total herds), S. Livingstone (20%), S. Weltevreden (18%), S. Derby (12%), S. Rissen (10%), S. Typhimurium (10%), S. Newport (10%), S. Amsterdam (2%), S. Enteritidis (2%), S. Durban (2%) and S. Kisangani (2%).

Risk indicators : Fourteen risk indicators were identified (Table 1). The modality of the variables for which the number of positive farms is higher was identified as risk indicator for *Salmonella* infection of fattening pigs. These results have to be confirmed by logistic regression analysis.

Discussion

In our study, 78% of herds at the end of the fattening period were positive for *Salmonella*. Compared to results from previous studies in Europe, the prevalence is higher in Reunion Island. Indeed, in 2004 Beloeil *et al.* detected a pig herd *Salmonella* contamination around 36% in France. This difference could be explained by tropical climate (temperature and humidity) which is favorable for *Salmonella* growth and persistence. A prospective study, carried out by Cardinale *et al.* (2010), bring out a prevalence of *Salmonella* in farms around 60%, which is close to our result.

The most predominant serotype was S. 4,[5],12,i:-; monophasic emergent serotype often recovered from other studies concerning pigs (EFSA, 2013). S. Typhimurium, S. Weltevreden, S. Derby and S. Livingstone, had ever been identified in pigs in Reunion Island (Cardinale *et al.*, 2010).

The risk of *Salmonella* infection of farms decreases when there are more than 3 pig houses in the farm; farmers with several houses probably dissociate the different stage of growing in different houses and limit then transmission between pig stages. The risk of *Salmonella* contamination of farms decreases when the farm was located higher than 600 meters above sea level; in altitude in Reunion Island, there are less animal productions which can limit *Salmonella* dissemination or *Salmonella* is sensitive to low temperatures and higher moisture. However, no association between farm density and *Salmonella* risk was found in the study of Benschop *et al.* (2008). But inactivation of *Salmonella* was observed with low temperature and increasing moisture (Villa-Rojas, *et al.*, 2013).

Ebeling (1997) hypothesized that mice, poultry and pig could be contaminated by cockroach faeces where *Salmonella* is stable for years. Rodents can also introduce *Salmonella* and participate to maintain infection on farms.

An effective pest control system is important; indeed, in Reunion Island, farms without disinsectization or only done by farmers have more risk to be contaminated by *Salmonella*.

Cleaning and disinfection of ceiling and of the natural service area were associated with decreased risk of infection. However, it is surprising that there is no other practice associated with *Salmonella* infection. The low *Salmonella* prevalence in farms using trough in fattening room is contrary to risk factors identified by (van der Wolf et al., 1999). Farmers with trough gave wet feed; (Fablet et al., 2003) showed that dry feed enhanced the risk of *Salmonella* shedding compared to wet feed. Use of antibiotic treatment during post-weaning period seems to be protective against *Salmonella* infection in pigs. However, studies showed that preventive antibiotic treatment during fattening period could enhance the risk of *Salmonella* shedding (Rossel et al., 2006).

Conclusion

These previous analysis allowed to identify risk indicators for *Salmonella* infection in fattening pigs in Reunion Island. Logistic multiple-regression analysis will confirm risk factors. PFGE on all isolates and MLVA on *S. Typhimurium* and *S. 4,[5],12;i:-* is in progress and will highlight the potential sources of contamination. This survey will help to identify measures which could be applied in farm in order to decrease *Salmonella* prevalence in pig production in Reunion Island.

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Table 1: Definition of risk indicators for *Salmonella* infection in fattening pig herds in Reunion Island

Definition of variables	Level	% of flocks	% of positive flocks	p-value
<i>General consideration</i>				
Frequency of entry in farm by day	1 to 2	48	92	0,020
	≥3	30	63	
Number of houses	1	40	91	0,014
	2	26	81	
	≥3	12	50	
Altitude	<250m	32	94	0,048
	250-600	24	75	
	>600	22	65	
Number of sows by band	≤6	54	90	0,042
	>6	34	60	
<i>Biosecurity</i>				
Disinsectization	Not done or by farmer	74	84	0,015
	By professional	12	33	
Equipment lending	Yes	36	91	0,050
	No	42	67	
<i>Cleaning and disinfection</i>				
Natural service area	Yes	10	50	0,043
	No	69	87	
Ceiling	Yes	18	56	0,016
	No	60	88	
<i>Gestation/service stage</i>				
Distribution feeding	Automatic	12	55	0,043
	Manual	66	85	
Maternity stage				
Material for feeder	Plastic	34	64	0,036
	Other	45	95	
<i>Post-weaning stage</i>				
Weighing distribution feed	Yes	34	65	0,037
	No	44	92	
<i>Fattening stage</i>				
Container	Trough	10	50	0,025
	Feeder	68	85	
<i>Treatment</i>				
Injection method	Only restraining	36	67	0,049
	Other methods	42	91	
Antibiotic treatment during post-weaning	Yes	2	25	0,029
	No	76	83	

References

- Arnold, M.E., Cook, A.J.C., Davies, R.H., 2006. A modelling approach to estimate the sensitivity of pooled pen faecal samples of isolation *Salmonella* in pig. *Journal of the Royal Society Interface* 2, 365-372.
- Beloil, P.A., Fravallo, P., Fablet, C., Jolly, J.P., Eveno, E., Hascoet, Y., Chauvin, C., Salvat, G., Madec, F., 2004, Risk factors for *Salmonella enterica* subsp. *enterica* shedding by market-age pigs in French farrow-to-finish herds. *Preventive Veterinary Medicine* 63, 103-120.
- Benschop J., Hazelton M.L., Stevenson M.A., Dahl J., Morris R.S., French N.P., 2008. Descriptive spatial epidemiology of subclinical *Salmonella* infection in finisher pig herds: application of a novel method of spatially adaptive smoothing. *Veterinary Research* 39, 2.
- Berends, B.R., Van Knapen, F., Mossel, D.A., Burt, S.A., Snijders, J.M., 1998, Impact on human health of *Salmonella* spp. on pork in The Netherlands and the anticipated effects of some currently proposed control strategies. *Int J Food Microbiol* 44, 219-229.
- Cardinale, E., Maeder, S., Porphyre, V., Debin, M., 2010, *Salmonella* in fattening pigs in Reunion Island: Herd prevalence and risk factors for infection. *Preventive Veterinary Medicine* 96, 281-285.
- Ebeling, W., 1997. Cockroaches as reservoirs and vector of drug resistant *Salmonella* spp. *Applied Bacteriology*
- EFSA, 2013. The European union summary report on Trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. *EFSA Journal* 11, 1-250.
- Fablet, C., Fravallo, P., Jolly, J.P., Eveno, E., Madec, F., Beloil, P.-A., 2003, Recherche des facteurs de risque de l'excrétion de *Salmonella enterica* par les porcs en croissance. Enquête épidémiologique analytique en élevage naisseur-engraisseur. *Epidémiologie et Santé animale* 43, 61-73.
- Rossel, R., Rouillier, J., Beloil, P.A., Chauvin, C., Basta, F., Crabos, J.P., Theau-Audin, S., 2006. *Salmonella* en élevages de porcs du Sud-Ouest de la France: séroprévalence en fin d'engraissement et facteurs de risque associés. In: *Journées Recherche Porcine*, Paris, pp. 371-378.
- Villa-Rojas R., Tang J., Wang S., Gao M., Kang D.H., Mah J.H., Gray P., Sosa-Morales M.E., López-Malo A., 2013. Thermal inactivation of *Salmonella enteritidis* PT 30 in almond kernels as influenced by water activity. *Journal of Food Protection* 76, 26-32.
- van der Wolf, P.J., Bongers, J.H., Elbers, A.R., Franssen, F.M., Hunneman, W.A., van Exsel, A.C., Tielen, M.J., 1999, *Salmonella* infections in finishing pigs in The Netherlands: bacteriological herd prevalence, serogroup and antibiotic resistance of isolates and risk factors for infection. *Vet Microbiol* 67, 263-275.

Prevalence and risk factors susceptibility of *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. isolated from pork and poultry sausages, in Reunion Island, France

Trimoulinard, A.^(1,2,3), Tessier, C.^(1,2,3,4), Beral, M.^(1,2,3), Atiana, L.^(1,2), Henry, I.⁽⁵⁾, Cardinale, E.^(1,2,3).

1. Centre de coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), UMR 15 CMAEE, F-97490 Sainte Clotilde, La Réunion, France
2. Institut national de la recherche agronomique (INRA), UMR 1309 CMAEE, F-97490 Sainte Clotilde, La Réunion, France
3. Centre de Recherche et de Veille des maladies émergentes dans l'Océan Indien (CRVOI), plateforme de recherche CYROI, F-97490 Sainte Clotilde, La Réunion, France
4. Coopérative des Producteurs de Porcs de la Réunion, -F-97410 Saint-Pierre, La Réunion, France
5. Crête d'Or Entreprise, F- 97490, Sainte Clotilde, La Réunion, France.

*corresponding author : ctessier@cpr.fr

Abstract

Zoonoses are a public health burden in France. The most predominant pathogens incriminated in TIAC are *Salmonella* spp., *Campylobacter* spp., and *Listeria* spp. The population of Reunion Island eats a lot of pork and poultry meats. One of the local cooking characteristic is the sausage 100% poultry or 100% pig. Actually, no similar study has been conducted in Reunion Island. We aimed to determine the health risk related to the consumption of this kind of products, through a transversal analysis, by identification and quantification of bacteria in pork and poultry sausages. Meat characteristics and sale practices associated with these three bacteria in outlets of Reunion Island were identified by a binomial regression. The microbiological culture was carried out according to AFNOR methods.

Two hundred and three pork or poultry sausages were sampled randomly from 67 local distributors in Reunion Island. *Salmonella enterica* was detected in 11.87% (95% confidence interval: [7.80-17.32]) of sample and in 26.87% (CI 95% [17.11-39.2]) of the outlets, with a most probable number count ranging from 6.00 bacteria per gram to 380 bacteria per gram. *Salmonella* serotypes isolated from pork or poultry sausage were S. Typhimurium (45.83 %), S. London (20.83 %), S. Derby (16.67 %), S. Newport (8.33 %), S. Blockley (4.17 %) and S. Weltevreden (4.17 %). We found that *Salmonella* spp. infection was positively associated with two things. The first one is the packaging for sausages (plastic or paper) and the second one is the fact that there is no fight against rodents. High surface areas of sale (> 250 m²) decrease the risk.

Only 4.48% of the outlets studied and 1.48% of sausages were contaminated with *Campylobacter* spp. Risk factors couldn't be determined because of the low prevalence.

Listeria spp. was isolated in 64.18% of the outlets studied and in 30.00% of sausages. Dirty clothes of restaurant employees increased risks of *Listeria* spp. contamination whereas the use of a disinfectant to clean the refrigerated displays decreased risks of contamination.

Introduction

Poultry and pigs breeding are very important in Reunion Island. Pork and poultry products should be protected from any bacteriological contamination to avoid risks for human infection. Contamination of pigs and poultry production by *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. is a major public health burden and also an economic problem. It should be noted that *Campylobacter* spp. and *Salmonella* spp. are the main bacteria responsible for collective food-borne infections in industrialized countries. (EFSA., 2012).

One of the notable features of the local cooking remains 100% pork and 100% poultry sausages which are composed of lean, fat, but also skin. This last feature is not without consequence on the health risks associated with the consumption of this type of product since the skin is the headquarters of *Salmonella* spp., *Campylobacter* spp and *Listeria* spp and is being used for the manufacture of sausages (Chamber of Trades and Crafts, 2012). In Reunion Island, except bacteriological controls conducted on farms and in slaughterhouses, there is not enough available information about the impact of this bacterium in this type of food preparation on the consumers.

This study aimed to determine the health risk related to the consumption of this kind of products, through a transversal analysis. We determine the presence *Salmonella spp.*, *Campylobacter spp.* and *Listeria spp.* and the quantification of *Salmonella spp.* in pork and poultry sausages. Products' characteristics and risk factors for sale practices associated with the *Salmonella spp.*, *Campylobacter spp.*, and *Listeria spp.* seroprevalence in outlets in Reunion Island were identified.

Material and Methods

Study sample

To obtain a representative and randomly sample, a random drawing on an exhaustive list was done. Our study included 67 outlets (supermarkets, grocery trades and butcher shops) in Reunion Island.

Data collection

In each outlet, we took 1 to 5 samples of different categories of sausages following availability (fresh, frozen or packed sausages). For each outlet, data on manufacturing practices and conservation were collected from a questionnaire on the following topics: general characteristics, cleaning and disinfection procedures, staff hygiene, presence of rodents and other domestic animals and waste management.

Microbiological analysis

On samples, we determined the presence and identification of *Salmonella spp.*, *Campylobacter spp.* and *Listeria spp.* After identification of *Salmonella spp.*, the most probable number (MPN) technique was used to estimate microbial populations. For *Salmonella spp.* this whole procedure referred to the French AFNOR (French Agency of Standardization) procedure NF EN ISO 6579 which is the reference method. For *Campylobacter spp.* isolation and identification, procedure used the reference method NF EN ISO 11272-1. For *Listeria spp.* isolation and identification, procedure used the reference method NF EN ISO 11290-1.

Data analysis

One outlet is declared infected by *S. enterica* subsp. *enterica*, *Campylobacter spp.*, and *Listeria spp.* if at least one sausage sample is tested positive. Binomial regression was used to assess the relationship between explanatory variables and *Salmonella spp.*, *Campylobacter spp.* and *Listeria spp.* status of the outlet. The contribution of each factor to the model was tested with a likelihood-ratio χ^2 through a backward stepwise procedure. At the same time, the best parsimonious model was compared to the full model by the Akaike information criterion.

Results

Sample description. In total, we investigated 67 outlets including 15 butchers shops, 4 butchers shops & grocery trades, 28 supermarkets and 20 grocery trades.

Salmonella spp. The global prevalence of pork sausage was 11.82% (CI 95% [7.80-17.32]). 26.87% (CI 95% [17.11-39.24]) of the outlets studied. The most prevalent serotypes isolated were *Salmonella* Typhimurium and *Salmonella* London. Pork sausages, smoked sausages, fresh sausages and butcher shops were more significant infected.

Average of quantity of *Salmonella spp.* per sample infected is 72.90 bacteria per gram (Min: 6.00 bacteria per gram; Max: 380.00 bacteria per gram). Butcher shops associated with grocery trade shows a higher probable number than the others outlets.

The risk of outlet infection with *Salmonella spp.* was increased when sausages are sold in plastic bags (OR = 26.63; CI 95% [11.85 -752.55]) or in paper bags (OR = 9.00; CI 95% [1.59-171.89]). As well, no rodents control increased the risk of *Salmonella spp.* infection (OR = 5.42; CI 95% [1.48-20.43]). However, high surface area sale (> 250 m²) decreased the risk (OR = 0.99; CI 95% [0.98-0.99]).

Campylobacter spp. Only 4.48% of the outlets studied and 1.48% of sausages were contaminated with *Campylobacter spp.* Risk factors couldn't be determined because of the low prevalence. The results can be explained by the sensitivity of this bacterium to desiccation and cold treatment.

Listeria spp. *Listeria spp.* was isolated in 64.18% of the outlets studied and in 30.08% of sausages. Dirty clothes for restaurant employees (OR=1.96 ; CI 95% [1.01-3.79]) increased risks of *Listeria* contamination whereas the use of a disinfectant for cleaning of the refrigerated displays (OR=0.13 ; CI 95% [0.01-0.64]) decreased the risk of contamination.

Discussion

Salmonella spp. This study is the first one achieved in Reunion Island. The most prevalent *Salmonella* serotype among outlets was *Salmonella* Typhimurium. This is the first serotype detected on humans (CNRSS., 2009). This *Salmonella* serotype was also isolated frequently from pigs (Cardinale et al., 2010) rearing in Reunion Island. Quantities of *Salmonella* identified using MPN method doesn't exceed 1 100 bacteria per gram, which minimum concentration to cause infection non-typhoid *Salmonella* (Spricigo et al., 2008).

The fight against rodents is a major problem in Reunion Island. These rodents are known to be carriers of *Salmonella* spp. (Meerburg and Kijlstra, 2007; Meerburg, 2006) and represent a significant risk of transmission in outlets and in environment. In addition, paper or plastic packaging already contaminated, a cross-contaminated by a lack of personal hygiene (Norrung, 2000) or raw products sold in the refrigerated display next to the sausages are all possible hypotheses for the risk of contamination.

A bigger retail store could be related to the practical cleaning and disinfection. In addition, a bigger retail store limits the possibilities of cross-contamination because there is more space between products.

Campylobacter spp. The prevalence of *Campylobacter* spp. in outlets of Reunion Island is very low (1.48 %). This result is surprising since contamination of pork and poultry production is important (Henry et al., 2011).

Listeria spp. In Reunion Island, prevalence of *Listeria* spp. is high (30.00 %), which can be explained by a good ability of *Listeria* spp. to withstand cold because it's a psychrophilic bacteria (Rosset et al., 2002). The personal hygiene practices are very important because they can be the source of cross-contamination (Kahraman et al., 2010). Using a detergent to clean refrigerated displays can limit contamination or persistence of *Listeria* spp. in pork and poultry sausages.

Conclusion

In view of the results of our study, there may be a health risk to the consumer, considering that some people consume these products slightly cooked. Hygiene practices are necessary and essential in the fight against *Salmonella* spp., *Campylobacter* spp., and *Listeria* spp. in each sector of pork and poultry. The Chamber of Trades and Crafts carried important work by proposing guide to good practice, HACCP and to conduct an assessment of risks in the butchers. However, butchers and grocers are not sensitive to all these arguments and many efforts are still needed.

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References

- Cardinale, E., Maeder, S., Porphyre, V., Debin, M., 2010. *Salmonella* in fattening pigs in Reunion Island: Herd prevalence and risk factors for infection. Preventive Veterinary Medicine 96,, 281-285.
- Chamber of Trades and Crafts 2012 (Saint Denis, Reunion island).
- CNRSS. 2009. Rapport d'activité annuel (Paris, Laboratoire des Bactéries Pathogènes Entériques Institut Pasteur.), 71.
- EFSA. 2012. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010 (European Food Safety Authority), 378.
- Henry, I., Reichardt, J., Denis, M., Cardinale, E., 2011. Prevalence and risk factors for *Campylobacter* spp. in chicken broiler flocks in Reunion Island (Indian Ocean). Preventive Veterinary Medicine 100, 64-70.
- Kahraman, T., Cetin, O., Dumen, E., Buyukunal, S.K., 2010. Incidence of *Salmonella* spp. and *Listeria monocytogenes* on equipment surfaces and personnel hands in meat plants. Revue Méd. Vét. 161, 108-113.
- Meerburg, B.G., Kijlstra, A., 2007. Role of rodents in transmission of *Salmonella* and *Campylobacter*. Journal of the Science of Food and Agriculture 87, 2774-2781.
- Meerburg, J.-R.W., Wagenaar JA, Kijlstra A., 2006. Presence of *Salmonella* and *Campylobacter* spp. in wild small mammals on organic farms. Appl Environ Microbiol 72, 960-962.
- Norrung, B., 2000. Microbiological criteria for *Listeria monocytogenes* in foods under special consideration of risk assessment approaches. International Journal of Food Microbiology 62,, 217-221.
- Rosset, P., Beaufort, A., Cornu, M., Poumeyrol, G., 2002. La chaîne du froid en agroalimentaire. Cahier de Nutrition et de Diététique 37, 124-130.
- Spricigo, D.A., Matsumoto, S.R., Espindola, M.L., Ferraz, S.M., 2008. Prevalence, quantification, and antimicrobial drug resistance of *Salmonella* serotypes isolated from fresh pork sausage. Ciencia E Tecnologia De Alimentos 28, 779-785.

Effect of age and anatomic site on likelihood of detecting *S. aureus* in pigs

Linhares, L., Yang, M., Sreevatsan, S., Davies, P.R.*

Department of Veterinary Population Medicine, University of Minnesota, Saint Paul, MN, USA

*corresponding author: davie001@umn.edu

Abstract

Intensive sampling of two swine farms in Minnesota was conducted to obtain basic information about the ecology and epidemiology of *S. aureus* in modern multiple site swine production. The farms were selected by convenience, and two cohorts of animals were sampled in each system. Samples were collected from the nose, tonsils, feces, and skin of suckling pigs, weaned pigs, and finishing pigs in each of two birth cohorts raised in each system, and from the same sites plus the vagina of sows nursing the selected piglets. Highest prevalence of *S. aureus* was in nasal, tonsil and skin samples (59 – 65%), and lowest prevalence (27%) was in vaginal samples from sows. No MRSA were detected on the farms. Diverse spa types of *S. aureus* were found on both farms and all age groups. The predominant spa types found belonged to MLST sequence type ST398, ST9, and ST5. *S. aureus* was also frequently detected in environmental samples, including air, and in people working on the farms. The 3 predominant spa types encountered (t034/ST398; t337/ST9; t002/ST5) have previously been identified as MRSA isolates of pigs in various international studies. The use of the term ‘livestock associated MRSA’ should be broadened to include certain variants belonging to the ST9 and ST5 sequence types (and likely others) rather than be synonymous with ST398 MRSA.

Introduction

Recognition of the common occurrence of livestock associated *Staphylococcus aureus* (LA-MRSA) in food animals has raised concerns about the potential occupational and public health risks associated with these organisms. Although there has now been extensive research into the occurrence of MRSA in many countries, there are remarkably few studies of generic *S. aureus*. We contend that understanding of the ecology and epidemiology of LA-MRSA cannot be achieved in a void of information about the parent organism, regardless of antimicrobial resistance status. That is, the ability to understand the origins and implications of LA-MRSA in pigs is limited by lack of basic information about the ecology of *Staphylococcus aureus* in pork production.

Published studies in pigs have almost universally used nasal swabbing, which is the standard method (with or without pharyngeal swabbing) for detecting *S. aureus* in humans. Environmental samples (such as dust) are effective for determining herd status for MRSA, but are of limited value for studying the ecology of an organism that is deemed to be normal flora in swine. There are reports of *S. aureus* being detected in various anatomical sites including sites of pigs including skin, tonsils, feces, nose, arthritic joints and internal organs. However, there has been no systematic study comparing sampling protocols for detecting *S. aureus* in pigs. We conducted a pilot study of two production systems to obtain basic information about *S. aureus* prevalence in pigs, and the relative sensitivity of detection of *S. aureus* by anatomical site. Specific aims were to (a) compare the effect of sample site on detection of *S. aureus* in pigs and (b) compare *S. aureus* prevalence and spa types in sows, suckling pigs, weaned pigs and finishing pigs.

Materials and Methods

A longitudinal study was conducted in 2 independent pig production systems in Minnesota. Eligibility of farms for the study was limited only by production type, as we wanted to study farms using multiple site systems (breeding herd and growing pigs reared on separate sites) that predominate in the US industry. The farms were selected purposively based on willingness of the producers to participate. The core assumption in design of the project was that *S. aureus* is part of the normal flora of pigs, and expected prevalence in preferential sites of carriage should exceed 50%. In the absence of prior information, and given resource limitations, we chose to conduct detailed sampling (4 – 5 anatomical sites per animal) in a relatively small number of pigs per sampling event. Samples were collected from recently farrowed sows (n=12 spread through a room) and one piglet from each respective litter (n=12). Subsequent sampling of the same birth cohorts of pigs occurred 4 and 20 weeks later in the “nursery” (n=12) and “market-age” (n=12) phases of pig production, respectively. Two pig cohorts were sampled in each flow, with at least 6 months between cohorts. Swab samples were collected from the nose, tonsil, feces, and skin (axilla) of each pig (plus vagina in sows).

Swabs were submitted to a double enrichment procedure using Mueller Hinton enrichment broth containing 6.5% NaCl, then Phenol red mannitol (PRM) broth with and without oxacillin (4mg/ml). Samples showing color change in PRM broths were and cultured on CNA agar and MRSA-selective plates. Putative *S. aureus* isolates were confirmed using the catalase and tube coagulase tests. Putative MRSA isolates were confirmed using PCR for *mecA* gene. DNA from each iso-

late was extracted with 10mM Tris-HCl and Lysostaphin and stored at -20°C. All confirmed *S. aureus* isolates were sub-typed using spa typing, and randomly selected isolates of all spa types were characterized using MLST following published methods. Spa types were determined using both the Ridom and egeonomics systems, and MLST types were obtained from the website <http://saureus.beta.mlst.net>.

Results

A total of 542 *S. aureus* isolates were obtained in the study, none of which were MRSA. Prevalence differed among anatomical sites ($p < 0.001$), and was higher in nose (68%), skin (62%) and tonsil (62%) samples than in fecal (42%) and vaginal (27%) samples. Prevalence of positive pigs (at least one site positive) was similar (from 88% to 96%) in all age-groups ($p = 0.55$). No MRSA were detected, but multiple spa types were detected in each system. Multiple spa types were also detected in many individual pigs, but there was no apparent association between spa type and anatomical location. Spa types t034 (ST398), t337 (ST9) and t002 (ST5) were most common. Spa type distributions were very similar between sows and their associated piglets, suggesting transmission from sow to offspring occurs commonly early after birth. Spa type patterns in growing pigs tended to vary between cohorts and farms

Discussion

This study confirms that *S. aureus* can indeed be considered part of the normal flora of pigs, and that the upper respiratory tract and skin are the sites where the bacteria can be most readily isolated. Vaginal colonization was common in sows, and colonization of piglets occurs early in life. Within farms, cohorts and even pigs, considerable diversity of spa types and sequence types was found. Interestingly, the most predominant sequence types (ST398, ST9, ST5) of the MSSA isolates detected on these two farms correspond with those reported most commonly in studies of MRSA in pigs. Since the initial detection of sequence type (ST) 398 MRSA in pigs in Europe, (Voss et al. 2005) research in other regions has revealed considerable genetic diversity among MRSA found in pigs. In particular, isolates of ST9 have been predominant in studies of swine in Asia, (Neela et al. 2009; Lo et al. 2012) while ST5 appear to be common in North America. (Khanna et al. 2008; Molla et al. 2012; Smith et al. 2013; Frana et al. 2013) The occurrence of these same sequence types and spa types among MSSA in this small pilot study suggest that these lineages of *S. aureus* are likely to have a long evolutionary relationship with pig. We also suggest that the term livestock association MRSA should not be restricted to the ST398 lineage, and that some variants of ST9 and ST5 *S. aureus* should be included under this 'umbrella' term.

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References

- Frana, T.S., Beahm, A.R., Hanson, B.M., Kinyon, J.M., Layman, L.L., Karriker, L.A., Ramirez, A., Smith, T.C., 2013. Isolation and Characterization of Methicillin-Resistant Staphylococcus Aureus from Pork Farms and Visiting Veterinary Students. PLoS One 8, e53738.
- Khanna, T., Friendship, R., Dewey, C., Weese, J.S., 2008. Methicillin Resistant Staphylococcus Aureus Colonization in Pigs and Pig Farmers. Vet. Microbiol. 128, 298-303.
- Lo, Y.P., Wan, M.T., Chen, M.M., Su, H.Y., Lauderdale, T.L., Chou, C.C., 2012. Molecular Characterization and Clonal Genetic Diversity of Methicillin-Resistant Staphylococcus Aureus of Pig Origin in Taiwan. Comp. Immunol. Microbiol. Infect. Dis. 35, 513-521.
- Molla, B., Byrne, M., Abley, M., Mathews, J., Jackson, C.R., Fedorka-Cray, P., Sreevatsan, S., Wang, P., Gebreyes, W.A., 2012. Epidemiology and Genotypic Characteristics of Methicillin-Resistant Staphylococcus Aureus Strains of Porcine Origin. J. Clin. Microbiol. 50, 3687-3693.
- Neela, V., Mohd Zafrul, A., Mariana, N.S., van Belkum, A., Liew, Y.K., Rad, E.G., 2009. Prevalence of ST9 Methicillin-Resistant Staphylococcus Aureus among Pigs and Pig Handlers in Malaysia. J. Clin. Microbiol. 47, 4138-4140.
- Smith, T.C., Gebreyes, W.A., Abley, M.J., Harper, A.L., Forshey, B.M., Male, M.J., Martin, H.W., Molla, B.Z., Sreevatsan, S., Thakur, S., Thiruvengadam, M., Davies, P.R., 2013. Methicillin-Resistant Staphylococcus Aureus in Pigs and Farm Workers on Conventional and Antibiotic-Free Swine Farms in the USA. PLoS One 8, e63704.
- Voss, A., Loeffen, F., Bakker, J., Klaassen, C., Wulf, M., 2005. Methicillin-Resistant Staphylococcus Aureus in Pig Farming. Emerg. Infect. Dis. 11, 1965-1966.

Longitudinal study of *Staphylococcus aureus* and MRSA colonization of US swine veterinarians

Sun J., Linhares, L., Yang, M., Sreevatsan, S., Davies, P.R.*

Department of Veterinary Population Medicine. University of Minnesota, Saint Paul, MN, USA

*corresponding author: davie001@umn.edu

Abstract

Patterns of detection of *S. aureus* are being evaluated in a longitudinal study of a cohort of 67 swine veterinarians in the USA. This report presents interim data from the initial period of the study. Overall, approximately 70% of sampling events yielded *S. aureus* in nasal swabs from veterinarians, and 8% yielded MRSA isolates. The majority of isolates belonged to ST398, ST9 and ST5 which together comprise the sequence types that have predominated among MRSA isolates from pigs in Europe, Asia and North America. Detection of *S. aureus* was negatively associated with time since pig contact. Patterns of colonization varied greatly among participants, with approximately one-fifth showing evidence of long term colonization, while transient colonization was suggested for most participants

Introduction

Recognition of the common occurrence of livestock associated *Staphylococcus aureus* (LA-MRSA) in food animals has raised concerns about the potential occupational and public health risks associated with these organisms. Since the initial detection of sequence type (ST) 398 MRSA in pigs in Europe, (Voss et al. 2005) more extensive research has revealed greater genetic diversity among MRSA found in pigs. In particular, isolates of ST9 have been predominant in studies of swine in Asia, (Neela et al. 2009; Lo et al. 2012) while ST5 appear to be common in North America. (Khanna et al. 2008; Molla et al. 2012; Smith et al. 2013; Frana et al. 2013) Although nasal colonization with LA-MRSA is common in livestock workers, questions remain about whether workers are permanently or transiently colonized, and whether this represents any substantial risks to their health.

Numerous studies have documented that individuals with occupational exposure to livestock are at elevated risk of nasal colonization with MRSA, and that the variants of MRSA detected are predominantly those found in animals with which they have contact. Most studies have been cross-sectional and therefore give little insight into the dynamics of colonization in humans, or whether the culture positive nasal swabs represent transient contamination of the nasal epithelium from contaminated environments rather than genuine persistent colonization. There are several studies indicating that many short term exposures result in only transient contamination. (Graveland et al. 2011; van Cleef et al. 2011; Frana et al. 2013) However, these studies do not necessarily represent the situation of workers with prolonged daily contact with livestock. Longitudinal studies in farm workers are problematic as these groups generally have almost daily exposure to barn environments, and mostly to the same groups of animals. Therefore persistent culture positive results in farm workers provide limited insight into patterns of LA-MRSA colonization vs. contamination, as they could simply reflect repeated recent contamination.

Swine veterinarians represent a unique population to investigate this question. They are known to be at elevated risk of MRSA colonization and have regular exposure to pigs. However, unlike farm workers, in the USA they are mostly exposed to diverse swine populations (i.e. multiple client farms). Another shortcoming of most research to date has been an almost myopic focus on MRSA, with minimal information available to date on the general ecology of *S. aureus* (MSSA and MRSA) in animals. The goal of this prospective longitudinal study of US swine veterinarians was to describe patterns of MSSA and MRSA detection in monthly nasal swabs in relation to the frequency and diversity of animal contact and the incidence of associated health events.

Materials and methods

A cohort of swine veterinarians was recruited at the 2012 meeting of the American Association of Swine Veterinarians. To be eligible for the study, volunteers had to be active swine veterinarians having regular (2 per week or more on average) contact with pigs. The target population range for the study was 50 – 70 veterinarians. After giving informed consent, participants were mailed materials for sample collection and transport, and shown a video to standardize the swabbing procedure. Commencing in July 2012, participants were contacted monthly by email to remind them to collect a nasal swab, and to complete an on-line survey. Survey questions related to time since pig exposure and use of personal protective equipment; recent (1 week) history of exposure to pigs including average daily contact and number of herds visited; contact with other animal species; incidence occupationally related injuries; incidence of skin or soft tissue injuries; occurrence on

any staphylococcal infections; and the date of swab collection. For logistic reasons, no attempt was made to standardize the interval from pig contact to nasal swabbing.

Nasal swabs were submitted to a double enrichment procedure using Mueller Hinton enrichment broth containing 6.5% NaCl, then Phenol red mannitol (PRM) broth with and without oxacillin (4mg/ml). Samples showing color change in PRM broths were and cultured on CNA agar and MRSA-selective plates. Putative *S. aureus* isolates were confirmed using the catalase and tube coagulase tests. Putative MRSA isolates were confirmed using PCR for *mecA* gene. DNA from each isolate was extracted with 10mM Tris-HCl and Lysostaphin and stored at -20°C. All confirmed *S. aureus* isolates were sub-typed using spa typing, and randomly selected isolates of all spa types were characterized using MLST following published methods. Spa types were determined using both the Ridom and eGenomics systems, and MLST types were obtained from the website <http://saureus.beta.mlst.net>.

Results

A total of 72 veterinarians were recruited to the study. Four withdrew before sample collection began due to concerns about frequency of pig contact, and 2 commenced sampling at the second (August 2012) sampling. One participant withdrew after 4 months due to migration from the USA. A cohort of 67 veterinarians is continuing to participate in the 18 month study. Sample submission and survey completion rates exceed 95%. The veterinarians reside in 15 major swine producing states of the USA and typically interact with diverse swine populations in their work.

Over the first 7 months of sampling, the prevalence of *S. aureus* among all samples was 70.7% (325/460), and varied among months 54% to 84%. MRSA prevalence varied from 4.4 to 10.6% among months, and MRSA isolates accounted for 7% of all isolates (32/460). Colonization patterns varied greatly among veterinarians, with 12 (21%) participants consistently colonized with the same spa type. Others participants showed considerable variability in spa types detected over time, while only two participants were consistently culture negative. The probability of culture positivity was associated with time since pig exposure, but delay in sample processing did not significantly affect the probability of a positive culture. The most prevalent isolates were t034-ST398 and t002-ST5, which comprised 74.2% (241/325) of all isolates. Several ST9 spa types (t337, t3446, t2498) were also common among MSSA isolates.

Discussion

The study described is ongoing and will be completed after 18 consecutive months of sampling. These interim results are of interest in relation to those from a recently published longitudinal study of swine veterinarians in Holland.¹⁰ Prevalence of *S. aureus* was similar (around 70%) in both studies, but the prevalence of MRSA was markedly higher (44%) in Dutch veterinarians than in our study in the USA (8%).(Verkade et al. 2013)

This observation adds to evidence suggesting that MRSA prevalence in the swine industry in the USA may be lower than seen in many European countries.(Smith et al. 2013) Our data also indicated that ST398, ST9 and ST5 variants are predominant among isolates from swine veterinarians, and likely indicate that most isolates were acquired from occupational exposure to animals. Finally, variable patterns of colonization were observed among veterinarians, with about 20% of veterinarians being consistently colonized with the same spa type. Verkade et al (2013) similarly found 14% of their study subjects appeared to be 'colonized' whereas different isolates were obtained from most individuals over the course of their study (5 samplings over 2 years). One could speculate that individual host determinants are likely to influence likelihood of genuine colonization of people with *S. aureus* of livestock origin, and that permanent colonization is not likely for a majority of people exposed to livestock.

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References

- Frana, T.S., Beahm, A.R., Hanson, B.M., Kinyon, J.M., Layman, L.L., Karriker, L.A., Ramirez, A., Smith, T.C., 2013. Isolation and Characterization of Methicillin-Resistant Staphylococcus Aureus from Pork Farms and Visiting Veterinary Students. PLoS One 8, e53738.

- Graveland, H., Wagenaar, J.A., Bergs, K., Heesterbeek, H., Heederik, D., 2011. Persistence of Livestock Associated MRSA CC398 in Humans is Dependent on Intensity of Animal Contact. *PLoS One* 6, e16830.
- Khanna, T., Friendship, R., Dewey, C., Weese, J.S., 2008. Methicillin Resistant Staphylococcus Aureus Colonization in Pigs and Pig Farmers. *Vet. Microbiol.* 128, 298-303.
- Lo, Y.P., Wan, M.T., Chen, M.M., Su, H.Y., Lauderdale, T.L., Chou, C.C., 2012. Molecular Characterization and Clonal Genetic Diversity of Methicillin-Resistant Staphylococcus Aureus of Pig Origin in Taiwan. *Comp. Immunol. Microbiol. Infect. Dis.* 35, 513-521.
- Molla, B., Byrne, M., Abley, M., Mathews, J., Jackson, C.R., Fedorka-Cray, P., Sreevatsan, S., Wang, P., Gebreyes, W.A., 2012. Epidemiology and Genotypic Characteristics of Methicillin-Resistant Staphylococcus Aureus Strains of Porcine Origin. *J. Clin. Microbiol.* 50, 3687-3693.
- Neela, V., Mohd Zafrul, A., Mariana, N.S., van Belkum, A., Liew, Y.K., Rad, E.G., 2009. Prevalence of ST9 Methicillin-Resistant Staphylococcus Aureus among Pigs and Pig Handlers in Malaysia. *J. Clin. Microbiol.* 47, 4138-4140.
- Smith, T.C., Gebreyes, W.A., Abley, M.J., Harper, A.L., Forshey, B.M., Male, M.J., Martin, H.W., Molla, B.Z., Sreevatsan, S., Thakur, S., Thiruvengadam, M., Davies, P.R., 2013. Methicillin-Resistant Staphylococcus Aureus in Pigs and Farm Workers on Conventional and Antibiotic-Free Swine Farms in the USA. *PLoS One* 8, e63704.
- van Cleef, B.A., Graveland, H., Haenen, A.P., van de Giessen, A.W., Heederik, D., Wagenaar, J.A., Kluytmans, J.A., 2011. Persistence of Livestock-Associated Methicillin-Resistant Staphylococcus Aureus in Field Workers After Short-Term Occupational Exposure to Pigs and Veal Calves. *J. Clin. Microbiol.* 49, 1030-1033.
- Verkade, E., van Benthem, B., den Bergh, M.K., van Cleef, B., van Rijen, M., Bosch, T., Kluytmans, J., 2013. Dynamics and Determinants of Staphylococcus Aureus Carriage in Livestock Veterinarians: A Prospective Cohort Study. *Clin. Infect. Dis.* 57, e11-7.
- Voss, A., Loeffen, F., Bakker, J., Klaassen, C., Wulf, M., 2005. Methicillin-Resistant Staphylococcus Aureus in Pig Farming. *Emerg. Infect. Dis.* 11, 1965-1966.

Use of a slaughter hygiene indicator (*Escherichia coli*) to quantify the risk of human salmonellosis related to pork in Denmark – an approach for risk based meat control?

Anne Mette Bollerslev¹, Maarten Nauta², Tine Hald², Tina Beck Hansen¹, Søren Aabo¹

Divisions of ¹Food Microbiology and ²Epidemiology and Microbial Genomics, Technical University of Denmark, Denmark.

Abstract

Food chain information does not per se allow an effective distinction of herds according to shedding of *Salmonella*. Thus, no effective sorting of pigs at slaughter according to *Salmonella* risk is possible and hygiene improvement is the only effective mitigating tool so far at the slaughter. From a large study of 1906 slaughter pigs we quantified *Salmonella* and established quantitative hygiene data (*E. coli*) on pig carcasses (paired data) at slaughter. Based on the results, we found a positive correlation between level of *E. coli* and the prevalence of *Salmonella* positive carcasses. The odds ratio for *Salmonella* being present on the carcass was found to increase by 1.87 for every one log₁₀ unit increase of *E. coli* found on the carcass. A simple *Salmonella* consumer risk model was constructed using the observed levels of *E. coli* contamination as input and the model, established a positive correlation between slaughter hygiene (*E. coli*) and consumer risk. Further, we analysed two years' own control data on *Salmonella* (prevalence data) and *E. coli* (quantitative data) from four large slaughterhouses in Denmark and found a similar positive correlation between the *E. coli* level and the carcass prevalence of *Salmonella*. The aim of this study was to propose a principle for setting risk based hygiene targets on *E. coli* on carcasses at pig slaughter. As such we provide input to the discussion on how to develop a risk based meat control procedure, based on statistical process control.

Introduction

The *Salmonella* is widespread in the slaughter pig production in Europe, and *Salmonella* from pork constitutes a significant risk for consumers. In recent years the ability of the classical meat control to provide consumer protection against food borne pathogens, has been discussed. A consensus of basing the meat control on food chain information of the pathogens is emerging in Europe. Food chain information do however not per se allow for an effective distinction between pigs from *Salmonella* positive and negative herds, and improvement of the general slaughter hygiene is the only mitigating tool to use. A modernisation of the meat control could include a risk based statistical process control but so far there have been no reports describing how the hygiene level at slaughter associates to *Salmonella* risk. We have established quantitative hygiene data (*E. coli*) and quantified *Salmonella* on pig carcasses at from 2880 pigs slaughter. Moreover we have analysed two years own control data on *E. coli* and *Salmonella* from five large pig slaughter houses in in Denmark. The objective is to establish the correlation between the hygiene level and presence of *Salmonella* and to provide the first suggestion for a method to set risk based process hygiene criteria at pig slaughter.

Material and methods

Sample collection

1906 carcasses from pigs slaughtered at five large Danish pig slaughterhouses were sampled in the period May 2005 to June 2007. Carcass swabs (2800 cm²) from was taken just before cooling and analysed both quantitatively for *E. coli* and semi-quantitatively for *Salmonella*. A total of 75 ml peptone water was added to stomacher bags with carcass swabs containing approximately 12.5 ml of peptone water and tissue fluid. The sample was stomached and one millilitre of 10-fold dilutions were spread on Petrifilm and subsequently incubated at 41.5 °C for 23-25 h. The number of *E. coli* was determined using Select *E. coli* Count Plate Petrifilm (3M Microbiology, St. Paul, MN, USA) in accordance with the supplier's instructions. Cell counts were determined by automated reading using a Petrifilm plate reader MI649 9 (3M Microbiology, St. Paul, MN, USA). From a the ten-fold dilution of the homogenate, a semi-quantitative analysis for *Salmonella* was performed. All stomached samples were analysed for *Salmonella* using MSRVR agar (ISO 6579, Annex D, Anonymous, 2007). Own control data from the same five slaughter houses were obtained based on swabbing of 300 cm² mandatory for slaughter-houses exporting to USA. Each day one pooled sample of five swab samples were analysed for *Salmonella* and one sample per 1000 carcasses were analysed for *E. coli* with a range of 5-12 samples per day depending of slaughterhouse.

Statistical analyses

All statistical analyses were performed with the software R (ver. 2.15.1) and RStudio (ver. 0.96.331). Bacterial counts of *E. coli* were \log_{10} -transformed to obtain approximately normally distributed data. Samples with *E. coli* below the detection limit of 1 CFU/ml were assigned a value of 0.5 CFU/ml to allow \log_{10} transformation.

Means and standard deviations were calculated for the \log_{10} -transformed *E. coli* data. The corresponding *Salmonella* prevalence was calculated after dichotomisation of the results (0 = *Salmonella* negative; 1 = *Salmonella* positive). A box-and-whisker plot was made to illustrate the correlation between the concentration of *Salmonella* and *E. coli* found in swab samples.

To determine the association between *E. coli* and *Salmonella*, univariable analyses were carried out. Variables with $p \leq 0.25$ were included in a multivariable logistic regression analysis. Selection of explanatory variables for the final model was done by stepwise backwards elimination of the least significant variable until only significant variables remained. In the analysis, p-values lower or equal to 0.01 were considered as statistically different. The final explanatory variables were tested for interaction and confounding.

Risk model

The risk model takes into account both the prevalence of *Salmonella* on carcasses and the estimated number of *Salmonella* bacteria present. The number of *Salmonella* bacteria per cm^2 was estimated from the observed contamination of *E. coli* on the carcass and the established regression between number of *E. coli* and number of *Salmonella* bacteria on the carcass. A simple exposure model was developed assuming that: 1) the concentration of bacteria per cm^2 was even on the whole carcass 2) the whole carcass was consumed raw in 200 gram portions and 3) all 101 human illnesses associated to pork in 2006 in Denmark could be associated to this. Additionally, the risk model included three factors: a correction factor, which adjusted the dose-response relationship provided by FAO/WHO (2002); an underreporting factor (Havelaar et al., 2012) and a factor accounting for preparation of pork which adjusted the model output to the number of registered cases in Denmark in 2006.

Results

The average level of *E. coli* found on the skin of the carcasses was 0.8 log CFU/ cm^2 , from all five slaughterhouses. The corresponding prevalence of *Salmonella* was found to be 2.5%.

The odds ratio for *Salmonella* being present on the carcass was found to

increase by 1.87 for every one \log_{10} unit increase of *E. coli*. The correspondence between *Salmonella* prevalence and *E. coli* carcass contamination across all 5 slaughterhouses is shown in Table 1.

Table 1 – Correspondence between *Salmonella* prevalence (%) and the level of *E. coli* (log cfu/38 cm^2)

<i>E. coli</i> [log CFU/38 cm^2]	0-1	1-2	2-3	3-4	4-5	5-6
<i>Salmonella</i> positive	1	6	12	22	3	3
<i>Salmonella</i> negative	233	536	605	337	107	41
Prevalence [%]	0.4	1.1	1.9	6.1	2.7	6.8
95 % of confidence interval	0,01-2,36	0,41-2,39	1,01-3,37	3,88-9,13	0,57-7,76	1,43-18,66

The correlation between the concentration of *E. coli* and the concentration of *Salmonella* is depicted in Figure 1.

By applying the observed *E. coli* and *Salmonella* data to the risk model, it was possible to make an estimate on the relationship between hygiene level measured by *E. coli* and the *Salmonella* consumer risk. Table 2 shows that the number of human cases could have been reduced by approx. 50% (from 101 to 48.6), if the *E. coli* level at slaughter had not exceeded 3-4 \log_{10} CFU per 38 cm^2 .

The analysis of the own control data showed a positive correlation between the number of samples being positive for *E. coli* and a positive analysis for *Salmonella* (Table 3).

Discussion

Our project data showed that it is possible to associate a certain *Salmonella* prevalence and the *Salmonella* concentration to a certain hygiene indicator level (*E. coli*). From the outcome of the risk model, developed on the project data, a risk based

criteria of 3-4 log₁₀ CFU per 38 cm² could be suggested to significantly reduce the number of human illnesses. However, in order to be operational a risk based process hygiene criteria should be embedded into the own control data from the slaughterhouse. The own control data is few per slaughter day but analysed across a number of slaughter days and across all five slaughterhouses it was possible also to identify a positive correlation between *Salmonella* and level of hygiene. The observed relationship between *Salmonella* and *E. coli* in the own control data opens for the opportunity to set risk based process hygiene criteria at least under the conditions similar to the five slaughterhouses studied.

Conclusion

This is to our knowledge the first report, which estimates consumer risk of human salmonellosis from the hygiene level at pig slaughter. This holds the perspective to establish risk based process hygiene criteria based on this principle.

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References

Anonymous, 2007. ISO 6579:2002/Amd 1:2007, Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage. International Organisation for Standardization.

Havelaar, A.H., Ivarsson, S., Löfdahl, M., Nauta, M.J. (2012). Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009. *Epidemiol. Infect.*, Page 1 to 10, Cambridge University Press 2012.

WHO/FAO, 2002. Risk assessments of *Salmonella* in eggs and broiler chickens. Microbiological risk assessment series; no. 2. World Health Organization; Food and Agriculture Organization of the United Nations.

Figure 1 – Box-and-whisker plot of the level of *E. coli* stratified by the concentration of *Salmonella*. The letters represents the following concentration intervals: K < 0.10 CFU/ml, A: 0.10 – 0.91 CFU/ml, B: 0.91 – 10.1 CFU/ml, C: 10.1 – 101 CFU/ml, D: 101 – 909 CFU/ml, DD > 909 CFU/ml.

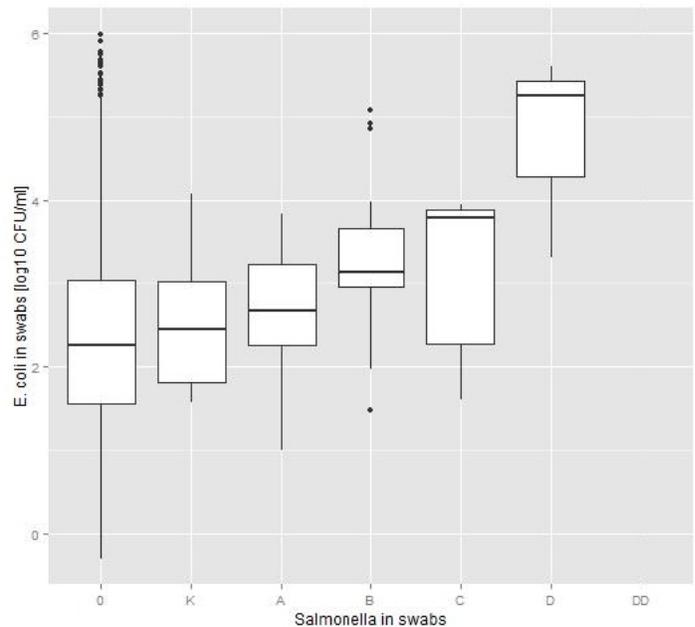


Table 2 – Modelled estimation of the total number of human salmonellosis cases in Denmark in 2006 depending on the maximum level of *E. coli* on pig carcasses before cooling at slaughter.

Maximal level of <i>E. coli</i> at carcass [log ₁₀ CFU 38cm ₂]	0-1	1-2	2-3	3-4	4-5	5-6
No of cases	0.0	6.3	1.6	40.7	0.6	51.8
Accumulated no. cases	0.0	6.3	7.9	48.6	49.2	101.0

Table 3 - Relationship between the number of days with a positive *Salmonella* analysis and the prevalence of *E. coli* positive swab samples per day of slaughter for a total of 1.839 days.

Own control data	Prevalence of <i>E. coli</i> positive swab samples per day				
	0%	0,1–25 %	25,1–50%	50,1–75%	75,1–100%
<i>Salmonella</i> positive days	9	20	17	2	1
<i>Salmonella</i> negative days	710	554	408	97	21
<i>Salmonella</i> positive days [%]	1,3	3,5	4,0	2,0	4,6 95
% confidence interval	0,57–2,36	2,14–5,33	2,35–6,33	0,25–7,11	0,12–22,84

Assessing the role of feed as a risk factor for *Salmonella* in pig production

A.M. Burns^{1,2,3*}, P.G. Lawlor³, G.E. Gardiner², U. Tiwari¹ and G. Duffy¹

1. Teagasc Food Research Centre, Food Safety Department, Ashtown, Dublin 15, Ireland
2. Department of Chemical & Life Sciences, Waterford Institute of Technology, Waterford, Ireland
3. Teagasc Pig Development Department, Animal & Grassland Research & Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland

Corresponding author: annemarie.burns@teagasc.ie

Abstract

The objectives of this study were to investigate *Salmonella* carriage at each stage of pig production (farrow to finish) on 10 commercial pig farms having a historically high *Salmonella* seroprevalence and to establish the role of feed as a source of *Salmonella* on the farms. Pig faecal (n=458) and feed (n=321) samples were taken across all pig production stages and analysed for the presence of *Salmonella*. The pathogen was detected in pigs on nine farms, in 58/458 (12.7%) faecal samples, with a high prevalence among gilts, weaners and finishers. Only 7/321 (2%) of feed samples were *Salmonella*-positive, with four farms having at least one *Salmonella*-positive feed sample. The serovar recovered (4, 12:i:-) was also detected in pigs on the same farms, suggesting that it may have originated in the feed supplied to the farm.. On the other hand, the feed may have been contaminated on the farm and in this way play a role in transmission of *Salmonella*

Introduction

Salmonella carriage in pigs is a significant food safety issue. The European Food Safety Authority has highlighted that feed is a risk factor for *Salmonella* prevalence in pigs (EFSA, 2008). The presence of *Salmonella* in feed can lead to the introduction of *Salmonella* into pathogen-free herds, an increase in shedding prevalence and the spread of *Salmonella* in pigs (EFSA, 2007). A study by Molla *et al.* (2010) showed genotypically related and in some cases clonal *Salmonella* strains including multidrug resistant isolates in commercially processed pig feed and pig faecal samples.

Due to the high prevalence of *Salmonella* in pigs, a number of countries have introduced *Salmonella* surveillance and control programmes. A revised National Pig *Salmonella* Control Programme was implemented in Ireland in January 2010, with monitoring based on determining the *Salmonella* status of pig herds by serological testing of meat juice at slaughter. However, a recent study has shown that 45% of pigs presented for slaughter in Ireland are caecally positive (Duggan *et al.*, 2010). Furthermore, Ireland had the highest carcass contamination rate (20 %) in a 2006-2007 EU baseline survey of *Salmonella* in slaughter pigs (EFSA, 2008). In a 'Farm to Fork' food safety concept, safe feed is the first step in ensuring safe food. Therefore, the aim of this study was to carry out an in-depth study on 10 commercial pig farms having a historically high *Salmonella* seroprevalence to firstly identify which production stages are the principal harbours of *Salmonella* infection in pigs and secondly, to assess the occurrence of *Salmonella* in pig feed throughout the different production stages on these farms and thereby assess potential risks as well as epidemiological relationships.

Material and Methods

On-farm sampling

The number of farms studied and the number of samples taken was in accordance with statistical advice. Farms (n=10) identified for sampling were selected from those with a history of high (>50%) *Salmonella* sero-prevalence in the Department of Agriculture, Food and the Marine National Pig *Salmonella* Control Programme. On-farm sampling was carried out from March-August 2012 with each farm being visited on one occasion between these dates. On each farm, a composite faecal sample was collected at random from at least 3 pigs per production stage, directly from the rectum via digital stimulation or from freshly voided faeces. For all stages of production, where insufficient faecal samples could be obtained, sterile pairs of gauze socks were used to swab the pen. Feed samples (50-100g) which included wet and pelleted dry feed depending on the farm and production stage from troughs, hoppers and storage areas (silos, feed tanks) on the farms. After collection, all samples were immediately transported on ice to the laboratory, where they were stored at 4°C until analysis (within 24 h).

Microbiological analysis of samples

The presence/absence of *Salmonella* in 10g samples was determined according to standard microbiological procedures (EN ISO 6579:2008) with modified brilliant green agar (Oxoid, Basingstoke, Hampshire, UK) used for additional selective plating. Presumptive *Salmonella* isolates were tested using a *Salmonella* latex agglutination kit (Oxoid) and confirmed as

Salmonella using real-time PCR (Prendergast *et al.*, 2012, McCabe *et al.*, 2011, Fricker *et al.*, 2007. One *Salmonella* isolate per sample were serotyped based on O- and H-group antigens according to the White-Kaufmann- Le Minor scheme.

Results

Salmonella was detected in 58/458 (12.7%) faecal samples across all production stages on 9 farms (Table 1) with an overall prevalence of 10.0% (95% confidence interval). Only farm H had no *Salmonella*-positive faecal samples. Six different serotypes were recovered, with a monophasic variant of Typhimurium (4,12:i:-) predominating, accounting for 40.9% (18/44) of all isolates recovered. The other serotypes recovered were Derby (18.2%; 8/44), Typhimurium (18.2%; 8/44), Typhimurium Copenhagen (11.4%; 5/44), Infantis (9.1%; 4/44), and Typhimurium 4,5,12:i:- (2.3%; 1/44), each from one herd. There was no consistent pattern of infection; however, large numbers of positive animals were detected within gilts, weaners and finishers (16.7, 15.3, 16.7 and 16.7% respectively). Three farms (A, E and G) had notably higher prevalence than the other farms (22.9, 24.4 and 17.1% respectively). Only 7/321 (2%) feed samples taken across all production stages were *Salmonella*-positive (Table 2). These *Salmonella*-positive feed samples originated on four farms (A, B, F and G) and all were found to harbour the monophasic variant of Typhimurium (4,12:i:-). Three of the positive feed samples originated on farms using wet feed practices (farms A and F). The *Salmonella*-positive feed samples were generally recovered at only one stage of production, although on farms A and B they were found at two stages (farrowing and 2nd stage weaner on farm A; and 1st stage weaner and finisher on farm B). Feed sampled from gilts had the highest *Salmonella* prevalence (Table 2).

Discussion

Salmonella was recovered from 9 of 10 commercial farms (feed, faecal) in this in depth study. This was to be expected, as all had been chosen from those with a history of high *Salmonella* seroprevalence. The monophasic *Salmonella* 4,12:i:- variant that was found to be predominating in the feed is one of a number of monophasic variants of the serovar Typhimurium, that have been emerging in Europe and are of increasing food safety concern (EFSA, 2010). The lack of recovery of *Salmonella* from any production stage on one of the farms (farm H) may be accounted for by the fact that this farm had low seroprevalence during the study period, highlighting the cyclical nature of *Salmonella* contamination on farms (White *et al.*, 2006). Although there is a lack of understanding as to why this occurs attributing factors are, persistence in the environment and of risk factors, carrier state or prolonged shedding, or reinfection of susceptible animals. Of the three farms with the highest *Salmonella* prevalence, only one had high seroprevalence during the study period, demonstrating the lack of correlation between bacteriological and serological data. Across all the farms large numbers of positive

Table 1: *Salmonella* prevalence in faecal samples taken from different stages of pig production

Farm	Number of faecal samples positive for <i>Salmonella</i>						Prevalence %
	Gilts	Dry Sow	Farrowing Sow	1 st Stage Weaner	2 nd Stage Weaner	Finisher	
A	1/8	0/6	1/8	4/8	5/8	5/10	22.9
B	1/8	1/6	0/8	4/8	2/8	1/10	12.5
C	2/6	0/6	2/6	0/6	0/6	0/8	5.3
D	0/6	1/6	0/8	0/6	0/6	1/8	2.4
E	5/6	2/6	0/8	0/6	0/6	5/8	24.4
F	0/8	0/6	0/8	0/8	1/8	0/10	2.0
G	2/6	0/6	0/8	0/6	3/6	2/8	17.1
H	0/6	0/6	0/6	0/6	0/6	0/6	0.0
I	1/8	0/6	0/8	0/8	0/8	3/10	8.2
J	0/10	0/6	0/12	3/10	1/10	0/24	5.5
Total	12/72	4/60	3/80	11/72	12/72	17/102	
Prevalence %	16.7	6.7	3.8	15.3	16.7	16.7	10.0

Table 2: *Salmonella* prevalence from feed samples from different stages of pig production

Farm	Number of feed samples positive for <i>Salmonella</i>						Prevalence %
	Gilts	Dry Sow	Farrowing Sow	1 st Stage Weaner	2 nd Stage Weaner	Finisher	
A	0/3	0/4	1/6	0/6	1/7	0/8	5.9
B	0/6	0/3	0/6	1/9	0/6	1/7	5.4
C	0/1	0/2	0/3	0/5	0/5	0/6	0.0
D	0/2	0/3	0/6	0/4	0/5	0/6	0.0
E	0/3	0/3	0/4	0/5	0/5	0/6	0.0
F	0/4	1/4	0/6	0/8	0/6	0/7	2.9
G	2/3	0/5	0/4	0/6	0/5	0/6	6.9
H	0/0	0/2	0/5	0/4	0/6	0/5	0.0
I	0/4	0/5	0/6	0/7	0/6	0/7	0.0
J	0/7	0/5	0/8	0/11	0/5	0/19	0.0
Total	2/33	1/36	1/54	1/65	1/56	1/77	2.2
Prevalence %	6.1	2.8	1.9	1.5	1.8	1.3	

animals were detected within the production stages of gilts, weaners and finishers. High carriage rates are commonly seen in weaners and finishers however; few studies have investigated *Salmonella* carriage from farrow to finish. The high prevalence within the replacement breeding stock (gilts) indicates that these animals may be an important source of on-farm *Salmonella* infection.

Salmonella prevalence in feed sampled on-farm was low. However, finding *Salmonella* in at least one of the feed samples tested from almost half of the farms examined could indicate that the organism was quite ubiquitous considering the large volume of feed contained on-site and the relatively small portion of feed sampled for testing. However, as all of the *Salmonella*-positive feed samples were taken from troughs and hoppers, the possibility of on-farm contamination cannot be ruled out. Therefore, we cannot ascertain if the feed is the cause of infection or rather a vector for its transmission. However, as the pig faeces harboured the same serovar as the feed from the same production stage on three of the farms it is likely that the feed became contaminated by the pigs on-farm.

In order to establish if the *Salmonella* contamination originated from the purchased feed or if on-farm contamination occurred genetic subtyping using pulsed field gel electrophoresis (PFGE) and multi-locus variable number tandem repeat analysis will be used to ascertain if the same *Salmonella* strains were found in the feed and the pigs. This will provide important additional epidemiological information. In addition, sampling of feed and feed ingredients from the commercial feed mills supplying our study farms is on-going and will help to further assess the risk posed by *Salmonella* in pig feed. It is interesting to note that three of the seven *Salmonella*-positive feed samples were from wet feed systems, as many investigators have shown that swine herds which were fed dry vs. wet diets were at increased risk of having high *Salmonella* seroprevalence (van der Wolf et al., 2001). Overall, the results indicate that the risk from feed is low with the detection of *Salmonella*-positive pigs on farms with *Salmonella*-negative feed samples demonstrating that there are multiple sources of *Salmonella* infection on pig farms. The importance of these sources may vary by production stage, farm and over time. Although feed could not be singled out as the source of the *Salmonella* isolated from the pigs in the present study, it nonetheless cannot be ruled out as a risk factor in the transmission of *Salmonella* and therefore, its control in feed should be considered an essential component of any control program.

Conclusion

Salmonella prevalence in feed samples taken on-farm was low however further research is needed to ascertain whether it originated in commercial feed supplied to the farms or if its presence in feed was as a result of on-farm contamination

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References

- Duggan, S.J., Mannion, C., Prendergast, D.M., Leonard, N., Fanning, S., Gonzales-Barron, U., Egan, J., Butler, F., Duffy, G., 2010. Tracking the Salmonella Status of Pigs and Pork from Lairage through the Slaughter Process in the Republic of Ireland. *Journal of Food Protection* 73, 2148-2160.
- European Food Safety Authority, 2007. Food safety aspects of different pig housing and husbandry systems. *The EFSA Journal* 613, 1-20.
- European Food Safety Authority, 2008. Microbiological risk assessment in feeding stuffs for food producing animals. *The EFSA Journal* 720, 1-84.
- European Food Safety Authority, 2010. Scientific Opinion on monitoring and assessment of the public health risk of “*Salmonella* Typhimurium-like” strains. *The EFSA Journal* 8(10):1826.
- Fricker, M., Messelhauser, U., Busch, U., Scherer, S., Ehling-Schulz, M., 2007. Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Applied and Environmental Microbiology* 73, 1892-1898.
- McCabe, E.M., Burgess, C.M., O'Regan, E., McGuinness, S., Barry, T., Fanning, S., Duffy, G., 2011. Development and evaluation of DNA and RNA real-time assays for food analysis using the *hlyA* gene of *Salmonella enterica* subspecies enterica. *Food Microbiology* 28, 447-456.

Molla, B., Sterman, A., Mathews, J., Artuso-Ponte, V., Abley, M., Farmer, W., Rajala-Schultz, P., Morrow, W.E.M., Gebreyes, W.A., 2010. Salmonella enterica in Commercial Swine Feed and Subsequent Isolation of Phenotypically and Genotypically Related Strains from Fecal Samples. *Applied and Environmental Microbiology* 76, 7188-7193.

Prendergast, D.M., Grady, D.O., McCann, A., McCabe, E., Fanning, S., Egan, J., Fanning, J., Gutierrez, M., 2012. Application of PCR for rapid detection and serotyping of Salmonella spp. from porcine carcass swabs following enrichment in semi-solid agar. *Food Research International* 45, 993-999.

van der Wolf, P.J., Wolbers, W.B., Elbers, A.R.W., van der Heijden, H., Koppen, J., Hunneman, W.A., van Schie, F.W., Tielen, M.J.M., 2001. Herd level husbandry factors associated with the serological Salmonella prevalence in finishing pig herds in The Netherlands. *Veterinary Microbiology* 78, 205-219.

White, A.P., Gibson, D.L., Kim, W., Kay, W.W., Surette, M.G., 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella. *Journal of Bacteriology* 188, 3219-3227.

Comparison of organic and conventional pig productions on prevalence, antibiotic resistance and genetic diversity of *Escherichia coli*

Kerouanton, A.^{1,3*}, Rose, V.^{1,3}, Chidaine B.^{1,3}, Kempf I.^{2,3} and Denis, M.^{1,3}

⁽¹⁾ Anses Laboratory of Ploufragan/Plouzané, Unit of Health and Quality of Poultry And Pig Products, BP 53, 22440 Ploufragan, France.

⁽²⁾ Anses Laboratory of Ploufragan/Plouzané, Unit of Mycoplasma and Bacteriology, BP 53, 22440 Ploufragan, France.

⁽³⁾ European University of Brittany, France.

*corresponding author: Annaelle.KEROUANTON@anses.fr

Abstract

The objectives of this study were to assess the prevalence, the tetracycline resistance level and the genetic diversity of *Escherichia coli* isolated from organic pigs in comparison with conventional pigs. This study is integrated in a global European project, Safeorganic, funded through CORE Organic II call. 25 organic and 25 conventional herds were considered in one slaughterhouse from April to October 2012. Colon content of 2 pigs per herds was sampled. For each pig, enumeration of *E. coli* and of tetracycline resistant *E. coli* (TET⁺*E. coli*) was determined. Level of tetracycline resistance was then calculated. Isolates were typed by PFGE using *Xba*I enzyme. *E. coli* was detected for all the organic (n=50) and conventional pigs (n=50). TET⁺*E. coli* was detected for 49 organic (98%) and 48 conventional pigs (96%). Number of *E. coli* per gram of colon content were significantly higher (p=0.0033) for conventional (6.81 log₁₀ UFC/g) than for organic pigs (6.19 log₁₀ UFC/g) as well as number of TET⁺*E. coli* (p=0.00021) with 6.33 log₁₀ UFC/g for conventional pigs and 5.68 log₁₀ UFC/g for organic pigs. Finally, the level of tetracycline resistance was significantly higher (p=0.0033) for conventional (57.4%) than for organic pigs (37.9%). PFGE was done on 373 *E. coli*; they were distributed in 277 PFGE profiles. The genetic diversity was very high (*D value*=0.998). No pulsotype was common between organic and conventional pigs. Difference for the level of tetracycline resistance between organic and conventional pigs suggests that farm managements may have an impact on the amount of *E. coli* excreted and on their antibiotic resistance. However, it is difficult to estimate the impact on human health with 0.65 log₁₀ UFC/g difference between the two productions. Diversity of strains is so high that it is difficult to associate strains to a production.

Introduction

Spread of antibiotic resistance along the food-chain is a major food safety concern due to the risk of treatment failure of human foodborne infections. Reports suggest that organic animals carry lower levels of antibiotic resistant bacteria in faeces, including lower levels of multiresistant bacteria (Nulsen *et al.*, 2008; Young *et al.*, 2009). This is, however, scarcely documented, particularly for swine. Organic pig production differs in many aspects from the conventional pig farming. Several of these differences may influence the pattern of the microbiological flora including the pattern of antimicrobial resistance. For example, prophylactic use of antimicrobials and growth hormones is prohibited, although antimicrobials can be used to treat ill animals when all other options fail. There are also differences in feeding regimes, a lower animal density and access to outdoor areas (Bonde & Sørensen, 2004).

As the food animal production is a primary reservoir for foodborne pathogens and antibiotic resistance, there is a continuous need to regularly document the status in the primary production. Caecal/colon content, sampled at slaughter, can be used to characterize the herd status in relation to antibiotic resistance (Rosenquist *et al.*, 2009). The bacterial population of these samples can reflect those originating from the farm and particularly *Escherichia coli* commonly used as indicator of faecal contamination and antimicrobial resistance. Thus, it may be used to compare antimicrobial resistance among populations. In the other hand, genetic profiles may be used to characterize also *E. coli* populations. Greater genetic diversity can be expected for organic animals due to the presumable lower antibiotic selection pressure and access to an open area.

The aims of this study were to assess the prevalence, the tetracycline resistance level and the genetic diversity of *E. coli* isolated at slaughter from colon of organic pigs in comparison with conventional pigs. Tetracycline was used as resistance indicator, as resistance to this antimicrobial is the more frequent for *E. coli* (EFSA 2012).

Material and Methods

Sampling

To evaluate the bacteriological status of the pigs at herd level, sampling of colon content from pigs was done at slaughter. Sampling was realized in one slaughterhouse from April to October 2012. In total, 25 organic herds and 25 conventional herds were considered; at each visit, and at each organic herd sampled, a conventional herd was also sampled. Colon content of 2 pigs per herd was collected at the evisceration step. Finally, 50 organic pigs and 50 conventional pigs were analyzed.

Enumeration of total *E. coli* and tetracycline resistant *E. coli*

Ten to 25 g of each colon content were diluted in 90 ml of tryptone salt (AES Chemunex, Bruz, France) and a 1:10 serial dilution was realized until the dilution -6. One ml of each dilution was dropped on a 3M™ Petrifilm™ Select *E. coli* Count Plate (SEC plate) (3M, Cergy-Pontoise, France). Characteristic colonies were counted in order to determine the quantity of *E. coli* per gram of colon content.

In parallel, and as described by Wu *et al.* (2008), 1 ml of each dilution was supplemented with oxytetracycline for a final concentration of 64mg/L and dropped on a 3M™ Petrifilm™ Select *E. coli* Count Plate. Characteristic colonies were counted in order to determine the quantity of tetracycline resistant *E. coli* (TET+*E. coli*) per gram of colon content.

Level of tetracycline resistance for each sample was then determined by the percentage of TET+*E. coli* from the total number of *E. coli*.

A multiplex-PCR was performed (Perrin-Guyomard *et al.*, 2008) to confirm isolates as *E. coli*.

Statistical Analysis

Statistical analyses were performed using SAS software. The variables to be explained were enumeration (\log_{10} UFC *E. coli* total/g, (\log_{10} T) and \log_{10} UFC *E. coli* resistant/g (\log_{10} R)) and the resistance level (%_UFGR). Test used was Anova when possible or Wilcoxon otherwise. To determine if differences observed between results were significant, a variance analysis was performed.

PFGE typing and analyzes of the genetic profiles

When possible, two isolates per positive 'SEC plate' and positive 'SEC plate+TET' (4 per sample) were characterized by RFLP-PFGE using *Xba*I enzyme (Ribot *et al.*, 2006). *Salmonella enterica* serotype Braenderup H9812 was used as molecular size marker (Hunter *et al.*, 2005).

*Xba*I profiles were analyzed on BioNumerics® software (V 6.5, Applied Maths, Kortrijk, Belgium). The similarities between profiles, based on the position of the restricted fragments, were calculated using the coefficient of Dice with a maximum tolerance of 1% (Struelens, 1996). The Simpson's index (Hunter, 1990) was calculated to estimate the diversity of the sample.

Results

From colon content, on the 100 sampled pigs, *E. coli* was detected for all the organic pigs (n=50) and conventional (n=50). TET+*E. coli* was detected for 49 organic pigs (98%) and 48 conventional pigs (96%). The number of *E. coli* per gram of colon content were significantly higher (6.81 \log_{10} UFC/g) for conventional pig (Table 1) than for organic pigs (6.19 \log_{10} UFC/g) (p=0.0033). A significant difference for the number of TET+*E. coli* per gram of colon content was also observed between organic (5.68 \log_{10} UFC/g) and conventional pigs (6.33 \log_{10} UFC/g) (p=0.00021). The level of tetracycline resistance is higher for conventional pigs (57.4%) than for organic pigs (37.9%); this is also significant (p=0.0033).

Table 1: Enumeration of total *E. coli* and TET+*E. coli* from colon content of organic and conventional pigs and level of tetracycline resistance.

Type of production	Total <i>E. coli</i>		TET+ <i>E. coli</i>		Level of tetracycline resistance
	N° of positive samples (%)	\log_{10} UFC/g	N° of positive samples (%)	\log_{10} UFC/g	
Organic	50 (100%)	6.19	48 (98%)	5.68	37.9%
Conventional	50 (100%)	6.81	49 (99%)	6.33	54.4%

A total of 373 *E. coli* and TET+*E.coli* isolates were collected from colon content, 195 from organic pigs and 178 from conventional pigs (Table 2). Isolates were distributed in 277 PFGE profiles after *XbaI* restriction: 142 for isolates from organic pigs and 135 from conventional pigs (Table 2). For individual sample, the diversity ranged from 0.25 (one subtype found among 4 isolates) to 1.00 (4 subtypes found among 4 isolates).

Table 2: PFGE diversity after *XbaI* restriction

Type of production	Number of isolates	Number of PFGE profiles	Index of diversity [95% confidence intervals]
Organic	195	142	0.99 [0.99-1.00]
Conventional	178	135	0.99 [0.99-1.00]
Total	373	277	0.99 [0.99-1.00]

No PFGE profile was common to the two type of production, and, no specific genetic cluster was observed within the dendrogram, even using a cut-off value of 80% genetic similarity.

Thus, the diversity index was very high whatever the type of production (ID³0.99). Most of the time, isolates harboring a same PFGE profile were from a same sample or a same herd. Only 1 PFGE profile was common to isolates from 2 different organic herds sampled at different days.

Discussion

E. coli was detected for all the pigs; these commensal bacteria colonize intestinal tract of almost warm-blood animals and are present throughout the environment. So, the access to an outdoor area of organic pigs may contribute to maintain a high level of contamination.

Difference for the level of tetracycline resistance between organic and conventional pigs suggests that farm managements may have an impact on the amount of *E. coli* excreted and on their antibiotic resistance. The lower level of tetracycline resistance for organic pigs could be explained by contamination in the outdoor area with wild strains supposed to be more sensitive to antibiotic and overall by the restricted antimicrobial practices in organic farm.

Difference in antibiotic resistance was previously observed by Tadesse *et al.*, (2011) between farms with or without antibiotic treatments. They observed a higher erythromycine resistance for *Campylobacter* in farm with antibiotic treatment. For our study, after contact with farmers, only one of the herds sampled in our study has been treated with antimicrobials which could explain our results. However, some studies in North America showed that antimicrobial resistance can remain in faecal *E. coli* population in pig decades after the drug was banned from food-animal production (Maynard *et al.*, 2003).

The genetic diversity of *E.coli* was very high for each production and it was difficult to associate strains or genetic clusters to a production. PFGE demonstrated to be an interesting epidemiological tool to differentiate *E. coli* within a specific serotype, as O157:H7 (Watabe *et al.*, 2008) or to differentiate *E. coli* isolates from piglets with diarrhea (Vu-Khac, *et al.*, 2007). But finally, for the commensal *E. coli* population, this method cannot be used as origin population marker.

Conclusion

Difference between organic and conventional pigs suggests that farm managements may have an impact on commensal bacteria. Nevertheless, while difference in the level of tetracycline resistance between organic and conventional pigs was statistically significant, it is difficult however to estimate the impact on human health with a difference of 0.65 log₁₀ UFC/g of *E. coli* tetracycline resistant between the two productions. Strains are being tested with a panel of antibiotics; the results will clarify those previously obtained.

PFGE is not a good tool to associate genetic profiles to production. Other typing methods should be considered.

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References

- BONDE, M. and SØRENSEN, J.T., 2007. *Salmonella* Infection Level in Danish Indoor and Outdoor Pig Production Systems measured by Antibodies in Meat Juice and Faecal Shedding on-farm and at Slaughter. XIII international Congress in Animal Hygiene ISAH-2007, Tartu, Estonia, June 17-21, 2007. *In: Animal health, animal welfare and biosecurity proceedings 2*, pp. 729-734.
- EFSA, 2012. The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2010. *The EFSA Journal* 10(3), 2598.
- HUNTER P., 1990. Reproducibility and indices of discriminatory power of microbial typing methods. *Journal of Clinical Microbiology*, 28 : 1903-5.
- HUNTER, S.B., VAUTERIN, P., LAMBERT-FAIR, M.A. *et al.*, 2005. Establishment of an universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols. Converting the national databases to the new size standard. *Journal of Clinical Microbiology*, 43:1045-1050
- MAYNARD C, FAIRBROTHER JM, BEKAL S, SANSCHAGRIN F, LEVESQUE RC, BROUSSEAU R, MASSON L, LARIVIERE S, HAREL J., 2003. Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. *Antimicrob Agents Chemother* 47:3214-21.
- NULSEN, M.F., MOR, M.B., and LAWTON, D.E.B., 2008. Antibiotic resistance among indicator bacteria isolated from healthy pigs in New Zealand. *New Zealand Vet. J.* 56, 29-35.
- PERRIN-GUYOMARD, A., GICQUEL-BRUNEAU, M., LOUAPRE, P., LAURENTIE, M. and SANDERS, P., 2008. Characterization of *Escherichia coli* by polymerase chain reaction for French monitoring programme on antimicrobial resistance in indicator bacteria. In *4th conference on AAVM*. Prague.
- RIBOT, E.M., FAIR, M.A., GAUTOM, R., *et al.*, 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogene Disease*, 3:59-67
- ROSENQUIST, H., SANDØE, P., TVEIT, G., WINGSTRAND, A. and AABO, S., 2009. Fremtidens fødevarerikkerhed [*in Danish*, Future Food Safety – new ways towards safer meat in Denmark], pp. 160. Schultz Grafisk, Copenhagen Denmark.
- STRUELENS, M.J., Members of the European Study Group on Epidemiological Markers (ESGEM), 1996. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. *Clinical Microbiology and Infection*, 2: 2-11
- VU-KHAC H, HOLODA E, PILIPCINEC E, BLANCO M, BLANCO JE, DAHBI G, MORA A, LOPEZ C, GONZALEZ EA, BLANCO J. Serotypes, virulence genes, intimin types and PFGE profiles of *Escherichia coli* isolated from piglets with diarrhoea in Slovakia. *Vet J* 2007;174:176-87.
- WU, S., CHOULIARA, E., JENSEN, L.B. and DALSGAARD, A. 2008. Evaluation of Petrifilm Select *E. coli* Count Plate medium to discriminate antimicrobial resistant *Escherichia coli*. *Acta Vet Scand* 50, 38
- WATABE M, HOGG GM, MILLAR BC, CROTHERS L, ROONEY PJ, LOUGHREY A, GOLDSMITH CE, McMahan MA, McDowell DA, Moore JE. Epidemiological study of *E. coli* O157:H7 isolated in Northern Ireland using pulsed-field gel electrophoresis (PFGE). *Ulster Med J* 2008;77:168-74.
- YOUNG, I., RAJIC, A., WILHELM, B.J., WADDELL, L., PARKER, S. and MCEVEN, S.A., 2009. Comparison of the prevalence of bacterial enteropathogens, potentially zoonotic bacteria and bacterial resistance to antimicrobials in organic and conventional poultry, swine and beef production: a systematic review and meta-analysis. *Epidemiol. Infect.* 137, 1217–1232.

Similarities in seroprevalence of *Toxoplasma gondii*, *Trichinella* spp., *Trichuris suis* and *Ascaris suum* in swine in the conventional and antibiotic free swine production systems.

Thakur, S.*⁽¹⁾, Hill, D.⁽²⁾, Susick, E. ⁽¹⁾

⁽¹⁾ Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27607

⁽²⁾ USDA-ARS, 10300 Baltimore Avenue, Building 1040 BARC-East, Beltsville, MD, 20706

*Siddhartha Thakur : sthakur@ncsu.edu

INTRODUCTION

Helminth parasite infections in swine represent a significant, but understudied health concern for both the swine industry and consumers. While many parasitic infections cause subclinical infections, infected swine pose a public health risk from consumption of contaminated meat products. In swine, infection with parasites has also been shown to negatively impact pig performance and impede weight gain (Kipper et al., 2011; Pittman et al., 2010). *Toxoplasma gondii* is one of the top pathogens responsible for fatal foodborne infections in the US (Mead et al., 1999). While *T. gondii* has been recovered from retail pork products, the importance of direct transmission from the consumption of contaminated pork products is still unclear (Dubey et al., 2008). Transmission occurs through ingestion of infected meat containing parasitic, infective cysts (Davies et al., 1998). *Trichuris suis* and *Ascaris suum* are both common nematodes identified in swine, with prevalence widely ranging and depending on location and farm practices (Nedjsum et al., 2012). Trichinellosis, while rare in humans, can be associated with consumption of undercooked noncommercial, or antibiotic free (ABF), pork contaminated with *Trichinella* (Davies et al., 2011). From 2002-2007 there were 54 reported cases of trichinellosis in the US, with 19% of those cases attributed to the consumption of pork products (Kennedy et al., 2009). Nearly half of the worldwide population is infected with one of the above mentioned parasites, with symptoms ranging from diarrhea to malnutrition and occasionally death (Hall et al., 2008). The purpose of this study was to determine the seroprevalence of *T. gondii*, *Trichinella*, *T. suis*, and *A. suum* in indoor conventionally raised and outdoor Antimicrobial free (ABF) swine.

MATERIALS AND METHODS

Origin of Meat Tissue Fluid Samples

Meat samples were collected as part of a larger longitudinal study on the prevalence of antimicrobial resistant bacteria in conventional and ABF swine production systems in North Carolina (Quintana-Hayashi et al., 2012; Keelara et al., 2013). Briefly, ten cohorts of conventionally-raised pigs and eight cohorts of ABF-raised pigs were sampled five times at farm and at slaughter. One ABF cohort could not be sampled at slaughter and is therefore not represented in the meat tissue samples. Conventionally-raised pigs followed an all-in-all-out production system, where pigs were housed indoors and moved to different farms together as cohorts at different stages of production (farrowing, nursery, and finishing). Conventionally-raised pigs were given antimicrobials for therapeutic and prophylaxis purposes. ABF pigs were raised outdoors on the same farm location for all production stages and were not given antimicrobials for any purpose. At slaughter, all conventionally-raised pigs were sent to a single, large-scale slaughter facility whereas ABF pigs were sent to one of two small-scale slaughter facilities that processed only ABF pigs. Approximately 50 g of meat was taken from each carcass just above the diaphragm after evisceration using a sterile knife, transported on ice, and stored at -20°C until processed. A questionnaire detailing farm management practices was given to producers at each production stage (farrowing, nursery, slaughter). In addition, a similar questionnaire was filled out by lab personnel to document farm conditions and practices while samples were collected.

Serology

Tissue fluids were isolated from 50 g pieces of muscle from individual pigs by freezing at -20°C overnight, thawing at room temperature, and centrifuging at 1g to collect released fluids; tissue fluids were stored at -20°C until use. Tissue fluid samples were tested in duplicate for the presence of antibodies to *Trichinella* spp. and *T. gondii* using two 96-well commercial ELISA kits as recommended by the manufacturer (SafePath Laboratories, Carlsbad, CA). The optical density (OD) of each sample was determined at 450_{nm} using a dual wavelength (450/620_{nm}) microplate reader (Molecular Devices, Sunnyvale, CA). Specific parasite positive and negative control pig sera supplied by the manufacturer were included on each ELISA plate. ELISA values were reported as the mean optical density (OD) of duplicate wells after subtraction of the OD for the negative control well. Optical densities in both tests which exceeded 0.30 after subtraction of the negative control OD were considered positive.

Tissue fluids were also tested for the presence of specific antibodies to *Trichuris suis* and *Ascaris suum* using 2 in-house ELISAs. *Ascaris* E/S antigens were derived from 72 hour *in vitro* culture of *A. suum* 4th stage larvae (16 days post infection), while *Trichuris* E/S antigens were derived from 72 hour *in vitro* culture of adult worms (42 days post infection) as previously described (Hill et al., 1990). Analysis by spectroscopy to determine the 280:260_{nm} absorbance ratio was used to determine protein concentration. Antigens were passively absorbed onto 96-well microtitre plates, 1µg/well of *T. suis* or *A. suum* excretory/secretory (E/S) antigens, in coating buffer (0.2 M carbonate/bicarbonate buffer, pH 9.6) for 60 minutes at 37°C. Antigen-coated wells were washed three times with wash buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5.0% non-fat milk powder and 0.5% Tween-20 (Tris-NaCl w/T). Tissue fluid was diluted 1:10 in wash buffer without Tween-20 (Tris-NaCl wo/T), and 100µl of diluted tissue fluid was added to each antigen-coated well. Known positive and known negative tissue fluid (diluted 1:10) and serum samples (diluted 1:50) were included on each plate. Plates were incubated for 2 hours at room temperature, then washed 3 times as above with Tris-NaCl w/T. Affinity-purified rabbit anti-swine IgG-peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, 1mg/ml), diluted 1:1000 in Tris-NaCl wo/T, was added to each well (100 µl/well) and incubated at room temperature for 2 hours. The plates were washed three times in Tris-NaCl w/T, and rinsed once with distilled water. Chromogen/peroxide substrate was added to each well (100 µl tetramethylbenzidine (TMB), Safepath Laboratories, Carlsbad, CA, USA) and incubated for 10 minutes at room temperature.

The optical density (OD) of each sample was determined at 450_{nm} using a dual wavelength (450/620_{nm}) microplate reader (Molecular Devices, Sunnyvale, CA). ELISA values for each sample were reported as the mean optical density (OD) of duplicate wells after subtraction of the OD for the negative control well. Optical densities in the *Trichuris* ELISA which exceeded 0.30 after subtraction of the negative control OD were considered positive, while an OD which exceeded 0.391 after subtraction of the negative control OD in the *Ascaris* ELISA were considered positive.

Statistics

T-tests were used to calculate significant differences in parasite prevalence between farm types (SigmaPlot). Odds ratios were calculated to determine any increased risk associated with various farm management practices, including presence of various animals or pests on farm, anthelmintic administration, herd density, and biosecurity practice. Responses from each stage were combined into an overall farm data set, where a single positive response (i.e. presence of dogs/cats) at any stage marked the overall farm as positive.

RESULTS

Seroprevalence

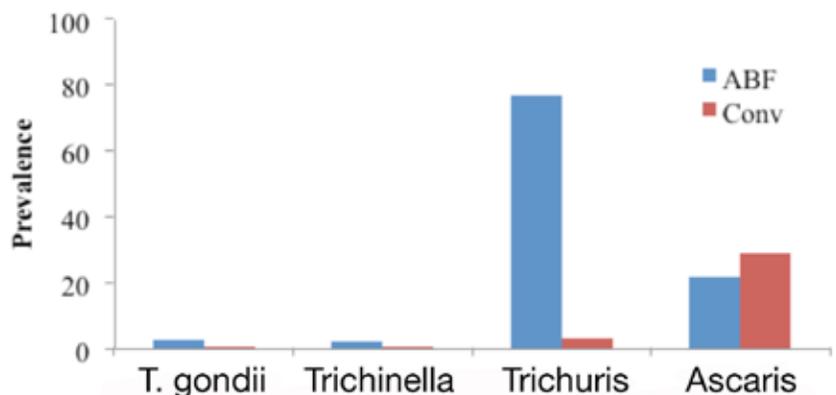
A total of 443 meat samples (conventional: 263, ABF: 180) were collected at three slaughter facilities in North Carolina. Meat tissue fluid was tested for serological evidence of *T. gondii*, *T. suis*, *Trichinella spp*, and *A. suum*, Figure 1. Seroprevalence of *T. gondii* and *Trichinella* was low and not significantly different between the conventional (0.7%, 0.3% respectively) and the ABF (2.6%, 2.2% respectively) production systems (P=0.275, P=0.132 respectively). However, swine in the ABF system (77.7%, 132/170) had a significantly higher seroprevalence of *T. suis* than swine in the conventional system (3.0%, 8/263; P<0.001).

In the conventional system, *A. suum* was detected in all cohorts with seroprevalence ranging from 8.7% to 65.5%. Similarly, *A. suum* was detected in all but one ABF cohort, with seroprevalence ranging from 0% to 44%.

Farm Questionnaire

No significant associations were detected between presence of parasites and farm questionnaire items, with the exception of *T. suis* and farm type (OR=0, P=0.002) due to 100% of ABF farms being positive for *T. suis*. Risk factors for *A. suum* were difficult to detect due to all but one farm being positive for the parasite. This lack of significant risk factors is likely influenced by the similar answers given on the questionnaires (Table 1). The presence of one parasite was not significantly associated with an increased risk of that farm being positive for a different parasite.

Figure 1. Seroprevalence of parasites in meat tissue fluid.



DISCUSSION

While seroprevalence of *T. gondii* and *Trichinella* was higher in ABF compared to conventional swine tissue samples, the difference was not statistically significant. This is likely due to the low number of positive samples. Such low prevalence in the conventional swine system has been reported previously (Davies et al., 1998). Previous studies have detected a significantly higher prevalence of *T. gondii* and *Trichinella* in outdoor, ABF swine compared to conventional swine, some at statistically significant levels (Gebreyes et al., 2008; Giessen et al., 2007). This is likely due to exposure to the outdoor environment in ABF/extensively raised pigs and the presence of biosecurity measures and lack of access to the outdoors in conventional pigs. The seroprevalence of *T. suis* was significantly higher in ABF swine than conventional. This trend has also been seen in previous studies comparing the prevalence of gastrointestinal parasites in different swine management systems (Eijck and Borgsteede, 2005). This may be the result of differences in environmental and farm management factors. Interestingly, we detected a higher, but not statistically significant, seroprevalence of *A. suum* conventional swine compared to ABF. This is in contrast with previous reports that identified a higher prevalence in outdoor and organic swine systems (Eijck and Borgsteede, 2005).

CONCLUSION

These results highlight the similarity of *T. gondii*, *Trichinella*, and *A. suum* seroprevalence rates in conventional and ABF production systems despite significant differences in management practices. While *T. suis* was detected in a significantly higher percentage of ABF swine tissue samples compared to conventional swine, the zoonotic potential of *T. suis* to cause disease in humans is still unclear.

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REFERENCES

- Davies PR, Worrow WEM, Deen J, Gamble HR, Patton S. Seroprevalence of *Toxoplasma gondii* and *Trichinella spiralis* in finishing swine raised in different production systems in North Carolina, USA. *Prev Vet Med* 1998; 36: 67-76.
- Davies PR. Intensive swine production and pork safety. *Food Path Dis* 2011; 8(2): 189-201.
- Dubey JP and Jones JL. *Toxoplasma gondii* infection in humans and animals in the United States. *Int J Parasitol* 2008; 38: 1257-1278
- Eijck IAJM and Borgsteede FHM. A Survey of Gastrointestinal Pig Parasites on Free-range, Organic, and Conventional Pig Farms in The Netherlands. *Vet Res Commun* 2005; 29: 407-414.
- Gebreyes WA, Bahnson PB, Funk JA, McKean J, Patchanee P. Seroprevalence of *Trichinella*, *Toxoplasma* and *Salmonella* in Antimicrobial-Free and Conventional Swine Production Systems. *Foodborne Pathog Dis* 2008; 5(2): 199-203.
- Giessen J, Fonville M, Bouwknegt M, Langelaar M, Vollema A. Seroprevalence of *Trichinella spiralis* and *Toxoplasma gondii* in pigs from different housing systems in The Netherlands. *Vet Parasitol* 2007; 148: 371-374.
- Hall A, Hewitt G, Tuffrey V, de Silva N. A review and meta-analysis of the impact of intestinal worms on child growth and nutrition. *Matern Child Nutr* 2008; 4(S1): 118-236.
- Keelara S, Scott HM, Morrow WM, Gebreyes WA, Correa M, Nayak R, Stefanova R, Thakur S. Longitudinal Study Comparing the Distribution of Phenotypically and Genotypically Similar Antimicrobial Resistant *Salmonella* Serovars Between Pigs and Their Environment in Two Distinct Swine Production Systems. *Appl Environ Microbiol* 2013; [Epub ahead of print]
- Kennedy ED, Hall RLH, Montgomery SP, Pyburn DG, Jones JL. *Trichinellosis Surveillance --- United States, 2002—2007*.

Table 1. Producer response to farm management practices questionnaire.

Management Practice	Conv (n=10)	ABF (n=7)
Anthelmintic use	2	3
Presence of dog/cat	4	6
Presence of insects	10	6
Presence of birds	5	5
Presence of cattle	10	6
Animal/insect access to storage bins	4	2

MMWR Surveillance Summaries Dec. 4, 2009, 58(SS09); 1-7.

Kipper M, Andretta I, Monterio SG, Lovatto PA, Lehnen CR. Meta-analysis of the effects of endoparasites on pig performance. *Vet Parasitol* 2011; 181: 316-320.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. Food related illness and death in the United States. *Emerg Infect Dis* 1999; 5: 607-625.

Nejsum P, Beton M, Bendall RP, Thamsborg SM, Stothard JR. Assessing the zoonotic potential of *Ascaris suum* and *Trichuris suis*: looking to the future from an analysis of the past. *J Helminthol* 2012; 86 (2): 148-155.

Pittman JS, Shepherd G, Thacker BJ, Myers GH. *Trichuris suis* in finishing pigs: Case report and review. *J Swine Health Prod* 2010; 18(6): 306-313.

Quintana-Hayashi MP and Thakur S. Longitudinal study of the persistence of antimicrobial-resistant *Campylobacter* strains in distinct swine production systems on farm, at slaughter, and in the environment. *Appl Environ Microbiol* 2012; 78(8): 2698-2705.

Feed size and texture influence propionic and butyric acid concentrations and *Escherichia coli* populations in the pig gastrointestinal tract

Longpré, J.^(1,4), LeBel, P.^(1,4), Massé, D.⁽²⁾, Fravallo, P.^(1,4), Fairbrother, J.M.^(3,4) and Letellier, A.*^(1,4)

⁽¹⁾NSERC Industrial Research Chair in Meat Safety, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

⁽²⁾Dairy and Swine Research and Development Center, Agriculture and Agri-Food Canada, Sherbrooke, Canada

⁽³⁾*Escherichia coli* Laboratory, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

⁽⁴⁾Swine and Avian Infectious Disease Research Center, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

*Corresponding author: ann.letellier@umontreal.ca

Abstract

Natural approaches are now being considered to replace antimicrobials to reduce the risk of antimicrobial resistance development. This has put new emphasis on using diet to control bacterial infections in pigs, some of which having recently demonstrated a zoonotic disease potential. Moreover, dietary modifications can lead to a modulation of the bioregulation of volatile fatty acids (VFA). Our objective was to assess the effect of feed size and texture on intestinal VFA profiles and concentrations, *E. coli* populations, and on growth performance. Fattening pigs (n=840) received one of six different diets (mash feed 500, 750 and 1250 µm and pellet feed 500, 750 and 1250 µm). Weight gain of pigs was monitored for each diet formulation over the fattening period. At the slaughterhouse, caecal and colon contents from 165 pigs were sampled for enumeration of *E. coli* by quantitative PCR (qPCR) and for VFA quantification. Acetic, propionic, and butyric acids were quantified by capillary gas chromatography. The *yccT* gene was used to enumerate total *E. coli*. A decrease in feed conversion associated with pellet texture and/or 500 µm particle size was observed for each diet formulation (p<0,05). In addition, caecal (p=0,0271) and colon (p=0,0012) propionic acid concentrations were lower for pigs receiving pellet rather than mash feed. Similarly, caecal (p=0,0167) and colon (p=0,0008) butyric acid concentrations were also lower for pigs receiving pellet rather than mash feed. Moreover, caecal (p=0,0208) and colon (p=0,0006) butyric acid concentrations were higher for pigs receiving a feed with a 1250 µm rather than 500 µm particle size. For total *E. coli* enumeration, caecal (p=0,01) and colon (p=0,04) *yccT* gene copy numbers were higher for pigs receiving pellet rather than mash feed. Taken together, results showed that mash feed is associated with favourable intestinal changes (VFA levels) and with a reduction of *E. coli* in the pig.

Introduction

Certain *Escherichia coli* are associated with postweaning diarrhoea and oedema disease in pigs, others are important zoonotic pathogens in the food chain. Antibiotics are commonly used to control this pathogen at the farm level, but natural approaches, such as feed strategies, are now being considered to increase the gut health and reduce the use of antimicrobials as growth promoters, and then, reduce the risk of antimicrobial resistance development. This has put new emphasis on using diet to control bacterial infections in pigs. It is now shown that the porcine intestinal health and intestinal microbiota composition can be modified by feed strategies, specifically by feed texture and feed particles size changes of the pig's diet (1). Moreover, dietary modifications can lead to a modulation of the bioregulation of volatile fatty acids. Particularly, propionic and butyric acids are important metabolites because of their bactericidal potential (2, 3). The aim of this study was to better understand the effect of feed particle size and texture on intestinal VFA profiles and concentrations, *E. coli* populations, and on growth performance of fattening pigs.

Material and Methods

A total of 840 crossbred Yorkshire-Landrace fattening pigs received one of six different diets (mash feed 500, 750 or 1250 µm or pellet feed 500, 750 or 1250 µm). Pigs were assigned a diet randomly and were fattened for a total of 120 days. Weight gain of pigs was monitored for each diet formulation over the fattening period. At the slaughterhouse, ileal, caecal and colon contents from 165 pigs were sampled individually. Contents were collected using conical 15 ml plastic tubes. The tubes were filled and stored at -20°C for analysis of volatile fatty acids (VFA). VFA concentrations were measured with a Perkin-Elmer gas chromatograph model 8310 (Perkin-Elmer, Waltham, Mass.), equipped with a DB-FFAP high resolution column. Caecal contents were also sampled for multiplex PCR. A group of 12 virulence genes reported in the literature to be associated with different *E. coli* pathotypes, were selected to be used in our study (4). A series of 4 multiplex PCR procedures were performed according to a protocol of the Reference Laboratory for *Escherichia coli* (Ecl – Faculty of Veterinary

Medicine from the Université de Montréal) available at http://www.apzec.ca/en/APZEC/Protocols/APZEC_PCR_en.aspx. Also, caecal and colon contents were sampled individually and stored at -80°C for bacterial DNA analysis. Total DNA was extracted from caecal and colon contents of pigs by use of a physical-chemical method with phenol-chloroform essentially as previously described. Quantitative PCR was performed on a Eco Real-Time PCR System (Illumina, San Diego, CA) using the Eco Software (version 4.1). All standard curves were constructed using PCR products and each reaction was run in triplicate. Volatile fatty acids and qPCR data were analysed according to multiple linear regression of the Statistical Analysis System version 9.3 (SAS Institute Inc., Cary, NC).

VFA	Feed texture			Feed particle size			
	Mash	Pellet	P value	500 µm	750 µm	1250 µm	P value
Ileum							
Acetic	20,3±3,0	23,8±2,9	0,09	22,4±3,7	23,0±3,6	20,7±3,5	0,65
Propionic	2,9±0,9	3,4±0,8	0,44	3,7±1,1	3,0±1,0	2,8±1,0	0,47
Butyric	3,02±0,6	3,50±0,6	0,26	3,46±0,7	3,14±0,7	3,19±0,7	0,81
Caecum							
Acetic	85,9±4,6	82,6±4,5	0,31	85,1±4,1	85,8±5,5	81,9±5,4	0,56
Propionic	36,8±2,1	33,4±2,1	0,03	34,8±2,7	33,4±2,5	37,1±2,5	0,12
Butyric	16,3±1,2	14,3±1,2	0,02	14,3±1,5	14,9±1,4	16,7±1,4	0,05
Colon							
Acetic	83,4±4,0	83,4±3,9	0,99	84,5±5,0	85,9±4,8	79,7±4,7	0,16
Propionic	37,1±2,1	32,2±2,0	<0,01	33,6±2,6	33,7±2,5	36,6±2,4	0,16
Butyric	20,7±1,3	17,4±1,3	<0,01	17,5±1,7	18,1±1,6	21,6±1,6	<0,01

Results

Ileal, caecal and colon volatile fatty acid production is shown in Table 1. Caecal ($p=0,03$) and colon ($p<0,01$) propionic acid concentrations were higher for mash than for pellet fed animals. Similarly, caecal ($p=0,02$) and colon ($p<0,01$) butyric acid concentrations were also higher for mash than pellet fed animals. With respect to the feed particle size, caecal ($p=0,05$) and colon ($p<0,01$) butyric acid concentrations were higher for the 1250 µm diet than for that of 500 µm diet. Ileal acid production was similar for all feed textures and feed particle sizes.

Caecal *E. coli* virulence factors detection by multiplex PCR showed interesting results, especially concerning the F4 fimbriae (Table 2). Presence of the F4 fimbriae gene was detected only in pellet fed animals. Moreover, the genes for two virulence factors, F18 and Stx1, were not detected in any of the samples.

Feed texture	N	Virulence factor									
		LT	STa	STb	F4	Stx2	EAE	CNF	P	Aero	Tsh
Mash	81	43(53)	69(85)	21(26)	0(0)	20(25)	17(21)	56(69)	30(37)	58(72)	75(93)
Pellet	84	42(50)	59(70)	23(27)	6(7)	16(19)	14(17)	58(69)	32(38)	63(75)	73(87)

For total *E. coli* enumeration by real-time PCR (Table 3), caecal ($p=0,01$) and colon ($p<0,01$) *yccT* gene copy numbers were higher for pellet than mash fed animals. On the other hand, the enumeration of the genes *faeG*, *estB* and *cnf1*, amplified for the quantification of virulent *E. coli* populations, showed no differences between the pellet and the mash fed animals.

In addition, a decrease in feed conversion associated with pellet texture and/or 500 µm particle size was observed for each diet formulation ($p<0,05$).

Discussion

In the present study, we demonstrated that administration of mash rather than pellet feed was related to higher intestinal propionic and butyric acid levels. Furthermore, a 1250 µm diet is also associated with higher intestinal butyric acid production. Mash feeding may possibly be associated with an increase of bacteria producing volatile fatty acids and contribute to gut health by preventing the proliferation of injurious bacteria (5). Total *E. coli* enumeration was lower in mash fed animals. This could result in minimizing the contamination of the carcass with *E. coli* at the slaughterhouse. However, there was no evidence that mash feed affected the virulent *E. coli* populations. Consistent effects of feed texture and feed particles size were not observed for *cnf1*, *faeG* and *estB* gene copies in caecal and colon contents. Surprisingly, a feed texture effect was observed on the F4 fimbriae (*faeG* gene) caecal prevalence by multiplex PCR. In addition, feed conversion rates associated with mash feed were higher to those associated with pellet feed. As already demonstrated, administration of

pellet feed results in improved feed conversion but not necessarily average daily gain for pigs of all ages (6).

Conclusion

Mash feed diet is associated with higher propionic and butyric acid levels and a reduction of total *E. coli* numbers in the digestive

tract of pigs. Moreover, economic disadvantages of mash feeding can be countered by optimizing strategies, such as the use of mash feed for curative purposes or in the maternity to reduce the piglets exposure to potential pathogens and during stressful periods associated with greater vulnerability of animals. Thus, such strategies provide interesting alternatives to antibiotic use.

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References

1. **Canibe N, Højberg O, Højsgaard S, Jensen BB.** 2005. Feed physical form and formic acid addition to the feed affect the gastrointestinal ecology and growth performance of growing pigs. *J. Anim. Sci.* **83**:1287-1302.
2. **Prohászka L.** 1986. Antibacterial Mechanism of Volatile Fatty Acids in the Intestinal Tract of Pigs against *Escherichia coli*. *Journal of Veterinary Medicine, Series B* **33**:166-173.
3. **Boyen F, Haesebrouck F, Vanparrys A, Volf J, Mahu M, Van Immerseel F, Rychlik I, Dewulf J, Ducatelle R, Pasmans F.** 2008. Coated fatty acids alter virulence properties of *Salmonella* Typhimurium and decrease intestinal colonization of pigs. *Veterinary microbiology* **132**:319-327.
4. **Chapman TA, Wu X-Y, Barchia I, Bettelheim KA, Driesen S, Trott D, Wilson M, Chin JJ-C.** 2006. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Appl. Environ. Microbiol.* **72**:4782-4795.
5. **Mikkelsen LL, Højberg O, Jensen BB.** 2007. Coarse structured feed stimulates members of the genera *Lactobacillus* and *Mitsuokella* as well as propionate and butyrate producers in the pig stomach. *Livestock Science* **109**:153-156.
6. **Medel P, Latorre M, De Blas C, Lázaro R, Mateos G.** 2004. Heat processing of cereals in mash or pellet diets for young pigs. *Anim. Feed Sci. Technol.* **113**:127-140.

TABLE 3. Effect of feed texture and feed particle size on *Escherichia coli* populations (log gene copies/g faeces)

Gene	N*	Feed texture			Feed particle size			
		Mash	Pellet	P value	500 µm	750 µm	1250 µm	P value
Caecum								
<i>yccT</i>	111	14,45±0,6	15,53±0,6	0,01	15,67±0,8	14,54±0,7	14,87±0,7	0,07
<i>cnf1</i>	87	10,54±1,0	11,58±0,8	0,11	10,95±1,0	11,13±1,2	11,10±1,1	0,97
<i>faeG</i>	1	10,11	-	-	-	10,11	-	-
<i>estB</i>	105	7,97±0,7	8,08±0,7	0,81	8,59±0,8	7,68±0,8	7,81±0,9	0,24
Colon								
<i>yccT</i>	131	12,66±0,5	13,64±0,5	<0,01	13,70±0,6	12,80±0,6	12,97±0,6	0,10
<i>cnf1</i>	118	8,50±0,7	8,61±0,7	0,83	8,61±0,9	8,68±0,9	8,37±0,9	0,88
<i>faeG</i>	18	8,59±1,8	8,39±1,9	0,87	7,79±2,3	8,01±2,3	9,66±2,3	0,41
<i>estB</i>	119	7,07±0,5	7,12±0,5	0,88	7,27±0,6	6,72±0,6	7,31±0,6	0,28

*Positive samples by real-time PCR

Identification and distribution of *E. coli* virulence gene profiles in an operating swine production network.

Desmarais, G.⁽¹⁾, Letellier, A.*⁽²⁾, Fairbrother, JM.⁽²⁾, Fravallo, P.⁽³⁾

(1) Rue Sicotte 3200, J2S 2M2, St-Hyacinthe (Qc), Canada: gabriel.desmarais@umontreal.ca

(2) Rue Sicotte 3200, 08, J2S 2M2, St-Hyacinthe (Qc), Canada: john.morris.fairbrother@umontreal.ca

(3) Rue Sicotte 3200, SPV-1, J2S 2M2, St-Hyacinthe (Qc), Canada: philippe.fravallo@umontreal.ca

*Rue Sicotte 3200, SPV-1, J2S 2M2, St-Hyacinthe (Qc), Canada: ann.letellier@umontreal.ca

Abstract: A number of studies have demonstrated a link between the detection of potentially pathogenic *Escherichia coli* strains and economic loss in the swine industry. *E. coli* strains belong to different commensal or pathogenic clonal groups, the latter being characterized by the presence of specific virulence genes. The transmission of such *E. coli* between herds and a slaughterhouse in a production network, in particular to illustrate the dissemination of *E. coli* strains in a zoonotic perspective, has not been well characterized. The presence of certain virulence genes could be used as indicators of contamination between herds and the slaughterhouse. The objective of this study was to examine some transmission modes of such *E. coli* in a well defined swine production network. A defined region containing 10 farms, a slaughterhouse, and a transportation network was selected. Samples (feces, dust, soil...) were collected at various sites on the farms (3 visits), at the slaughterhouse (2 visits), and on the vehicles of stakeholders linking the farms and slaughterhouse, such as animal transporters. Three consecutive production batches were followed during 8 months. The presence in the samples of virulence genes (*eltB*, *estA*, *estB*, *faeG*, *stxA*, *stx2A*, *eae*, *cnf*, *papC*, *iucD*, and *tsh*) commonly associated with pathogenic *E. coli* was examined by conventional multiplex PCR. The monitoring of the virulence gene profiles both temporally and spatially resulted in the identification of an ETEC: F4 profile as such a marker. The distribution of ETEC:F4 suggests that the slaughterhouse yard acts as a reservoir of contamination in the network, ETEC:F4 being transmitted back to the farms by mechanical vectors. These results illustrated the need to improve the biosecurity relationship between herds and slaughterhouse, both playing a role in distribution of pathogens in pig production.

Introduction: *E. coli* is known to be an important constituent of the pig intestinal microflora. Most of the isolates colonize the small intestine and are commensal. However, some isolates encode virulence genes thus may be pathogenic and potentially zoonotic. Certain *E. coli* pathotypes cause post-weaning diarrhea, an important cause of mortality in pigs (Fairbrother et al., 2007). The transmission of such *E. coli* between herds and the slaughterhouse in a given network, in particular to illustrate the dissemination of *E. coli* strains in a zoonotic perspective, has not been well characterized. The presence of certain virulence genes could be used as an indicator of contamination between herds and the slaughterhouse. The objective of this study was to examine some transmission modes of such *E. coli* in a well defined swine production network. The monitoring of the virulence gene profiles both temporally and spatially resulted in the identification of an ETEC: F4 contamination profile.

Material and Methods: A swine production network consisting of 10 finishing farms, a transportation network and a slaughterhouse was selected. A total of 388 environmental samples were taken at the farms during 3 visits representing respectively 3 successive production batches. The farm environmental samples consisted of pools of feces from healthy pigs, swabs of objects and tools inside the farm buildings (panels, scales, working desks, high places, loading dock floor) and some surfaces of vehicle tracks outside the farm (animal departure, feed, knacker). Also, 272 environmental samples were taken in the slaughterhouse field following transport of 2 production batches from each of 9 of the 10 farms. The slaughterhouse environmental samples were taken on the departure dock, inside the truck cabin before loading and on the truck mud-guards and vehicle tracks at different times during the departure procedure. Samples were incubated 24 hours in a non-selective pre-enrichment broth at 37°C then transferred, respecting a 1:10 ratio, in an enrichment broth for another 24 hours. The cultured samples were centrifuged at 12000 rpm for 5 min, washed in buffer and placed at 100°C for 10 min to prepare the DNA templates for PCR. The presence in the samples of virulence genes (*eltB*, *estA*, *estB*, *faeG*, *stxA*, *stx2A*, *eae*, *cnf*, *papC*, *iucD*, and *tsh*) commonly associated with pathogenic *E. coli* (ETEC, ExPEC, STEC and EPEC) was examined by conventional multiplex PCR according to a protocol of the Reference Laboratory for *Escherichia coli* (ECL – Faculté de Médecine Vétérinaire de L'Université de Montréal) available at http://www.apzec.ca/en/APZEC/Protocols/APZEC_PCR_en.aspx. The distribution of *E. coli* virulence genes present in the samples was monitored and analyzed in a spatial and a temporal perspective.

Results: The distribution of *E. coli* virulence genes in the farm samples differed between the pathotypes and the farms. The ETEC virulence gene distribution on the farms was non-homogenous and showed a different contamination profile for each farm. The virulence genes encoding STb and STa toxins are detected throughout the network. On the other hand, the genes encoding for F4 and LT were only detected on certain farms. The detection of environmental samples positive for at least one ETEC toxin in combination with the gene encoding the fimbriae F4 suggests the presence of ETEC: F4+ strains in these samples. The distribution of samples positive for ETEC and F4 was analysed spatially and temporally to describe events of contamination in the network.

Samples positive for ETEC and F4 were detected in feces, objects and the vehicle tracks in most farms in the network, most commonly on farms A, B and H (Table 1). Few or no samples positive for ETEC and F4 were observed on farms D, E, F, G, I and J (results not shown). The spatial distribution of such samples positive for ETEC and F4 indicates the presence of contamination in the intestinal microflora of the pigs, but also in the farm environment and on vehicle tracks.

Most samples positive for ETEC and F4 were associated with the second farm visit (Table 2). This visit occurred in approximately the same period of time for every farm. These results suggest that most farms in the network were more contaminated by ETEC: F4 strains in that same period. Nevertheless, farm A still showed the highest level of ETEC: F4 contamination.

In the slaughterhouse environment, STEC and EPEC virulence genes were detected in a low proportion of samples, although Stx1 was detected more frequently than on the farms, particularly for departure visits associated with farms I and J. Interestingly, in contrast to farm results, ETEC virulence genes were detected on every departure visit to the slaughterhouse (Table 3).

The spatial distribution analysis of samples positive for ETEC and F4 permitted the identification of possible contamination sources in the network. ETEC and F4 were detected on objects and tracks at every delivery visit, but less frequently (one detection or less) for visits on farms D, E, and I (results not shown). Interestingly, delivery visits for farms A, B, C, G and J demonstrated higher ETEC: F4 contamination levels than other visits (Table 3). Hence, ETEC: F4 strains present in the environment could be transmitted by cross contamination to a vector such as the swine transporter and be brought to other farms and/or slaughterhouses.

Table 1. Spatial distribution of samples positive for ETEC and F4 in the farm environment on 3 visits.

	Total of samples	No. of samples positive for ETEC and F4 (%)			
		Feces	Objects	Tracks	Total samples positive for ETEC and F4
Farm A	41	4 (10)	5 (12)	1 (2)	10 (24)
Farm B	40	2 (5)	2 (5)	2 (5)	6 (15)
Farm C	38	0 (0)	1 (3)	1 (3)	2 (5)
Farm H	39	3 (8)	3 (8)	1 (3)	7 (18)
Total	388	9 (2)	13 (3)	6 (2)	28 (7)

Table 2. Temporal distribution of samples positive for ETEC and F4 in the farm environment.

	Total of samples	No. of samples positive for ETEC and F4 (%)			
		Visit 1	Visit 2	Visit 3	Total samples positive for ETEC and F4
Farm A	41	0 (0)	9 (22)	1 (2)	10 (24)
Farm B	40	0 (0)	5 (13)	1 (3)	6 (15)
Farm C	38	0 (0)	2 (5)	0 (0)	2 (5)
Farm H	39	1 (3)	6 (15)	0 (0)	7 (18)
Total	388	1 (1)	22 (6)	2 (1)	28 (7)

Table 3. Spatial distribution of samples positive for ETEC and F4 in the slaughterhouse environment on 2 visits.

	Total of samples	No. of samples positive for ETEC and F4 (%)		
		Objects	Tracks	Total samples positive for ETEC and F4
Delivery farm A	26	4 (15)	3 (12)	7 (27)
Delivery farm B	27	3 (11)	0 (0)	3 (11)
Delivery farm C	31	2 (6)	1 (3)	3 (10)
Delivery farm G	30	0 (0)	5 (17)	5 (17)
Delivery farm H	29	0 (0)	2 (7)	2 (7)
Delivery farm J	32	3 (9)	3 (9)	6 (19)
Total	272	13 (5)	15 (6)	28 (11)

The temporal ETEC: F4 virulence gene distribution permitted the identification of the visits when the contamination by the ETEC: F4 profile was higher with respect to the farms associated with the visits. The analyse of this distribution showed that most samples positive for ETEC and F4 were found on the first delivery associated with farms A, B, G and J (Table 4). This result suggests these farms were a contamination source in the network at this time.

Discussion: ETEC: F4 is responsible for post-weaning diarrhea in pigs and its presence can have a great economic impact on

production. Hence, it is considered that ETEC: F4 would be a good candidate to describe the transmission of *E. coli* contamination in a production network. Interestingly, the ETEC: F4 profile was more frequently observed in the environment or on objects less accessible for cleaning and disinfection, such as the top of the feeding conveyor and also in the vehicle tracks. This suggests that ETEC: F4 strains may be resistant to desiccation and persist in the farm environment. Such environments could act as a reservoir, permitting transmission of pathogenic *E. coli* strains to pigs of successive batches and from one establishment to another via contamination vectors such as swine transporters. The Stx1 STEC virulence gene was sporadically detected in the slaughterhouse fields. Interestingly, this gene is usually associated with STEC strains found in cattle (Beutin et al., 1993). As the slaughterhouse in our study is only processing swine, it would be important to investigate possibilities explaining the presence of cattle associated STEC isolates in the slaughterhouse environment. A plausible explanation is that swine transporters carry other animal species in other production networks outside of our study.

ETEC virulence genes were detected on every delivery visit to the slaughterhouse. This suggests that the holding field is frequently contaminated with ETEC strains throughout the year. It also strengthens the idea that ETEC: F4 strains are persistent in the environment since their virulence genes are detected in the yards of the slaughterhouse. Also, the slaughterhouse could act as a reservoir of ETEC: F4 strains, as the yards are known to be frequently visited by transporters. Hence, there is a possibility of ETEC: F4 transmission back to the production network via the slaughterhouse field.

Conclusions and perspectives: Our approach gives the opportunity to study the distribution of pathogenic *E. coli* virulence genes in a defined network containing farms and a slaughterhouse over a one year period. The virulence gene spatial distribution permitted us to identify an ETEC: F4 profile contamination marker in the network to describe contamination events in the network. In addition, the virulence gene temporal distribution showed when the contamination marker was more or less present in the network. The study of spatial and temporal distribution of samples positive for ETEC and F4 in the production network helped to better understand the transmission mode of pathogenic *E. coli* strains and to prevent possible contamination events in the network. Some farms presented a higher ETEC: F4 level. The slaughterhouse yards appear to be a potential reservoir of contamination. Characterization and comparison of ETEC: F4 isolates from these farms and the slaughterhouse could attest a direct link of contamination. Thus, the next step in this study is to isolate the contamination marker strains in the positive samples taken in the environment of the network. The genotypic characterization of these strains will then link sources with vectors of contamination and contamination events in the network.

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References:

Beutin, L., Geier, D., Steinruck, H., Zimmermann, S., Scheutz, F., 1993. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J Clin Microbiol* 31, 2483-2488.

Fairbrother, J.M., Nadeau, É., Gyles, C.L., 2007. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews* 6, 17-39.

Table 4. Temporal distribution of samples positive for ETEC and F4 in the slaughterhouse environment.

	Total of samples	No. of samples positive for ETEC and F4 (%)		
		Visit 1	Visit 2	Total samples positive for ETEC and F4
Delivery farm A	26	6 (23)	1 (4)	7 (27)
Delivery farm B	27	3 (11)	0 (0)	3 (11)
Delivery farm C	31	0 (0)	3 (10)	3 (10)
Delivery farm G	30	3 (10)	2 (7)	5 (17)
Delivery farm H	29	2 (7)	0 (0)	2 (7)
Delivery farm J	32	6 (19)	0 (0)	6 (19)
Total	272	22 (8)	6 (2)	28 (10)

Assessment of the efficiency of ozonated water as bacterial contamination reduction tool in a pork cutting plant.

Larivière-Gauthier, G.^{1,2,3}, Letellier, A.^{1,2,3*}, Quessy, S.^{1,2,3}, Fournaise, S.⁴ and Fravallo, P.^{1,2,3}

1 NSERC, Industrial Research Chair in Meat Safety, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

2 CRIPA, Centre de recherche en infectiologie porcine et avicole, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

3 GRESA, Groupe de recherche et enseignement en salubrité alimentaire, Faculté de médecine vétérinaire, Université de Montréal, Saint-Hyacinthe, Canada

4 Olymel S.E.C., Boucherville, Canada

The food industry is constantly searching for new tools to reduce bacterial contamination in the plants. In this study we assessed the efficiency of ozonated water as a tool to improve reduction of residual bacterial contamination in a pork cutting plant as a complement of sanitation procedures. First, the effectiveness of the ozonated water was tested on conveyors to reduce residual *Salmonella*, coliforms and aerobic flora load. Three conveyors were selected in the cutting rooms. Ten samples of 300 cm² were collected before and after water or ozonated water rinse. Aerobic flora and coliforms counts were done using petrifilms (3M) and *Salmonella* were detected using the MFLP-75 Health Canada method. In all the samples, *Salmonella* couldn't be detected and coliforms counts were below technical threshold limit. Aerobic flora results were compared after water and ozonated water treatments. A statistically significant benefit of 0.64 cfu / 300 cm² for ozonated water rinse was measured on one of the conveyor (t-test $p < 0.05$). This strategy was also evaluated as a way to eliminate *Listeria monocytogenes* that persists after cleaning and disinfecting operations. Sixteen contaminated sites after these operations were selected. They consisted in surfaces on the equipment and conveyors of the cutting rooms and non-contact surfaces. Each site was divided in two parts; one half receiving a 3.5 ppm ozonated water treatment (10 seconds application), the second part received a water rinse as control. Two to eight swabs were collected on each site. *Listeria monocytogenes* detection was conducted using MFHPB-30 Health Canada method. No statistically significant differences ($\chi^2 > 0.05$) were measured with 62.5 % (10/16) of sites and 37.7 % (26/69) of samples contaminated without treatment and 75 % (12/16) and 39.1 % (27/69) respectively after treatment. Results show that, in these conditions, the benefit of a supplementary ozonated water treatment in the cutting room is low and has no industrial relevance. This could be caused by the presence of residual organic matter on the surfaces, which reacts with ozone molecules.

*3200, rue Sicotte, C.P. 5000, J2S 7C6, Saint-Hyacinthe, Québec, Canada

E-mail: ann.letellier@umontreal.ca, Fax : (450) 778-8157

Introduction

The food industry is constantly searching for new tools to reduce bacterial contamination in the plants. One of the most promising alternatives or complement to traditional disinfectant is ozone. This molecule composed of three oxygen atoms is a very powerful oxidative product (Oxidation potential of 2.07 mV) which gives it a strong bactericidal power (Guzel-Seydim *et al.*, 2004). Indeed, this molecule has been shown to kill a wide variety of microorganisms that are relevant in the food industry such as *Listeria monocytogenes*, *Salmonella* and *E. coli*, but also to reduce microbial spoilage (Choi *et al.*, 2012; Khadre *et al.*, 2001). The ozone has been shown to oxidize bacterial proteins, enzymes, peptides and cause damage to nucleic acid but also to cause a degradation of the cell envelope (Thanomsub *et al.*, 2002; Zhang *et al.*, 2011). This technology is already in use as tool for water disinfection and studies have shown its effectiveness in vegetable and fruits disinfection (Choi *et al.*, 2012). Since, ozonated water has been declared as generally regarded as safe (GRAS), leaves no toxic residues and can be produced directly on site for a low cost, it has been described as a good alternative to chlorine-based disinfectant (Aguayo *et al.*, 2013; Khadre *et al.*, 2001). However information is still missing on its effectiveness as a tool to reduce the surface contamination in a meat production context.

For the porcine industry, this technology is also interesting in the context of the reduction of *Listeria monocytogenes*. This microorganism is a major foodborne pathogen and causes outbreaks that have frequently been linked with processed pork meat (Pichler *et al.*, 2009). In 2008, an outbreak, associated with cold cuts occurred across Canada, affecting 57 persons and

causing 22 deaths (Weatherill, 2011). It has been shown that *Listeria monocytogenes* persists on the equipment and in the environment of the pork meat production and could be responsible for the contamination of the final product (Carpentier and Cerf, 2011). The biofilms formed by *Listeria monocytogenes* are hard to eliminate and can persist even after cleaning and disinfection of the plant (Carpentier and Cerf, 2011). Hence, in this context, adding an ozonated water wash could be beneficial.

The objective of this study is to assess the potential of the ozonated water as a new tool in the cleaning and disinfection procedure of the cutting room in a pork meat production plant but also to evaluate its use as a tool to reduce *Listeria monocytogenes* persistence in its environment.

Material and Methods

To assess the bactericidal potential of the ozonated water, three conveyor belts were selected and sampled in the cutting room of the plant. Each conveyor was separated in three parts. One part was sampled before treatment as control; one was sampled after a treatment with water only and the last one after a 3.5 ppm ozonated water treatment (10 seconds application). On three different occasions, 10 swabs of 300 cm² were collected on each part on the conveyor 1 and 2, and 5 swabs on the conveyor 3.

Salmonella detection was conducted on the 225 samples collected on the conveyors using a method based on the MFLP-75 Health Canada method. A pre-enrichment was conducted in nutrient broth for 24h at 37°C and was followed by a selective enrichment on MSR/V semi-solid media 48h at 42°C. Plates with suspect migration were inoculated on BGS agar and incubated for 48h at 37°C. Presumptive colonies were tested biochemically using TSI and Urea test and slide agglutination. Coliform and aerobic flora counts were conducted using petrifilms (3M).

Contamination reduction was then compared for the two treatments using t-test (Graphpad 6.0 prism).

To assess the effect of adding an ozonated water wash to the cleaning and disinfection protocol as a tool to reduce *Listeria monocytogenes* persistence, sites that were previously identified as contaminated by this microorganism after cleaning and disinfection were sampled in the cutting room. The selected sites mostly consisted of equipment and environmental surfaces. Each identified site was separated in two with one part treated with a supplementary 3.5 ppm ozonated water treatment for 10 sec. Two to eight swabs of 300 cm² were collected on the treated and non-treated part of sites on three different visits in the plant.

Listeria monocytogenes detection was conducted on the 138 samples using a method based on the MFHPB-30 Health Canada standard technique. Briefly, the first enrichment was conducted in UVM-1 broth for 24h at 30°C and the second in Fraser broth for 48h at 37°C. Both broths were inoculated on ALOA agar and incubated for 48h at 37°C. Confirmation of the species of the typical isolate was conducted by evaluating hemolysis, CAMP test, motility in semi-solid agar and carbohydrates use in broths (xylose, rhamnose, mannitol).

The effect of the treatment on the presence of persisting *Listeria monocytogenes* was analysed using the Chi-squared test (Graphpad 6.0 prism).

Results

On the three sampled conveyors, no *Salmonella* was detected and Coliform contamination was below the detection technical threshold of 30 cfu / 300 cm².

Initial aerobic flora contamination of the conveyors' surfaces was between 2.84 and 4.53 log cfu / 300 cm² and there was a significant difference of contamination between these different conveyors (ANOVA p < 0.05). For the conveyor no. 1 and 2, no significant difference of reduction was measured between the ozonated water treatment and the water only treatment. However on the conveyor 3 (presenting a higher initial residual contamination), a significant difference of reduction of 0.64 log cfu was measured, showing a bactericidal effect of the ozone (t-test p<0.05). (Table no.1)

Table no 1: Aerobic flora reduction after water or ozonated water wash on three different conveyor belts.

Conveyor	Treatment	Mean (log cfu / 300 cm ²)	Standard deviation	n
1	Ø	3.72	1.23	30
	Water	3.87	0.53	30
	O3 Water	4.03	0.62	30
2	Ø	2.84	1.101	30
	Water	2.7	0.87	30
	O3 Water	2.44	0.83	30
3	Ø	4.53	0.99	15
	Water	4.46	0.62	15
	O3 Water	3.82	0.62	15

Listeria monocytogenes was detected on a total of 13 of the 16 sites that were sampled (81.3%) and in 53 of the 138 of the individual samples. It was found on 10 of the 16 sites and 26 of the 69 individual samples before treatment and on 12 of the 16 sites and 27 of the 69 samples after ozonated water treatment. No significant difference was measured when comparing the sites with or without ozonated water treatment (Chi squared $p > 0.05$) (Table no 2).

Table no 2 : *Listeria monocytogenes* presence on the selected sites before with or without ozonated water treatment

	Sites			Samples		
	positive	n	%	positive	n	%
No treatment	10	16	62.5	26	69	37.7
O ₃ treatment	12	16	75	27	69	39.1
Total	13	16	81.3	53	138	38.4

Discussion

The results obtained on the conveyors first showed that after the cleaning and disinfection protocol, these working surfaces are exempt of *Salmonella* and that if coliforms are present it is only in very low levels. Hence we were not able to measure any effect of the ozonated water on these microorganisms.

For the aerobic flora the three conveyors had different levels of initial contamination. The products that are processed on these conveyors that have various level of contamination or their ease of washing during cleaning and disinfection could explain these differences. On the first conveyor a non-significant augmentation was measured after the two treatments that could be explained by a resuspension of bacterial cells from biofilm by the water. However on the second one, a non-significant reduction was obtained using ozonated water and on the third conveyor a significant difference between the water wash and the ozonated water wash was observed with a higher reduction obtained using the ozonated water treatment. This shows that, in some context, the added ozone molecules have an added bactericidal potential to the physical effect of the water. The variable results obtained on the three conveyors could be explained by the presence of different levels of organic matter that the different processed product leave on the tested surface. Since ozone is an extremely reactive molecule, it can easily react with the fat or meat residues lowering its availability to a level where it has no antibacterial effect (Yousef *et al.*, 2001). However with an added reduction of only 0.64 log cfu / 300 cm² this reduction has no real industrial relevance.

Since the bactericidal effect of the ozonated water treatment was show in the previous experiment, it was also tested as a tool that can be applied on selected sites that are at risk for *Listeria monocytogenes* persistence. As expected, a large part (13 of the 16) of the sites that we had pre-selected were contaminated after cleaning and disinfection indicating that this microorganism can persist in the cutting room environment of this plant as described in the literature (Carpentier and Cerf, 2011). However our results showed that adding ozonated water rinsing that targeted these sites had no significant effect on the presence of persisting *Listeria monocytogenes* for the individual samples or sites in the plant. Once again it could be explained by the presence of residual organic matter in the plant's environment.

Conclusion

The results obtained in this study showed that, in the conditions found in the cutting plant, ozonated water can have an added bactericidal effect when compared to tap water. However with a maximum reduction of 0.64 log cfu / 300 cm² the efficacy of an ozonated water treatment is low and has no industrial relevance. Furthermore we found no reduction on persisting *Listeria monocytogenes* presence after the addition of an ozonated water treatment step on targeted sites. In the context of this study, these results could be caused by residual presence of organic matter on the surfaces, which reacts with ozone molecules.

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References

- Aguayo, E., Escalona, V., Silveira, A.C., Artés, F., 2013. Quality of tomato slices disinfected with ozonated water. *Food Science and Technology International*.
- Carpentier, B., Cerf, O., 2011. Review--Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International journal of food microbiology* 145, 1-8.
- Choi, M.-R., Liu, Q., Lee, S.-Y., Jin, J.-H., Ryu, S., Kang, D.-H., 2012. Inactivation of *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes* in apple juice with gaseous ozone. *Food Microbiology* 32, 191-195.
- Guzel-Seydim, Z.B., Greene, A.K., Seydim, A.C., 2004. Use of ozone in the food industry. *LWT - Food Science and Technology* 37, 453-460.
- Khadre, M.A., Yousef, A.E., Kim, J.G., 2001. Microbiological Aspects of Ozone Applications in Food: A Review. *Journal of Food Science* 66, 1242-1252.
- Pichler, J., Much, P., Kasper, S., Fretz, R., Auer, B., Kathan, J., Mann, M., Huhulescu, S., Ruppitsch, W., Pietzka, A., Silberbauer, K., Neumann, C., Gschiel, E., de Martin, A., Schuetz, A., Gindl, J., Neugschwandtner, E., Allerberger, F., 2009. An outbreak of febrile gastroenteritis associated with jellied pork contaminated with *Listeria monocytogenes*. *Wien Klin Wochenschr* 121, 149-156.
- Thanomsub, B., Anupunpisit, V., Chanphetch, S., Watcharachaipong, T., Poonkhum, R., Srisukonth, C., 2002. Effects of ozone treatment on cell growth and ultrastructural changes in bacteria. *The Journal of general and applied microbiology* 48, 193-199.
- Weatherill, S. 2011. Report of the Independent Investigator into the 2008 Listeriosis Outbreak. (Gouvernement du Canada).
- Yousef, A.E., Khadre, M.A., Kim, J.G., 2001. Microbiological aspects of ozone applications in food: A review. *Journal of Food Science* 66, 1242-1252.
- Zhang, Y.Q., Wu, Q.P., Zhang, J.M., Yang, X.H., 2011. Effects of ozone on membrane permeability and ultrastructure in *Pseudomonas aeruginosa*. *Journal of Applied Microbiology* 111, 1006-1015.

Digestive microbiota changes during application of an effective, feed presentation based, mitigation option against *Salmonella* shedding in pigs.

LeBel, P.^{1,2,3}; Fravallo, P.^{1,2,3}; Longpré, J.^{1,2,3}; Yergeau, É.⁴; Laplante, B.⁵; Letellier, A.*^{1,2,3}

- (1) NSERC Industrial Research Chair in Meat Safety, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, Qc, Ca;
- (2) Groupe de Recherche et Enseignement en Salubrité Alimentaire, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, Qc, Ca;
- (3) Swine and Poultry Infectious Diseases Research Center, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, Qc, Ca;
- (4) National Research Council Canada, Energy, Mining and Environment, Montréal, Qc, Ca;
- (5) F. Ménard, L'Ange-Gardien, Qc, Ca.

*Ann Letellier: ann.letellier@umontreal.ca

Abstract

If some studies have attempted to mitigate the *Salmonella* spp. excretion in pigs by feed related interventions, none clearly demonstrated the impact of the presentation (mash or pellet and particular size). Thus this study aimed to determine if the modification of the pigs feed presentation alone can lower the *Salmonella* spp. excretion. To do so, 144 eight weeks aged piglets, previously confirmed as homogeneously in contact with *Salmonella* during post-weaning, were given diets that varied only by the particle size (500, 750 or 1250 μm) and/or the texture (mash or pellet). **During the fattening period, they were individually sampled for blood and feces collection after 0, 21, 46 and 88 days of specific diet. Colons and caecums content were also sampled at the slaughterhouse. There were more pigs from the pellet groups shedding *Salmonella* spp. in their feces after 21 (p=0.012) and 88 days (p=0.002) and in their colon content at slaughter (p=0.026) than from the mash feed groups.** In contrast with the literature, no seroconversion significant differences were found between the different groups. Real-time PCR analyses revealed that pigs from the pellet groups had significantly less *Bifidobacterium* spp. in their feces than those from the mash feed groups at day 21. At the same date, a 16S rRNA gene amplicon analysis of the fecal microbiota using Ion Torrent™ sequencing revealed a significantly lower representation of the *Spirochaetes* phylum in the feces of pigs from mash feed groups. It also indicated significantly more bacteria of the *Fibrobacteres* phylum and less Chloroplast in the feces of pigs from pellet feed groups. Correlation between *Salmonella* mitigation efficiency and changes in microbiota will be tested. Our data are compatible with the fact that a mash feed would promote a healthier gut microbiota while pellet feed would promote better digestion efficiency.

Introduction

Modifying the pigs feed to mitigate the presence of *Salmonella* spp. in herds at the farm level while increasing gut health and reducing the use of antibiotics is gaining in popularity over the years. Many solutions have been explored but none resulted in the elimination of the pathogen. These strategies are believed to be associated with beneficial modifications of the gut microbiota and its production of antibacterial element (Mountzouris, *et al.*, 2006). One promising strategy is the use of mash feed instead of the commonly used pellet feed (Lo Fo Wong, *et al.* 2004). In their review comparing feed management practices and feed characteristics associated with *Salmonella* prevalence, O'Connor *et al.* (2008) came to similar conclusions based on serological data. Unfortunately, none demonstrated a *Salmonella* spp. shedding reduction or any modification of the microbiota that would go along with it. It is also important to notice that in most studies many variables were different between the compared diets (composition, particle size, texture, heating process, etc.). The goal of this study was to investigate on modifications of the pigs gut microbiota that would explain or go along with a reduction of *Salmonella* spp. shedding on the farm when only the feed presentation is modified (mash or pellet/particle size).

Material and Methods

On farm

A batch of nine hundred 5 weeks old piglets, known to have been in contact with *Salmonella* spp. at the nursery, were split (10 per pen) into 6 groups. Each group received a different diets varying only by their particle size (500, 750 or 1250 μm) or texture (mash or pellet). Pellet 500 being the reference group from the industry. Individual blood and feces samples were taken as well as colon content at slaughter on 144 of the pigs (24 per diet, 2 per pen) on days: 0, 21, 46 and 86. An aliquot

of the feces was put into liquid nitrogen and later kept at -80° C for molecular biology analysis and the rest was kept at 4° C until beginning of the analyses.

Blood analysis

Salmonella spp. seroconversion was followed by a commercial ELISA kit (Maxivet, St-Hyacinthe, Quebec, Canada) developed to detect serological response to more than 95% of *Salmonella* serotypes commonly found in Canada (Letellier, A, 2009). Sera were analysed by the diagnostic service of the Faculté de Médecine Vétérinaire of the University of Montreal.

Salmonella spp. detection

We used a modified version of the ISO 6579 annexe D for the detection of *Salmonella* spp. in feces and environment (BPW 18-24h at 37° C, MRSV 48h at 42° C, isolation on BGS and XLD 24h at 37° C followed by biochemical and sero-agglutination confirmation).

Real-time PCR

DNA extraction (mechanical lysis followed by a phenol-chloroform extraction) was performed on all samples. Real-time PCRs, using the parameters shown in table 1, were performed on the samples of interest using an Eco[®] Illumina[®] real-time PCR with EvaGreen[®] qPCR mix as recommended by the manufacturer. For lactobacilli and enterobacteria, 15 ng of DNA and a final primer concentration 1 µM was used. For the *Bifidobacterium* genus, the amplifications reactions consisted of 10 ng of DNA with a final primer concentration of 0.25 µM. The standard curves for each reaction were obtained by diluting the precipitate of PCR product realized in the same conditions with the following reference strains *L. acidophilus* ATCC314, *E. coli* 25922 and *B. Longum* ATCC 15707. Results were expressed as log of copies/10 ng of DNA used.

Table 1. Real-time PCR parameters.

Group	Primers	Length	Cycles
Lactobacilli (Castillo, <i>et al.</i> 2006)	LB-F : GCAGCAGTAGGGAATCTTCCA (Tm=57 °C) LB-R : GCATTYCACCGCTACACATG (Tm=57 °C)	≈200 pb	Init. Den : 10m@95°C 40 cycles: 15s@95° C 1m@60° C
Enterobacteria (Castillo, <i>et al.</i> 2006)	Ent-F : ATGGCTGTCGTCAGCTCGT (Tm=60 °C) Ent-R : CCTACTTCTTTTGCAACCCACTC (Tm=60 °C)	364 pb	Init. Den. : 10m@95°C 40 cycles : 15s@95° C 1m@60° C
<i>Bifidobacterium</i> spp. (Matsuki, <i>et al.</i> 2004)	Bif-F : CTCCTGGAAACGGGTGG (Tm=53 °C) Bif-R : GGTGTTCTTCCCGATATCTACA (Tm=53 °C)	550 pb	Init.: 5m@94°C 40 cycles : 20s@94°C 20s@55°C 50s @72°C

16S rRNA gene sequencing

PCR of the partial 16S rRNA was performed using the universal primers F343 (5'- TACGGRAGGCAGCAG-3') and R533 (5'- ATTACCGCGGCTGCTGGC-3') containing the 10-bp multiplex identifiers (MID) and adaptor sequences for Ion Torrent sequencing on DNA extract from the fecal samples of each pig (Yergeau, *et al.* 2012). PCR products were purified on 2% agarose gel and pooled in an equimolar ratio and sequenced together. A total of 3.50x10⁷ molecules were used in an emulsion PCR using the Ion OneTouch 200 Template Kit v2 (Life Technologies) and the OneTouch instruments (Life Technologies) according to the manufacturer's protocol. The sequencing of the pooled library was done using the Ion Torrent Personal Genome Machine (PGM) system and a 316 chip with the Ion Sequencing 200 kit according to the manufacturer's protocol. Sequence data were analyzed by using the Ribosomal Database Project Pyro-sequencing Pipeline (<http://pyro.cme.msu.edu/>, RDP release 10, update 26). The sequences, deconvoluted and binned, were trimmed by using the Pipeline Initial Process tool. Datasets containing MID sequences associated with the 16S rRNA gene amplifications were individually classified using the RDP Classifier tool with an 80% bootstrap cutoff (6).

Results

Salmonella spp. detection

As shown in tables 2, no significant differences in *Salmonella* spp. shedding was found at D0. At D21, D88 and in the colon content at slaughter, more pigs from the pellet feed diets were shedding *Salmonella* spp. ($p=0.012$, $p=0.002$ and $p=0.026$ respectively) than from pigs on mash diet (Table 2).

Real-time PCR

The lactobacilli and enterobacteria groups and their ratio (lactobacilli/enterobacteria) were similar from group to group. On the other side, *Bifidobacterium* spp. were more present in feces from the mash feed groups compared to the pellet feed groups (all particle size together) and higher values in the mash 1250 μm compared to the 3 other groups tested on day 21 ($p<0.05$) were noted (Table 3).

16S rRNA gene sequencing

At day 21, four phyla showed significant differences in between the pigs from the different diets. While *Spirochaetes* and *Fibrobacteres* were found in greater numbers in the feces from the pellet groups compared to the mash groups; *Firmicutes* and Cyanobacteria/Chloroplast phylums were found in lesser numbers in the same samples. The higher representation of *Spirochaetes* is mainly due to bacteria from the *Treponema* genus. Similar link can be made for the *Fibrobacteres*: *Fibrobacter* being the only representing genus of its phylum in this analysis. Unfortunately, such a link cannot be made for now to conclude on the higher presence of *Firmicutes* in the samples from the mash groups because of the higher diversity of bacterial from this phylum in the gut microbiota. Finally, the higher proportion of Cyanobacteria/chloroplast in the mash is caused by a greater presence of cells of the Chloroplast family.

Discussion

This study clearly showed that it is possible to modify the *Salmonella* spp. excretion in pigs by the modification of feed texture and size of particles without modification of the formula. This reduction was present at the beginning and the end of the fattening period (D21 and D88) and maintained in the colon content at slaughter despite the known increasing effect of transportation stress on this pathogen.

These reductions go along with the raise of *Bifidobacterium* spp. count observed in the mash feed groups. In fact, bacteria of this genus have often been associated with a raise in antibacterial compound such as volatile fatty acids (Mountzouris, *et al.*, 2006). A higher presence of *Treponema* spp., another potential pathogen, was also revealed in the feces from the pigs fed pellet feed by the metagenomic analyses. These three put together indicate a better gut health for the pigs fed the mash feed diets compared to those feed pellet feed diets (all particle size together).

On the other side, more *Fibrobacter* spp., an important agent in the degradation of cellulose was found in greater number in the groups fed pellet feed. Which also correspond with the lesser presence of chloroplast, an indicator of the presence of vegetal cells. Therefore, the higher presence of bacteria being able to digest vegetal cells in the pellet groups linked with a lower presence of vegetal cell fits with the observations made in other studies where pigs fed mash feed would get a better gut health but would suffer a lesser digestion efficiency.

Conclusion

Our study is the first one to demonstrate the benefit of the pigs feed presentation (texture or particle size) alone on *Salmonella* spp. fecal shedding. Other specific changes in the microbiota were measured as an effect of the different diets used. The data from this study are compatible with the fact that mash feed would promote a healthier microbiota compared to a

Table 2. Total of pigs in the mash or pellet groups excreting *Salmonella* spp. on days 0, 21, 46, 88 and positive colon content at slaughter. Legend: Data with (b) notice are significantly higher than data with (a) notice from the same date.

Texture	D0	D21	D46	D88	Colon content
Mash (n=72)	31	16 ^(a)	9	5 ^(a)	10 ^(a)
Pellet (n=72)	36	30 ^(b)	14	19 ^(b)	21 ^(b)

Table3. Log of copies of *Bifidobacterium* spp. 16S RNA gene/10 ng of DNA from pig feces on day 0 and 21. Legend: Data with (a) or (c) noticed are significantly higher than data with respectively (b) or (d) notice.

Date	Pellet 500 (n=24)	Pellet 1250 (n=24)	Mash 500 (n=24)	Mash 1250 (n=24)	Total Pellet (n=72)	Total Mash (n=72)
D0	3.52	3.62	3.40	3.52	3.57	3.46
D21	3.25 ^(b)	3.32 ^(b)	3.39 ^(b)	4.23 ^(a)	3.29 ^(d)	3.81 ^(c)

better digestion efficiency for pellet feed.

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References

- BELL T.H., YERGEAU E., MARTINEAU C., JUCK D., WHYTE L.G., GREER C.W., 2011. Identification of nitrogen-incorporating bacteria in petroleum-contaminated Arctic soils by using [¹⁵N] DNA-based stable isotope probing and pyro-sequencing., *Appl Environ Microbiol*, 2011, 77: 4163–4171.
- CASTILLO M., MARTIN-ORUC S.M., MANZANILLA E.G., BADIOLA I., MARTIN M., GASA J., 2006, Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR, *Veterinary Microbiology*, Vol. 114: 165-170.
- LO FO WONG D.M.A., DAHL J., STEGE H., VAN DER WOLF P.J., LEONTIDES L., VON ALTOROCK A., THORBERG B.M., 2004, Herd-level risk factors for subclinical Salmonella infection in European finishing-pig herds, *Preventive Veterinary Medicine*, Vol. 62: 253-266.
- MATSUKI T., WATANABE K., FUJIMOTO J., KADO Y., TAKADA T., MATSUMOTO K., TANAKA, R. 2004. Quantitative PCR 16s rRNA-Gene-Targeted Species-Specific Primers for Analysis of Human Intestinal *Bifidobacteria*. *Applied and Environmental Microbiology*. Vol. 70. No. 1. 167-173.
- MOUNTZOURIS K.C., BALASKAS C., FAVA F., TUOHY K. M., GIBSON G.R., FEGEROS K. 2006. Profiling of composition and metabolic activities of the colonic microflora of growing pigs fed diets supplemented with prebiotic oligosaccharides. *Anaerobe*. Vol. 12. 178-185.
- O'CONNOR A.M., DENAGAMAGE T., SARGEANT J.M., RAJIC A., MCKEAN J. 2008. Feeding management practices and feed characteristics associated with Salmonella prevalence in live and slaughtered market-weight finisher swine: A systematic review and summation of evidence from 1950 to 2005. *Preventive Veterinary Medicine*. Vol. 87: 213-228.
- YERGEAU E., 2012. Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities, *Appl. Environ. Microbiol.*, 78:7626-7637.

Evaluation of the impact of functional foods on the course of *Salmonella* infection in piglets

Bergeron N^{*}, ^(1,2), Lessard, M. ^(2,3), Quessy, S. ^(1,2), Letellier, A. ^(1,2)

⁽¹⁾ Research Chair in Meat Safety (CRSV), Research and Teaching Group in Food Safety (GRESA), Research group on Infectious Diseases of Swine (GREMIP), Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, Québec, Canada.

⁽²⁾ Swine and Poultry Infectious Diseases Research Center (CRIPA), Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, Québec, Canada.

⁽³⁾ Dairy and Swine R&D Centre, Agriculture and Agri-Food Canada (AAFC), Sherbrooke, Québec, Canada.

*3200 Sicotte, C.P. 5000, St-Hyacinthe, Québec, Canada, J2S 7C6

e-mail: nadia.bergeron@umontreal.ca; fax : 450-778-8128

Abstract

With the aim to improve growth of weaning piglets and to minimize incidence of intestinal diseases, the effect of a combination of functional foods able to stimulate the development of systemic and mucosal immune system and to modulate bacterial populations in the gut was evaluated. In this study, we assessed the impact of functional foods on the course of *Salmonella* infection in piglets. Piglets from different litters were weaned at 21 days of age and assigned to 1 of the 4 feed additives (8 litters per treatment) as follow: 1-control (CTRL), 2-antibiotic (ATB), 3-cocktail of functional foods (CFF), 4-bovine colostrum + cocktail of functional foods (COL-CFF). At 49 days of age (day 0), four piglets per litter were orally inoculated with 1×10^8 CFU of *Salmonella* Typhimurium. A clinical exam was done for each piglet twice a day. Fecal samples were taken to evaluate the *Salmonella* shedding before and post-infection (days 1, 3, and 7 post-infection). Before challenge and on days 2 and 6 post-infection, blood samples were taken from all piglets to evaluate serum level of prostaglandin E metabolite (PGEM), tumor-necrosis-factor- α (TNF- α) and interleukin-8 (IL-8). At days 3 and 4 post-infection, pigs from ATB group showed no diarrhea compared to pigs from CTRL and CFF groups. At days 4 post-infection, pigs from COL-CFF group showed no diarrhea compared to pigs from CTRL and CFF groups. Regarding fecal excretion at day 1 post-infection, in ATB group, pigs showed lower *Salmonella* fecal excretion than in CFF group and the lower weight piglets showed a higher fecal excretion than the higher weight piglets. The ATB group pigs showed a lower *Salmonella* fecal excretion level than pigs from CTRL group at 3 days post-infection. A significant time effect indicated that serum level of PGEM was significantly reduced 2 and 6 days post-infection in comparison to day 0 (before challenge) whereas TNF- α and IL-8 levels were significantly increased after *Salmonella* challenge.

Introduction

In our strategy to improve development and health of weaning pigs and to minimize incidence of diseases caused by different pathogens such as *Escherichia coli* (Fairbrother *et al.*, 2005) and *Salmonella* (Berger and Wierup, 2012), the effect of a combination of functional foods capable of modulating the development of systemic and mucosal immune system, physical barrier of the intestine and bacterial populations in the gut need to be further examined. Among functional foods, bovine milk by-products, including colostrum and whey, cranberry products, essential oil, probiotics, prebiotics, essential fatty acids and vitamins are considered as good candidates. For example, both colostrum and milk are rich in oligosaccharides and these molecules have the potential to inhibit binding of enteric pathogens to intestinal host cell receptors (Newburg *et al.*, 2005). Cranberry has been shown to have beneficial effects on health through their antioxidant property and antimicrobial activity against foodborne pathogens (Wu *et al.*, 2008). Recent study on essential oils as alternative to antibiotics have demonstrated that some essential oils demonstrated a good potential to antimicrobial activity towards bacterial pathogens found in pigs (including *S. Typhimurium*) (Si *et al.*, 2006). Probiotics are bacteria with beneficial effects on host gut health through immunomodulation (Lessard *et al.* 2009). In this study, we evaluated the impact of functional foods on *Salmonella* carriage in pigs, more specifically; we evaluated the presence of *Salmonella* in different organs, clinical signs and level of prostaglandin E metabolite (PGEM), tumor-necrosis-factor- α (TNF- α), and interleukin-8 (IL-8) in sera.

Material and Methods

***Salmonella* challenge strain dose and detection:** *Salmonella* Typhimurium DT-104 strain #4393 rifampicin resistant, isolated from a clinical case of salmonellosis (Côté *et al.*, 2004), was used for inoculation. The isolate was thawed on nutrient agar and was grown in Buffered Peptone Water medium in a shaking incubator at 150 rpm, both at 37°C. The first part of the project was to determine the proper dose of *S. Typhimurium* needed to establish the carrier state in piglets and

to characterize the *Salmonella* infection kinetic. Three groups of 10 piglets were exposed to different oral doses (1×10^4 , 1×10^6 or 1×10^8 CFU) of *S. Typhimurium* strain #4393. The research of *Salmonella* in feces, mesenteric lymph nodes and junction ileo-cecal was carried out using a modification of ISO6579 2002(E): Annex D, using BGS agar with rifampicin as the single isolation medium.

Design of the study: At the Dairy and Swine R&D Center, 48 sows were inseminated to obtain 32 litters of piglets. Four farrowing periods were planned to do 4 experimental infections. At each farrowing period, 8/12 sows and their litter were chosen for the experimental infection. After 14 days of lactation, the 4 smaller and 4 bigger piglets were identified. At weaning (20-21 days-old), the 4 smaller piglets were put in the same pen and the 4 bigger piglets were in another pen. Then, they were assigned to 1 of the 4 feed additives as follow: 1- Basic diet + 3,5% plasma proteins (CTRL), 2- Basic diet + 3,5% plasma proteins + antibiotics (ATB), 3- Basic diet + 3,5% plasma proteins + cocktail of functional foods (cranberry extract, encapsulated calvacrol (kindly provided by Drs Wang and Gong, Guelph, AAFC), yeast extract, vitamins and mineral with organic Se, vitamins A, D and B, probiotic *Pediococcus acidilactici* MA18/5M) (CFF), and 4- Basic diet + bovine colostrum + cocktail of functional foods (COL-CFF).

Twenty-one days after weaning (day 42), all remaining piglets (2 smaller and 2 bigger piglets/litter) were transferred in level II facilities of the Veterinary School in St-Hyacinthe for 7 days of acclimation before the experiment. An individual clinical exam was done each day during the acclimation period: general state (score 1 to 6), respiration, body temperature, and consistency fecal score (0-3). Feces samples were collected 24 hrs after their arrival to be sure that piglets were *Salmonella* free before to start the experiment. One week after transfer (day 49), piglets were orally inoculated with *S. Typhimurium* DT-104 #4393 at 1×10^8 CFU, and an individual clinical exam was done twice a day as described above. Fecal samples were taken to evaluate the *Salmonella* post-infection shedding (days 1-3-7). On days 2 and 6 post-infection, blood samples were taken from all piglets to evaluate serum level of PGEM, TNF- α , and IL-8. Three and 7 days post-infection, two piglets per litter were euthanized (1 smaller/1 bigger). Ileo-cecal junction and mesenteric lymph nodes were collected to detect and quantify *Salmonella*.

Data analysis: Body temperatures and cytokines were analyzed using a repeated-measures linear model with treatment as a between-subject factor, time as a within-subject factor and block and id within the block as random factors. A priori contrasts were performed between pairs of means using the sequential Bonferroni procedure to adjust comparison-wise alpha levels. Categorical data (such as consistency fecal scores) were analyzed using the Cochran-Mantel-Haenszel test. An exact chi-square test was used to test the relationship between prevalence data in different compartments (feces, junction, and rectum) and treatment separately at each time period. Log10 transformed bacterial counts were analyzed using a mixed linear model with treatment as a fixed factor, id as a random factor and sex, weight and dose as co-factors. Tukey's post-hoc tests were used to compare pairs of treatment means. The level of statistical significance was set at 0.05 and analyses were carried out using SAS v.9.3 (Cary, N.C.).

Results

In the first part of the study, results showed that piglets from group 3 (1×10^8 CFU) demonstrated the higher level of carrier state during the *Salmonella* challenge and this dose was used for the second part of the study on the impact of functional foods on the course of *Salmonella* infection in piglets. All piglets were *Salmonella* free before to start the experiment. For the clinical exam, no significant observations were noted between the groups. The majority of animals presented a normal respiration and a general state score (1). The temperature varies significantly in the time ($p < 0.0001$) and it is similar from a group to other one, without any group effect. At days 3 and 4 post-infection, pigs from ATB group showed no diarrhea compared to pigs from CTRL and CFF groups. At days 4 post-infection, COL-CFF group pigs showed no diarrhea compared to pigs from CTRL and CFF groups. In a general way, the majority of piglets showed presence of *Salmonella* in feces, in mesenteric lymph nodes and in the ileo-cecal junction at days 1, 3 and 7 post-infection. Regarding fecal excretion level at day 1 post-infection, pigs from ATB group showed lower *Salmonella* fecal excretion level than CFF group ($P = 0.0494$) and the lower weight piglets showed a higher fecal excretion level than the higher weight piglets ($P = 0.0002$). The ATB group pigs showed a lower *Salmonella* fecal excretion level than CTRL group at 3 days post-infection ($p = 0.0157$).

Table 1: Effect of *Salmonella* infection on blood prostaglandin (PGEM), tumor-necrosis-factor- α (TNF- α) levels and interleukin-8 (IL-8).

Inflammatory Factor	Day 0	Day 2 post-infection	Day 6 post-infection	SEM	D0 vs. D2 (P value)	D2 vs. D6 (P value)
PGEM (pg/ml)	151.0	107.1	85.9	10.5	<0.001	<.0001
TNF- α (pg/ml)	44.4	59.7	60.1	4.9	<0.001	0.60
IL-8 (pg/ml)	238.2	225.8	323.45	42.0	0.20	0.02

At 7 days post-infection, a significant interaction ($p=0.0146$) was observed between the treatment and the weight on the quantification of *Salmonella* in the lymph nodes. The lower weight piglets in the ATB group pigs were more colonized by *Salmonella* in lymphatic nodes than the higher weigh piglets. The opposite scenario was observed for piglets in the CFF group pigs.

A significant time effect showed that PGEM level was significantly reduced 2 and 6 days post *Salmonella* challenge whereas TNF- α and IL-8 were respectively increased on day 2 and day 6 after *Salmonella* challenge (Table 1). Dietary treatments had no effect on PGEM, TNF- α , and IL-8 serum level.

Discussion

In the first part of this study, we developed a reproducible model for future studies on strategies to control *Salmonella* in pigs. Piglets infected with 1×10^8 CFU of *Salmonella* Typhimurium DT104 showed the highest level of carrier state during the *Salmonella* challenge. During protection assays, the use of colostrums and probiotics in a functional food (COL-CFF) showed a reduction of diarrhea and this observation is also seen with the ATB group. Bovine milk by-products have been demonstrated to contain bioactive molecules with immuno-regulatory and antimicrobial properties (Cross and Gill, 2000; Schlimme *et al.*, 2000). Both colostrum and milk are rich in oligosaccharides and there is evidence that neutral oligosaccharides present in milk are not digested or absorbed into the small intestine, but instead delivered into the colon (Boehm and Stahl, 2007). These molecules have been shown to play an important role in the establishment of different bacterial populations in the gut (Kuntz *et al.*, 2008) and have the potential to inhibit binding of pathogenic Gram-negative bacteria such as *E. coli* to intestinal host cells (Newburg *et al.* 2005). Because of all these functional properties, bovine colostrum could then be more appropriate than plasma protein as protein source in weanling diet.

In this study, *Salmonella* infection was efficient in modulating the release of inflammatory mediators such as PGEM, TNF- α and IL-8. Our results are in contradiction with another study from Balaji *et al* (2000) indicating that *Salmonella* infection did not alter plasma TNF α and PGE₂ in pigs. These authors hypothesized that PGE₂ released by inflamed gut tissue remain sequestered locally and does not contribute to elevate PGE₂ in blood (Balaji *et al.*, 2000). However, it has been reported that the clinical state of *Salmonella* orally infected pigs correlated with bacterial translocation and levels of the inflammatory cytokines IL-8 and TNF- α in plasma and intestinal lavages (Splichalova *et al.* 2011). In our study, it is also interesting to note that plasma level of TNF- α and IL-8 differently varied with the course of infection.

Conclusion

This study permit to determine the proper dose of *S. Typhimurium* DT104 needed to establish the carrier state in piglets and to characterize the *Salmonella* infection kinetic. This model is useful to evaluate the mechanisms of action of natural feed additives strategies as alternative to antibiotics to control *Salmonella* infection and at the end reduce risk of foodborne infection in human but more investigations are needed to adjust the feed treatment and optimize the efficacy.

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References

- BALAJI R., Wright KJ., Hill CM., Drits SS., Knoppel EL., Minton JE., 2000, Acute phase responses of pigs challenged orally with *Salmonella* Typhimurium, *J Anim Sci*, 78:1885-1891.
- BERGER AC., Wierup M., 2012, Nutritional strategies to combat *Salmonella* in monogastric food animal production, *Animal*, 6: 557-564.
- BOEHM G., Stahl B., 2007, Oligosaccharides from milk, *J Nutr*, 137 (Suppl 2):847S-849S.
- CROSS ML., Gill HS., 2000, Immunomodulatory properties of milk, *Br J Nutr*, 84:S81-S89.

CÔTÉ S., Letellier A., Lessard L., Quessy S., 2004, Distribution of *Salmonella* in tissues following natural and experimental infection in pigs, *Can J Vet Res*, 68:241-248.

FAIRBROTHER JM., Nadeau E., Gyles CL., 2005, *Escherichia coli* in post-weaning diarrhea in pigs: an update on bacterial types, pathogens, and prevention strategies, *Anim Health Res Rev*, 6: 17-39.

KUNTZ S., Rudloff S., Kunz C., 2008, Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells, *Bri J Nutr*, 99: 462-471.

LESSARD M., Dupuis M., Gagnon N., Nadeau É., Matte JJ., Goulet J., Fairbrother JM., 2009, Administration of *Pediococcus acidilactici* or *Saccharomyces cerevisiae* boulardii modulates development of porcine mucosal immunity and reduces intestinal bacterial translocation after *Escherichia coli* challenge, *J Anim Sci*, 87: 922-934.

NEWBURG DS., Ruiz-Palacios GM., Morrow AL., 2005, Human milk glycans protect infants against Enteric Pathogens, *Annu Rev Nutr*, 25:37-58.

SCHLIMME E., Martin D., Meisel H., 2000, Nucleosides and nucleotides: natural bioactive substances in milk and colostrums, *Br J Nutr*, 84:S59-S68.

SI W., Gong J., Tsao R., Zhou T., Yu H., Poppe C., Johnson R., Du Z., 2006, Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria, *J Appl Microbiol*, 100:296-305.

SPLICHALOVAA., Trebichavsky I., Rada V., Vlkova E., Sonnenborn U., Splichal I., 2011, Interference of *Bifidobacterium choerinum* or *Escherichia coli* Nissle 1917 with *Salmonella* Typhimurium in gnotobiotic piglets correlates with cytokine patterns in blood and intestine, *Clin Exp Immunol*, 163: 242-249.

WU VC., Qiu X., Bushway A., Harperet L., 2008, Antibacterial effects of American cranberry (*vaccinium macrocarpon*) concentrate on foodborne pathogens, *LWT - Food Sci Tech*, 41: 1834-1841.

Simulation Model for *Salmonella* Typhimurium on a Farrow-to-Finish Herd

Correia-Gomes, C.^{1,2*}, Economou, T.³, Bailey, T.³, Brazdil, P.⁴, Vieira-Pinto, M.⁵, Niza-Ribeiro, J.^{1,6}

¹ICBAS-UP, Universidade do Porto, Rua de Jorge Viterbo Ferreira, n°228, 4050-313 Porto, Portugal

² ERU-SRUC, Scotland's Rural College, Drummondhill, Inverness, IV2 4JZ, UK

³ CEMPS, University of Exeter, Harrison Building, North Park Road, Exeter, EX4 4QE, UK

⁴ LIAAD – INESC Porto L.A. Universidade do Porto, Rua de Ceuta, 118, 6º, 4050-190 Porto, Portugal

⁵ UTAD, Veterinary Science Department, Apartado 1013, 5001-801 Vila Real, Portugal

⁶ ISPUP – Institute of Public Health – Universidade do Porto, Rua das Taipas, 4099-003 Porto, Portugal

*Corresponding author: carla.gomes@sruc.ac.uk

Abstract

A stochastic model which simulates the dynamics of *Salmonella* Typhimurium in moderate to highly infected farrow-to-finish farms in Portugal was developed. The model comprises six different stages: three at the reproductive phase (sows) and another three for pig growth. Infection dynamics of *Salmonella* are modelled for each stage with four infection transition parameters: susceptible to infectious (β), infectious to carriers (α), carriers to infectious (δ), and carriers to susceptible (θ); and the health status: susceptible, infectious and carrier. The infection transition parameters were estimated based on field conditions and the ones that influence the infectious state the most, at the end of the fattening stage, were: the transition rate from susceptible to infectious (β) in all pig-compartments, and the piglets' immunity protective factor. Several control measures can be suggested to reduce the effect from those parameters. The simulation model, if coupled with an economic model can then be used to test control measures, in terms of their cost-benefit, and the reduction of the prevalence in these moderated to highly infected farms will have an impact on human burden. The simulation model is flexible enough to introduce changes in the parameter values appropriately if future research and changes in the legislation so require. The model can also be adapted to different types of production (e.g. breeding, weaners or finishers units) as it was built in a compartmental way.

Introduction

Salmonella spp. is one of the major causes of food-borne outbreaks in the world (the second cause in Europe). As such, its control was considered necessary by the European food-safety policy makers under the EC Regulation 2160/2003. In practice, however, the control of this agent has proved to be difficult and expensive at farm level. Consequently the evaluation of the efficiency of control strategies for this agent has become an important and stringent issue, as stated in recent reports (Consortium, F. 2010). Modelling the dynamics of *Salmonella* spp. in pigs can be useful when assessing alternative control strategies. Susceptible – Infectious – Resistant (SIR) models are attractive tools in assessing the disease dynamics. The SIR model describes the dynamic of different states of individuals in the population in terms of a system of ordinary differential equations. The variables in the system are given by three compartments: susceptible group (S), infectious group (I) and carrier group (R). The aims of this study were: a) to develop a stochastic model which incorporates a production model with an infection model (the production model simulates the management procedures of an average farrow-to-finish Portuguese pig farm, while the infection model simulates the *Salmonella* Typhimurium infection in the farm); and b) to identify the parameters which influence most the model results at different compartments and stages of life within these compartments. The ones which influence most the infectious state at fattening stage were: the transition rate from susceptible to infectious (β) in all pig-compartments, and the piglets' immunity protective factor.

Material and Methods

The model simulates a farrowing-to-finish herd in which batch farrowing is applied to sows, leading to batch management of pigs. In these herds the complete life cycle of sows is considered, from the time they are recruited until they are culled; also the same for pigs, from their birth till slaughter. The duration of the sow reproduction cycle depends of the weaning time of the piglets which was fixed at 4 weeks. The pig growth period was fixed at 26 weeks. The modelling unit was the batch (for sows and pigs) and the time unit was one week. The reproduction cycle was divided in three stages (mating period, gestation period and farrowing/suckling period) corresponding to the occupation of three different types of rooms. Each batch of pigs composed of the litters from the batch of sows. The pig growth was divided in three stages (sucking period, post-weaning period and fattening period) corresponding to the occupation of three different types of rooms. All animals simultaneously leave the room they occupied except for the sows which abort at gestation. Mortality, culling, insemination

ination failure, abortion and litter size were the production variables modelled using a binomial distribution. The infection model was based on a SIR model for *Salmonella*. Direct transmission between the pigs in the batch was assumed as well as indirect transmission via contaminated floor, rodents, etc. The transmission parameters considered were the transition from S to I (β), the transition from I to R (α), the transition from R to I (δ), and the transition from R to S (θ). Due to the short life span of pigs, it was assumed that they could not experience the transition from carrier to susceptible. The binomial distribution was used to simulate the transition S to I, and I to R. For the transition R to I; and R to S, Poisson distributions were used. For the transition S to I, a cohort time-dependent random effect was included to emulate the dynamic structure of the spreading of infection within cohorts, where the velocity of infection depends on the number of susceptible and infectious animals in the previous time step. In the sensitivity analysis of the model, all the production variables and infection parameters were increased and decreased by 50% and the results were compared with the original results from the unperturbed parameters. The values and sources of the model variables and parameters are shown in Table 1.

Results

Results are shown in Table 2. The proportion of infectious sows was similar within the different rooms. There was an increase on the prevalence of infectious and carrier pigs along time for the pig compartment (piglets, growers and finishers), while the number of susceptible pigs went down. The prevalence of infectious animals in the pig compartment is lower than the prevalence of infectious sows in the sow

Table 1: Model variables and parameters

Variables and parameters		Random/fixed	Value	Reference
Average number of sows per herd		Fixed	264	a, b
Median number of pig per pen	Post-weaning	Fixed	25	a
	Fattening	Fixed	17	a
Duration (weeks)	Mating	Fixed	6	c
	Gestation	Fixed	10	c
	Farrowing - sows	Fixed	5	c
	Maternity - piglets	Fixed	4	b
	Post-weaning	Fixed	8	c
	Fattening	Fixed	14	c
Mortality probability (per week)	Mating	Fixed	0.000833	c
	Gestation	Fixed	0.00357	c
	Farrowing - sows	Fixed	0.001786	c
	Farrowing - piglets	Fixed	0.0275	b
	Post-Weaning	Fixed	0.00375	a
	Fattening	Fixed	0.00357	a
Artificial insemination success probability - applied in the end of mating (pins)		Squared root of a Weibull distribution	10.31 (mean), 0.77 (sd)	b
Abortion probability (per week)		Fixed	0.0025	c
Culling probability	After failing insemination	Fixed	0.017 at end of mating	c
	After abortion	Fixed	0.017/week	c
	Voluntary culling	Fixed	0.333 at end of farrowing	c
Litter size		Normal distribution (the final value was rounded)	10.45 (mean), 0.87 (sd)	b
Transmission parameter or transition rate from susceptible to infectious (β)		Random (posterior distribution)	0.34/week [0.17-0.66]	d
Transmission parameter or transition rate from infectious to carrier (α)		Random (posterior distribution)	0.27/week [0.24 - 0.30]	d
Transmission parameter or transition rate from carrier to infectious (δ)		Random (posterior distribution)	0.09/week [0.008 - 0.21]	d
Transmission parameter or transition rate from carrier to susceptible (θ)		Fixed	0.06/week	Soumpasis and Butler, 2009
Cohort time-dependent random effect (σ^2_{β})		Normal distribution	0 (mean), 1.29 (sd)	d
Piglets' protective factor due to sows passive immunity (pf)		Fixed (1/70 days)	0.1/week	Beloeil et al, 2003

Legend: sd - standard deviation, ^a Baptista et al, unpublished results of a survey to 109 herds in Portugal in 2009, ^b Production data of 200 Portuguese herds, collected by a software company from 2004 to 2006, ^c Expert opinion, ^d Correia-Gomes et al, unpublished.

compartment (mating, gestation and farrowing). The parameter α and θ were the most influential for the sow compartment. For the pig compartments, parameter β and variable “passive immunity” were most influential for the pig part.

Discussion

The predicted prevalence for the infectious animals, in the sow-compartment is higher than the predicted prevalence of infectious animals in the pig-compartment. The same trend was observed in the Portuguese Baseline Studies (EFSA, 2008 and EFSA, 2009) for *Salmonella* Typhimurium. The prevalence of infectious animals at the end of the fattening period can be reduced using several control measures (such as increasing the cleaning frequently of the pen floor, reducing stock density per pen, minimizing the mixture of litters at post-weaning and fattening, and the control of rodents and other vectors) which influence the β parameter. The increase of the piglets’ passive immunity can be achieved by allowing the correct consumption of colostrum by the piglets and decreasing the risk of concomitant diseases. The results of the sensitivity analysis have shown that the parameters which depended on expert opinion have not caused a major change in the results of the simulation model.

Conclusion

The simulation model potentially allows estimation of cost-benefit control measures if coupled to an economic model. The simulation model is flexible enough to introduce changes in the parameter distributions or values if future research and legislation so require. At the same time the model can be adapted to different types of production (e.g. breeding units, finisher units) as it was built in a compartmental way.

References

- Beloil P.A., Chauvin C., Proux K., Rose N., Queguiner S., Eveno E., Houdayer C., Rose V., Fravallo P., Madec F. 2003. Longitudinal serological responses to *Salmonella enterica* of growing pigs in a subclinically infected herd, *Prev Vet Med*, 60, 207-226.
- Consortium, F. 2010. Analysis of the costs and benefits of setting a target for the reduction of *Salmonella* in slaughter pigs for European Commission Health and Consumers Directorate-General SANCO/2008/E2/036 Final Report, 1-198.
- EFSA, 2008. Report of the Task Force on Zoonoses Data Collection on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, PartA. *The EFSA Journal* 135, 111.
- EFSA, 2009. Analysis of the baseline survey on the prevalence of *Salmonella* in holdings with breeding pigs, in the EU, 2008, Part A: *Salmonella* prevalence estimates. *EFSA Journal* 7, 1377.
- Soumpasis I., Butler F. 2009. Development and Application of a Stochastic Epidemic Model for the Transmission of *Salmonella* Typhimurium at the Farm Level of the Pork Production Chain, *Risk Anal.*, 29 (11), 1521-1533.

Table 2: Results for the infection state in each room for the sows and pigs

Production stage	Infection State	Results in proportions				Parameters with highest impact on the results
		Mean	SD	95% CI		
				LL	UL	
Mating	Susceptible	0.19	0.13	0.0	0.45	θ (mating/sows)
	Infectious	0.55	0.16	0.22	0.88	α (mating/sows)
	Carrier	0.26	0.14	0.0	0.56	α and θ (sows)
Gestation	Susceptible	0.23	0.16	0.0	0.60	α and θ (sows) + θ (gestation)
	Infectious	0.51	0.19	0.13	0.88	α (gestation/sows)
	Carrier	0.26	0.14	0.0	0.57	θ (gestation/sows)
Farrowing	Susceptible	0.16	0.16	0.0	0.50	β , α and θ (sows)
	Infectious	0.57	0.21	0.17	1.0	α (sows)
	Carrier	0.27	0.19	0.0	0.67	θ (sows)
Piglets	Susceptible	0.91	0.09	0.70	1.0	β (piglets)
	Infectious	0.09	0.09	0.0	0.30	β (piglets) + passive immunity
Growers	Susceptible	0.80	0.20	0.35	1.0	β (pigs)
	Infectious	0.19	0.19	0.0	0.64	β (pigs)
	Carrier	0.003	0.007	0.0	0.02	α (post-weaning)
Finishers	Susceptible	0.68	0.27	0.17	1.0	β (pigs)
	Infectious	0.31	0.26	0.0	0.8	β (pigs)
	Carrier	0.01	0.02	0.0	0.05	α (pigs)

Legend: SD – standard deviation, LL – lower limit, UL – upper limit, β – transition rate parameter from susceptible to infectious, α – transition rate parameter from infectious to carrier, δ – transition rate parameter from carrier to infectious, θ – transition rate parameter from carrier to susceptible

Assessment of the impact of omitting palpation of the lungs and the liver at meat inspection

Alban, L.*, Pacheco, G., Kruse, A.B., Petersen, J.V.

Danish Agriculture & Food Council, Copenhagen, Denmark

*corresponding author Lis Alban: lia@lf.dk

Abstract

Meat inspection of finisher pigs is gradually moving from traditional inspection involving palpation and incisions to a more visual inspection. However, what do we miss if we no longer palpate and incise? This is addressed in this paper which focuses specifically on palpation of the lungs and the liver. A risk assessment following international guidelines was undertaken. The assessment shows that omission of these routine palpations on finisher pigs from controlled housing (i.e. herds with high biosecurity) will have no significant impact on food safety. The reasoning for reaching these conclusions is presented in the following.

Introduction

The main aim of meat inspection is to ensure safe and savoury meat. However, much has changed since the birth of modern meat inspection 100 years ago. Today, *Campylobacter*, *Salmonella* and *Yersinia* fill up the human statistics for zoonotic infections. And it is well-known that meat inspection in itself can do only little to mitigate the risk associated with such agents, because they do no results in macroscopic lesions in affected carcasses or plucks.

In fact, palpation and incision might result in cross-contamination on the carcasses. In line, it has been suggested to omit unnecessary palpation and incisions to limit the cross-contamination with *Salmonella*.

However, what would we risk if we stopped the routine palpations and incisions? Two earlier risk assessments - following international guidelines - concluded that omitting routine palpation of mandibular and mesenteric lymph nodes and discontinue the routine opening of hearts did not increase any food safety risks (Alban et al., 2008; Alban et al., 2010).

Next, we have looked at the impact of omitting palpation of the lungs, the liver and their lymph nodes.

We looked at food safety as well as the impact on animal health (including the ability to identify notifiable diseases) and welfare of pigs. Only the food safety results are described in this paper. For the other aspects, the reader is kindly referred to the full risk assessment which can be found on the following link: http://www.lf.dk/~media/lf/Aktuelt/Publikationer/Svinekod/Risk%20assessment_lungs%20liver%202013%2002%2028.ashx

Material and Methods

The work followed OIE guidelines for risk assessment. This implies that the following steps were undertaken: Hazard identification, release assessment, exposure assessment, and consequence assessment. Finally, risk estimation was made based on an integration of the four previous steps.

Data consisted of a comparison study involving 3,000 plucks, own collection of slaughterhouse samples (N=104) sent for microbiological investigation, slaughterhouse statistics, literature and expert opinion.

Results

Hazard identification: embolic pneumonia and liver abscesses were identified as the two most important lesions that might escape detection if routine palpation is omitted. This was based on a literature review and discussions with experts in the field.

Release assessment: Two studies were conducted to assess the proportion of plucks with embolic pneumonia that might escape detection, if visual inspection is applied instead of traditional inspection. The first was a comparison study including 3,000 plucks (Table 1). The second consisted of an evaluation of 104 plucks with embolic pneumonia collected during meat inspection (Table 2). The comparison study showed that one out of three cases found in traditional inspection was missed by visual inspection (Table 1). Likewise, the evaluation study showed that around one out of five cases would be missed if visual inspection was used compared to traditional inspection.

Based on these data as well as slaughterhouse data, it was estimated that between 1,080 and 1,800 cases might be missed in a year, if visual only was applied. This figure is probably a worst case scenario because the visual inspection that formed part of the present risk assessment was conducted under suboptimal conditions: the chain was not set up to allow an easy visual inspection prior to the traditional inspection.

Exposure assessment: The distribution of the agents found in the lungs with embolic pneumonia is shown in Table 3. It is noted that *Staph. aureus* and *E. coli* were found predominantly. Only two livers were found with abscesses. These were large and easy recognizable. Here, *Staph. aureus* were found.

The human exposure risk related to the hazards identified in embolic pneumonia was assessed as negligible for the lungs since lungs are not considered edible tissue in Denmark. The human exposure risk related to meat from pigs with embolic pneumonia that escaped detection seems low, because the bacteria are normally not present in the muscle tissue – and if present it will be in low numbers.

Moreover, the low numbers of abscesses present in the carcass associated with pyaemia are most likely found during cutting. It was also shown that although presence of pyemia is a risk factor for abscesses in the carcass (RR=4.4, P<0.001) it was less than 1% of the abscesses that were found in pigs with embolic pneumonia (Table 4).

Livers for human consumption are handled individually which will make it easy to identify abscesses. Therefore, the exposure risk was assessed as low.

The exposure risk for pets was assessed to be negligible, because lungs destined for pet food is heat-treated. Exposure will only take place in case raw lungs are fed directly to pets, which is thought to occur only infrequently. Likewise, for fur animals, the exposure is very low because most animal by-products are heat-treated prior to being fed to fur animals in Denmark.

Consequence assessment: None of the agents involved in the development of embolic

Table 1. Association between visual and traditional inspection of plucks for embolic pneumonia, Denmark (N=3000 finisher pigs)

Number of plucks found with lesions			Assessment of association		
<u>Visual inspection</u>	<u>Traditional inspection</u>	<u>Sum</u>	<u>Visual/Traditional*</u>	<u>Kappa</u>	<u>P-value</u>
	+	-			
+	2	7	9	3.0	0.33
-	1	2990	2991		0.0339
Sum	3	2997	3000		

*: Prevalence of cases found at visual versus traditional inspection

Table 2. Summary statistics on the ability to detect by visual inspection the 104 plucks with lesions indicative of embolic pneumonia, found during meat inspection of finisher pigs from controlled housing herds, October-November 2012, Denmark

	<u>Yes</u>	<u>Maybe</u>	<u>No</u>
Lesion visually detectable?	65 (62.5%)	19 (18.3%)	20 (19.2%)

Table 3. Distribution(*) of agents found in 104 lungs with embolic pneumonia and 98 associated lymph nodes. The lungs and lymph nodes were found during meat inspection of finisher pigs from controlled housing herds, October-November 2012, Denmark

<u>Agents/growth</u>	<u>Lung samples</u>		<u>Lymph node samples</u>	
	Number	Percentage	Number	Percentage
Staphylococcus aureus	82	78.8	69	70.4
Escherichia coli	61	58.7	40	40.8
Proteus spp.	6	5.8	1	1.0
Pasteurella multocida	4	3.8	4	4.1
Bacillus cereus	20	19.2	11	11.2
Pseudomonas spp	6	5.8	7	7.1
Growth	96	92.3	79	80.6
No Growth	8	7.7	19	19.4
Total	104	-	98	-

*: More than one agent could be found in a sample. Therefore, the percentages do not add to 100.

Table 4. Association between pyaemia and abscesses found in finisher pigs from Danish Crown Horsens, October-December 2012

<u>Pyaemia</u>	<u>Abscesses*</u>		<u>Sum</u>	<u>Assessment of association</u>
	+	-		
+	97	620	717	Relative Risk = 4.4
-	37.195	1.172.660	1.209.855	P<0.0001
Sum	37.292	1.173.280	1.210.572	Pos. pred. value= 0.14

*: Defined as abscess on the carcass including legs and toes except the head and abdomen

pneumonia or liver abscesses in pigs had a significant zoonotic foodborne potential. This is supported by the fact that these hazards do not show up in the human statistics, where Salmonella, Yersinia, Toxoplasma and Trichinella have been identified by the European Food Safety Authority as the pig/pork-relevant hazards (EFSA, 2011).

Risk estimation: The prevalence of embolic pneumonia is low; however, because of the substantial size of the Danish pig production a non-negligible number of cases might be overlooked if visual inspection replaces traditional inspection. The food safety impact of this is very low, because lungs are not considered edible tissue and because the agents involved in these lesions have a limited foodborne impact.

The prevalence of liver abscesses is very low. The agent involved has a limited food-borne impact, and cases will be identified during the unique handling of the organ if destined for human consumption.

Discussion

Visual inspection does not necessarily result in a lower number of plucks being suspected of embolic pneumonia. This was also seen of other lesions in the pluck (results not shown here but in the risk assessment report. Registration intensity merely depends upon what we want the meat inspectors to record.

Consumers might expect the abattoir to do what is possible to detect pigs with pyaemia from an aesthetic point of view. Therefore, in case of doubt plucks should be palpated – or sent to the rework area for extended examination.

Conclusion

The assessment showed that routine palpation of the liver and lungs is an unnecessary part of meat inspection in finisher pigs, if there are no visual indications of infection or other data pointing to disease.

Epilogue

The EU Commission has recently (May 2013) amended the EU Meat inspection regulation 854(2004). The changes - which will come into force in June 2014 - open up for visual inspection as the standard for inspection of pigs unless ante mortem, post mortem or any other finding on the individual or food chain information or geographical data indicate otherwise.

The next step is to investigate how the results of this risk assessment might be used in practice by the abattoirs in their modernisation process of the meat inspection. Here, simultaneous inspection by one inspector of plucks hanging over the intestines might be of interest – if judged feasible. Communication with important trade partners prior to implementation of such changes is required to ensure recognition of equivalence.

Continued discussion about meat inspection is needed to ensure the most food safety for the resources spent. Next work will focus on an evaluation of the decision code “accepted for deboning” – does this code result in value for money?

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References

Alban, L., Vilstrup, C., Steenberg, B., Jensen, H.E., Aalbæk, B., Thune-Stephensen, Jensen, S., 2008c. Assessment of the risk for humans associated with Supply Chain Meat Inspection – The Danish way. Danish Veterinary and Food Administration /DMA Available online: http://www.lf.dk/Aktuelt/Publikationer/~media/lf/Aktuelt/Publikationer/Svinekod/Modernisation%20of%20Meat%20Inspection_DK.ashx 54 pp.

Alban, L., Steenberg, B., Petersen, J.V., Jensen, S., 2010. Is palpation of the intestinal lymph nodes a necessary part of meat inspection of finisher pigs? Landbrug og Fødevarer Available online: <http://www.lf.dk/Aktuelt/Publikationer/~media/lf/Aktuelt/Publikationer/Svinekod/UKRisikovurdering%20kroeslymfeknuder%20koedkontrol.ashx> 27pp.

EFSA, 2011. Scientific Opinion on the public health hazards to be covered by inspection of meat (swine). EFSA Journal 2011;9(10):2351. [198 pp.] doi:10.2903/j.efsa.2011.2351. Available online: <http://www.efsa.europa.eu/en/efsajournal/doc/2351.pdf>

Study on *Salmonella* sp. in the head part of carcasses from slaughtered pigs

Vieira-Pinto, M.M. ^{*(1)}, Morais, L. ⁽¹⁾, Reais, P. ⁽¹⁾, Themudo, P. ⁽²⁾

⁽¹⁾ Dept. Veterinary Science. CECAV. Lab. Food Inspection, University of Trás-os-Montes e Alto Douro. Apartado 1013. 5001 – 911. Vila Real. Portugal

⁽²⁾ INIAV-Animal Production and Health Unit -Dept. Bacteriology. *Salmonella* serotyping (LNR). Estrada de Benfica, 701, 1549-011 Portugal

*corresponding author: mmvpinto@utad.pt

Abstract

To define the importance of the head part of pigs' carcasses as a potential vehicle of *Salmonella*, 105 carcasses were sampled at one abattoir. The results revealed the presence of *Salmonella* sp. in 25 samples (23.8%), which corresponds to a higher value than those previously presented by the same authors in similar studies in pig carcasses (12,9%). By means of serotyping, were identified 5 different serotypes: S. Typhimurium (9, 36%), S. London (6 24%), S. Rissen (6 24%), S. Agona (3, 12%) and S. Goldcoast (1, 4%). This study underlines the importance that the head part of the pigs' carcass can take as a source of *Salmonella* throughout meat chain and a potential vehicle, direct or indirect, to the final consumer. For this reason, the authors suggest that increased hygienic measures should be adopted during head processing and cutting, especially if its meat will be subsequently used for sausage or smoked meat (Figure 1) production that could be consumed without any kind of heat treatment.

Introduction

According to EFSA report (2013), human salmonellosis in Europe, in 2011, still being the second most frequently reported zoonotic disease, accounting for 95,548 cases with a total of 56 fatal cases. Also, according to the same report, *Salmonella* remained the most frequently detected causative agent in foodborne outbreaks reported in the EU.

Recently, BIOHAZ Panel estimated that a rate of around 56.8 % of human salmonellosis cases could be attributable to pigs.

To protect human health against *Salmonella* infections transmissible between animals and humans, EU Regulation (EC) N.º 2160/200313 requires MSs to set up national control programmes for *Salmonella* serovars deemed to be of particular public health significance in animal species including pigs. The purpose of this Regulation is to ensure that proper and effective measures are taken to detect and to control *Salmonella* and other zoonotic agents at all relevant stages of production, processing and distribution, particularly at the level of primary production, including in feed, in order to reduce their prevalence and the risk they pose to public health

The slaughter of *Salmonella*-infected pigs represents a potential risk for contamination of carcasses and other edible products, introducing *Salmonella* into the food chain (Vieira-Pinto *et al.*, 2005).

At the slaughterhouse the swine can carry *Salmonella* on the skin and in several tissues. The tissues mostly referred as affected by *Salmonella* are those from the digestive tract and the corresponding lymphatic tissue such as tonsils and mesenteric- and mandibular lymph nodes (Jung *et al.*, 2001). According to Olsen *et al.* (2001), the spread of *Salmonella* during the slaughter process may occur especially through pharynx (tonsils) and intestinal contents. The (cross-) contamination of carcasses, through contaminated utensils, equipment or handlers, is basically a matter of redistributing the *Salmonella* bacteria from the positive pigs during the various slaughter processes (Lo Fo Wong *et al.*, 2002), even when the slaughter process is performed correctly, the contamination from these reservoirs can be transferred to the carcass (Olsen *et al.*, 2001).

The presence of *Salmonella* sp. in pigs' carcasses has been shown in several international and national research studies. However, no national data was found on the presence of *Salmonella* sp. in the head part of pigs' carcasses, whose muscles are often used for traditional sausages manufacturing or smoked meat products, some of which consumed raw or undercooked. As it was referred Bruun *et al.*, (2009), the ingestion of pig meat not submitted to a deep thermal treatment may enhance health risks for some food borne diseases like Salmonellosis.

Additionally, the presence of *Salmonella* sp. in the head part of pigs' carcasses can constitute a risk, since this tissues can be adversely incised and touched during sanitary inspection (incision of mandibular lymph nodes), during tonsils extraction as well as along some other slaughter procedures. All of these procedures may allow cross-contamination through the hands, knives and other utensils or equipment.

For these reasons, 105 head part of pigs' carcasses were sampled at one abattoir to define its importance as a potential vehicle of *Salmonella* sp.

The results revealed the presence of *Salmonella* sp. in 23.8% of the sampled carcasses and identified five different serotypes: S. Typhimurium (9, 36%), S. London (6 24%), S. Rissen (6 24%), S. Agona (3, 12%) and S. Goldcoast (1, 4%).

Material and Methods

During this study, 105 carcasses were sampled at one abattoir by means of swabbing the internal face of the head with a cotton sterilised gauze (hydrated in 25 ml of Buffered Peptone Water with 0.1% Tween) (Swanenburg, 2000) (Figure 2).

All the samples were individually packed in a sterile named recipient and transported under refrigerated conditions to the laboratory where *Salmonella* sp. isolation procedures were started on the same day.

Salmonella sp. isolation method was performed according to ISO 6579:2002 "Microbiology of food and animal feeding stuffs- Horizontal method for the detection of *Salmonella* spp. Briefly, the diluted samples in BPW were incubated at $37^{\circ}\text{C}\pm 1^{\circ}\text{C}$ for $18\pm 2\text{h}$, afterwards 0,1 ml and 1ml was respectively inoculated in Rappaport-Vassiliadis medium with soya (RVS broth, Oxoid® - 669) and in Muller-Kauffmann tetrathionate/novobiocin broth (MKTn broth, Merk® - 110863). The RVS broth was incubated at $41,5\pm 1^{\circ}\text{C}$ for $24\text{h}\pm 3\text{h}$, and the MKTn broth at $37^{\circ}\text{C}\pm 1^{\circ}\text{C}$ for $24\text{h}\pm 3\text{h}$. After that, one loop of each selective enrichment broth was streaked onto the surface of two selective solid media: Hektoen (Oxoid® - CM419) and XLD (Oxoid® - CM469) agar. Colonies of presumptive *Salmonella* were confirmed by biochemical tests (Oxidase reaction, Triple Sugar Iron Agar (Oxoid® - CM277), Urea broth (Merk® - 1.08483), L-Lysine decarboxylation medium (Oxoid® - CM308S) and serological agglutination with Poli A-I & Vi antiserum (Difco® - 222641).

Suspicious *Salmonella* sp. isolates were serotyped according to the Kauffmann-White scheme in the INIAV (Lisbon, Portugal), the National Reference Laboratory for *Salmonella*.



Figure 1 – Smoked head part of pigs' carcasses

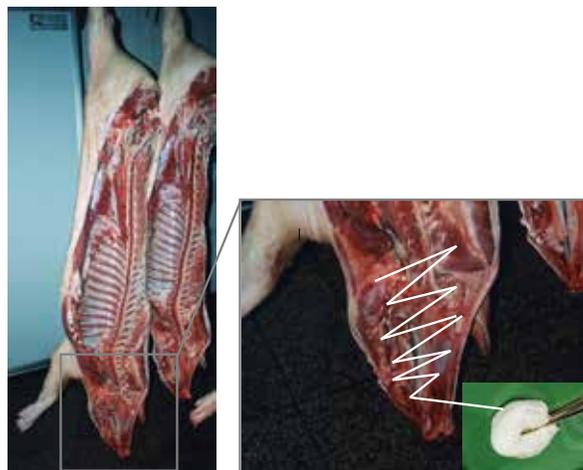


Figure 2 – Swabbing procedure

Results

The results revealed the presence of *Salmonella* sp. in 25 samples (23.8%).

Among these isolates were identified 5 different serotypes: S. Typhimurium (9, 36%), S. London (6 24%), S. Rissen (6 24%), S. Agona (3, 12%) and S. Goldcoast (1, 4%).

Discussion

The results revealed the presence of *Salmonella* sp. in 25 (23.8%) of swab samples from the internal face of the carcass head, which corresponds to a higher value than those previously presented by the same authors in similar studies (Vieira-Pinto *et al.*, 2005) in pig carcasses (12,9%).

The presence of *Salmonella* sp. in this part of the carcass can be associated to contamination processes during the slaughter and meat inspection procedures and also can be related to oral infection of pigs before slaughter (Swanenburg, 2000) which can be favoured by the typical exploratory and coprophagy behaviour. In fact, the faecal-related contamination of the pig's tonsils has already been described by Vieira-Pinto *et al.* (2006) showing a highly significant association ($p < 0.001$) between the presence of *Salmonella* in the ileum and/or ileocolic lymph nodes and its presence in the corresponding tonsils, as well

as the very high percentage (80%) of positive tonsils having the same *Salmonella* genotype as ileum and/or ileocolic lymph nodes.

According to Swanenburg *et al.* (1999), Hald (2001) and Olsen *et al.* (2001), oral cavity, particularly the tonsils, pharynx and tongue can accommodate large quantities of *Salmonella*, resulting from contamination during transport and at rest in the lairage, by regurgitation of gastric contents or by water scald. From these tissues, *Salmonella* sp. can be transferred to the carcass during removal of the tongue, together with the pluck, while cutting the head or through the incisions made during the meat inspection Olsen *et al.* (2001). These authors have also shown that not removing the tongue of the head intact reduced by 30% the presence of *Salmonella* positive-carcasses.

According to the results found in this research, attention should be paid to the handling procedures of the head part of pigs' carcasses in order to mitigate *Salmonella* sp. contamination to the other edible products.

Also, since many of the national monitoring programmes for *Salmonella* sp. in pig meat are based on sampling at the slaughterhouse (by means of carcass swabbing) and the *Salmonella* criteria laid down by the Regulation (EC) No 1441/2007 prescribe rules for sampling pig carcasses at slaughterhouse, it should be important to understand the influence of this part of the pig carcass on the sensitive sampling method and, consequently, on microbiological results.

Were identified 5 different serotypes: *S. Typhimurium* (9, 36%), *S. London* (6 24%), *S. Rissen* (6 24%), *S. Agona* (3, 12%) and *S. Goldcoast* (1, 4%).

The most prevalent serotype was *S. Typhimurium*. This serotype has been described as the main serotype identified in swine in several studies as well as in the baseline studies based on the EU Regulation 2160/2003, in Europe and in Portugal (EFSA, 2007). This serotype should be also under special attention due to its virulence to humans and animals and to its high resistance rate to antibiotics (Botteldoorn *et al.*, 2004). According to EFSA (2013), *S. Typhimurium* human cases are mostly associated with the consumption of contaminated pig, poultry and bovine meat.

Respecting to *Salmonella* Rissen, scarce references were found on its occurrence in swine, and in these references no particular attention to this serotype were found. But, in the fifth European Unionwide baseline survey carried out at farm level to determine the prevalence of *Salmonella* in pig breeding holdings (between January 2008 and December 2008), *S. Rissen* appeared to occur only in a few countries, but was one of the most common serotype in Portugal and Spain (EFSA, 2007). Nevertheless, this serotype doesn't seem to have implication in the number of Human salmonellosis in both countries.

Conclusion

This study underlines the importance that the head part of the pigs' carcass can take as a source of *Salmonella* sp. throughout meat chain and a potential vehicle, direct or indirect, to the final consumer. For this reason, the authors suggest that increased hygienic measures should be adopted during head processing and cutting, especially if its meat will be subsequently used for sausage production that is to be consumed without any kind of heat treatment.

References

Botteldoorn, N., Herman, L., Rijpens, N. & Heyndrickx, M. (2004). Phenotypic and molecular typing of *Salmonella* strains reveals different contamination sources in two commercial pig slaughterhouse. *Applied and Environmental Microbiology*, 70, 5305-5314.

Bruun, T., Sørensen, G., Forshell, L. P., Jensen, T., Nygard, K., Kapperud, G., Lindstedt, B. A., Berglund, T., Wingstrand, A., Petersen, R. F., Müller, L., Kjelsø, C., Ivarsson, S., Hjertqvist, M., Löfdahl, S. & Ethelberg, S. (2009). An outbreak of *Salmonella* Typhimurium infections in Denmark, Norway and Sweden. *Euro Surveillance*, 14 (10), 1-6.

EFSA (2013). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010; *EFSA Journal* 2012; 10(3):2597.

EFSA, 2007. Opinion Report of the Task Force on Zoonoses Data Collection on a proposal for technical specifications for a baseline survey on the prevalence of *Salmonella* in breeding pigs. *EFSA Journal* 2007, 99, 1-28.

Grimont, P. and Weill, F-X. (2007) Antigenic formulae of the *Salmonella* serovars, 9th edition. WHO Collaborating Centre for Reference and Research on *Salmonella*. Institute Pasteur, Paris.

Hald, T. (2001). *Salmonella* in pork. Epidemiology, control and the public health impact. Ph. D. Thesis. Royal Veterinary and Agricultural University. Copenhagen. Denmark, 310 Pp.

Jung, B. Y, Lee, W. W., Lee, H. S., Kim, O. K. & Kim, B. H. (2001). Prevalence of *Salmonella* in slaughter pigs in South Korea. Proceedings of the 4th International Symposium on the Epidemiology and Control of *Salmonella* and other food borne pathogens in Pork. Leipzig. Germany, 202-204.

Lo Fo Wong, D.M.A., Hald, T., van der Wolf, P.J. & Swanenburg, M. (2002). Epidemiology and control measures for *Salmonella* in pigs and pork. Livestock Production Science, 76, 215–222.

Olsen, A-M, Jensen, T., Dahl, J. & Christensen, H. (2001). Reduction in level of *Salmonella* on swine carcasses after slaughter without splitting the head. Proceedings of the 4th International Symposium on the Epidemiology and Control of *Salmonella* and other food borne pathogens in Pork. Leipzig. Germany, 124-126.

Swanenburg, M. (2000). *Salmonella* in the pork production chain: Sources of Salmonella on pork. PhD Thesis.161. Netherlands

Swanenburg, M., Urlings, H. A. P., Keuzenkamp, D. A. & Snijders, J. M. A. (1999). Tonsils of slaughtered pigs as marker sample for *Salmonella* positive pork. Proceedings of the 3rd International Symposium on the Epidemiology and Control of *Salmonella* in Pork. Washington, 264-265.

Vieira-Pinto, M., Tenreiro, R.; Martins, C. (2006). Unveiling contamination sources and dissemination routes of *Salmonella* sp. in pigs at a Portuguese slaughterhouse through macrorestriction profiling by Pulsed-Field Gel Electrophoresis. *International Journal of Food Microbiology*.110: 77-84.

Vieira-Pinto, M. M., Temudo, P. & Martins, C. (2005). Occurrence of *Salmonella* in the ileum, ileocolic lymph nodes, tonsils, mandibular lymph nodes and carcasses of pigs slaughtered for consumption. Journal of Veterinary Medicine Series B (Infectious Diseases and Veterinary Public Health), 52, 476-481. 264-265.

The first notification of *Salmonella* Budapest in Portuguese meat products: a case report

Santos, A.^{*Ω§}, Lourenço, M. [§], Vieira-Pinto, M.^Ω

^Ω Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal;

[§] Vetdiagnos, Diagnóstico Veterinário Lda, Cantanhede, Portugal.

^Ω Universidade de Trás-os-Montes e Alto Douro, Dept. Ciências Veterinárias. CEAV. Vila Real, Portugal.

*corresponding author : ana.santos@vetdiagnos.pt

Abstract

Salmonella is a bacterium that can cause an illness in humans called salmonellosis. In the European Union, over 100,000 human cases were reported last year. The EFSA has estimated that the overall economic cost of human salmonellosis could be as high as EUR 3 billion a year.

In the present study, *Salmonella* spp. analyses based on ISO 6579:2002, was derived as a part of a HACCP procedure in a meat processing company. From the four analyzed samples, dry cured sausage, black pudding, fresh sausage and meatloaf, *Salmonella* sp. was isolated in the last two samples. Both isolates were sent to the Portuguese Reference Laboratory for *Salmonella* (Instituto Nacional de Saúde Dr Ricardo Jorge – INSA) and serotyped according to the Kauffmann-White scheme. *Salmonella* Budapest was identified in both products. This result may indicate a common source of contamination for both products that may occurred in the production chain. Using rastreability data it was possible to observe that the meat used in both products was from national swine slaughtered in Portuguese abattoirs. According to data provided by INSA, until 2012, besides these two *Salmonella* Budapest isolates, were only recorded two more cases isolated from sausage and frozen gizzards from Brazil.

With regard to Portuguese human data, in the last decade, there was five cases of *S. enterica* serovar Budapest infection that were related to gastroenteritis and only one associated with pyogenic skin lesion. This data suggests that we may be facing an isolated case of cross-contamination in the production line due to poor equipment hygiene throughout the chain. Additional studies are being made to understand dangerous strain with regard to virulence profile and antimicrobial resistance.

Introduction

Salmonella is a bacterium that can cause an illness in humans called salmonellosis. It is an almost universally accepted dogma that human salmonellosis is a zoonosis^(1, 2, 3) and is a major cause of bacterial enteric illness in both humans and animals⁽⁴⁾. A high percentage of *Salmonella*-positive samples of ground meat from swine (40.3%) and cattle (46%) and of processed poultry products (56.3%) implies that a consumer has a 50:50 chance of carrying home live *Salmonella* with these products from the supermarkets⁽⁵⁾. *Salmonella* strains from food animals are passed to the human population via insufficiently cooked meat, eggs, and milk⁽³⁾. Since enteric infections with *Salmonella* in humans result in multiplication and excretion of the infectious agents in and from the human intestine, the animal-food-human spread must be regarded as an important contribution to the release of antibiotic-resistant bacteria from farm animals^(6, 7). In 1997, the World Health Organization for the first time ever published a report on the medical impact of the use of antimicrobials in food animals⁽⁸⁾. The main threats were formulated as: (a) an increase in the prevalence of resistant bacteria in animals; the transfer of resistant pathogens to humans via direct contact with animals, or through the consumption of contaminated food or water; (b) the transfer of resistance to human bacteria; (c) an increase in the incidence of human infections caused by resistant pathogens; (d) potential therapeutic failures in animals and humans; and (e) frightening resistance situations in farm animals regarding *Salmonella*, *Campylobacter*, *Enterococcus* species and *E. coli*. In these study was serotyped two strains of *Salmonella* Budapest according to the Kauffmann-White scheme, on the basis of serologic identification of O (somatic) and H (flagellar) antigens⁽⁹⁾ and was made an antimicrobial resistance profile based on CLSI information.

Material and Methods

1. Sampling

Sampling procedure was done randomly during the HACCP actions in a butcher for resale area from one meat processing company. Four meat samples were analyzed: dry cured sausage, black pudding, fresh sausage and meatloaf. The material was collected in full for sterile bags and transported to the laboratory under controlled temperature (4° C) during 24 hours.

2. Detection of *Salmonella* spp.

The detection of *Salmonella* spp. was made according to the method ISO 6579:2002 on accredited laboratory by IPAC (L0352) ⁽¹⁰⁾.

3. Serotyping of *Salmonella* spp.

The serotyping was performed at the National Reference Laboratory according to the methodology of slide agglutination and tube. This methodology is described to be unique and not disclosed by the laboratory where it is held, as it is the only laboratory at the national level to accomplish the same.

4. Susceptibility to antibiotics

The antimicrobial resistance profile was treated using the disc diffusion method based on NCCLS:M31-A2 (11).

Results

From the four analysed samples, two were positive for *Salmonella* spp (Table 1.). Both strains were serotyped (Table 2.).

It was confirmed that it was the same serovar and was done the antibiotic susceptibility profile. The results are presented in Table 3.

Discussion

Processed meat products analyzed were produced from portuguese pork meat, whose process of fattening, slaughtering and butchering was also carried out on national territory.

The fact that the same *Salmonella* serovar has been isolated from two different meat samples produced in the same production line, point out this step as a possible common source of contamination and suggests that should be an improvement in the disinfection scheme and further analysis to demonstrate the effectiveness of this process which, however, does not was held at the express wish of the customer.

After identification of *Salmonella* Budapest, there was a broad literature search and wasn't found any references to this serovar in Portugal. For that, INSA was personally contacted that confirmed that this was the first case of contaminated Portuguese meat with *Salmonella* Buda-

Table 1. Results of *Salmonella* spp. detection

Sample #	Sample name	Method	Result	Units
1	Dry cured sausage	ISO 6579:2002	Absent in 10g	Present/absent in 10g
2	Black pudding		Absent in 10g	
3	Fresh sausage		Present in 10g	
4	Meatloaf		Present in 10g	

Table 2. Results of the serotyping

Sample #	Sample name	Parameter	Method	Result
3	Fresh sausage	<i>Salmonella</i> spp serotyping	Agglutination	Salmonella enterica serotip Budapest
4	Meatloaf			

Table 3. Antibiotic susceptibility to *Salmonella* Budapest (S –susceptible; I – Intermediate; R- resistant).

CLSI Class	Antimicrobial Agent	Result
Aminoglycosides	Kanamycin	S
	Gentamicin	S
	Streptomycin	R
β-lactam	Amoxicilin	S
Penincilin	Ampicillin	S
	Penincilin	R
Cephems	Ceftiofur	S
Folate pathway inhibitors	Thrimethropin-Sulfametoxazol	I
Quinolones	Ciprofloxacin	S
	Enrofloxacin	S
	Nalidixic acid	S
Phenicols	Sulfamid	R
	Chloranphenicol	S
Tetracyclines	Tetracyclin	R
	Doxiciclin	R

pest. However, two cases from meat imported from Brazil were previously reported (personal communication). To draw the profile of susceptibility to antibiotics confirming that it was a resistant strain to important groups (Table 3).

Conclusion

The presence of one serovar in separate products in the same production line confirms ineffectiveness of the hygiene methods and suggests a revision of the HACCP since the presence of *Salmonella spp.* in meat products disrespects legislation (12).

The appearance of this new serovar in Portugal, confirms the high mobility of bacterial strains so far in the case of *Salmonella enterica* Budapest confined to northern Europe to countries further south. This mobilization may be due to high transaction of live animals and carcasses within Europe, especially between countries of Eastern Europe, where traceability systems are deficient.

The antimicrobial resistance profile obtained confirms that this is an highly harmful bacterial strain which corroborates the fears already shown in other studies that warn of the risk of ineffectiveness of antimicrobial treatments in human nature, these strains represent a risk for increasing public health.

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References

- 1 National Research Council & Institute of Medicine, 1998. The Use of Drugs in Food Animals: Benefits and Risks. National Academy Press, Washington, DC.
- 2 National Academy of Sciences, 1980. The Effects on Human Health of Subtherapeutic Use of Antimicrobials in Animal Feeds. National Academy of Sciences, Washington, DC.
- 3 D'Aoust J. Y., 1997. *Salmonella* species. In: Food Microbiology – Fundamentals and Frontiers, Doyle M. P., Beuchat L. R. and Montville T. J. (eds), ASM, Washington, DC, pp. 129–158.
- 4 Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R., Swaminathan., B., 2000. *Salmonella* Nomenclature. Journal of Clinical Microbiology. 38, 2465-2467.
- 5 Teuber, M., 1999. Spread of antibiotic resistance with food-borne pathogens. Cell. Mol. Life Sci. 56, 755-763.
- 6 WHO, 1998. Outbreak of quinilone-resistant, multiresistant *Salmonella typhimurium* DT104, Denmark. Weekly Epidemiol. Rec. 42, 327–328
- 7 WHO, 1998. Multiresistant *Salmonella typhimurium* DT104, Czech Republic. Weekly Epidemiol. Rec. 49, 385–386
- 8 WHO, 1997. The Medical Impact of the Use of Antimicrobials in Food Animals. Report of a WHO Meeting, Berlin, Germany, 13–17 October 1997. WHO, Geneva
- 9 Kauffmann, F., 1966. The bacteriology of Enterobacteriaceae. Munksgaard, Copenhagen, Denmark
- 10 Standardization, I. O. (Ed.), 2002. ISO 6579:2002 Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of *Salmonella* spp.
- 11 National Committee for Clinical Laboratory Standards (NCCLS), 2002. – Document M31-A2. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, approved standard, 2nd Edition. NCCLS, Wayne, Pennsylvania, 80 pp.
- 12 REGULAMENTO (CE) N.º 853/2004 DO PARLAMENTO EUROPEU E DO CONSELHO. Parlamento Europeu e Conselho da União Europeia, Bruxelas 2004 (in Portuguese).

Antimicrobial resistance and class 1 integrons in *Salmonella enterica* subsp. *enterica* serovar Derby isolates from pig abattoirs

Lopes, G.V. ^(1,2), Michael, G.B. ⁽²⁾, Schwarz, S. ⁽²⁾, Cardoso, M * ⁽¹⁾

⁽¹⁾ Departamento de Medicina Veterinária Preventiva, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

⁽²⁾ Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany

*corresponding author: mcardoso@ufrgs.br

Abstract

Salmonella enterica subsp. *enterica* (S.) serovar Derby is one of the most prevalent serovars in pigs. The aim of this study was the investigation of S. Derby isolates for the presence of antimicrobial resistance and class 1 integrons and their gene cassettes. Forty-nine S. Derby isolates, obtained from different sources at four pig abattoirs (A-D) in Southern Brazil were analysed. Five isolates were susceptible to all antimicrobial agents tested. Twenty-seven isolates were multi-resistant, showed a common resistance pattern to streptomycin/spectinomycin-sulphonamides-tetracycline and shared the same XbaI-pattern. Except for one isolate, all these multi-resistant isolates carried class 1 integrons. The integrons are most likely located in the chromosomal DNA of 16 and ten S. Derby isolates from abattoirs A and B, respectively. All amplicons for the variable part of class 1 integron showed the same EcoRI RFLP pattern. The integrons harboured a new *aadA* variant designated *aadA26*, which encodes combined resistance to streptomycin and spectinomycin. The presence of the same class 1 integron among related isolates, from the same or different abattoirs, points towards a dissemination of the integron by a clonal expansion of the isolates.

Introduction

Salmonella enterica subsp. *enterica* serovar (S.) Derby has been commonly isolated from slaughter pigs and pork products (Hauser et al., 2011). In 2011, S. Derby was among the top five serovars most frequently isolated from clinical and non-clinical isolates from non-human sources, which were submitted to the National Veterinary Services Laboratories (NVSL) in the U.S.A. Moreover, it was the second most isolated serovar from porcine sources (CDC, 2013). In Southern Brazil, this serovar was also among the most common serovars isolated from pigs and pork products (Bessa et al., 2007; Mürmann et al., 2009).

Multi-resistant (resistance to three or more classes of antimicrobial agents) S. Derby isolates have been obtained from different sources, and integrons with different gene cassette arrays have been identified in this serovar (Akiba et al., 2006; Beutlich et al., 2011). Integrons are genetic elements able to integrate and excise gene cassettes by site-specific recombination: They usually carry antimicrobial resistance gene cassettes and therefore contribute to maintenance and dissemination of antimicrobial resistance. The aim of this study was the investigation of S. Derby isolates from pigs for (a) the presence of antimicrobial resistance, (b) the detection of class 1 integrons and their gene cassettes and (c) the location of the class 1 integrons.

Material and Methods

A total of 49 S. Derby isolates obtained from lairage, pig carcasses and intestinal contents at four abattoirs (A-D) in 2008 in Southern Brazil were analyzed. They were tested for susceptibility to 12 antimicrobial agents by agar disk diffusion (CLSI, 2008). Multi-resistant isolates (n=27) were further investigated by XbaI-macrorestriction analysis (Ribot et al., 2006), plasmid profiling (Schwarz and Liebisch, 1994), and PCR assays for the detection of the resistance genes: *sul1*, *sul2* and *sul3* (sulphonamide resistance), *tet(A)* and *tet(B)* (tetracycline resistance) and *strA* (streptomycin resistance) and *aadA* variants (streptomycin/spectinomycin resistance) (Frech et al., 2003; Kadlec et al., 2005).

Integrons were screened by PCR assays for the presence of the *intI1* integrase gene and the variable part of class 1 integrons (Sandvang et al., 2007). The amplicons specific for the variable part of class 1 integrons were analysed by restriction fragment length polymorphism (RFLP) using the EcoRI restriction enzyme. A representative amplicon was chosen for cloning into pCR2.1-TOPO Vector (Invitrogen, Groningen, The Netherlands) and the recombinant vector was transformed into *Escherichia coli* recipient strain TOP10. Sequence analyses were conducted with the M13 forward and reverse primers (MWG, Ebersberg, Germany). Sequence comparisons were carried out using the BLAST programs blastn and blastp (<http://www.ncbi.nlm.nih.gov/BLAST/>) and with the ORF Finder program (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The nucleotide sequence of the amplicon for the variable part of class 1 integron has been deposited in the European Molecular Biology Laboratory (EMBL) database under the accession number HG314953.1. To confirm the linkage between *aadA26* and *sul1*, as well as, *aadA26* and *qacEΔ1*, specific PCR assays were used (Michael et al., 2005).

The location of the class 1 integrons was determined by Southern blot hybridization. For this, plasmid DNA was prepared by alkaline lysis and whole-cell DNA was digested using EcoRI restriction enzyme. The plasmid and whole-cell DNA were transferred from agarose gels to a nylon membrane by the capillary blot procedure. A sequenced amplicon for the variable part of the class 1 integron was enzymatically labelled by the Dig-Hib prime DNA labelling and detection system (Roche, Mannheim, Germany) and used as probe. Hybridization and signal detection were carried out according to the manufacturer's recommendations.

Results

Of the 49 *S. Derby* isolates originally tested, five isolates were susceptible to all antimicrobial agents tested and 27 isolates showed multi-resistance to streptomycin/spectinomycin, sulphonamides and tetracycline. The multi-resistant isolates were found in samples from lairage, pig carcasses and intestinal contents or from pig carcasses and intestinal contents at abattoir A (n=16) or B (n=11), respectively. Among the multi-resistant isolates five plasmid profiles and an indistinguishable XbaI-macrorestriction pattern were seen. Moreover, PCR analysis revealed that all multi-resistant isolates carried an *aadA* variant gene coding an aminoglycoside adenylyltransferase which confers combined resistance to streptomycin/spectinomycin, the gene *sul1* coding for a sulphonamide-resistant dihydropteroate synthase and the tetracycline exporter gene *tet(A)*. The PCR screening for class 1 integrons identified the class 1 integrase gene *intI1* in 26 isolates (abattoir A, n=16 and abattoir B, n=10). In addition, the PCR for the variable part of the integron showed an amplicon of approximately 1 kb. Restriction analysis of amplicons revealed the same fragment patterns in all isolates consisting of two EcoRI fragments of 560 and 449 bp. A representative amplicon was cloned and sequenced.

Sequence analysis of the class 1 integron confirmed the presence of a 1009 bp amplicon of which the first 118 bp were part of the 5' conserved segment (CS) and the final 111 bp were part of the 3' CS of the class 1 integron. The variable part (780 bp) showed a single gene cassette with a reading frame coding for a new variant of the AadA aminoglycoside adenylyltransferase, designated AadA26 (Fig 1). The *aadA26* gene cassette showed an amino acid substitution in the coding region, Ile® Val at the position 209, which is unique in the databases (NCBI database last accessed date: 11.07.2013). The *aadA26* gene has a putative GTG translational start codon (positions 119-121) and codes for an aminoglycoside 3'-(9)-O-adenylyltransferase of 259 amino acids. This gene cassette proved to be functionally active for combined resistance to streptomycin and spectinomycin. The 59-base element of the gene cassette was identified containing the binding sites 1L, 2L, 1R and 2R for the *intI1*-encoded integrase. The linkage of *aadA26* to *sul1* or *aadA26* to *qacEΔ1* was confirmed by the specific PCR assays for all isolates carrying class 1 integron. Since the hybridization experiments did not yield signals when using plasmid DNA as targets for the cassette-specific probe, the class 1 integrons are probably located in the chromosomal DNA of the *S. Derby* isolates. Additional studies are on-going to determine the location of the class 1 integrons in the chromosomal DNA and to investigate if the 3'-CS or 5'CS regions are absent in the single multi-resistant isolate, which was not positive for the detection of *intI1* gene and the variable part of class 1 integrons.

Discussion

In the present study, the same class 1 integron was detected in multi-resistant *S. Derby* isolates obtained during a survey study at two pig abattoirs in Southern Brazil. The same class 1 integron was detected in related isolates from different sources and different abattoirs, which may suggest the spread of a resistant clone of *S. Derby* in the pig production chain in the Southern of Brazil. An explanation for this clonal dissemination, within this particular geographic area in Brazil, could be the pig production system. It is a vertically integrated system, in which the abattoirs are supplied by specific finishing farms that purchase the piglets from common pig farms. Moreover, the feed is also supplied by a common feed industry. Interestingly, the multi-resistance pattern (streptomycin /spectinomycin-sulphonamides-tetracycline) found with these isolates was also the most common pattern found in a previous study (sampling period 1999-2000), in which 24 porcine *S. Derby* isolates from Southern Brazil were characterized (Michael et al, 2006). Comparing these two studies, almost all isolates that shared the same resistance phenotype showed also the same resistance genotype, except a single *sul2* resistance gene and the *aadA* variant of the gene cassette of the class 1 integrons. In the previous study, an *aadA2* variant was found (Michael et al., 2005) and in the present study a new variant, *aadA26*, was identified. The *aadA26* can be distinguished from *aadA2* variant by a single amino acid substitution. Moreover, the isolates from the present study shared the same XbaI macrorestriction pattern with one isolate or differed by only one fragment from another 23 isolates of the previous study (Michael et al, 2006). Although there is a 8-9 years interval between the two survey studies, the class 1 integrons and their gene cassettes proved to be highly conserved and stable. The association between the presence of class 1 integron and tetracycline resistance may be due to genetic linkage of integrons and transposons carrying the gene *tet(A)*. Although streptomycin and sulphonamide have not been administered to pigs in Southern Brazil anymore, and the use of tetracycline has been steadily declining in the last years, such resistance determinants are likely to persist in the *Salmonella* population.

Conclusions

Class 1 integrons are present in *S. Derby* isolates from apparently healthy pigs at slaughter and seem to represent genetic elements that are highly conserved and stable in this population in Southern Brazil. Moreover, the presence of the same class 1 integron among related isolates, from the same or different abattoirs, points towards a dissemination of the integron by a clonal expansion of the isolates in apparently healthy pigs. Noteworthy, such asymptomatic carriers may promote the dissemination of *S. Derby* not only to other animals, but also to humans when the isolates enter the food chain.

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References

- Akiba, M., Nakamura, K., Shinoda, D., Yoshii, N., Ito, H., Uchida, I., Nakazawa, M., 2006. Detection and characterization of a variant *Salmonella* Genomic Island 1s from *Salmonella* Derby isolates. *Jpn. J. Infect. Dis.* 59, 341-345.
- Bessa, M.C., Michael, G.B., Canu, N., Canal, C.W., Cardoso, M., Rabsch, W., Rubino, S., 2007. Phenotypic and genetic characterization of *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolated from pigs in Rio Grande do Sul, Brazil. *Res. Vet. Sci.* 83, 302-310.
- Beutlich, J., Jahn, S., Malorny, B., Hauser, E., Hühn, S., Schroeter, A., Rodicio, M.R., Appel, B., Threlfall, J., Mevius, D., Helmuth, R., Guerra, B., 2011. Antimicrobial resistance and virulence determinants in European *Salmonella* Genomic Island 1-positive *Salmonella enterica* isolates from different origins. *Appl. Environ. Microbiol.* 77, 5655-5664.
- Center for Disease Control and Prevention (CDC). National *Salmonella* Surveillance Annual Report, 2011. Atlanta, Georgia: US Department of Health and Human Services, CDC, 2013. <http://www.cdc.gov/ncezid/dfwed/PDFs/salmonella-annual-report-2011-508c.pdf>, and <http://www.cdc.gov/ncezid/dfwed/PDFs/salmonella-annual-report-appendices-2011-508c.pdf>. Accessed 27/06/2013.
- Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals-Third edition: Approved Standard M31-A3. Wayne, PA, USA: CLSI; 2008.
- French, G., Kehrenberg, C., Schwarz, S., 2003. Resistance phenotypes and genotypes of multiresistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium var. Copenhagen isolates from animal source. *J. Antimicrob. Chemother.* 51, 180-182.
- Hauser, E., Hebner, F., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A., Malorny, B., 2011. Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany. *Int. J. Food Microbiol.*, 151, 141-149.
- Kadlec, K., Kehrenberg, C., Schwarz, S., 2005: Molecular basis of resistance to trimethoprim, chloramphenicol and sulphonamides in *Bordetella bronchiseptica*. *J. Antimicrob. Chemother.* 56, 485-490.
- Michael, G.B., Cardoso, M., Schwarz, S., 2005. Class 1 integron associated gene cassettes in *Salmonella enterica* subsp. *enterica* serovar Agona isolated from pig carcasses in Brazil. *J. Antimicrob. Chemother.* 55, 776-779.
- Michael, G.B., Cardoso, M., Rabsch, W., Schwarz, S., 2006. Phenotypic and genotypic differentiation of porcine *Salmonella enterica* subsp. *enterica* serovar Derby isolates. *Vet. Microbiol.* 118, 312-318.
- Mürmann, L., Santos, M.C., Cardoso, M., 2009. Prevalence, genetic characterization and antimicrobial resistance of *Salmonella* isolates from fresh pork sausages in Porto Alegre, Brazil. *Food Control.* 20, 191-195.
- Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B., Barrett, T.J., 2006. Standardization of pulsed-field gel electrophoresis protocols for subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathog. Dis.* 3, 59-67.
- Sandvang, D., Aarestrup, F.M., Jensen, L.B. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104, 1997. *FEMS Microbiol. Lett.* 157, 177-181.

Schwarz, S., Liebisch, B., 1994. Use of ribotyping, IS200 typing and plasmid analysis for the identification of *Salmonella enterica* subsp. *enterica* serovar Typhimurium vaccine strain Zoosaloral H and its differentiation from wild type strains of the same serovar. Zentralb. Bakteriologie. 281, 442-450.

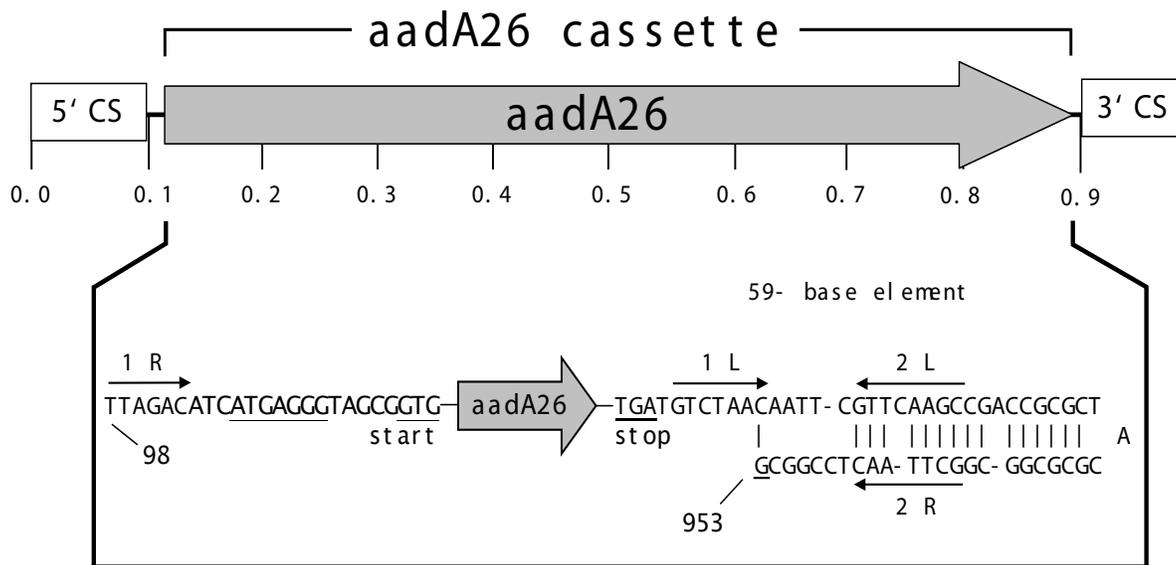


Fig. 1 Schematic presentation of the *aadA26* gene cassette from *S. Derby*. The *aadA26* reading frame is shown as an arrow whereas the 5' and 3' conserved segments (5' CS, 3' CS) of the class 1 integron are displayed as boxes. The beginning and the end of the gene cassette are shown in detail below. The translational start (GTG) and stop (TAA) codons are underlined. The 59-base element is presented as a stem-loop structure and the integrase 1 binding domains 1L, 2L, 2R, and 1R are indicated by arrows. The 59-base element of the gene cassette is shown in bold type. Numbers indicating important positions of bases in the 59-base element refer to the corresponding database entry (accession no. HG314953.1).

Abattoir-specific ways of implementing risk-based meat inspection methods in Germany

Blaha, T.*⁽¹⁾, Klein, G.⁽²⁾, Meemken, D.⁽²⁾

⁽¹⁾Field Station for Epidemiology, University of Veterinary Medicine Hannover, Buescheler Str. 9, D-49456 Bakum, Germany

⁽²⁾Institute for Food Quality and Food Safety, University of Veterinary Medicine, Hannover, Bischofsholer Damm 15, D-30173 Hannover, Germany

*corresponding author: thomas.blaha@tiho-bakum.de

Abstract

In the European Union serological and/or bacteriological monitoring results on zoonoses are to be taken into account for the risk assessment of slaughter pig herds in the framework of the risk-based meat inspection. Furthermore, the European food safety strategy pursues the additional goal to increase herd health. The new understanding of “One Health” (healthy animals and healthy people) and the new paradigm of assuring safe food (responsibility of food producers, prevention, risk-orientation, process-optimization, and continuous improvement) require new concepts for replacing the traditional meat inspection at the slaughter line with a risk-based meat inspection focusing at the whole food chain, and for modern and proactive veterinary diagnostics. The focus of such new concepts supporting continuous improvement systems is proactively acquiring knowledge on the herd health status, early warning and surveillance systems, instead of diagnosing diseases and causes of death in single animals. This paper describes how the basics of the risk-based meat inspection and especially the permit for the so-called “visual” meat inspection were implemented in three slaughterhouse companies taking into consideration the differing agricultural structures and differing qualities of the information exchange between the slaughterhouses and “their” farmers, but also differing points of view of the regional veterinary authorities on the preconditions for the permission of the visual meat inspection, i.e. inspection without palpations and incisions

Introduction

The need for restructuring the meat inspection procedures and the veterinary diagnostic system for food animals was identified by the “White Paper of the EU on Food Safety” in 2000. The resulting new European food safety concept with its basic Regulations (EC) No. 178/2002 (Anonym, 2002), and the so called “Hygiene Package” Regulations (EC) No. 852/2004, 853/2004, 854/2004 and 882/2004 (Anonym, 2004a, 2004b, 2004c), reflects the new paradigm targeted at improving not only food safety but also the health and welfare of all food animals (Hathaway and Richards, 1993). In contrast to the traditional sole responsibility of the state for providing safe food by final end product inspections at the slaughter line, the core elements of these new European regulations are:

a) Strengthened responsibility of the food producer: All persons that are involved in the production of food of animal origin share the food producers’ responsibility for food safety, animal health (notifiable and production diseases), and animal welfare, which is supervised by the official veterinary surveillance (i.e. public-private partnership). The food producers along the meat production chain are feed producers (farmers and feed mill operators), food animal producers (farmers supported by their consulting veterinarians), and slaughterhouse operators (supported by their quality management staff). The state still has the final responsibility, but not by inspecting the end products alone, but by enforcing the principle of the “control of the control”. This principle has greatly contributed to the establishment of industry-driven self-monitoring systems with independent auditing and certification procedures.

b) Prevention and process-optimization: In contrast to the past paradigm of protecting the consumer by just condemning carcasses and organs during the official meat inspection at the slaughter line for preventing products “not fit for consumption” from entering the food chain, the new goal is to assure production processes at farm level that result in healthy animals for slaughter, which in turn result in carcasses that are “fit for consumption”. The major tool for this is to implement systems for a continuous process optimization from feed to meat.

c) Risk-orientation and continuous improvement: Traditionally, official inspections of food production operations have been equally distributed at random with the same quantity and quality of the inspections, since no information on any differences in the compliance of the operations with current laws had been taken into account. The new approach recognizes that it is possible to gain information from existing data (feed mill and farm records, veterinary documentations on drug use, and slaughter check results) so that risk-oriented selections for inspecting operations are feasible: low-compliance (i.e.

“high-risk”) operations are inspected more frequently than full-compliance (i.e. “low-risk”) operations. This principle leads “automatically” into incentive systems, which encourages continuous improvements.

Consequently, the future food production system demands for a new proactive diagnostic strategy for food animals. The overall task is to build up new concepts that serve the holistic requirements of the new European food safety philosophy. Such new diagnostic strategy should enable both the responsible food producers (from feed to food) and the official control system to make cost-effective and risk-oriented decisions that results in targeted, information-based actions for the continuous improvement of food safety, animal health and animal welfare on the basis of the growing role of public-private partnerships.

After the so-called “Hygiene Package” of the new EU food safety concept was issued in 2004 and put in force in 2006, the German federal risk assessment authorities predominantly thought of ONE national “prescription” of how to implement the risk-based meat inspection in all slaughterhouses in the country. Furthermore, according to the traditional understanding of the food safety responsibility, the general expectation was that the new approach has to be implemented predominantly by the official veterinary food safety services. This was mainly due to the difficulty of switching from the traditional prescription of “what to do” to “reaching food safety goals”. Another difficulty was the intended shift of the food safety responsibility from the veterinary authority to the food business operator, with the veterinary authority mainly controlling the self-control mechanisms of the food operators including the farming community as part of the food chain.

In short: the task was and still is to make the responsible people (farmers, slaughterhouse operators and official veterinarians) understand that nowadays the food safety risks stem mainly from the production phases prior to slaughter (zoonotic microbes and residues), which cannot be dealt with by the traditional meat inspection, but by improving the herd health of all pig supplying herds including the latent infections especially with the relevant zoonotic pathogens as listed in the Scientific Opinion of the EFSA “On the public health hazards to be covered by inspection of meat (swine)” (EFSA, 2011): *Salmonella* spp. *Yersinia enterocolitica*, *Toxoplasma gondii* and *Trichinella* spp. (Meemken and Blaha, 2011).

Having these basic principles of the new food safety paradigm were better understood, it became obvious that there is not ONE way to implement the risk-based meat inspection in all slaughter facilities, but several ways to achieve the same goal. The reason for this flexibility is that slaughterhouses with their specific sets of pig supplying farmers have very different supply chain conditions (number and size of herds, quality of cooperation between suppliers and slaughterhouse, quality of information exchange, etc.). Even the risk patterns vary between regions and slaughterhouses (outdoor holdings vs. confinement; small vs. large pig units, straw or wood chips litter vs. slatted floor, etc.).

Material and Methods

In the framework of the recent research of the Field Station for Epidemiology of the University of Veterinary Medicine Hannover on improving the so-called “food chain information” (Reg. [EC] 853 and 854/2004) the authors combined data from the farm such as the mortality rate, the drug use measured by the “animal treatment index” (Blaha et al., 2006), and the slaughter check results to provide the official meat inspection service with meaningful information for the risk-based meat inspection procedure, the so-called “Herd Health Score” (Dickhaus et al., 2009). During these efforts it became obvious that there are general gaps in the knowledge about the health status and the zoonoses load of pig herds supplying pigs to the slaughterhouse. This led to considerations how to collect data on the occurrence of especially the subclinical infections in pig herds with relevance for the safety of meat, but also for the health and well-being of the food animals in question.

The resulting concept was developed into a general guideline for the implementation of the risk-based meat inspection. These guidelines cover the following steps:

- 1) A kick-off meeting with the management of the slaughter facility, the regional veterinary authority responsible for the meat inspection at the chosen slaughterhouse, and representatives of the pig supplying farmers for explaining the plan and getting the full commitment of these three “players”, with T. Blaha and D. Meemken acting as both moderators of the meeting and facilitators of the following implementation process.
- 2) Adaptation of the software packages of both the official veterinarians responsible for the meat inspection at the slaughter line and of the slaughterhouse to make the herd-wise recording of organ lesions possible so that at any time a “6-month rolling average” of the percentage of lesions per herd can be established. This rolling average is to define herds that can be “visually” inspected (low frequency of lesions) and herds that need additional inspection activities at a slower line speed or at the trimming line (very high frequency of lesions).

- 3) Developing forms for the so-called “food chain information” for informing the meat inspectors and the slaughterhouse about food-safety-relevant herd health status issues such as a controlled and integrated husbandry system, no out-door keeping of the animals, food-safety relevant diseases and/or laboratory results such as Salmonella serology, as well as the drug use prior to slaughter.
- 4) Preliminary permission of the visual meat inspection and carrying out a parallel inspection to compare the accuracy of the decision “fit for consumption” in both methods.
- 5) After making sure that the new meat inspection method achieves the same or even better level of food safety transferring the entire meat inspection procedure to the new risk-based approach: inspecting carcasses from herds with a high herd health status and a low frequency of lesions “visually”, and inspecting carcasses from herds with a poor herd health status and a high frequency of lesions “specifically more intensively” addressing the specific risks per herd.
- 6) Validating the functionality of the new system by double-checking carcasses from randomly chosen slaughter batches in the cooling facilities to make sure the “visually inspected” and the “specifically more intensively inspected” carcasses are likewise fit for consumption.

These general guidelines were used as basis for the implementation of the risk-based meat inspection in three different types of slaughter enterprises:

- a) A slaughterhouse in the North of Germany belonging to a multi-national big meat company with its headquarters in The Netherlands and, consequently, acting under the quality policy of the Dutch company.
- b) A Meat Cooperative in the West of Germany with 4 slaughterhouses acting under the cooperative’s quality policy, and
- c) A family-owned mid-sized slaughterhouse in the Northwest of Germany near the Dutch border, slaughtering also pigs raised and finished in The Netherlands.

Results

Although the same guidelines were used as basis for the implementation processes, due to the very different organisational structures, information systems, bondage between the slaughterhouses with the farmers and the differing points of views of the respective official veterinarians in these slaughterhouses, varying implementation steps had to be taken, and, accordingly, the finally routinely used ways of achieving the intended objectives of the risk-based meat inspection method are distinctly abattoir-specific:

- a) The multinational company focussed on: improving the QM-system in the slaughterhouse, adding information about the feed sources for the animals (taking into account that several food scandals took their start at the feed production level), implementing additionally to the serological Salmonella monitoring serological surveillance on *M. avium* and *Toxoplasma gondii*, and implementing additional testing for drug residues, and decision on “visual” or “specifically intensified” inspection at the following threshold: only the pigs of herds with a lesion frequency above the double value of the slaughterhouse average are “specifically intensified” inspected.
- b) The Meat Cooperative focussed on: improving the veterinary care and consultancy of their supplying herds by employing two cooperative-own veterinarians exclusively consulting any farmer with deficiencies of the health and welfare status of his or her herd, logistic slaughter by day-wise slaughtering the pigs for the “visual” inspection and those for the “specifically intensified” inspection, additionally logistic slaughter of pigs according to the Salmonella status of the herd in question, and selecting herds with an unusually high frequency of lesions for residue testing.
- c) The family-owned mid-sized slaughterhouse uses a “slaughterhouse index” (i.e. the average of the frequencies of lesions of all carcasses slaughtered in a certain period of time and compares this to every herd-specific (the “farmer’s index”) frequency of lesions in the same period of time, adding mortality rates to the “food chain information”, using the slaughter check results and the mortality rates as trigger of risk-oriented controls of pig herds with health deficiencies.

Although in each of the three pork-producing entities (the slaughterhouses with their set of pig supplying farmers) has finally implemented its own abattoir-specific way of using the risk-based meat inspection, they all achieved the same objective:

- 1) replacing the traditional hands-on meat inspection by inspecting the majority “visually”, i.e. in a hands-off manner for reducing the potential cross contamination of zoonotic pathogens,

- 2) improving the information flow between the farmers and the slaughterhouse (the “food chain information”) and between the slaughterhouse and the farmers (feed-back information of the herd specific slaughter check results), and
- 3) reducing the veterinary man power at the slaughter line and shifting the veterinary awareness more and more to the supplying farms focussing on the improvement of the health and the animal welfare of the animals produced for slaughter.

References

ANONYMOUS, 2002, Regulation (EC) No 178/2002 of the European parliament and of the council laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, O. J., L 31.

ANONYMOUS, 2004, Regulation (EC) No 853/2004 of the European parliament and of the council laying down specific hygiene rules for food of animal origin, O. J., L 139.

DICKHAUS C.-P., Meemken D. and Blaha T., 2009): Attempts to quantify the health status of pig herds: developing and validating a Herd Health Score (HHS). In: Aland and Madec, eds. Sustainable animal production: the challenges and potential developments for professional farming. Wageningen Academic Publishers, Wageningen, 191-201.

EFSA, 2011, Scientific Opinion on the public health hazards to be covered by inspection of meat (swine), EFSA J., 9: 2351.

HATHAWAY S.C. and RICHARDS M.S., 1993, Determination of performance attributes of post-mortem meat inspection procedures, Prev. Vet. Med., 16: 119-131.

MEEMKEN D., and BLAHA T., 2011, “Meat Juice Multi-Serology” - a tool for the continuous improvement of herd health and food safety in the framework of the risk-based meat inspection of slaughter pigs, J. Food Saf. Food Qual. 62: 192-199.

Evaluation of penicillin G residues by kidney inhibition swab tests in sow body fluids and tissues following intramuscular injection

Shelver, W.L.^{*(1)}, Lupton, S.J.⁽¹⁾, Newman, D.J.⁽²⁾, Larsen, S.⁽³⁾, Smith, D.J.⁽¹⁾

⁽¹⁾USDA-ARS, Biosciences Research Laboratory, Fargo, USA

⁽²⁾Department of Animal Sciences, North Dakota State University, Fargo, USA

⁽³⁾National Pork Board, Des Moines, USA

*Corresponding author: Weilin.Shelver@ars.usda.gov

Abstract

In 2011, the USDA-Food Safety and Inspection Service (FSIS) changed the method used for screening swine tissues for antimicrobial residues from the Fast Antimicrobial Screen Test to the Kidney Inhibition Swab (KIS™). Here, we describe the use of KIS™ test for the detection of penicillin G residues in kidney, liver, plasma, urine, and skeletal muscle of heavy sows following the administration of a 5x label dose of penicillin G procaine. Such off-label use is legal in the United States under the Animal Medicinal Drug Use Clarification Act (AMDUCA) when label routes or doses are ineffective at treating disease and is commonly used to treat bacterial infections in heavy sows. Heavy sows (n=126; 228 ± 30.1 kg) were administered intramuscular (IM) doses of penicillin G (33,000 U/kg bw) for 3 consecutive days using 3 different administration patterns. Within treatment, six sows each were slaughtered after 5, 10, 15, 20, 25, 32, or 39 withdrawal days. The IM administration pattern had no discernible effect on penicillin G depletion from kidney, skeletal muscle, serum, urine or liver. Residues were depleted most rapidly from liver and skeletal muscle and more slowly from kidney and urine. While kidney residues were a poor predictor of penicillin G residues in skeletal muscle, kidney was the most sensitive target tissue for detecting penicillin G residues, with two positive results even after a 39-day withdrawal period. The most suitable ante-mortem matrix to replace FSIS on-site tests using kidney was urine. Serum, another ante-mortem matrix predicted muscle residue well albeit showing more positives than muscle. These data support a 15-day withdrawal period suggested by Food Animal Residue Avoidance Databank for extra-label penicillin G treated heavy sows with the caveat that kidney tissues be excluded from human consumption.

Introduction

Penicillin G is active against a variety of Gram-positive pathogens affecting livestock production and is indicated for treatment of a number of bacterial diseases in a variety of animal species including erysipelas in swine (NADA 065-010). For most food animals, the typical route of penicillin G administration is by IM injection at daily dose of 6,600 IU/kg with treatment not to exceed 4 consecutive days. Under label conditions, approved pre-slaughter withdrawal periods are 7 days for swine with zero tolerance for penicillin G residues in tissues. However, sows are commonly treated with higher doses which is allowed under a veterinarian's supervision when labeled doses are ineffective. Under AMDUCA, the veterinarian prescribing the off-label use must recommend an appropriate pre-slaughter withdrawal period to ensure that drug residues remaining in edible tissues deplete to safe levels (21 CFR Part 530).

There are very few data which describe the depletion of penicillin G residues under off-label conditions. The studies available indicate increased doses increase the elimination time. Apley et al. (2009) conducted a residue depletion study in sows using a single 5x label dose of 33,000 IU/kg administered by intramuscular injection or with a needle free device. Intramuscular injection in the "hip" produced flip-flop kinetics (KuKanich et al., 2005; Riviere 2011) in which the terminal elimination rate is controlled by the rate of absorption rather than by the rate of elimination. Between-animal plasma half-lives were highly variable. After an 8-day withdrawal period two of five hogs had a quantifiable residue. From these data, they recommended of a 28 day withdrawal period based on a 95% confidence interval that 99% of treated animals would have no detectable residue in the kidney.

The US-FSIS changed the method for screening swine tissues for antimicrobial residues from the Fast Antimicrobial Screen Test to the KIS™ test in September of 2011 (FSIS notice 45-11). An increase in the detection of penicillin residues in sow tissues subsequent to the adoption of the new screening assay may be attributed to the survey method change. This paper describes the detection of penicillin G procaine residues with the CHARM-KIS test in kidney, liver, plasma, urine, and skeletal muscles of heavy sows after an extra-label penicillin-G procaine administration. Sows were treated IM with 33,000

IU/kg bw for 3 consecutive days and were slaughtered with withdrawal periods extending to 39 days post-treatment.

Material and Methods

Chemicals and Supplies. Penicillin G procaine (300,000 U/mL; Norocillin; Norbrook Pharmaceuticals, Lenexa, KS) injection solution was purchased from Ivesco, LLC (Iowa Falls, Iowa). Penicillin G procaine monohydrate reference standard was purchased from U. S. Pharmacopeia, Rockville, MD. Kidney inhibition swabs, neutralization tablets, penicillin G controls, and heating blocks were obtained from Charm Laboratories (Lawrence, MA). Driploss containers were purchased from the Danish Meat Research Institute, Taastrup, Denmark.

Animal Housing and Treatment Assignment. A study protocol was approved by the North Dakota State University Institutional Animal Care and Use Committee prior to the initiation of the live-phase of the study. Heavy sows were purchased from the North Dakota Pig Cooperative (Larimore, ND) and delivered to the North Dakota State University Animal Research Center (Fargo, ND) and acclimatized for at least 14 days. Animals were randomly assigned to one of three treatments and were each provided unique identification numbers by ear tag. For treatment 1, sows received injections (10 mL on each side) in a single location for 3 days at the same location. For treatment 2, sows received consecutive injections (10 mL on each side) for 3 consecutive days at locations separated by approximately 2 inch intervals. For Treatment 3, sows received 20 mL in the one side with overflow injections occurring on the other side of the neck. Injections occurring on consecutive days were separated by approximately 2-3 inches.

Sows were slaughtered with 5, 10, 15, 20, 25, 32, or 39 day withdrawal periods relative to the last off-label dosing day. Positive control sows (n = 2 per withdrawal period) were treated with the label dose (6,600 U/kg bw) of penicillin G via IM administration for 3 consecutive days and were euthanized after a 7-day withdrawal period, consistent with the product label; or a 15-day withdrawal period. Negative control sows (n = 2) were dosed IM with sterile saline (1 mL per 45.5 kg) for 3 consecutive days and were slaughtered 5 days after the last saline injection.

On-Site Analysis of Kidneys. Kidney samples were screened for penicillin residues on the kill floor using the Charm-KIS microbial inhibition test according to FSIS procedures (FSIS CLG-ADD 3.01, 2011); the manufacturer's recommended incubation time, without the automatic shut-off option, was followed. All samples were determined in duplicate by separate operators and each result scored independently by both operators.

Sample Collection and Treatment. At collection, skeletal muscle and additional kidney were collected using a 3-cm diameter tissue coring device driven by a cordless drill and trimmed to approximately 3 x 3 cm. Samples were placed into Driploss containers and were frozen at -80 °C until analysis. Samples were thawed at room temperature for 1 hr and the tissue juices were collected from the drip tubes after centrifuging at 1,200 x g for 10 min. The collected tissue juices were used to saturate Charm-KIS swabs. Microbial inhibition tests for tissues were then conducted as described by the FSIS (2011) for determination of the presence of penicillin G. Liver required the addition of an equal volume of water followed by boiling (1 min) and centrifugation at 14,000 x g prior to swab. Urine or serum aliquots (500 µL) were combined with a single Charm-KIS neutralization tablet and vortexed; particulates were allowed to settle for 1 min. Serum/urine supernatant was adsorbed for 10 seconds with a cotton swab, after which, the CHARM-KIS microbial inhibition assay was performed.

Assay Sensitivity Determination. Control serum, urine, skeletal muscle juice, and kidney juice were prepared as previously described and tissue matrix aliquots were spiked with 0, 10, 20, 30, 40, and 50 ppb of penicillin G procaine. Liver juice was spiked with 0, 10, 25, 50, 75, and 100 ppb of penicillin G procaine. Fortification of each matrix was repeated on three separate days.

Results and Discussion

Typical color indication for positive, negative, and "caution" KIS results are shown in Figure 1. Sensitivity for penicillin residues in kidney juice, muscle juice, urine, and serum was 20, 30, 20, and 30 ppb respectively and can be lot dependent. When dilution factor is accounted for, liver juice sensitivity was 100 ppb.

Negative control sows that received normal saline and which were slaughtered on withdraw day 5 had negative readings for all matrices tested. The positive control sows, which received the label dose and were slaughtered at withdrawal day 7 showed positive Charm-KIS results for all tissues. For withdrawal day 15, one out of two positive control animals tested positive using Charm-KIS in kidney and urine samples but the

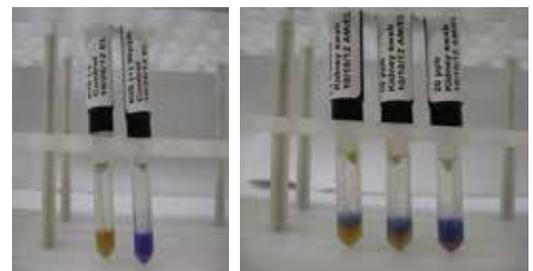


Figure 1. Typical results returned from the Charm-KIS rapid screening assay, from left to right, KIS negative control, KIS positive control, negative, caution, and positive results from kidney swabs. A caution result is considered to be negative by FSIS.

rest of matrices returned negative results. As seen in Figure 2 there is no difference in the various treatments indicating the differences from the various injection patterns are not discernibly different. The variability is probably due to the variability in absorption since penicillin G procaine would demonstrate flip-flop kinetics. Apley's (2009) estimate of a 28 day withdrawal period is at least reasonable and our data suggest it may be longer before kidney levels become non-detectable as we observed positives even at day 39. Slow penicillin depletion could be attributed to absorption from the injection sites where residues remain and the fact that penicillin concentrated in the kidney where it is excreted by an active process. From sow penicillin results reported by Korsrud et al (1998), penicillin concentrations in kidney were approximately 40-70 times greater than residues in corresponding muscle samples. The Charm-KIS assay returned smaller numbers of muscle positives than in kidneys for the same animal and time point, in agreement with Korsrud et al's findings. Charm-KIS assays of liver returned fewer positive results than the assays of other tissues, even at day 5 (Figure 3) possibly caused by the much poorer assay sensitivity with liver compared to other matrices.

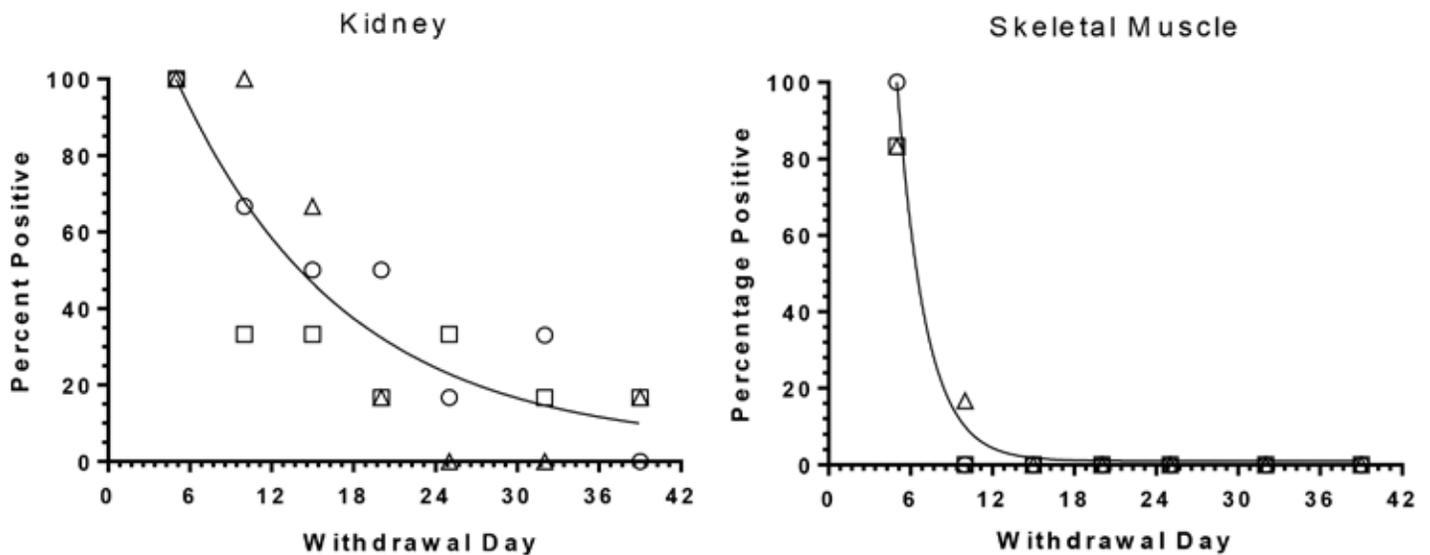


Figure 2. Depletion of Penicillin G residues in kidneys (left panel) and muscle (right panel) of heavy sows as detected by the Charm-KIS rapid screening assay. Line is a first order depletion fit of combined data from Treatments; false positive results (where there is no measureable residues from LC-MS/MS, data not shown) were removed from the skeletal muscle data prior to plotting.

Applicability of using the Charm-KIS for pre-slaughter screening of treated animals was tested with assays of serum and urine. No false-positive Charm-KIS assay results were returned for either matrices (LC-MSMS data not shown). The urine was a much better surrogate matrix for detecting potential violative penicillin G residues in kidney (Figure 3) than was serum. Serum results clearly demonstrate that serum is an inadequate surrogate for kidney because serum is more rapidly cleared of penicillin than kidney.

Conclusion

Based on kidney results a prolonged withdrawal period is needed prior to culling sows that have been previously treated with high doses of penicillin G procaine. The labeled dose can also return positive kidney penicillin tests beyond the prescribed withdrawal period. Urine can serve as an ante-mortem test reflecting the levels which will be observed in the kidney.

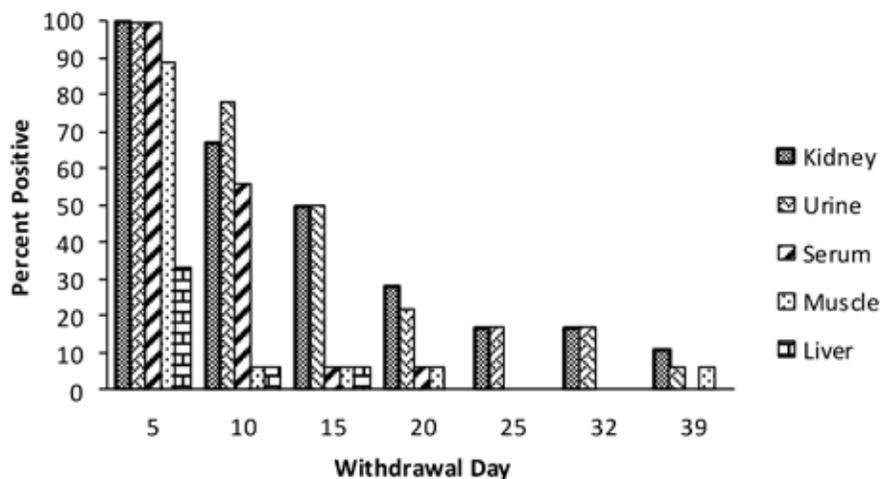


Figure 3. Comparison of Charm-KIS test results in kidneys, urine, serum, muscle, and liver of off-label treated sows as a function of withdrawal day.

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References

- APLEY M., GOETZEE H., GEHRING R., KARRIKER L., 2009. Pharmacokinetics and tissue residues of procaine penicillin G in sows after administration of 33,000 IU/kg intramuscularly and by needle-free injection in the hip. National Pork Board Research Report NPB #07-234.
- Food Safety and Inspection Service, Swab test for antimicrobial drug detection, CLG-ADD 3.01. Effective 05/29/2011.
- Food Safety and Inspection Service Notice 44-11, Using the kidney inhibition swab (KIS™) test to detect antimicrobial drug residues in swine in selected establishments - Phase III.
- Food Safety and Inspection Service Notice 43-12, Using the kidney inhibition swab (KIS™) test to detect antimicrobial drug residues in all livestock in slaughter establishments – phase IV. 6/26/12.
- KUKANICH B., GEHRING R., WEBB A. I., CRAIGMILL A. L., RIVIERE J. E, 2005. Effect of formulation and route of administration on tissue residues and withdrawal times. *JAVMA*. 227, 1574-1577.
- KORSRUD G. O., SALISBURY C. D. C., RHODES C. S., PAPICH M. G., YATES W. D. G., et al.1998, Depletion of penicillin G residues in tissues, plasma, and injection sites of market pigs injected intramuscularly with procaine penicillin G. *Food Additives Contam.* 15, 421-426.

The change of plasma C-reactive protein and metabolite concentrations, and MPS sick degree score in Landrase selected for resistance to MPS, Large Yorkshire selected for immune performances and the crossbreed

Suda, Y.⁽¹⁾, Sakuma, A.⁽²⁾, Shimazu, T.⁽³⁾, Borjigin, L.⁽³⁾, Yu, H.⁽³⁾, Katayama, Y.⁽³⁾, Nakajo, M.⁽²⁾, Kitazawa, H.⁽³⁾, Aso, H.⁽³⁾, Kato, K.⁽³⁾, Suzuki, K.⁽³⁾

⁽¹⁾ Department of food, agricultural and environmental sciences, Miyagi University, 2-2-1 hatatate, Taihaku, Sendai, Miyagi prefecture, Japan.

⁽²⁾ Miyagi Prefectural Animal Industry Experimental Station, 1 Iwadeyama Minamisawa Aza hiwata, Osaki, Miyagi prefecture, Japan.

⁽³⁾ Graduate school of agriculture, Tohoku University, 1-1 Tsutsumidori Amemiya, Aoba, Sendai, Miyagi prefecture, Japan.

* suda@myu.ac.jp

Abstract

Swine *Mycoplasma Hyopneumoniae*, *hp*, is known as a major factor to affect for the specific pneumonia (MPS). This damages is very serious because carrier rate of *hp* in piglets from 3 to 4 months of age is very high, the rate of piglets that the response of antibody to *hp* shows positive is 80 % over, and the rate that has very terrible tissue from MPS is 51% in Japanese pig farm. We bred a resistant strain to MPS by selection to decrease MPS pathogenic condition over 5 generations using Landrase (MPS strain), and a high immune performance strain by selection for peripheral phagocytosis, complement activity and antibody production against erysipelatos vaccine using Large Yorkshire (HI strain). And then, the crossbreed (F1) strain was produced by mating MPS and HI strains. In this study, we examined the selection response considering with the inflammation response in MPS, HI and the crossbreed strains. As 1st examination, 18 heads of two MPS strain and 12 of general strain were supplied at about 60kg of body weight. Body weight measurement, and taking blood and swab from nasal cavity were carried out from 70kg to 110kg by about 7 days each. Direct PCR method was performed for the detection of *hp* in nasal cavity, and plasma C-reactive protein (CRP) and metabolite concentrations were measured by using Dry Chem analyzer with a specific kit. The degree of MPS disease was evaluated by a general method. As 2nd examination, the same examinations in HI and F1 strain were performed respectively. All statistics and ANOVA were calculated by using SAS program. Body weight did not significantly differ among selection strains and general. Although most of animals were recognized as carriers of *hp*, plasma CRP concentration and degree of disease in MPS strains was low significantly in normal condition compared with general one ($p < 0.01$). In HI and F1 strains, significant differences were not recognized.

Introduction

Swine *Mycoplasma Pneumonia* (MPS) is one of the representative chronic respiratory disease caused by the main infection of *Mycoplasma Hyopneumoniae*, *hp*. Although MPS is known to decrease the daily gain and feed efficiency, resulting in huge economic losses in the productivity (Sarradell et al., 2003). This damages is very serious because carrier rate of *hp* in piglets from 3 to 4 months of age is very high, the rate that the response of antibody to *hp* shows positive is 80 % over, and the rate that has very terrible tissue from MPS is 51% in Japanese pig farm. *Hp* infection might complete by invasion to the host animal through the oral and/or nasal pathway. And *hp* would adhere to the bronchial and pulmonary alveolus epithelial cells and firstly induce inflammation in adhesion site and around (DeBey M.C., 1992). The *hp* infection and its inflammation are recognized via the Toll-like receptors on the porcine alveolar macrophages (Muneta Y., 2003). So *hp* itself on the epithelial cells is possible to form a lesion of pulmonary inflammation. In the previous study, we evaluated MPS pathogenic condition and bred a resistance strain to MPS by selection to decrease MPS lesion over 5 generations using Landrase (MPS strain) (Katayama M., 2011). Also we bred high immune performance strain by selection for peripheral phagocytosis, complement activity and antibody production against erysipelatos vaccine using Large Yorkshire (HI strain). And then, the crossbreed (F1) strain was produced by mating MPS and HI strains. In this study, we examined the selection response, plasma CRP and metabolite concentrations, and MPS lesion degree considering with the inflammation response in the MPS, HI and the crossbreed strains.

Material and Methods

All animal managements were carried out at the School of Food, Agricultural and Environmental Science, at Miyagi University according to the animal handling guidelines for animal experiments. As 1st examination, 18 heads of two MPS strain which was genetically selected to show lower incidence of the pulmonary MPS lesion in Miyagi Prefecture Animal Industry Experiment Station, and 12 heads of general strain were supplied at about 60kg of body weight. In brief, this MPS strain was established based on the genetic selection aggregated breeding value of 4 traits (average daily gain, back fat thickness, MPS lesion, and cortisol concentration). And 12 heads of high immune performance strain by selection to increase peripheral

phagocytosis, complement activity and antibody production against erysipelatous vaccine using Large Yorkshire (HI strain) and 12 heads of crossbreed (F1) strain produced by mating MPS and HI strains were supplied at the same time with MPS strain and at same body weight level. As control strain, general Large Yorkshire and crossbreed between Large Yorkshire and Landrase were used, respectively. Body weight measurement, and taking blood and swab from nasal cavity were carried out from 70kg to 110kg by about 7 days each from trial start, day 0. In this study, we examined the selection response considering with the inflammation response in the MPS, HI and the F1 strains compared to each control pigs. Direct PCR method with *hp* specific primer sets was performed to detect *hp* in nasal cavity, and plasma CRP and metabolite concentrations were measured by using Dry Chem analyzer (Fujifilm Co.Ltd.) with a specific kit. The degree of MPS lesion was evaluated by a general method. All statistics and ANOVA was calculated by GLM procedure of SAS program (Version 9.0).

Results

Body weight did not significantly differ among selection strains and general one. Including to a previous reserach, *hp* was detected from nasal cavity in all animals with no relationship to strains. Interestingly, the carrying condition of *hp* in MSP strain was lower compared to general Landrase (Figure 1). Plasma CRP concentration and degree of disease in MPS strains were lower significantly in normal condition compared to general one ($p < 0.05$) (Figure 2). In HI and F1 strains, significant differences were not recognized. MPS lesion degrees in MPS strain, F1 strain and general Large Yorkshire were lower significantly compared to general Landrase ($p < 0.05$).

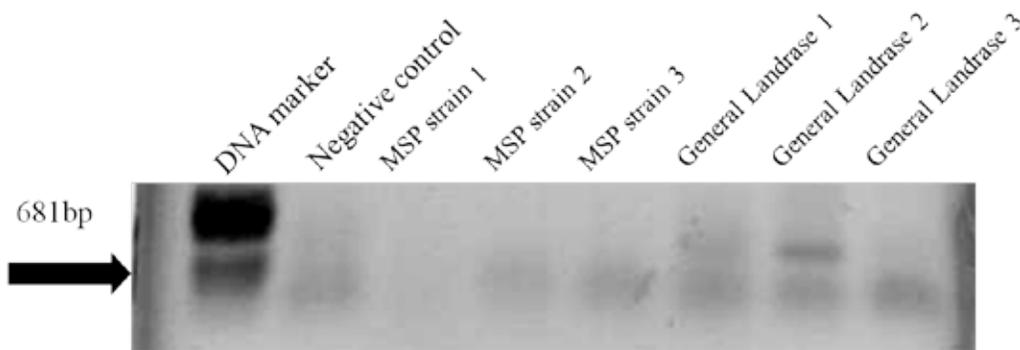


Figure 1. Infection condition on pulmonary epithelial tissue.

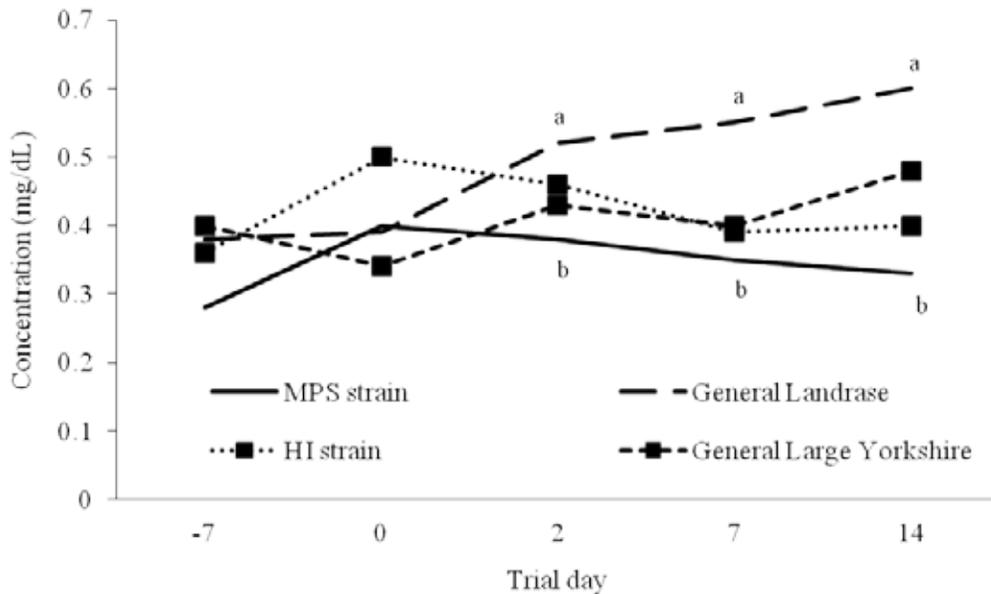


Figure 2. The changes for plasma CRP concentration. a, b; The differences between means of same breeds in the same trial day are significant at 5% level.

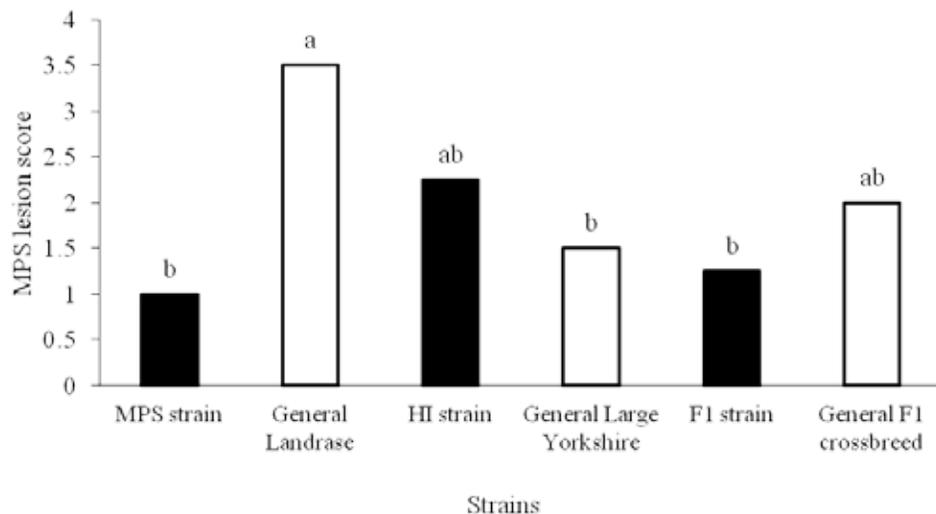


Figure 3. The MPS lesion score in each strain. a,b; The differences between means with different superscripts are significant at 5% level.

Discussion

Especially, MPS strain selected for decreasing MPS sick degree might have a performance to resist to inflammation from *hp* infection. However the selection responses to decrease MPS lesion did not be recognized in HI strain selected for increasing 4 kinds of immune performances. Also considering to the results in F1 strain, selection specificity to resist to inflammation from *hp* in MPS strain might not be dominant.

Acknowledgements

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References

- Sarradell J., Andrada M., Ramírez A.S., Fernández A., Gómez-Villamandos J.C., Jover A., Lorenzo H., Herráez P., Rodríguez E., 2003. A morphologic and immunohistochemical study of the bronchus-associated lymphoid tissue of pigs naturally infected with *Mycoplasma hyopneumoniae*., *Vet. Pathol.* 40(4), 395-404.
- DeBey M.C., Jacobson C.D., Ross R.F., 1992. Histochemical and morphologic changes of porcine airway epithelial cells in response to infection with *Mycoplasma hyopneumoniae*., *Am. J. Vet. Res.* 53(9), 1705-1710.
- Muneta Y., Uenishi H., Kikuma R., Yoshihara K., Shimoji Y., Yamamoto R., Hamashima N., Yokomizo Y., Mori Y., 2003. Porcine TLR2 and TLR6: identification and their involvement in *Mycoplasma hyopneumoniae* infection., *J. Interferon Cytokine Res.* 23(10), 583-590.
- Katayama M., Fukuda T., Okamura T., Suda Y., Suzuki E., Uenishi H., Suzuki K., 2011. Immunophenotype characterization for swine selected line, which is resistant to *Mycoplasma Pneumoniae*., *Asian-Aust. J. Anim. Sci.* 24(7), 889-897.
- Shimazu T., Borjigin L., Katayama Y., Li M., Satoh T., Watanabe K., Kitazawa H., Roh S.G., Aso H., Katoh K., Suda Y., Sakuma A., Nakajo M., Suzuki K., 2013. Immunological characterization of peripheral blood leukocytes using vaccine for mycoplasmal pneumonia of swine (MPS) in swine line selected for resistance to MPS., *Anim. Sci. J.* 8 April, in press.

Dynamic of *Campylobacter* infection within pig farms from sows to fattening pigs

Leblanc-Maridor M. ^(1,2), Denis M. ^(3,4), Chidaine B. ^(3,4), Belloc C. ^(1,2)

¹INRA, UMR1300 Biology, Epidemiology and Risk Analysis in animal health, F-44307 Nantes, France

²LUNAM Université, Oniris, Nantes-Atlantic College of veterinary medicine and food sciences and engineering, UMR Bio-EpAR, BP 40706, F-44307 Nantes, France

³Anses, Unité Hygiène et Qualité des Produits Avicoles et Porcins, BP 53, 22440 Ploufragan, France

⁴Université Européenne de Bretagne, France

*Corresponding author : mily.leblanc-maridor@oniris-nantes.fr

Abstract

This work aimed at (i) describing *Campylobacter* excretion by conventional pigs in field conditions from sows to fattening pigs, and (ii) assessing the role of the environment as a source of pig contamination. Five sows and six piglets per sow were individually followed in two farrow-to-finish farms. Faecal shedding, contamination of pens (empty or with animals), feed and water were monitored from birth to finishing for pigs and during one production cycle for sows. *Campylobacter*, mainly *C. coli*, was highly prevalent for sows and their piglets. All the sows excreted *Campylobacter*. Piglets became infected early: 84 and 86% of them excreted *Campylobacter* three to five days after their birth. The prevalences increased progressively through the rearing period with some young pigs becoming punctually negative. The amount of *Campylobacter* in faeces was highly variable between pigs (from 0 to 10⁹ CFU/g of faeces) and between sampling times for a given pig, with sometimes no detection. Samples of the pigs' environment during the down period were always negative and became positive when pigs were housed in the pens. Nevertheless, no correlation was established between the excretion level of the pigs and the contamination level of their environment. Some samples of feed, initially free of *Campylobacter*, became positive due to contamination by faecal material. Finally, our study underlines the role of the sows as a *Campylobacter* contamination source for their piglets.

Introduction

Campylobacter, a major cause of food-borne human infection, is commonly carried in the intestinal tract of a wide range of animals, including livestock animals, without causing clinical signs (EFSA, 2007). Pigs are known to be frequently infected by *Campylobacter* with prevalence between 50% and 100% and excretion levels ranging from 10² to 10⁷ Colony Forming Units (CFU) of *Campylobacter* per gram of faeces (Alter *et al.*, 2005). For the implementation of control measures in farms, dynamics of *Campylobacter* excretion and sources of contamination have to be known. Previous studies describe that piglets became infected by their mothers within the first weeks of life (Hume *et al.*, 2002). Other sources could be suspected among which the environment (Alter *et al.*, 2005). This work aimed at (i) describing *Campylobacter* excretion (presence/absence, main species and quantity) by conventional pigs in field conditions along one production cycle from sows to fattening pigs and (ii) assessing the role of the environment as a potential source of pig contamination.

Material and Methods

Farms and animals individual follow up

This study was carried out in two farrow-to-finish farms. In each farm, five sows from the same batch have been followed along one production cycle (before farrowing and until the slaughter of their offspring). After farrowing, 6 piglets per sow have been individually followed from their first week of life until their departure to the slaughterhouse. Sows were monitored weekly 2 weeks before farrowing. Piglets and sows were followed weekly from the first week to 5 weeks of age then growing pigs were monitored every three weeks until slaughter whereas the sows have been sampled at the 8th, 11th and 17th weeks after farrowing.

Samples and analysis

Fresh faecal samples from sows and young pigs were collected individually and 10 g of each were diluted to 1:10 in Preston broth for detection and enumeration. For the environment, surface swabs (floors, walls, structures) were collected from each pen and from the floors and walls around each pen (i) in empty rooms after cleaning/disinfection (during the down period) and (ii) in presence of the animals. Surfaces swabbed were measured to detect and quantify the number of *Campylobacter* per square meter. Swabs were humidified with 45ml Preston broth. Enumeration for all the samples was done using a 10-fold serial dilution on Karmali plates.

Presence/absence of *Campylobacter* was checked in feed and water. Feed samples were collected before the distribution to the animals (in bags or silos) and in presence of pigs (into the trough of each studied pens). Water samples were collected upstream to the distribution network and at the faucet in each unit (service, gestation, farrowing, post-weaning, fattening).

Species of isolates were identified by real-time PCR (Leblanc-Maridor *et al.*, 2011).

For the statistical analysis, the correlation between the contamination level of the environment and the excretion level of *Campylobacter* by the animals was estimated.

Results

For one farm (Farm I), all the faecal samples from sows were positive for *Campylobacter* whatever the sampling times while in the second farm (Farm II), no *Campylobacter* were detected in the faecal samples of one sow at three different sampling times. Level of *Campylobacter* excretion varied from less than 10^2 to 10^7 CFU of per gram of faeces, and varied between sows at one sampling time and also for a given animal at different sampling times.

At 3 to 5 days after birth (first faecal sample), 84% and 86% of the piglets (respectively Farm I and Farm II) excreted *Campylobacter* (Figure 1). These prevalences increased progressively through the rearing period except at three weeks of age where only 60 % of the piglets excreted *Campylobacter*. Afterwards, prevalences were above 90 % with some pigs becoming punctually negative.

The *Campylobacter* faecal excretion of young pigs was on average 10^5 to 10^7 CFU of *Campylobacter* per gram of faeces with a high variability in the quantities between two different samples for a given animal or between pigs at the same sampling time (from 0 to 10^9 CFU/g) (Figure 2). A decrease of the quantity of *Campylobacter* excreted by piglets was observed between the 4th and the 5th weeks of age for both farms after treatment with tylosine given to the piglets at the entrance into the post-weaning unit.

No *Campylobacter* was ever found in the environmental samples taken after the cleaning-disinfection process (without animals) as well as in the feed samples taken before distribution to the animals or in the water. When the animals were in the pens,

feed samples and environmental samples were positive with a high variability of the contamination level (from less than 100 to 10^9 CFU/square meter). Concerning the environmental samples (walls or floors), the number of positive samples

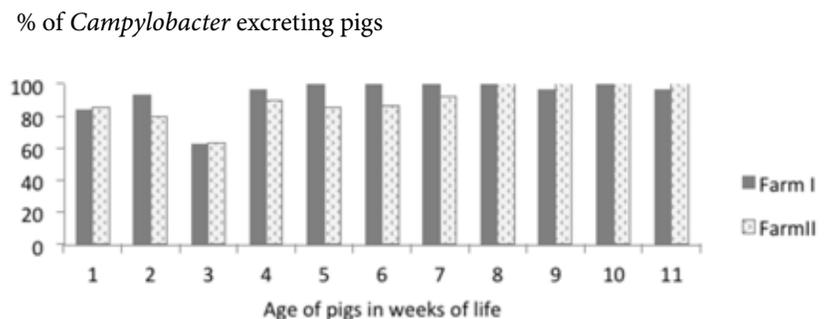


Figure 1: Evolution of the percentage of young pigs excreting *Campylobacter* in their faeces

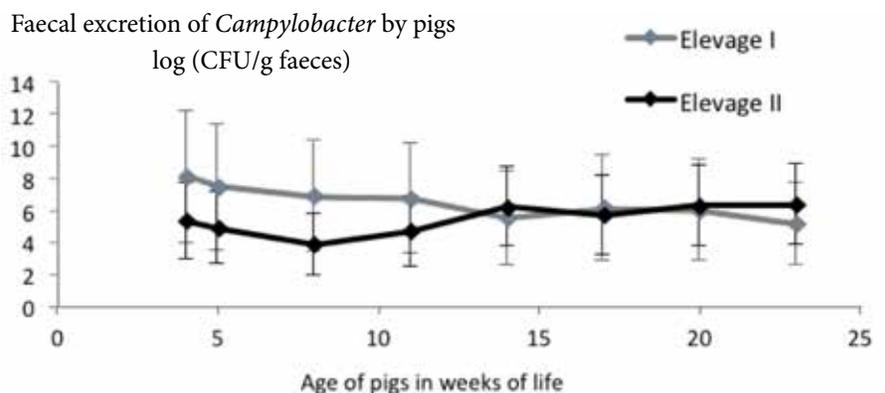


Figure 2: Faecal excretion of *Campylobacter* by young pigs

increased with the age of the pigs. There is no significant correlation between the level of *Campylobacter* excretion by pigs and the contamination level of the environment, whatever the samples (walls or floors of the pens and food) ($P>0.05$).

All *Campylobacter* isolates from faecal samples of sows and pigs, environmental samples and feed were identified as *C. coli*.

Discussion

The objective of this study was to describe the dynamics of *Campylobacter* excretion in a conventional farrow-to-finish farm and to assess the role of the environment as a potential source of contamination. The originality of this study was the individual follow-up of the same animals all along a production. Indeed, in the other studies, the comparisons of prevalence or excretion levels of *Campylobacter* between pigs at various stages have been done on different animals, at one sampling time or on a grouped way (Alter *et al.*, 2005; Weijtens *et al.*, 1993; Weijtens *et al.*, 1999).

In this survey, the high prevalence observed for sows throughout a production cycle is close to that found in the literature. Nevertheless, this result has to be taken with caution considering the low number of individuals sampled (5 sows per farm). Regarding the piglets, 84 and 86% of them are contaminated at the first sampling time, 3 to 4 days after their birth. This early contamination of piglets was similar to the results previously described (Weijtens *et al.*, 1997; Weijtens *et al.*, 1999).

In our study, the early contamination of the piglets seems to be due to the contact with their mothers as the environment was negative during the down period (after the cleaning and disinfection process) as well as feed and water of the farrowing unit. Moreover, sows excrete high quantities of *Campylobacter* which could promote the contamination of the piglets. The role of the sows as the first contamination source of *Campylobacter* for their piglets was previously described (Weijtens *et al.* 1999; Hume *et al.*, 2002; Alter *et al.* 2005).

Besides, the progressive increase of the prevalence of young pigs' infection is in accordance with the results of Weijtens *et al.* (1997). However, like Alter *et al.* (2005) we did not observe a decrease of the percentage of carrier pigs during the fattening period as observed by Weijtens *et al.* (1993). To our knowledge, the punctual decrease of prevalence (60% of the piglets) observed in both farms at the third week of life has not been reported previously. The origin of this decrease is difficult to explain and not linked to bias of bacteriological method as the positive sows samples have been treated at the same time.

For sows and for piglets, the amount of *Campylobacter* in faeces was highly variable (from 0 to 10^9 CFU/g). After the 5th week of life, all young pigs became shedders until the end of the study with a level of excretion similar to those found in previous studies, except for one animal. Nevertheless, similar to previous findings (Weijtens *et al.*, 1999), variations in the average colony count of *Campylobacter* in the faeces between both animals and samples from a given animal were observed in our trial. *Campylobacter* could not be detected in one animal at one time point whereas high counts were observed in faeces from the same pigs at previous and following sampling times. This situation was previously described in an experimental trial (Leblanc-Maridor *et al.*, 2008). These observations suggest an intermittent excretion of *Campylobacter* or elimination followed by re-contamination of pigs by *Campylobacter* (Weijtens *et al.*, 1999).

No significant correlation has been found in our study between the contamination level of the environment and the excretion level of the pigs. However, the environment can play a role as source of indirect contamination for pigs, especially due to pig having frequent oral contacts with their environment. In this work, the resistance of *Campylobacter* in the environment in a pig farm seems limited as shown by a high number of negative environmental samples in the presence of the animals on dirty soil. In the same way, no positive environmental samples have been observed after the down period showing that good measures of hygiene between two batches (cleaning, disinfection, down period) allow the elimination of *Campylobacter* in the concerned rooms. The epidemiological role of the environment in the infection dynamics and the excretion pattern of *Campylobacter* by pigs might be limited if this environment is not constantly recontaminated by pigs faeces. Weijtens *et al.* (2000) underlined in a study that *Campylobacter* infection in pig farm can be reduced even eliminated through the implementation of strict hygienic measures associated with a repopulation with *Campylobacter* non-carriers pigs. Among the feed samples taken into the troughs in presence of animals, few were positive due probably to a contamination *via* the faecal material (the presence of faeces into the trough has been sometimes observed or strongly suspected). These results highlight that feed could play a role in indirect transmission of *Campylobacter* between pigs.

Conclusion

Our study confirmed the high prevalence of *Campylobacter* infection for sows and pigs in conventional farms all along a production cycle. The early contamination of the piglets could be mostly due to the contact with their mothers. Even if no significant correlation has been shown between the environment contamination and the excretion levels of *Campylobacter*

by the animals, the environment appeared frequently contaminated notably by faecal material (walls, floor and structures of the pen/room, feed in the troughs) and is probably an element of *Campylobacter* transmission between animals.

References

- Alter, T., Gaull, F., Kasimir, S., Gurtler, M., Mielke, H., Linnebur, M., Fehlhaber, K., 2005, Prevalences and transmission routes of *Campylobacter* spp. strains within multiple pig farms. *Vet. Microbiol.* 108, 251-261.
- EFSA 2007. The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2006, Journal, T.E., ed. (EFSA), p. 130.
- Hume, M.E., Droleskey, R.E., Sheffield, C.L., Harvey, R.B., 2002, *Campylobacter coli* pulsed field gel electrophoresis genotypic diversity among sows and piglets in a farrowing barn. *Curr. Microbiol.* 45, 128-132.
- Leblanc Maridor, M., Denis, M., Lalande, F., Beaurepaire, B., Cariolet, R., Fravallo, P., Federighi, M., Seegers, H., Belloc, C., 2008, Experimental infection of specific pathogen-free pigs with *Campylobacter*: excretion in faeces and transmission to non-inoculated pigs. *Vet. Microbiol.* 131, 309-317.
- Leblanc-Maridor, M., Beaudeau, F., Seegers, H., Denis, M., Belloc, C., 2011, Rapid identification and quantification of *Campylobacter coli* and *Campylobacter jejuni* by real-time PCR in pure cultures and in complex samples. *BMC Microbiol.* 11, 113.
- Weijtens, M.J., Bijker, P.G., Van der Plas, J., Urlings, H.A., Biesheuvel, M.H., 1993, Prevalence of *Campylobacter* in pigs during fattening; an epidemiological study. *Vet. Q* 15, 138-143.
- Weijtens, M.J., van der Plas, J., Bijker, P.G., Urlings, H.A., Koster, D., van Logtestijn, J.G., Huis in't Veld, J.H., 1997, The transmission of *Campylobacter* in piggeries; an epidemiological study. *J. Appl. Microbiol.* 83, 693-698.
- Weijtens, M.J.B.M., Reinders, R.D., Urlings, H.A.P., Van der Plas, J., 1999, *Campylobacter* infections in fattening pigs; excretion pattern and genetic diversity. *J. Appl. Microbiol.* 86, 63-70.
- Weijtens, M.J.B.M., Urlings, H.A.P., Van der Plas, J., 2000, Establishing a *Campylobacter*-free pig population through a top-down approach. *Letters in Appl. Microbiol.* 30, 479-484.

Potential alternatives to antimicrobials in pig production based on perceived effectiveness, feasibility and return on investment.

Merel Postma ^{(1)*}, Elisabeth grosse Beilage ⁽²⁾, Catherine Belloc ⁽³⁾, Denise Iten ⁽⁴⁾, Elisabeth Okholm Nielsen ⁽⁵⁾, Annette Backhans ⁽⁶⁾, Katharina D.C. Stärk ⁽⁷⁾, Jeroen Dewulf ⁽¹⁾

⁽¹⁾ Ghent University, Faculty of Veterinary Medicine, Department of Reproduction, Obstetrics and Herd Health, Unit of Veterinary Epidemiology, Merelbeke, Belgium

⁽²⁾ Field Station for Epidemiology, University of Veterinary Medicine Hannover, Bakum, Germany,

⁽³⁾ Oniris, Nantes, France

⁽⁴⁾ ETH Zürich, Institute for Environmental Decisions (IED) Consumer Behavior, Zürich, Switzerland,

⁽⁵⁾ Danish Agriculture & Food Council, Pig Research Centre, Copenhagen, Denmark,

⁽⁶⁾ Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden,

⁽⁷⁾ SAFOSO AG, Bern, Switzerland

* Salisburylaan 133, 9820, Merelbeke, Belgium.

E-mail: Merel.Postma@UGent.be; tel: 0032 9 264 75 48; www.minapig.eu

Abstract

Based on a questionnaire returned by 111 pig health experts from six European countries a list of potential alternatives to antimicrobials was ranked. These alternatives provide input for further studies to find strategies to reduce the widely discussed use of antimicrobials and the potential risk from antimicrobial resistance. Based on their scores for effectiveness, feasibility and return on investment, the most promising alternatives according to the consulted experts are believed to be: improvement of biosecurity measures, increased/improved vaccination, use of zinc/metals, improvement of feed quality and the use of regular diagnostic testing combined with a clear action plan. The MINAPIG consortium will use these results as input for future studies.

Introduction

Reduced antimicrobial usage in livestock is widely discussed and highly promoted in Europe and worldwide as a measure to reduce antimicrobial resistance. However, to guarantee animal health, welfare and economic viability, effective and efficient alternatives are necessary. Pig health experts were asked to provide their perception of the usefulness of different alternatives to antimicrobials. The aim of this study was to distillate a list of potential alternatives that could be used as input for further studies.

Material and Methods

A paper or web-based questionnaire was sent to porcine veterinary experts in Belgium, Denmark, France, Germany, Sweden and Switzerland. The participants were asked to score pre-listed alternatives to antimicrobial usage regarding expected effectiveness, feasibility and return on investment (ROI) between 0= not effective/feasible/economical and 10= highly effective/feasible/economical. The pre-listed alternatives were: Financial/tax (increased price of products), Benchmarking of farmers/veterinarians, Communication/unified advice (improved communication between farmer and veterinarian, all herd advisors promote same strategy), Diagnostics/action plan (herd specific action plan based on diagnostics and historical data), High health/SPF/eradication programs, Genetics, Increased vaccination, Strict euthanasia policy, Increased use of anti-inflammatory products, Improved external biosecurity, Improved internal biosecurity, Age and transfer management (minimized number of movements and at older age), Reduced stocking density, Improved climate/environmental conditions, Improved water quality, Acidification of feed or water, Optimization of feed quality, Use of feed additives and Use of Zinc/metals. The alternatives were provided with a short explanation. The experts were also asked to provide their professional occupation. Data were analyzed using Microsoft Excel 2010 and IBM SPSS version 21.

Results

A total of 111 experts returned the questionnaire, ranking at least 8 out of 19 alternatives (Belgium n=24, Denmark n=30, France n=8, Germany n=17, Sweden n=23, Switzerland n=9). Response rates were minimum 40% and maximum 94%. The majority of respondents were veterinary practitioners (n=53). The other respondents were: professors/teachers (n=13), researchers (n=20), nutritionists (n=6), technical consultants of the pharmaceutical industry (n=8) and other advisors (n=11).

The average scores of all respondents resulted in the following top 5 per scoring parameter:

Top 5 Effectiveness: Improved internal biosecurity ($\mu=8.21$, $SD=1.91$), improved external biosecurity ($\mu=7.85$, $SD=1.83$), improved climate/environmental conditions ($\mu=7.75$, $SD=1.63$), increased vaccination ($\mu=7.64$, $SD=1.58$) and high health/SPF/disease eradication ($\mu=7.64$, $SD=2.13$).

Top 5 Feasibility: Increased vaccination ($\mu=7.32$, $SD=1.79$), increased use anti-inflammatory products ($\mu=7.30$, $SD=2.11$), improved water quality ($\mu=7.18$, $SD=2.10$), feed quality/optimization ($\mu=7.15$, $SD=1.96$) and use of zinc/metals ($\mu=7.13$, $SD=2.85$).

Top 5 ROI: Improved internal biosecurity ($\mu=7.61$, $SD=1.75$), use of zinc/metals ($\mu=6.99$, $SD=2.39$), diagnostics/action plan ($\mu=6.94$, $SD=2.07$), feed quality/optimization ($\mu=6.90$, $SD=2.28$) and climate/environmental improvements ($\mu=6.86$, $SD=1.96$).

Combining the scores for Effectiveness, Feasibility and ROI resulted in the following top 5:

Improved internal biosecurity ($\mu=7.49$), increased vaccination ($\mu=7.24$), use of zinc/metals ($\mu=7.21$), feed quality/optimization ($\mu=7.20$) and diagnostics/action plan ($\mu=7.04$).

On average researchers and professors/teachers preferred diagnostics and action plans as respectively the best or second best alternative related to the overall score of the three scoring parameters. While for example practitioners placed this alternative in sixth position.

Nutritionists and other advisors, such as pig consultants and veterinary managers, saw the use of zinc/metals as the most important alternative. Those experts focused in general more on the alternatives related to feed, water and zootechnical improvements such as climate and environmental, while professors, teachers and consultants from the pharmaceutical industry gave higher rankings to biosecurity measures and increased vaccination.

Financial/tax as an alternative was in the bottom three ranking for all profession categories. Benchmarking of veterinarians and farmers ended up in the middle regions of the ranking for most profession categories, except for the practitioners, they placed this alternative fourth last.

Discussion

Response bias might be relevant in this study, although the order of the pre-listed alternatives and the additional explanatory information were carefully designed to minimize this bias risk.

Some countries, such as The Netherlands (SDa, 2013) and Denmark (Alban et al., 2013), already have

some benchmarking measures in place, combined with a low or even obligated strict reduction of antimicrobial usage in livestock production. Furthermore some EU countries allow the usage of zinc in weaned piglets. However, use of zinc will or legally might not be implementable in all countries. As a single measure they are unlikely to sufficiently reduce antimicrobial usage without affecting production. Other (combinations of) alternatives, focusing on the production of healthy animals in an optimized environment are necessary. The experts ranked improvement in biosecurity and climate/environment highest in effectiveness, indicating that there is room for improvement in these areas.

For practitioners the average scores vary less, with the lowest maximum average score and highest minimum average score, indicating that they perceive relatively more importance for all proposed alternatives.

Although a limited number of experts from a limited number of EU countries were included in this study, conclusions can be drawn from their perceptions on alternatives to antimicrobial usage.

Conclusion

These results provide a first impression on the experts' opinion on possible alternatives to antimicrobial usage in pig production. Biosecurity improvements, increased vaccination, use of zinc/metals, improvement of feed quality and use of regular diagnostics testing and a clear action plan score high on all criteria. Based on our results, these measures are believed to be the most promising alternatives to antimicrobial usage. The MINAPIG consortium will add complementary information and use these results to design future field studies.

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References

Alban, L., Dahl, J., Andreasen, M., Petersen, J.V., Sandberg, M., 2013. Possible impact of the yellow card antimicrobial scheme on meat inspection lesions in Danish finisher pigs. *Preventive Veterinary Medicine* 108, 334-341.

SDa, 2013. The Netherlands Veterinary Medicines Authority.

Assigning Defined Daily Doses Animal: a European multi-country experience.

Merel Postma^{(1)*}, Marie Sjölund⁽²⁾, Lucie Collineau⁽³⁾, Svenja Loesken⁽⁴⁾, [Elisabeth Okholm Nielsen](#)⁽⁵⁾, Katharina D.C. Stärk⁽³⁾, Jeroen Dewulf⁽¹⁾ on behalf of the MINApig consortium.

⁽¹⁾ *Veterinary Epidemiology Unit, Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Belgium*

⁽²⁾ *Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute, Uppsala, Sweden*

⁽³⁾ *SAFOSO AG, Bern, Switzerland*

⁽⁴⁾ *Field Station for Epidemiology, University of Veterinary Medicine Hannover, Bakum, Germany*

⁽⁵⁾ *Danish Agriculture & Food Council, Pig Research Centre, Copenhagen, Denmark*

* Salisburylaan 133, 9820, Merelbeke, Belgium.

E-mail: Merel.Postma@UGent.be; tel: 0032 9 264 75 48; www.minapig.eu

Abstract

In order to be able to compare antimicrobial usage data between countries with a uniform quantification method a Defined Daily Dose Animal (DDDA) per active substance was defined. Information of 731 antimicrobial products licensed for use in porcine medicine in four EU countries was used to establish mean DDDAs for 83 unique active substances (AS) including combinations of different AS. Common DDDAs were defined in spite of large variations in the authorized dosages for the same active substances and administration routes. These DDDAs will be used to quantify and compare antimicrobial usage in pig production in four EU countries.

Introduction

High antimicrobial usage in food producing animals is of major concern. To compare usage data between countries, a uniform quantification method is needed. This requires the definition of a Defined Daily Dose Animal (DDDA) for each active substance (AS) to be able to compare the number of animals treated based on the amount of antimicrobials used. Such DDDAs have not been defined in most countries. Therefore, we attempted to assign DDDAs for antimicrobials used in pig production in four EU countries.

Material and Methods

Based on the Summary of Product Characteristics (SPC), DDDAs (Dewulf.J. et al., 2012) were calculated for antimicrobial products licensed in Sweden (n=51), Germany (n=281), France (n=240) and Belgium (n=159). Products were categorized based on their AS(s), long acting characteristics and administration route. Dosages for the main indication were used to calculate the mean, modus and median doses for each AS including combinations of AS. When a dose range was indicated for an AS without stating a main indication, the minimum and maximum doses were used to calculate the mean dose for the particular AS. Dosages for combination products were calculated after adding all included ASs for each product. The mean dose obtained for each AS including combination products for the four countries was then determined to be the consensus DDDA. Long acting (LA) products received a LA factor depending on the duration of the activity to determine the number of DDDAs resulting from one treatment. Whether the product was categorized as critically important by the World Health Organization (WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2011) or World Organisation for Animal Health (75th International Committee, 2007) was registered as additional information.

Results

A total of 83 unique (including combinations of) AS were categorized. The most common administration routes were feed/water (n=360) and parenteral (n=327). Colistin (n=53) and amoxicillin (n=49) were the most commonly licensed antimicrobials. However, enrofloxacin (n=44) and ceftiofur (n=33), WHO and OIE critically important antimicrobials, were also well represented.

Large differences between SPC dosages were observed for the same AS and the same administration route. Differences were more prominent for older products in comparison to more recently authorized products. Large differences were seen in feed/water tetracycline (n=6, min=20 mg/kg, max=85 mg/kg), chlortetracycline (n=15, min=15 mg/kg, max=85 mg/kg) and oxytetracycline (n=19, min=20 mg/kg, max=100 mg/kg). There were also remarkable differences between countries

(i.e. spectinomycin oral drench, Belgium (n=1), authorized dose = 40 mg/kg, Germany (n=1), authorized dose = 150 mg/kg). Even within the same country the differences for some AS were huge (i.e. sulphaguanidin + sulphadimidin France, n=2, min=38.40 mg/kg, max=160 mg/kg and tylosin Belgium, n=7, min=4.5 mg/kg, max= 45 mg/kg). More recently registered products like enrofloxacin show minor deviations in authorized doses between countries (n= 38, min=2.5 mg/kg, max=3.8 mg/kg). European Union harmonized products like ceftiofur have, as harmonization suggests, the same authorized dose in all four countries. Parenteral dosages were generally lower compared to those for oral preparations.

Discussion

This report is the first attempt to define general DDDAs as far as the authors are aware. The listing and comparison of available products and their SPCs revealed remarkably huge variations in authorized doses of the same compound between countries and even between commercial products within countries making the selection of one defined daily dose difficult. Therefore criteria as described above were determined to allow an objective selection of the DDDAs. Since a large number of products from several countries were included, we consider the determined DDDAs as representative; however including more products from other countries will likely further influence the mean DDDAs. Also the numbers and kinds of marketed products changes over time which may further influence the mean DDDAs. To enhance comparability between countries it would be hugely beneficial if one, preferably global, list of DDDAs would be determined. Our effort may be a first step towards such a harmonization.

Conclusion

Comparison of antimicrobials licensed for pigs in four EU countries showed large variations in number and types of products registered as well as differences in dosages. Despite the large variations, a methodology was developed to define common DDDAs for used AS. These DDDAs will be used to quantify and compare antimicrobial usage in pig production in four EU countries.

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References

75th International Committee, 2007. OIE List of Antimicrobials of Veterinary Importance.

Dewulf, J., Moulin G., Catry B, Chauvin C., Greko C, Heederik D, Jacobsen E., van Geijlswijk I., McEwen S., Muentener C., Litleskare I., 2012. ESVAC reflection paper on collecting data on consumption of antimicrobial agents per animal species, on technical units of measurements and indication for reporting consumption of antimicrobial agents in animals.

WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), 2011. WHO list of Critically Important Antimicrobials (CIA) - Critically Important Antimicrobials for Human Medicine.

InVitro characterization of colistin resistance and transfer of neomycin resistance in *Escherichia coli* O149 strains

Thériault, W.^{1,2}, L-Lewandowski, S.¹, Archambault, M.², Letellier, A.^{*1,2}

¹ NSERC Industrial Research Chair in Meat Safety, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

² GREMIP Research Group on Infectious Diseases of Swine, CRIPA Centre de recherche en infectiologie porcine et aviaire and GRESA, groupe de recherche et d'enseignement en salubrité alimentaire, Faculty of veterinary medicine, Université de Montréal, Saint-Hyacinthe, Canada

*3190 rue sicotte, St-hyacinthe, Québec, Canada, J2S 8H5

e-mail: ann.letellier@umontreal.ca

Tel :450-778-7128

Abstract

Neomycin, an aminoglycoside, is used at farm level for *Escherichia coli* treatment of piglets postweaning diarrhea, with 40% of unsuccessfully treated due to the antimicrobial resistance. To overcome this situation, veterinarians use colistin also called polymyxin E, but this antibiotic is not homologated in Canada, even if it seems to be effective against *E.coli*. The described resistance to polymyxin is associated to a modification of the LPS core and the lipid A regions in the bacteria. For *Salmonella*, those modifications are associated to the two components system PmrA-PmrB. This system is known in *E.coli* but not yet reported to be implicated in colistin resistance. The neomycin resistance is linked to enzymatic modifications associated to genes located on plasmids. This study has for objectives to investigate the acquisition of colistin resistance and study the transfer of neomycin resistance between *E.coli* strains and other enterobacteria. *E.coli* O149 strains isolated from clinical cases (2008 to 2011) were used and susceptibility testing of strains was performed by the disk diffusion method. E-test and a micro-dilution method were used to determine the minimal inhibitory concentration (MIC) of colistin and neomycin respectively. All tested strains had a MIC higher than 128 ppm for neomycin whereas MICs were between 0.064 to 0.128 ppm for colistin, indicating that all strains were resistant to neomycin but all susceptible to colistin. In these isolates, neomycin antimicrobial resistance genes *aac(3)-IV*, *apkA1* and *aphA2* have been detected using PCR. Conjugation experiments are presently being performed. Colistin natural mutants ($n=22$) were created through serial passages on LB agar with 25xMIC. Sequencing of the PmrA-PmrB region of these mutants was performed to identify mutations. Three PmrA and seven PmrB mutations were for the first time reported in *E.coli* O149. Other mutants are still under investigation for other possible resistance mechanisms.

Introduction

Escherichia coli O149 is the major cause of post-weaning diarrhea with an important economic impact. Veterinarians use neomycin, an aminoglycoside for treatment of this condition. Unfortunately near 40% of the treatments are unsuccessfully due to *E.coli* resistance to this antibiotic (Maynard et al., 2003). The most frequently associated mechanism of resistance is by enzymatic modification, acetylation and phosphorylation, of the neomycin. Three genes are mostly associated with neomycin resistance; *acc(3)-IV*, *apk(3')-I*, *aph(3')-II* and most of the time, we find those genes on plasmids of different size (4 to 93kb) (Shaw et al., 1993). To overcome this situation, veterinarians use colistin sulphate (CS), a polypeptide antimicrobial also called polymyxin E, but this antibiotic is not authorized in Canada even if it seems to be effective against *E.coli*, because it is used in human medicine for *Pseudomonas aeruginosa* in cystic fibrosis. In other countries, this polypeptide antibiotic is recommended for the oral therapy of intestinal infections in pig. The target of CS is the Gram negative bacteria lipopolysaccharide (LPS) molecule by a cationic interaction leading to a displacement of cation magnesium Mg^{2+} and calcium Ca^{2+} causing a leakage of intracellular contents and bacterial death (Wang and Quinn, 2010). Resistance to colistin has been reported in *Salmonella* sp. by the two components system PmrAB. This system is associated with a modification of the LPS by adding a L-ara4N group to LPS core causing a reduction of negative charge, leading to a less negative LPS charge (Boll et al., 1994). A mutation in those genes could lead to a constitutively active system leading to a CS resistance by decreasing the cationic liaison between CS and LPS. Therefore, the aim of this study was to first evaluate the *in vitro* acquisition of CS resistance and second to assess the *in vitro* transfer of neomycin resistance.

Material & method

Eight strains of *E.coli* O149 isolated from clinical cases (2008 to 2011) by ECL lab were selected for neomycin resistance. Susceptibility testing was performed by the disk diffusion method. Briefly, a dilution corresponding to a McFarland 0,5 was made in saline solution and uniformly plated on Mueller-Hinton (MH) agar, disks were aseptically added and plates were incubated overnight at 37°C. E-test was used to determine the MIC of colistin, briefly an uniformly plate was made with a dilution corresponding to a McFarland 0,5 on MH agar, E-test bands were aseptically added and plates were incubated overnight at 37°C. For neomycin MIC, a micro-dilution method was used. Briefly, in sterile 96-well polystyrene microtitre plates, serial double dilution were made in MH broth from 128 mg/L to 0.25 mg/L. Each well was inoculated with approximately 5×10^4 CFU/ml of *E.coli* strains in MH broth. The MIC was determined as the lowest concentration that resulted in inhibition of bacterial growth. In all MIC experiments, ATCC 25922 *E.coli* strain was used as control. For the 8 *E. coli* strains, PCR was used to determine the presence of antimicrobial resistance genes *acc(3)-IV* (F-GTGTGCTGCTGGTCCACAGC R-AGTT-GACCCAGGGCTGTCGC), *apk(3')-I* (F-ATGGGCTCGCGATAATGTC R-CTCACCGAGGCAGTTCAT) and *aph(3')-II* (F-GAACAAAGATGGATTGCACGC R-GCTCTTCAGCAATATCACGG).

Colistin mutants were created by incubation of each strain overnight at 37°C, on Luria-Bertani broth (LB) with a colistin concentration under the MIC. Serial passages were done on LB with 25xMIC for mutants selection. Mutant DNA was extracted by standard boiling method. Amplification of genes *pmrA* and *pmrB* was performed by PCR using respectively primers F-CAAACCTGACAGGAGAGT-GAG R-GCTGATCAGCTCAAACACCA and F-GGCTTTGGCTATATGCTGGT R-TTAACTACCGTGTTCAGCGT. PCR products were purified with PureLink® PCR Purification Kit (Invitrogen, Canada). Sequencing was performed by Sanger (Genome Québec). Alignments of sequence were done with Clustal X software.

Results

For the first objective on antimicrobial resistance, all tested strains had a MIC higher than 128 ppm for neomycin whereas MICs were between 0.064 to 0.128 ppm for colistin, indicating that all strains were resistant to neomycin but all susceptible to colistin. PCR detection of neomycin resistance genes showed that *apk(3')-I* was found in six strains. Strains 1000 and 2000 have two resistance genes respectively *acc(3)-IV*, *apk(3')-I* and *acc(3)-IV*, *aph(3')-II*. (Fig 1)

Mutants were created (n=24) and sequencing showed 7 different mutation sites in *pmrAB* region leading to a colistin resistance phenotype. Most of the mutations were found in mutants from *E.coli* ATCC 25922 strain and these mutations were observed in the *pmrB* gene. (Fig 2)

Strain ID	Years	Virotype	MIC Neomycin (µg/ml)	Resistance genes	MIC Colistin (µg/ml)
1000	2008	LT:STb:Paa:F4	512	<i>acc(3)-IV</i> , <i>apk(3')-I</i>	0,064- 0,094
2000	2009	LT:STb:East1:F4	128	<i>acc(3)-IV</i> , <i>aph(3')-II</i>	0,094- 0,125
3000	2009	LT:STb:Paa:F4	128	<i>apk(3')-I</i>	0,047- 0,064
4000	2009	LT:STb:Paa:F4	128	<i>apk(3')-I</i>	0,094
5000	2010	LT:STb:East1:F4	128	<i>apk(3')-I</i>	0,125
7000	2010	LT:STb:F4	256	<i>apk(3')-I</i>	0,125
8000	2010	LT:STb:East1:Paa:F4	512	<i>apk(3')-I</i>	0,064

Fig 1: Table of seven strains of *E.coli* O149 isolated from field with years of isolation, virotype, MIC to neomycin (µg/ml), MIC to colistin (µg/ml) and neomycin resistance genes

Mutant ID	Gene	Genotype	MIC (µg/ml)
<i>E.coli</i> Lab strain ATCC 25922			
0004	<i>pmrA</i>	A80V	6
0001	<i>pmrA</i>	N128I	6
0001	<i>pmrA</i>	S144G	6
0001	<i>pmrB</i>	D65E	6
0003	<i>pmrB</i>	T98M	12
0006	<i>pmrB</i>	V103G	3
O149 field strain causing post-weaning diarrhea			
5001	<i>pmrB</i>	V87E	3-4
2003	<i>pmrB</i>	F145Y	6
2003	<i>pmrB</i>	D148Y	6
7003	<i>pmrB</i>	D148V	8

Fig 2: Table of *E.coli* mutants colistin resistant with the mutation genes, the genotype and the MIC of colistin in (µg/ml). A: Alanine, V: Valine, N: Asparagine, I: Isoleucine, T: Threonine, M: Methionine, G: Glycine, E: Glutamate, D: Aspartate

Discussion

Study of colistin resistance

This study is the first to report exact mutation in *pmrAB* genes leading to colistin resistance in *E.coli* O149 field strains and *E.coli* ATCC 25922 strain. In another study (Sun et al., 2009), *pmrAB* mutations were identified for *Salmonella* sp. In our study, mutants had higher MIC values (30 to 80x) comparatively to *Salmonella* mutants (2 to 35x) suggesting that the site of mutation in *pmrAB* is probably linked to the degree of resistance. We also create mutants without a *pmrAB* mutation suggesting another mechanism of resistance. Other genes have been reported to have a higher expression in a bile salt medium leading to colistin resistance (Kus et al., 2011). The *arn* operon genes which are involved in the synthesis and transport of the L-ara4N subunit (Wang and Quinn, 2010) and *acrAB* genes who are involved in a multidrug efflux system are potential other genes implicated in the colistin resistance without a *pmrA* or *pmrB* mutation.

Conclusion

This study showed mutations in *pmrAB* genes leading to colistin resistance in *E.coli* strains. A better understanding of the mechanism of resistance to colistin is needed for a better use of this antibiotic and avoid therapeutic practices having an impact of antimicrobial resistance of *E.coli* associated to postweaning diarrhea or other disease. Further studies are on going to characterized mutations not associated to *prmA* / *prmB* genes.

References

- Boll, M., Radziejewska-Lebrecht, J., Warth, C., Krajewska-Pietrasik, D., Mayer, H., 1994. 4-Amino-4-deoxy-L-arabinose in LPS of enterobacterial R-mutants and its possible role for their polymyxin reactivity. *FEMS Immunol. Med. Microbiol.* 8, 329-341.
- Kus, J.V., Gebremedhin, A., Dang, V., Tran, S.L., Serbanescu, A., Barnett Foster, D., 2011. Bile salts induce resistance to polymyxin in enterohemorrhagic *Escherichia coli* O157:H7. *J. Bacteriol.* 193, 4509-4515.
- Maynard, C., Fairbrother, J.M., Bekal, S., Sanschagrín, F., Levesque, R.C., Brousseau, R., Masson, L., Larivière, S., Harel, J., 2003. Antimicrobial resistance genes in enterotoxigenic *Escherichia coli* O149:K91 isolates obtained over a 23-year period from pigs. *Antimicrob. Agents Chemother.* 47, 3214-3221.
- Shaw, K.J., Rather, P.N., Hare, R.S., Miller, G.H., 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* 57, 138-163.
- Sun, S., Negrea, A., Rhen, M., Andersson, D.I., 2009. Genetic analysis of colistin resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob. Agents Chemother.* 53, 2298-2305.
- Wang, X., Quinn, P.J., 2010. Lipopolysaccharide: Biosynthetic pathway and structure modification. *Prog. Lipid Res.* 49, 97-107.

Assessment of human risk for yersinosis after consumption of Danish produced non-heat-treated ready-to-eat pork products.

Sandberg, M.*⁽¹⁾, Hansen, F.⁽²⁾, Christensen, H.⁽²⁾, Alban, L.⁽¹⁾

⁽¹⁾ Danish Agriculture and Food Council, Axeltorv 3, 1609 Copenhagen, Denmark

⁽²⁾ Danish Technological Institute - Danish Meat Research Institute, Maglegårdsvej 2

4000 Roskilde, Denmark

*corresponding author: msa@lf.dk

Abstract

The objectives were to evaluate the risk of obtaining an infective dose of *Yersinia enterocolitica* (*Y. enterocolitica*) after consuming fermented sausages (made in a controlled process) and smoked filet made of Danish pork. For fermented sausages it was estimated that a maximum of two bacteria would be present in a serving of up to 40 g. However, most likely only one bacterium would be present per serving (4,000 times of one million simulations of 40 g servings = 0.4 %). Only in 27 times of one million simulated serving's there were two bacteria present. For smoked filet it was estimated a presence of a maximum of one *Y. enterocolitica* per 40 g servings. The results showed that this would happen in 39 times of a total of one million simulations of 40 g servings. It has been shown that *Y. enterocolitica* does not grow within the given shelf-life of these products, and the infection dose for *Y. enterocolitica* is assessed to be relatively high. Given this, it is therefore unlikely that Danish produced fermented sausages (made in a controlled process) and smoked filet are the cause of any of the annually reported 200-300 cases of human yersinosis in Denmark.

Introduction

There are reported 200-300 cases of human yersinosis annually in Denmark.

A series of challenge tests in pork, using a real-time PCR-method, were conducted in the project «*Yersinia* in non-heat treated meat». The aim of these challenge-test studies was to measure reduction of the concentration of *Y. enterocolitica* during fermentation of sausages and production of the Danish speciality; «smoked filet» made of respectively pork shoulder and loin.

In this study the collected data in the above described projects, was used as inputs in a simulation model with the objective of evaluating the risk of acquiring an infective dose of *Y. enterocolitica* by consuming non-heat treated meat products made of Danish pork.

Material and Methods

The model:

A simulation model originally developed for estimating human risk of *Salmonella* Typhimurium DT104 infections caused by consumption of fermented sausage was used (Alban et al. 2002).

Modelling Y. enterocolitica in a batch of minced meat

Semi-quantitative data obtained from a screening of Danish and German pork conducted in 2010-2011 was used to model the prevalence and concentration of *Y. enterocolitica* in a 240 kg's batch of minced meat - a standard size when producing fermented sausages and smoked filet (Larsen et. al 2012).

A Pert distribution was used to model the prevalence of the contaminated meat obtained from the different abattoirs: minimum = 21 %, most likely = 42 % and maximum = 63 % for shoulder meat, and minimum = 4 %, most likely = 8 % and maximum = 12 % for loin.

The number of bacteria (in the final products) was estimated under the assumption that a batch of pork of 240 kg contained 960 pieces of meat. A risk discrete distribution was used for all the 960 pieces, where the possible number of *Y.*

enterocolitica was combined with the frequency distribution of samples and the “abattoir-prevalence”. The resulting number of *Y. enterocolitica* for each of the 960 pieces was then summed up to a total number of bacteria’s present in a positive batch.

There was also assumed that *Y. enterocolitica* was homogenously distributed in the minced meat/sausage and in the meat that would be used to make smoked filet. The latter was put in a salt water/solution and salt/water solution was injected into the filet – also assumed to fulfil the assumption of homogenous distribution of the *Y. enterocolitica* present.

Modelling reduction of Y. enterocolitica concentration during fermentation/smoking-process of pork

The *Y. enterocolitica* reduction obtained during production of fermented sausages and smoked filet were estimated, in the model, based on inputs of results obtained in the challenge-tests (Hansen 2011a and Hansen 2011b).

The reduction obtained during fermentation of sausages under controlled conditions were; from 2.0 to 4.5 log cfu– of which we modelled by a Pert distribution; 2.0 log cfu = minimum, 2.4 log cfu = most likely, 3.5 log cfu = maximum (Hansen 2011b).

For a standard smoked filet, reduction of 1.9 to 4.7 log cfu were obtained, and the values put into the Pert distribution in the model were hence; minimum = 1.9 log cfu, most likely = 2.2 log cfu and maximum = 3.2 log cfu (Hansen 2011a).

The probable number of bacteria in the raw material was, in the model, compared with the obtained reduction in concentration measured in the challenge tests. By doing that, an estimate for the number of *Y. enterocolitica* in one gram of the final products was obtained.

Modelling of Y. enterocolitica in servings

Further the number of *Y. enterocolitica* in servings of 10 g, 25 g and 40 g fermented sausage/smoked filet were estimated. A 10 g serving is a typical weight of a slice on a sandwich made for children, whereas 25 g is a standard weight of a slice on a sandwich for adults. Further, a typical weight of a slice of fermented sausage/smoked filet on a large sandwich for adults would be 40 g.

Dose response for Y. enterocolitica in humans

An estimation of the likely annual number of human cases was not conducted since there is, as far as the authors know, no dose response model available for *Y. enterocolitica*. Nor does outbreak data exists that could have been used to make attack rates. Neither could we find experimental data published, except from the case-story reporting about a person turning ill after eating a dose of 3.5×10^9 yersinia-bacteria (Lake & Hudson 2004). In the literature it is given that the infective dose for *Y. enterocolitica* is $10^6 - 10^7$ - and it is not very likely that a human being could acquire infection when exposed to a low number of Yersinia ($< 10^3$). The assumption could therefore be that the infective dose is relatively high for healthy adults. For children, not previously exposed, the infective dose could, however, be lower.

Table 1. The distributions of samples collected from pork shoulder meat and loin with the estimated concentration (cfu/g) of *Y. enterocolitica*, and the estimated frequency distribution (Larsen et al. 2012)

Cuts	<i>Y. enterocolitica</i> /g	No. of samples	% distribution of samples
Shoulder	<0,05	67	53,2
	0,25	24	19,0
	2,5	21	16,7
	25	8	6,3
	100	6	3,3
	1000	-	1,0*
	5000	-	0,5*
Loin	<0,05	84	88,4
	0,25	11	10,0
	2,5	0	1,0
	25	0	0,5*
	100	0	0,1*
	1000	-	-
	5000	-	-

*Estimated based upon assumptions of a Normal distribution and assumptions of that a few samples could contain a high number of Yersinia.

Technical issues and software:

The data were modelled in @RISK (Palisade Corporation, Newfield NY), and the results are based on 1.000.000 simulations. The variation in the prevalence of contaminated chilled pork between abattoirs, and the variation in the bacteria concentration on the contaminated pieces of pork used for production of fermented sausage and smoked filet are included stochastically in the model. The Proc. lifereg (SAS[®]) was used to estimate the frequency-distribution of the different *Y. enterocolitica* concentrations obtained in the study of *Y. enterocolitica* in pork shoulder and loin by Larsen et al. (2012)

Results

There was, by the model, estimated an average content of 0.00013 *Y. enterocolitica* /g in a fermented sausage made of shoulder meat with a confidence interval of: 1.84×10^{-5} to 2.46×10^{-4} . Similarly, the estimated content of *Y. enterocolitica* in a smoked filet made of loin was 0.0000011 bacteria /g with a confidence interval of 2.7×10^{-7} to 2.89×10^{-6} . Table 2 sums up the estimated frequencies of servings of fermented sausages and smoked filet that contains *Y. enterocolitica*. The estimated distributions indicate that it is very unlikely that there will be more than two *Y. enterocolitica* per 10 g, 25 g or 40 g servings of fermented sausages. The frequency of servings with one bacterium is, on the other hand higher, depending on serving sizes; from 0.1 % to 0.4 %.

Discussion

Even though the results shows that servings of fermented sausages relatively often will contain *Y. enterocolitica*, the number of bacteria in a standard size of a serving of the final products would, however, be very low.

It is of importance to mention that it was shown that *Y. enterocolitica* does not grow within the given shelf-life of these products. Therefore, even in a situation with a very high daily intake of fermented sausages and smoked filets, it would not lead to a dose high enough (over days) to infect healthy human beings. However, for immune- suppressive people or people suffering from other disease, the situation could be different. Hence, in our opinion one have to seek elsewhere for cause of the annually reported 200-300 cases of human yersinose in Denmark.

Conclusion

By using the model, it was estimated; that a maximum of two bacteria would be present in a serving of up to 40 g of fermented sausages. However, more likely only one bacterium will be present per serving (4,000 times of one million simulations of 40 g serving's = 0.4 %). Only in 27 times of one million simulated serving's there were two bacteria present. For smoked filet it was estimated a presence of a maximum of one *Y. enterocolitica* per 40 g servings, of which could happen in 39 times of a total of one million simulations of 40 g servings.

It was shown that *Y. enterocolitica* does not grow within the given shelf-life of these products, and the infection dose for *Y. enterocolitica* is assessed to be relatively high. Given this, it is therefore unlikely that Danish produced fermented sausages (made in a controlled process) and smoked filet are the cause of any of the annually reported 200-300 cases of human yersinosis in Denmark.

Table 2. The estimated number of servings (10 g, 25 g, 40 g) with one or two *Y. enterocolitica* present.

Size of serving	No. of servings with <i>Y. enterocolitica</i> of a total of 1.000.000servings		
	0 bacteria	1 bacterium	2 bacteria
10 g fermented sausages	998999	1000	1
25 g fermented sausages	996987	3000	13
40 g fermented sausages	995973	4000	27
10 g smoked filet	999986	14	0
25 g smoked filet	999972	28	0
40 g smoked filet	999961	39	0

References

Anonym 1999. Fakta om fødevarehygiejne, Levnedsmiddelstyrelsen, Danmark.

Alban, L., et. al. 2002. Qualitative and quantitative risk assesment for human salmonellosis due to multi-resistant *Salmonella Typhimurium* DT104 from consumption of Danish dry-cured pork sausages, Prev. Vet. Med. 52, p. 251-265.

Larsen, H., Hansen, F. & Christensen, H. 2012. ”Forekomst af *Yersinia enterocolitica* i udskæringer af fersk svinekød af dansk og tysk oprindelse”, DMRI Rapport af 17. april 2012, Proj. Nr. 200245.

Hansen, F. 2011a. ”Pilot Plant forsøg med røget filet. Dokumentation af reduktion under nuværende processer/recepter” DMRI Rapport af 12. oktober 2011, Proj. Nr. 200247.

Hansen, F. 2011b. ”Pilot Plant forsøg med spegepølser. Dokumentation af reduktion under nuværende processer/recepter” DMRI Rapport af 12. oktober 2011, Proj. Nr. 200247.

Lake, R., & Hudson A. 2004. Risk profile: *Yersinia enterocolitica* in pork report prepared as part of a New Zealand Food Safety Authority contract for scientific services, made by Institute of Environmental Science & Research Limited Christchurch Science Centre, Website: www.esr.cri.nz, pp. 48.

Oral colistin sulfate in pigs: pharmacokinetics and effect on fecal *Escherichia coli* excretion of weaned pigs challenged with *Escherichia coli* F4 (K88)

Rhouma M^{1,2}, Thériault W^{1,2}, Bergeron N^{1,2}, Lewandowski S.L^{1,2}, Fairbrother J.M², Beaudry F³,

Letellier A^{*1,2}

¹Chaire de recherche en salubrité des viandes (CRSV), ²Groupe de recherche sur les maladies infectieuses du porc (GRE-MIP), ³ Groupe de recherche en pharmacologie animale du Québec (GREPAQ). Faculté de médecine vétérinaire – Université de Montréal (3200 Sicotte, Saint-Hyacinthe, QC, J2S 7C6, Canada)

*Corresponding author: ann.letellier@umontreal.ca

Abstract

Colistin sulfate (CS), a polymyxin antibiotic, is used in Canada for the treatment of post-weaning diarrhea in pigs as an alternative to neomycin. The aim of the present study was to evaluate some pharmacokinetics parameters of CS and its effect on the evolution of the intestinal *Escherichia coli* population in pigs challenged with enterotoxigenic *E. coli* (ETEC): F4. A total of 14 weaned piglets were divided into two groups, a non-challenged, treated group (n=7) and a challenged, treated group (n=7). Both groups received a single oral dose of CS at 50,000 IU/Kg. Challenge was carried out by oral administration of 10⁹ CFU of a hemolytic ETEC: F4 strain resistant to nalidixic acid. Blood samples were taken at 30 min and 1, 2, 4, 6, 8, 12, 24, 36 and 48 hours post treatment from each pig and CS quantification was performed by LC-MS/MS. In the challenged group, severity of diarrhea was monitored and the presence of the ETEC:F4 strain in the feces was enumerated using 5% bovine blood agar plates containing nalidixic acid. In both groups, total *E. coli* counts were carried out using Petrifilm *E. coli*/Coliform count plates. In both groups, the plasma concentration of CS was below the lower limit of quantification (20 ng/ml). Following CS treatment, a decrease in the total *E. coli* and ETEC: F4 fecal counts were observed at 24 h post treatment. The fecal consistency was not affected by CS treatment.

For the first time, a study of some CS pharmacokinetics parameters with a highly sensitive method showed that CS levels are not detectable in systemic circulation following oral administration, and concurrent oral challenge with an ETEC strain did not affect CS absorption. A single dose of CS resulted in reduced bacterial counts of the total *E. coli* and ETEC: F4 populations in the feces.

Introduction

Colistin (polymyxin E), a cationic antimicrobial peptide produced by *Bacillus polymyxa subsp. Colistinus*. This antibiotic is used in Canada for the oral therapy of intestinal infections in pigs, particularly those caused by *E. coli*. Colistin is used clinically in the form of its water-soluble colistin sulphate (CS) (Chauvin, Beloeil, Orand, Sanders, & Madec, 2002). In pigs receiving therapeutic doses by the oral route, it was found that CS was poorly absorbed, plasma concentrations being less than the lower limit of quantitation (0.250 µg/mL) of high-pressure liquid chromatography (Guyonnet et al., 2010).

The objective of the present study was to develop an analytical technique with high sensitivity for monitoring plasma concentrations of CS following oral administration, and to determine the effect of experimental infection with enterotoxigenic *E. coli* (ETEC):F4 (K88) on CS absorption level. And we also determined the effect of an oral dose of colistin (50.000 UI/kg), on the level of fecal shedding of total *E. coli* and of ETEC: F4 (K88).

Materials and methods

1. Animals

Fourteen clinically healthy piglets, 21 days of age at the beginning of experimentation, selected for the presence of F4 gene by PCR-RLFP were used in this study. Each pig was individually housed in a pen, fed a standard non-medicated ration for post-weaning pigs and received water *ad libitum*. Piglets were weighed at the beginning and end of the experiments. The temperature was kept at 24–26°C. Animals were allowed to acclimatize for 2 days before beginning of experiments.

2. Jugular catheterization of pigs and blood sampling

After the 2 days of acclimatization (23 d old), animals were restrained on a V-shaped table, and a catheter was inserted over the wire guide in the jugular vein as previously described (Matte, 1999). Blood samples (3mL) from the catheter were collected into EDTA tube, from one day after catheter placement (24 d old) until animal's euthanasia (32 d old). After CS oral administration (30 d old), blood samples (3mL) were collected, 30 min and 1, 2, 4, 6, 8, 12, 24, 36 and 48h following

CS administration. These samples were used to determine CS plasma concentrations by LC-MS/MS.

3. Experimental infection, antibiotic administration and health status

The challenge strain for experimental infection of pigs, was a nalidixic acid-resistant (Nal^r) variant of ETEC F4 strain ECL8559 (0149:LT: STa: STb: East1: paa: hemβ: F4), as previously described (Daudelin et al., 2011). At 27 days of age, 7 pigs were each orally challenged with 5 mL of trypticase soy broth containing 10⁹ CFU of the ETEC F4 challenge strain following the administration of 10 mL CaCO₃. At 30 days of age, each pig of the two groups of piglets received a single oral dose of colistin sulfate (50,000 IU/kg). Clinical examination included observation of fecal consistency, behavioural disturbances and presence of cyanosis. The severity of diarrhea was quantified by using a fecal consistency scoring (0, normal; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea).

4. Analytical methods

A high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) was used for CS quantification in plasma following an oral administration. The HPLC system was a Perkin-Elmer liquid chromatography apparatus (Series 200, Boston, MA), and the spectrometry system used was an API 2000 QTRAP (AB-Sciex, Concord, Canada). CS was extracted from pig's plasma by using a protein precipitation method. The lower limits of quantitation (LLOQ) of the method were 20 ng/mL of plasma. Chromatographic separation and the mass spectrometry detection were derived and optimized based on (Ma, Wang, Gerber, & Milne, 2008).

5. Microbiological analysis of fecal samples

Faecal samples were collected before challenge and 24, 48, 72, 84, 96, 108, 120 h post challenge. These samples were used to examine the presence of the challenge strain ETEC: F4 strain and total *E. coli* population. A quantity of 10 g of fecal samples was diluted 10-fold in peptone water and selected dilutions were plated on to Petrifilm *E. coli*/Coliform count plates, and 5% bovine blood agar plates, containing nalidixic acid, for counting of the total *E. coli* population and the haemolytic challenge ETEC F4 strain respectively. The plates were incubated aerobically for 24 h at 37°C.

Results

1. Quantification of plasmatic colistin sulfate concentration

For the two treated groups (infected and not infected), in all samples, the plasma concentration of CS was less than the lower limit of quantitation (20ng/mL). In the non-infected treated group the concentration of CS was greater than the limit of detection (LOD) after 30 minutes of CS administration but less than LLOQ (Figure 1). However, in the non-infected treated group, at all sampling times, the concentration of CS was less than the LOD (figure 2).

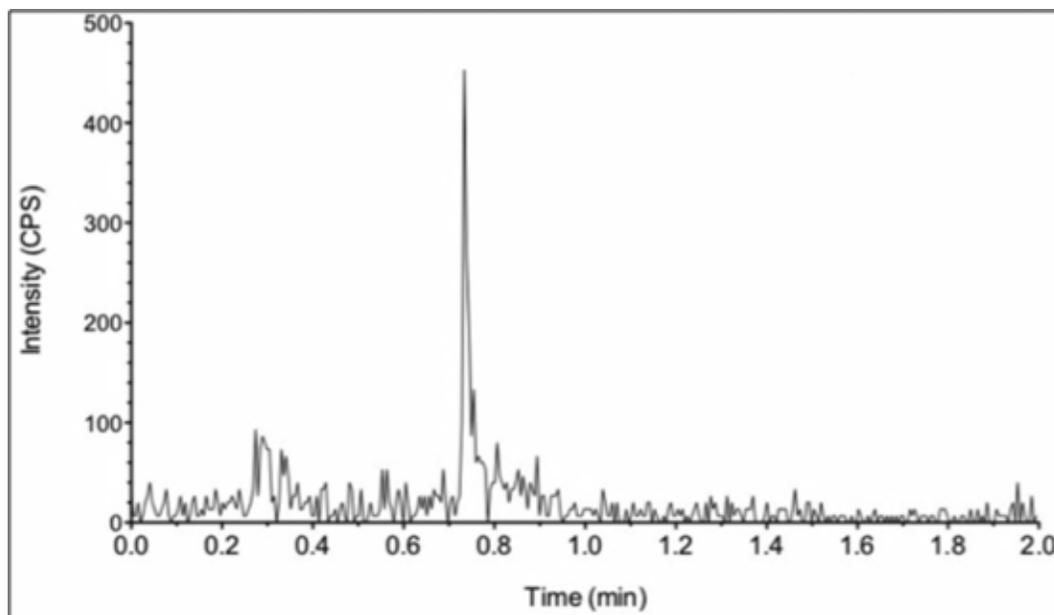


Figure 1: LC-MS/MS mass chromatogram of a typical sample from the non-infected group at 30 min following CS administration. Plasmatic concentrations of CS were above the limit of detection but significantly less than the limit of quantitation (20ng/mL). CS was not detected at other time points.

2. Analysis of Bacterial Shedding and Clinical Scoring of Diarrhea

None of the animals showed clinical diarrhea or had to be removed from the experiment due to illness. However, one animal in the infected treatment group was not used due to poor feed intake, and was consequently removed from the experiment. The mean number of ETEC: F4 colonies recovered from the faeces of the 6 experimentally challenged treatment pigs is shown in Figure 3. Experimental challenge was performed at 0 hour, and treatment with CS was carried out at 72h post challenge. The administration of a single oral dose of CS reduced mean fecal ETEC F4 counts during the next day following treatment; with a maximum effect at 24h post-treatment (96h post challenge). Nevertheless, the mean count following treatment was not significantly lower than the of baseline values (72h post challenge). At 24h post treatment, the fecal ETEC F4 numbers were increased to regain baseline values (Figure 3). In both treated groups, that is in challenged and non- challenged animals, total *E. coli* counts demonstrated the same trend of decline as observed for ETEC F4 (i.e maximum effect was observed at 24h post-treatment (96h post challenge). The highest mean diarrhea score of the challenged-treated groups was observed at 72h post challenge. After CS treatment, there was a tendency for score of diarrhea to decrease, with a maximum effect observed at 48h post treatment (120 h post challenge).

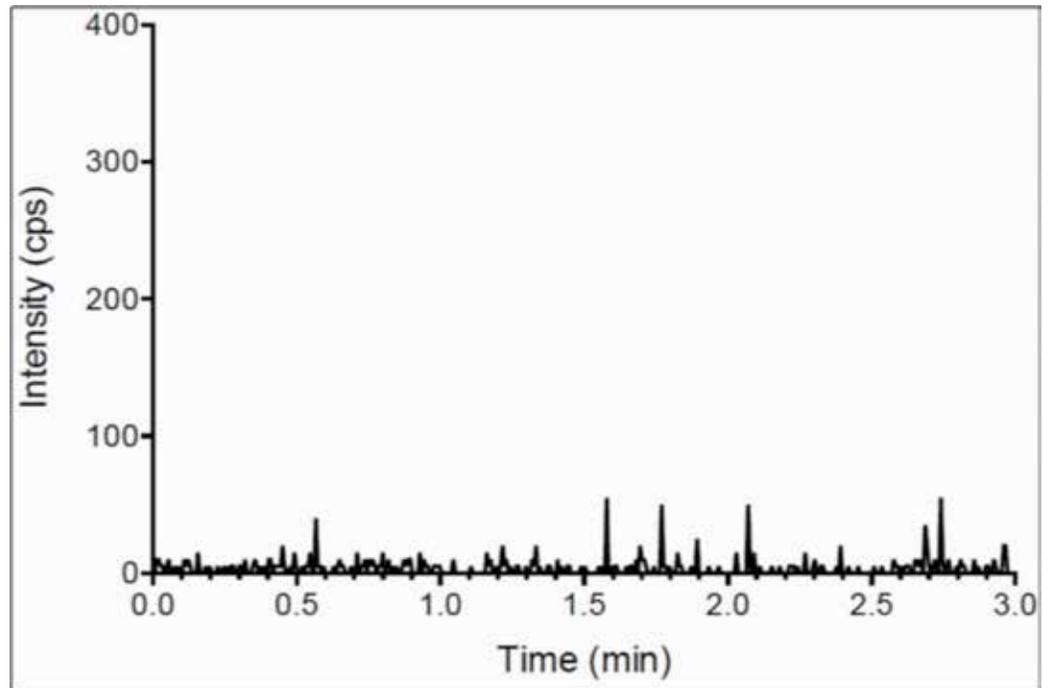


Figure 2: LC-MS/MS mass chromatogram of a typical sample from the infected group at 30 min following CS administration. Plasmatic concentrations of CS were less than the limit of detection. Furthermore, CS was not detected at other time points.

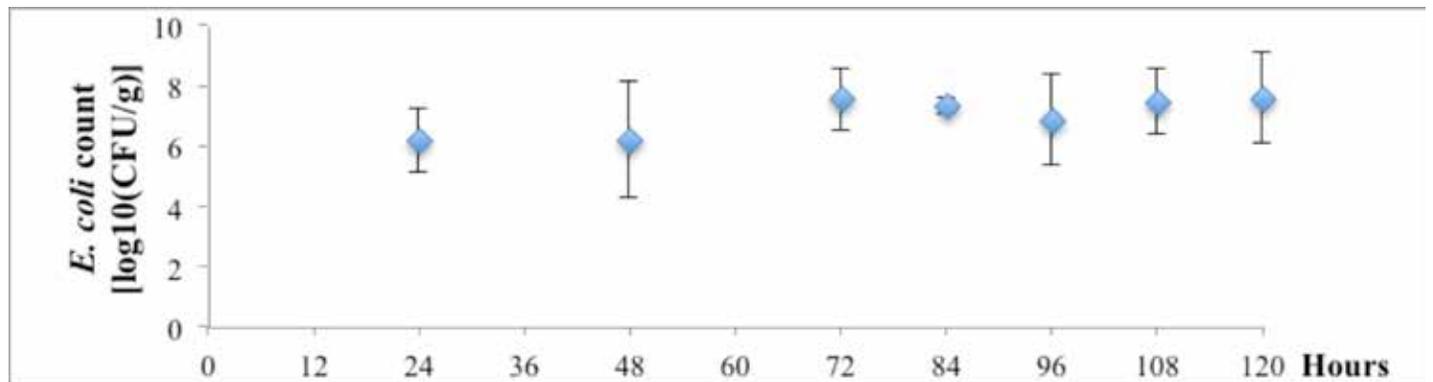


Figure 3: Evolution of the fecal ETEC F4 count (means, and standard deviation). Experimental inoculation was performed at 0 hour, and treatment with CS was carried out at 72 hours post challenge. Maximum effect of CS was observed at 24h post-treatment (96h post challenge).

Discussion

To the best of our knowledge, non other experimental studies focussing on CS systemic residues in pigs using LC-MS/MS as an analytic method. In our study, healthy pigs showed a very small level of systemic CS at 30 minutes after its oral administration, and these quantities were lower than the LLOQ. Thus, the absence of CS absorption in healthy pig after oral administration despite the use of a sensitive analytic method (LOQ=20 mg/ml), confirms the reports of previous workers

who demonstrated that colistin was poorly absorbed and systemic residue levels were usually undetectable (Guyonnet et al., 2010). In challenged treated group, CS systemic concentrations were not detected in any of the samples analyzed. These results differ from those of studies that have shown that infectious gastroenteritis increases gut permeability, and inflammation appears to act via mast cells, to an increase intestinal permeability (Camilleri, Madsen, Spiller, Greenwood-Van Meerveld, & Verne, 2012). In our study, the enterotoxins produced by the ETEC: F4 strain cause secretion of water and electrolytes leading to diarrhea with few microscopic lesions (Fairbrother & Gyles, 2012). Thus, diarrhea and the effect of enterotoxins may explain the non detection of CS systemic concentration. Our study showed that antibacterial effect of CS in bacterial shedding was most important in the 24-h post treatment period, beyond this range, its antimicrobial activity being absent. Thereby, one dose per day of CS is ineffective to produce significant diminution of bacteria counts.

Conclusion

For the first time, a study of some CS pharmacokinetics parameters with a highly sensitive method showed that CS levels are not detectable in the systemic circulation following oral administration in pigs, and concurrent oral challenge with an ETEC F4 strain did not affect CS absorption. A single dose of CS resulted in reduced bacterial counts of the total *E. coli* and ETEC: F4 populations in the feces. Further studies are needed to evaluate the effect of CS on *E.coli* populations and potential antimicrobial resistance.

References

- Camilleri, M., Madsen, K., Spiller, R., Greenwood-Van Meerveld, B., & Verne, G. N. (2012). Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterol Motil*, 24(6), 503-512. doi: 10.1111/j.1365-2982.2012.01921.x
- Chauvin, C., Beloeil, P. A., Orand, J. P., Sanders, P., & Madec, F. (2002). A survey of group-level antibiotic prescriptions in pig production in France. *Prev Vet Med*, 55(2), 109-120.
- Daudelin, J. F., Lessard, M., Beaudoin, F., Nadeau, E., Bissonnette, N., Boutin, Y., . . . Fairbrother, J. M. (2011). Administration of probiotics influences F4 (K88)-positive enterotoxigenic *Escherichia coli* attachment and intestinal cytokine expression in weaned pigs. *Vet Res*, 42(1), 69. doi: 10.1186/1297-9716-42-69
- Fairbrother, J. M., & Gyles, C. L. (2012). Colibacillosis. In J. J. Zimmerman & H. W. Dunne (Eds.), *Diseases of swine* (10 ed., pp. 723-749). Chichester, West Sussex: Wiley-Blackwell.
- Guyonnet, J., Manco, B., Baduel, L., Kaltsatos, V., Aliabadi, M. H., & Lees, P. (2010). Determination of a dosage regimen of colistin by pharmacokinetic/pharmacodynamic integration and modeling for treatment of G.I.T. disease in pigs. *Res Vet Sci*, 88(2), 307-314. doi: 10.1016/j.rvsc.2009.09.001
- Ma, Z., Wang, J., Gerber, J. P., & Milne, R. W. (2008). Determination of colistin in human plasma, urine and other biological samples using LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci*, 862(1-2), 205-212. doi: 10.1016/j.jchromb.2007.12.009
- Matte, J. J. (1999). A rapid and non-surgical procedure for jugular catheterization of pigs. *Lab Anim*, 33(3), 258-264.

Antimicrobial resistance in *Escherichia coli* and *Enterococcus* sp. isolated from swine carcasses at the pre-chill stage

Pissetti, C.⁽¹⁾, Campos, T.⁽¹⁾, Werlang, G.O.⁽¹⁾, Kich, J.D.⁽²⁾, Cardoso, M.^{(1)*}

⁽¹⁾ Preventive Veterinary Medicine Department, Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

⁽²⁾ Embrapa Swine and Poultry Research Center, Concórdia, Brazil

*corresponding author: mcardoso@ufrgs.br

Abstract

The prevalence of antimicrobial resistant bacteria has been increasingly monitored in animals in order to prevent the spread of these strains through the food supply chain. Particularly, the emergence of vancomycin-resistant *Enterococcus* and Extended-spectrum beta-lactamases (ESBL) producing *Enterobacteriaceae* has been investigated worldwide. In the current study, the frequency of antimicrobial resistance of generic *Escherichia coli* and *Enterococcus* isolated from swine carcasses sampled at the pre-chill stage was assessed. A total of 319 strains of *E. coli* and 240 strains of *Enterococcus* sp. from carcasses sampled in three Brazilian slaughterhouses were tested by the agar diffusion test, according to the guidelines of Clinical and Laboratory Standards Institute. *Escherichia coli* strains showed high frequency of resistance against tetracycline (79.3%), ampicillin (64.3%), sulfonamide and florfenicol (64.9%). Five isolates were resistant to either cefotaxime or ceftazidime, but only one of them displayed a positive result in the confirmatory phenotypic test recommended by CLSI. The five isolates were subjected to PCR for detection of bla_{TEM} and bla_{PSE} genes and three strains presented amplification for bla_{TEM} . The most prevalent resistance profiles found among *Enterococcus* strains were tetracycline (42.5%), erythromycin (26.7%) and high level streptomycin (HLAR, 20.4%). All isolates were susceptible to vancomycin, teicoplanin and ampicillin. Minimum inhibitory concentration (MIC) was determined for resistant and intermediate erythromycin *Enterococcus* strains. The determined MIC ranged from 1 µg/mL to 4 µg/mL for erythromycin intermediate strains, while among the resistant strains it ranged from 6 µg/mL to >256 µg/mL. The results indicate that vancomycin-resistant *Enterococcus* isolates are not prevalent in pig carcasses; however ESBL producing *E. coli* may be present.

Introduction

Monitoring of antimicrobial resistance in pathogenic or commensal bacteria is essential to evaluate the risk of antimicrobial resistance genes spread through the food supply chain. Resistant bacteria present on carcasses that were able to surpass the food processing hurdles, may colonize the consumer's gastrointestinal tract (Apley, 2001). In particular, extended-spectrum β-lactamases (ESBL) resistant bacteria and vancomycin-resistant *Enterococcus* have been investigated worldwide, due to its importance for human therapy. Therefore, the aim of this study was to investigate the frequency of antimicrobial resistance in *Escherichia coli* and *Enterococcus* sp. isolated from pig carcasses sampled at the pre-chill stage.

Material and Methods

Two sampling cycles were conducted in three slaughterhouses located in the state of Santa Catarina, Brazil. A total of 252 pre-chill carcasses were sampled by rubbing individual sterile sponges (Nasco ®) on a 400 cm²-carcass area (Brazil, 2007). After the addition of 40 ml of Buffered Peptone Water (BPW 0.1%) to each sample, aliquots were transferred to Violet Red Bile Agar (Oxoid Brazil Ltda.) and KF *Streptococcus* Agar (Bento, Dickson & Company) for isolation of *E. coli* and *Enterococcus* sp., respectively. After incubation (37°C/48h) typical colonies were identified according to Quinn et al. (2011). The *Enterococcus* genus was confirmed by amplification of *tuf* gene (Ke et al., 1999) and the identification of *E. faecalis* was performed by amplification of $ddl_{E.faecalis}$ gene (Dutka-Malen et al. 1995).

The isolates were tested for antimicrobial resistance by disk-diffusion test in Müller-Hinton Agar (Oxoid), performed and interpreted according to "Clinical and Laboratory Standards Institute", document M100-S22 and M31-A2 (CLSI, 2008; 2012). *Escherichia coli* strains were tested using disks (Oxoid) of the following antimicrobials: ampicillin (10µg), ceftazidime (30µg), cefotaxime (30µg), gentamicin (10µg), florfenicol (30µg), nalidixic acid (30µg) tetracycline (30µg) and sulfonamide (300µg). Resistant strains to cefotaxime and/or ceftazidime were confirmed phenotypically for ESBL resistance, using disks of cefotaxime and ceftazidime alone and in combination with clavulanic acid (CLSI, 2012). Additionally, genotypic confirmation was conducted by PCR-detection of genes bla_{TEM} and bla_{PSE} (Sandvang et al. 2002). The *Enterococcus* isolates were tested against: ampicillin (10µg), ciprofloxacin (5µg), chloramphenicol (30µg), erythromycin (15µ), teicoplanin (30µg), tetracycline (30µg), vancomycin (30µg), streptomycin (300µg) and gentamicin (120µg). For isolates displaying erythromycin intermediate and resistant profiles, the minimum inhibitory concentration (MIC) was determined, using the

Etest® (BioMerieux). The frequency of *E. coli* and *Enterococcus* resistant strains among slaughterhouses was compared by chi-square, using the software SPSS 1.8 with a confidence level of 95%.

Results

From 319 tested *E. coli* strains, 12.8% were susceptible to all antimicrobials. The highest frequency of resistance was found against tetracycline (79.3%), sulfonamide, florfenicol (64.9%), and ampicillin (64.3%) (Table 1). Five strains were resistant or intermediate to cefotaxime or ceftazidime (screening test), but only one of them was confirmed in the ESBL phenotypic test. However, three strains were positive in the PCR for *bla*_{TEM} gene. No strain showed amplification for *bla*_{PSE} gene.

All the 240 typical colonies were confirmed by PCR as belonging to *Enterococcus* genus. From them, 217 (90.8%) were identified as *E. faecalis* by PCR. All isolates were susceptible to glycopeptides and ampicillin. The frequency of resistant strains against the tested antimicrobial is depicted in Table 2. Among the seventy-four erythromycin-intermediate strains, two had borderline MIC (4 µg/mL) while the other strains presented MIC between 1 µg/mL and 3 µg/mL. Among the sixty-four resistant strains only six presented MIC between 6 and 64 µg/mL, and 58 presented MIC ≥ 256 µg/mL.

Discussion

Tetracycline resistance was the most frequent in both *E. coli* and *Enterococcus* isolated from all slaughterhouses included in this study. In Brazil, tetracycline resistance is widespread in *Salmonella enterica* isolated from pigs as demonstrated in previous studies (Bessa et al., 2004; Mürmann et al., 2009). Since all the three bacteria species colonize the intestine, they may be exposed to the same selection pressure exerted by the antimicrobial administration. Regarding *E. coli*, those tetracycline-resistant strains presented resistance to sulfonamides and ampicillin as well, suggesting that genes the resistance may be located in mobile elements (Thorsteinsdottir et al., 2010). In our study this association was also observed in most *E. coli* strains tested, although the use of those antimicrobials as growth promoter has been banned since 1998 (Brasil, 2009). However, those antimicrobials are still used for treatment of highly prevalent respiratory and enteric diseases of swine. In this sense, the therapeutic use may have contributed to keep the selective pressure on bacterial population.

The frequency of antimicrobial use influences directly the selection of resistant strains (Wang et al., 2010); therefore variations in the protocol of their administration may justify differences in resistance profiles detected among the slaughterhouses. In Brazil, the pork supply chain is typically organized in a vertical integration system between large companies and small farms. Companies supply medicines and give technical assistance to the farmers and later on transport the market pigs to their own slaughterhouses. The large variation among slaughterhouses in the frequency of resistance against florfenicol and aminoglycosides in *E. coli* and *Enterococcus* strains, respectively, may illustrate the difference in treatment protocols. Specifically the increasing therapeutic use of florfenicol that has occurred in the last years may have contributed for the high percentage of resistant and intermediate strains observed.

Studies conducted in other countries demonstrated ESBL-resistant *E. coli* strains in feces of healthy pigs (Geser et al., 2011), pointing out the risk for carcass contamination. In our study, a low number of ESBL-resistant *E. coli* isolates was observed, indicating that this resistance phenotype is still not highly widespread in the region. Among five resistant strains, one was phenotypically confirmed, and three carried the *bla*_{TEM} gene, demonstrating that they were potentially ESBL producers. In spite of the low frequency, the presence of ESBL-resistant strains in pigs highlights that further studies should be conducted to determine the prevalence and distribution of these strains.

In this study, we included the genus *Enterococcus*, because of its importance as a reservoir of resistance genes against antimicrobials used for treatment of Gram-positive bacteria (Dzidic et al., 2008). In this regard, the reported increase of vancomycin-resistant enterococci in animals have led to the ban of avoparcin administration to pigs in many countries. In Brazil, avoparcin had not been widely administered to pigs up until 1998, when its use was prohibited (Brasil, 1998). Therefore, the absence of resistant strains against glycopeptides was expected. Similar results have been reported in a nationwide monitoring program of poultry carcasses at retail level, where less than 1% of the *Enterococcus* strains were resistant to vancomycin and teicoplanin (ANVISA, 2008).

On the other hand, the high frequency of erythromycin-resistant strains and the high MIC-levels (≥ 256 µg/mL) found is a matter of concern, since *erm* genes confer cross-resistance to the entire macrolide-lincosamides-streptogramins (MLS_B) group, which constitute an important alternative for human treatment (Emanieni et al., 2008). Considering that studies demonstrated that common clones can colonize pigs and humans (Larsen et al., 2011), the widespread of resistant *E. faecalis* strains may constitute a hazard to public health. Recently the use of erythromycin as growth promoter has been banned in Brazil (Brazil, 2012), in order to avoid the selection of resistant strains. Nevertheless, erythromycin-resistance pattern should be kept under monitoring.

Conclusion

The results indicate that vancomycin-resistant *Enterococcus* isolates were not prevalent in pig carcasses; however ESBL producing *E. coli* may be present.

References

- ANVISA. 2008. Programa Nacional de Monitoramento da Prevalência e da Resistência Bacteriana em Frangos – PREBAF.
- Apley, M., 2001. Role of veterinary therapeutics in bacterial resistance development: Animal and public perspectives. *JAVMA*, 212, 1209-1213.
- Bessa, M. C. et al.; 2004. Prevalência de Salmonella em suínos abatidos em frigoríficos do RS. *Pesquisa Veterinária Brasileira*. 24, 80-84.
- Brasil, Ministério da Agricultura, Pecuária e Abastecimento. 1998. Portaria SVS/MS 818 de 16 de outubro de 1998.
- Brasil, Ministério da Agricultura, Pecuária e Abastecimento. 2007. Circular Nº 130/2007/CGPE/DIPOA.
- Brasil, Ministério da Agricultura, Pecuária e Abastecimento. 2009. Instrução Normativa Nº26/2009.
- Brasil, Ministério da Agricultura, Pecuária e Abastecimento. 2012. Instrução Normativa n.14 de 17 de maio de 2012.
- CLSI, Clinical and Laboratory Standards Institute. 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, CLSI/M31-A3.
- CLSI, Clinical and Laboratory Standards Institute. 2012. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational supplement, CLSI/ M100-S22, 32, 3.
- Dutka-Malen, S. et al. 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *Journal Clinical Microbiology*. 33, 24-27.
- Dzidic, S. et al. 2008. Antibiotic Resistance Mechanisms in Bacteria: Biochemical and Genetic aspects. *Food Technology and Biotechnologic*. 46, 11-21.
- Emaneimi, M. 2008. Characterization of glycopeptides, aminoglycosides and macrolide resistance among *Enterococcus faecalis* e *Enterococcus faecium* isolates from hospitals in Tehran. *Polish Journal of Microbiology*. 57, 173-178.
- Geser, N. et al. 2011. Fecal carriage of extended-spectrum B-lactamase-producing Enterobacteriaceae in swine and cattle at slaughter in Switzerland. *Journal of Food Protection*. 74, 446-449.
- Ke, D. et al. 1999. Development of a PCR assay for rapid detection of Enterococci. *Journal Clinical Microbiology*. 37, 3497-3503.
- Larsen, J. et al. 2011. Porcine and human community reservoirs of *Enterococcus faecalis*, Denmark. *Emerging Infectious Diseases*. 17, 2395-97.
- Mürmann, L. et al. 2009. Prevalence, genetic characterization and antimicrobial resistance of Salmonella isolated from fresh pork sausages in Porto Alegre, Brazil. *Food Control*. 20, 191-195.
- Quinn, P.J. et al. 2011. *Veterinary Microbiology and Microbial Disease*. 2ª ed. Iowa: Wiley-Blackwell, 1231p.
- Sandvang, S. et al. 2002. Translocation of integron-associated resistance in a natural system: acquisition of resistance determinants by inc P and inc W plasmids from Salmonella enterica Typhimurium DT104. *Microbial Drug Resistance*. 8, 151-160.
- Thorsteinsdottir, T.R. et al. 2010. Prevalence and genetic relatedness of antimicrobial-resistant *E. coli* isolated from animals, foods and humans in Iceland. *Zoonoses and Public Health*. 57, 189-196.
- Wang, X.M. et al. 2010. Antimicrobial resistance, virulence genes, and phylogenetic background in Escherichia coli isolates from diseased pigs. *FEMS Microbiology Letters*. 306, 15-21.

Table 1 - Frequency of antimicrobial resistance in *Escherichia coli* isolated from pre-chill pig carcasses from three slaughterhouses (A, B, C) of Santa Catarina, Brazil.

	A (n = 102)		B (n = 122)		C (n = 95)		A, B, C (n = 319)		P value
	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	
Ampicillin	71.57	1.96	65.57	10.65	54.74	11.58	64.26	8.15	0.023
Cefotaxime	-	0.98	1.64	-	1.05	-	0.94	0.31	0.443
Ceftazime	-	-	1.64	-	-	-	0.62	-	0.197
Florfenicol	87.25	11.76	60.65	29.51	46.31	37.89	64.89	26.33	<0.001
Gentamicin	5.88	2.94	6.55	7.37	9.47	3.16	7.21	4.7	0.382
Nalidixic acid	75.49	0.98	48.36	4.1	50.53	5.26	57.68	3.44	<0.001
Sulfonamide	81.37	-	50.82	0.82	65.26	-	64.89	4.07	<0.001
Tetracycline	93.14	-	69.67	0.82	76.84	-	79.31	0.31	<0.001

Table 2 - Frequency of antimicrobial resistant *Enterococcus* isolated from pre-chill pig carcasses from three slaughterhouses (A, B, C) of Santa Catarina, Brazil.

	A (n = 84)		B (n = 74)		C (n = 82)		A, B, C (n = 240)		P value
	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	
Ciprofloxacin	3.6	13.1	17.6	9.5	20.7	8.5	13.8	10.4	0.018
Cloramphenicol	27.4	3.6	2.7	1.4	4.9	2.4	12.1	2.5	<0.001
Erythromycin	41.7	42.9	10.8	20.2	25.6	25.6	26.7	30.8	<0.001
Gentamicin (HLAR)	17.9	-	2.7	-	9.8	-	10.4	-	0.008
Streptomycin (HLAR)	34.5	-	6.8	-	18.3	-	20.4	-	<0.001
Tetracycline	45.2	-	48.6	2.7	34.1	2.4	42.5	1.7	0.209

Austrian model approach to assess quality of post-mortem feedback-information systems in pigs

Wanda, S.*⁽¹⁾, Schleicher, C.⁽²⁾, Scheriau, S.⁽²⁾, Koefer, J.⁽¹⁾

⁽¹⁾ Institute of Veterinary Public Health, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

⁽²⁾ Division of Data, Statistics and Risk Assessment, Austrian Agency for Health and Food Safety, Graz, Austria

*corresponding author : Sabine.Wanda@vetmeduni.ac.at

Abstract

A novel quality assurance approach was tested for its applicability to assess data validity and meat inspection performance by means of modeling and training of official meat inspectors (OMIs). General linear mixed models (GLMM) were used to estimate the variance in 20 selected lesions assessed by 12 official meat inspectors for 247.507 pigs. The pigs originated from 72 conventional farms and were slaughtered at one abattoir in Austria in the period January 2008 to June 2011. The heterogeneity in the health state of the pigs, variation due to the farms of origin, piglet producer, quarter and medication was considered in the statistical model. Based on the model meat inspection showed hardly any variation for post-mortem findings such as pericarditis, arthritis and milkspots, whereas meat inspection of blood aspiration, scalding water lungs, skin lesions and hepatitis can be deemed as not sufficiently standardized. Training of OMIs resulted in better detection rates of blood aspiration and pleurisy, but not in the detection of skin lesions related to slaughter technology and animal welfare. Grading of pneumonia has to be further improved in future training sessions. An improved data record system was established based on the outcome of the model and training of OMIs. In future research the benefit of the new code system and training effect to standardize meat inspection will be assessed in form of statistical re-evaluations.

Introduction

In order to revise meat inspection towards the introduction of a risk-based approach, information feedback systems have been established throughout Europe, legally required by European Commission regulations (EC) 854/2004 and (EC) 2074/2005 to grant safety along the production chain. It became mandatory for official meat inspectors (OMI) to record slaughter lesion data, and provide information about the predominant diseases affecting finishing pigs from the slaughter house to the pig producer and farm veterinarian, thus assist in monitoring disease in national herds. Although the variability in scoring lesions among OMIs is believed to be a major contributor to variability in lesion prevalence among slaughter prevalence in monitoring programs, hardly anyone has questioned the quality of data recorded in such databanks for reporting.

In Austria a novel quality assurance (QA) approach was evaluated for its applicability to assess data validity and meat inspection performance by means of modeling and training of OMIs. As described in Schleicher et al. (2013) statistical models were fitted to estimate the probabilities of a positive finding.

Furthermore, the amount of variation among these probabilities that contribute to the OMIs was determined by calculating variance partitioning coefficients (VPCs). Primary aim was to assess those lesions with the highest variability among OMIs for further training and to assess practically at the slaughter plant the feasibility of the model as training base.

Material and Methods

Study population

The population under study comprised all conventional pigs from 72 farms located in the province of Styria, Austria, that were slaughtered in the period January 2008 to June 2011. Farms included 21 (29.2%) fattening farms and 51 (70.8%) farms with “farrow to finish units”. Herd size ranged from 70 to 2025 finishing pigs. All farms participating in the study had no “all in/all out” management. Each farm sent batches of finishing pigs to slaughter according to growth performance. Additionally, only farms which sent more than 400 pigs to slaughter in the study period were included in the study. A total of 247.507 pigs were examined.

Meat inspection and Data recording

The study was conducted at one slaughter plant located in the province of Styria, Austria, slaughtering approximately 2000 – 2300 pigs per week. Finishing pigs were sent to slaughter with about 115 – 120 kg (253.5 – 264.6 lb). The abattoir killed about 115 -120 pigs per hour and used carbon dioxide stunning followed by conventional sticking with the animals lying

on the side. At the slaughterhouse post-mortem data were recorded by a total of 12 official meat inspectors (OMI) with 11-19 years of experience in meat inspection. Each veterinarian was registered under a certain “vetcode” in the system of the slaughterhouse. Along the pluck- and carcass line the veterinarian was able to select post-mortem findings on a touch screen out of 55 pre-defined parameters. In the study focus was laid on 20 lesions according to their frequency and animal health significance from the abattoir company’s database. The parameters were assessed for their validity, thus quality in post-mortem feedback-information systems.

Statistical analysis

General linear mixed models (GLMM) were used to estimate the variability due to the OMIs for each of the 20 selected lesions as described in Schleicher et al. (2013). The heterogeneity in the health state of the pigs, the variation due to farms of origin, piglet producer, quarter and medication (PCV2 vaccination, deworming, scabies treatment) were considered in the statistical models. For each lesion the amount of dispersion among OMIs and the farm of origin were quantified in the model using Variance Partitioning Coefficients (VPCs). Special care was taken that a balanced sample was given for the models. Each of the 12 OMIs examined more than 6.000 plucks and more than 7.000 carcasses. The latter ensured that each OMI inspected plucks and carcasses of at least 51 out of 72 farms, and that pigs originating from one farm were examined by at least 7 OMIs. The models were implemented in R (version 2.14.2) using the package lme4.

Training of veterinarians in meat inspection

Eleven of the twelve OMIs participated in the practical training. Each of the OMIs had to examine 12 preselected carcasses and plucks within 12 minutes. They were asked to record lesions out of a list of 14 pre-defined pathological abnormalities, namely: mild, moderate and severe forms of pneumonia, pleuritis visceralis, blood aspiration, scalding water lungs, pericarditis, milkspots, perihepatitis, hepatitis, skin lesion related to a) slaughter technology b) infectious agent and c) animal welfare, scabies, pleuritis parietalis and arthritis. Precise definitions and guidelines were given beforehand. Additionally, the OMIs were asked to record multiple lesions, not only the most predominant one. Subsequently, a photo documentation of each lesion was completed.

Results

In the study period January 2008 to June 2011 247.507 pigs were examined by 12 OMIs. In total about 70% of the plucks and approximately 40-50% of the carcasses were recorded with lesions. A detailed list on slaughter statistics is given in Table 1.

The abattoir company’s database was screened and finally 20 lesions, namely 18 pathological abnormalities and 2 findings related to slaughter technology chosen for further evaluation according to their relative frequency (Table 2) and animal health significance.

Table. 1 Number of pigs [%] slaughtered in the study period and number [%] of plucks, carcasses and number of pigs (carcass + pluck) recorded without lesion.

criteria	2008	2009	2010	2011*
	number [%]			
Pigs total	56.760 [22.9]	68.013 [27.5]	74.890 [30.3]	47.842 [19.3]
Pigs (fattening ¹)	18.348 [7.4]	21.713 [8.8]	26.673 [10.8]	16.045 [6.5]
Pigs (farrow ²)	38.412 [15.5]	46.300 [18.7]	48.217 [19.5]	31.797 [12.8]
Plucks (P) no lesion	16.089 [28.3]	20.267 [29.8]	23.541 [31.4]	13.770 [28.8]
Carcasses (C) no lesion	27.191 [47.9]	33.318 [49]	42.822 [57.2]	28.271 [59.1]
C + P no lesion	8.984 [15.8]	11.672 [17.2]	15.759 [21]	9.397 9.6]

¹originating from fattening farms ²originating from farms with farrow to finish units

*6months

Range of relative frequencies in lesions was found highest for blood aspiration, followed by scalding water, hepatitis, bursitis and skin lesion. The most frequent lesion was bursitis in carcasses (13.8%) and pneumonia (+/++/+++ in plucks (30.4%). However, a simple descriptive analysis will not allow a distinction between lesion variability due to farm/herd management and/or OMI performance. Therefore, a statistical analysis (GLMM) was conducted to estimate the influence of OMI and

farm on each of the 20 lesions and to determine the level of standardization and homogeneity in meat inspection as an indicator for data quality. The results of the models include fixed effects (influence of quarterly time effect, farm type and piglet producer). Largest variance among OMIs (VPC estimates) was given for the finding skin lesion (3.9-20.8%), followed by blood aspiration (8.2-19.8%) and hepatitis (2.9-18.9%). A negligible amount of variation was determined concerning pericarditis (0 – 0.1%), peritonitis (0-0.6%) and arthritis (0-0.4%). Particularly large variation on farm level was shown concerning milkspots. Subsequent analysis of the raw data revealed that the pigs from 2 of the considered piglet producers showed a noticeably larger risk for severe milkspots (>3). Training of the OMIs improved the detection rate of blood aspiration (90.9-100%) and pleuritis visceralis (63.6-90.9%). However, hardly any improvement was given for the detection of skin lesions. Localization and severity of the skin lesions affected highly the detection rate of the OMIs (18.2-100%). In addition, a certain disagreement in recording multiple lesions was noted between OMIs. The performance among meat inspectors to assess different forms of pneumonia were found acceptable, but need to be further improved. Lowest variability was given for pneumonia +++, which was found consistent with the model.

Discussion

Meat inspection might be considered standardized and homogeneous if the probability of a specific post-mortem finding is independent of the OMI carrying out the examination. Emphasis was laid to establish a model fitted for real work conditions. In contrast to trial designs no repeated measurements or reference standards were available. Focus of the work was not the estimation of the rater's sensitivity and specificity, but rather the analysis of the variation of the probabilities of a finding between different OMIs. It was considered essential in the model to take the variation between farms and seasonal effects into account to reduce the influence of the heterogeneity in the health state of pigs. Otherwise, the model fit might be poor, in particular for infrequent findings with low relative frequency (e.g. scabies). Findings for which there were different levels of gradation to choose from (i.e. pneumonia). typically exhibited larger variation among OMIs. In general good cooperation between trainer and OMIs was given. OMIs, who participated in the study, voluntarily gave feedback on how to improve the abattoirs' data recording system.

Table. 2 Overview on the total relative frequency (r.f., %) assessed by 12 OMIs and range of r.f. (min.—max.) for each OMI for 20 selected lesions over the full study period (01/08 – 06/11).

Carcass		%	Pluck		%
finding	r.f. (%)	min.-max.	finding	r.f. (%)	min.-max.
bursitis	13,8	4.9-27.1	pneumonia +	13,8	3.2-26.8
arthritis	0,6	0.2-1.4	pneumonia ++	11,9	4.8-23.5
scabies	0,5	0-6,3	pneumonia +++	4,7	1.5-16.2
skin lesion	8,6	0-19,8	pleuritis visc.	14,1	4.9-22.2
pleuritis +	7,3	3.6-13.2	blood aspiration	17,7	1.2-41.5
pleuritis ++	8,3	3,9-12	scalding water	12,9	2.1-35.2
abscess	1,5	0.3-3.0	lung adhesion carcass	0,5	0.1-0.9
peritonitis	0,1	0-0,5	pericarditis	3,1	2.5-3.9
			milk spots ≤3	2,5	1.0-3.9
			milk spots >3	4,7	2.0-10.1
			hepatitis	8,3	0.9-29.1
			perihepatitis	2,7	0.9-6.1

Conclusion

Inspection of pigs at slaughter has been widely used in epidemiological studies of risk factors associated with raised prevalence of lesions. However, the prevalence of lesions recorded in databanks must be based on valid data and reflect a certain consistency in data recording of OMIs to establish a functional post-mortem feedback information system. The statistical model (GLMM) was found an essential and helpful tool to estimate on the one hand the amount of variation in post-mortem findings that can be accredited to the OMI and on the other hand as a training base for OMIs. As a consequence of the statistical analysis and training of OMIs an improved code record system will be established in the abattoir. Focus was laid to assess fewer findings, but with high animal health significance in the future. Precise guidelines and definitions on each lesion were provided for the OMIs in cooperation with the veterinary section of the local government. Frequent training sessions and a re-evaluation of the OMIs' performance might be considered in the future to standardize meat inspection on the long term.

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References

EUROPEAN COMMISSION REGULATIONS (EC) 854/2004 of the European parliament and of the council of 29 April 2004 laying down specific rules for the organization of official controls on products of animal origin intended for human consumption

EUROPEAN COMMISSION REGULATIONS (EC) 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organization of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004

SCHLEICHER, C., SCHERIAU, S., KOPACKA, I., WANDA, S., HOFRICHTER, J., KOEFER, J., 2013, Analysis of the variation in meat inspection of pigs using variance partitioning, Preventive Veterinary Medicine, <http://dx.doi.org/10.1016/j.prevetmed.2013.05.018>

Assessing the role of feed as a risk factor for *Salmonella* in pig production

A.M. Burns^{1,2,3*}, **P.G. Lawlor**³, **G.E. Gardiner**², **U. Tiwari**¹ and **G. Duffy**¹

1. Teagasc Food Research Centre, Food Safety Department, Ashtown, Dublin 15, Ireland
2. Department of Chemical & Life Sciences, Waterford Institute of Technology, Waterford, Ireland
3. Teagasc Pig Development Department, Animal & Grassland Research & Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland

Corresponding author: annemarie.burns@teagasc.ie

Abstract

The objectives of this study were to investigate *Salmonella* carriage at each stage of pig production (farrow to finish) on 10 commercial pig farms having a historically high *Salmonella* seroprevalence and to establish the role of feed as a source of *Salmonella* on the farms. Pig faecal (n=458) and feed (n=321) samples were taken across all pig production stages and analysed for the presence of *Salmonella*. The pathogen was detected in pigs on nine farms, in 58/458 (12.7%) faecal samples, with a high prevalence among gilts, weaners and finishers. Only 7/321 (2%) of feed samples were *Salmonella*-positive, with four farms having at least one *Salmonella*-positive feed sample. The serovar recovered (4, 12:i:-) was also detected in pigs on the same farms, suggesting that it may have originated in the feed supplied to the farm. On the other hand, the feed may have been contaminated on the farm and in this way played a role in transmission of *Salmonella*.

Introduction

Salmonella carriage in pigs is a significant food safety issue. The European Food Safety Authority has highlighted that feed is a risk factor for *Salmonella* prevalence in pigs (EFSA, 2008a). The presence of *Salmonella* in feed can lead to the introduction of *Salmonella* into pathogen-free herds, an increase in shedding prevalence and the spread of *Salmonella* in pigs (EFSA, 2007). A study by Molla *et al.* (2010) showed genotypically related and in some cases clonal *Salmonella* strains including multidrug resistant isolates in commercially processed pig feed and pig faecal samples.

A revised National Pig *Salmonella* Control Programme was implemented in Ireland in January 2010, with monitoring based on determining the *Salmonella* status of pig herds by serological testing of meat juice at slaughter. However, a recent study has shown that 45% of pigs presented for slaughter in Ireland are caecally positive (Duggan *et al.*, 2010). Furthermore, Ireland had the highest carcass contamination rate (20%) in a 2006-2007 EU baseline survey of *Salmonella* in slaughter pigs (EFSA, 2008b). In a 'Farm to Fork' food safety concept, safe feed is the first step in ensuring safe food. Therefore, the aim of this study was to carry out an in-depth study on 10 commercial pig farms having a historically high *Salmonella* seroprevalence to firstly identify which production stages are the principal harbours of *Salmonella* infection in pigs and secondly, to assess the occurrence of *Salmonella* in pig feed throughout the different production stages on these farms and thereby assess potential risks as well as epidemiological relationships.

Material and Methods

On-farm sampling

The number of farms studied and the number of samples taken were in accordance with statistical advice. Farms (n=10) identified for sampling were selected from those with a history of high (>50%) *Salmonella* sero-prevalence in the Department of Agriculture, Food and the Marine Irish Pig *Salmonella* Control Programme. On-farm sampling was carried out from March-August 2012 with each farm being visited on one occasion between these dates. On each farm, a number of composite faecal samples were collected at random from at least 3 pigs per production stage, directly from the rectum via digital stimulation or from freshly voided faeces. For all stages of production, where insufficient faecal samples were obtained, sterile pairs of gauze socks were used to swab the pen. Feed samples (50-100g) which included liquid and pelleted dry feed, depending on the farm and production stage were taken from troughs, hoppers and storage areas (feed bins, feed tanks) on the farms. After collection, all samples were immediately transported on ice to the laboratory, where they were stored at 4°C until analysis (within 24 h).

Microbiological analysis of samples

The presence/absence of *Salmonella* in 10g samples was determined according to standard microbiological procedures (EN ISO 6579:2008) with modified brilliant green agar (Oxoid, Basingstoke, Hampshire, UK) used for additional selective plating. Presumptive *Salmonella* isolates were tested using a *Salmonella* latex agglutination kit (Oxoid) and confirmed as *Salmonella*

using real-time PCR (Prendergast *et al.*, 2012; McCabe *et al.*, 2011; Fricker *et al.*, 2007). One *Salmonella* isolate per positive sample was serotyped based on O- and H-group antigens according to the White-Kaufmann- Le Minor scheme.

Results

Salmonella was detected in 58/458 (12.7%) faecal samples across all production stages on 9 farms (Table 1) with an overall prevalence of 10% (95% confidence interval). Only farm H had no *Salmonella*-positive faecal samples. Six different serotypes were recovered, with a monophasic variant of Typhimurium (4,12:i:-) predominating, accounting for 40.9% (18/44) of all isolates recovered. The other serotypes recovered were Derby (18.2%; 8/44), Typhimurium (18.2%; 8/44), Typhimurium Copenhagen (11.4%; 5/44), Infantis (9.1%; 4/44), and 4,5,12:i:- (2.3%; 1/44), each from one herd. There was no consistent pattern of infection; however, large numbers of positive animals were detected within gilts, weaners and finishers (16.7, 15.3, 16.7 and 16.7% respectively). Three farms (A, E and G) had notably higher prevalence than the other farms (22.9, 24.4 and 17.1% respectively). Only 7/321 (2%) feed samples taken across all production stages were *Salmonella*-positive (Table 2). These *Salmonella*-positive feed samples originated on four farms (A, B, F and G) and the *Salmonella* isolated from them were identified as 4,12:i:-, Typhimurium and Typhimurium Copenhagen. Three of the positive feed samples originated on farms using liquid feed (farms A and F). The *Salmonella*-positive feed samples were generally recovered at only one stage of production, although on farms A and B they were found at two stages (farrowing and 2nd stage weaner on farm A; and 1st stage weaner and finisher on farm B). Feed sampled from gilts had the highest *Salmonella* prevalence (Table 2).

Discussion

Salmonella was recovered from the pigs on 9 of 10 commercial farms. This was to be expected, as all had been chosen from those with a history of high *Salmonella* seroprevalence. The *Salmonella* 4,12:i:- variant that predominated in the pigs is one of a number of monophasic variants of the serovar Typhimurium, that have been emerging in Europe and are of increasing food safety concern (EFSA, 2010). The lack of recovery of *Salmonella* from any production stage on one of the farms may be accounted for by the fact that this farm had low seroprevalence during the study period, highlighting the cyclical nature of *Salmonella* contamination on farms (White *et al.*, 2006). Of the three farms with the highest *Salmonella* prevalence, only one had high seroprevalence during the study period, demonstrating the lack of correlation between bacteriological and serological data. Across all of the farms large numbers of positive animals were detected within the gilt, weaner and finisher production stages. High carriage rates are commonly seen in weaners and finishers (Davies *et al.*, 1999); however, few studies have investigated *Salmonella* carriage from farrow to finish. The high prevalence within the replacement breeding stock (gilts) indicates that these animals may be an important source of on-farm *Salmonella* infection and this is of particular

Table 1: *Salmonella* prevalence in faecal samples taken from different stages of pig production

Farm	Number of faecal samples positive for <i>Salmonella</i>						Prevalence %
	Gilts	Dry Sow	Farrowing Sow	1 st Stage Weaner	2 nd Stage Weaner	Finisher	
A	1/8	0/6	1/8	4/8	5/8	5/10	22.9
B	1/8	1/6	0/8	4/8	2/8	1/10	12.5
C	2/6	0/6	2/6	0/6	0/6	0/8	5.3
D	0/6	1/6	0/8	0/6	0/6	1/8	2.4
E	5/6	2/6	0/8	0/6	0/6	5/8	24.4
F	0/8	0/6	0/8	0/8	1/8	0/10	2.0
G	2/6	0/6	0/8	0/6	3/6	2/8	17.1
H	0/6	0/6	0/6	0/6	0/6	0/6	0.0
I	1/8	0/6	0/8	0/8	0/8	3/10	8.2
J	0/10	0/6	0/12	3/10	1/10	0/24	5.5
Total	12/72	4/60	3/80	11/72	12/72	17/102	
Prevalence %	16.7	6.7	3.8	15.3	16.7	16.7	10.0

Table 2: *Salmonella* prevalence from feed samples from different stages of pig production

Farm	Number of feed samples positive for <i>Salmonella</i>						Prevalence %
	Gilts	Dry Sow	Farrowing Sow	1 st Stage Weaner	2 nd Stage Weaner	Finisher	
A	0/3	0/4	1/6	0/6	1/7	0/8	5.9
B	0/6	0/3	0/6	1/9	0/6	1/7	5.4
C	0/1	0/2	0/3	0/5	0/5	0/6	0.0
D	0/2	0/3	0/6	0/4	0/5	0/6	0.0
E	0/3	0/3	0/4	0/5	0/5	0/6	0.0
F	0/4	1/4	0/6	0/8	0/6	0/7	2.9
G	2/3	0/5	0/4	0/6	0/5	0/6	6.9
H	0/0	0/2	0/5	0/4	0/6	0/5	0.0
I	0/4	0/5	0/6	0/7	0/6	0/7	0.0
J	0/7	0/5	0/8	0/11	0/5	0/19	0.0
Total	2/33	1/36	1/54	1/65	1/56	1/77	2.2
Prevalence %	6.1	2.8	1.9	1.5	1.8	1.3	

importance on farms where replacement breeding stock are purchased onto the unit.

Salmonella prevalence in feed sampled on-farm was low. However, finding *Salmonella* in at least one of the feed samples tested from almost half of the farms examined could indicate that the organism was quite ubiquitous considering the large volume of feed contained on-site and the relatively small portion of feed sampled for testing. However, as all except one of the *Salmonella*-positive feed samples were taken from troughs within the animal pens, the possibility of on-farm contamination by the pigs is highly likely. Therefore, we cannot ascertain if the feed is the cause of infection or rather a vector for its transmission. However, as the pig faeces harboured the same serovar as the feed from the same production stage on three of the farms it is likely that the feed became contaminated by the pigs on-farm.

In order to establish if the *Salmonella* contamination originated from the purchased feed or if on-farm contamination occurred, genetic subtyping using pulsed field gel electrophoresis (PFGE) and multi-locus variable number tandem repeat analysis will be used to ascertain if the same *Salmonella* strains were found in the feed and the pigs. This will provide important additional epidemiological information. In addition, sampling of feed and feed ingredients from the commercial feed mills supplying our study farms is on-going and will help to further assess the risk posed by *Salmonella* in pig feed. It is interesting to note that three of the seven *Salmonella*-positive feed samples were from liquid feed systems, as many investigators have shown that pig herds fed dry vs. wet diets are at increased risk of having high *Salmonella* seroprevalence (van der Wolf *et al.*, 2001). Overall, the results from the present study indicate that the risk from feed is low with the detection of *Salmonella*-positive pigs on farms with *Salmonella*-negative feed samples demonstrating that there are multiple sources of *Salmonella* infection on pig farms. The importance of these sources may vary by production stage, farm and over time. Although feed could not be singled out as the main source of the *Salmonella* isolated from the pigs in the present study, it nonetheless cannot be ruled out as a risk factor in the transmission of *Salmonella* and therefore, its control in feed should be considered an essential component of any control program.

Conclusion

Salmonella prevalence in feed samples taken on-farm was low. However, further research is needed to ascertain whether it originated in commercial feed supplied to the farms or if its presence in feed was as a result of on-farm contamination.

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References

- Davies, P., Funk, J., Morrow, W.E.M., 1999. Fecal shedding of *Salmonella* by a cohort of finishing pigs in North Carolina. *Swine Health and Production* 7, 231-234.
- Duggan, S.J., Mannion, C., Prendergast, D.M., Leonard, N., Fanning, S., Gonzales-Barron, U., Egan, J., Butler, F., Duffy, G., 2010. Tracking the *Salmonella* Status of Pigs and Pork from Lairage through the Slaughter Process in the Republic of Ireland. *Journal of Food Protection* 73, 2148-2160.
- European Food Safety Authority, 2007, Food safety aspects of different pig housing and husbandry systems. *The EFSA Journal* 613, 1-20.
- European Food Safety Authority, 2008a, Microbiological risk assessment in feeding stuffs for food producing animals. *The EFSA Journal* 720, 1-84.
- European Food Safety Authority, 2008b, Analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, in the EU, 2006-2007. *The EFSA Journal / EFSA Scientific Report* 206, 1-111
- European Food Safety Authority, 2010. Scientific Opinion on monitoring and assessment of the public health risk of “*Salmonella* Typhimurium-like” strains. *The EFSA Journal* 8(10):1826.
- Fricker, M., Messelhauser, U., Busch, U., Scherer, S., Ehling-Schulz, M., 2007. Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Applied and Environmental Microbiology* 73, 1892-1898.

- McCabe, E.M., Burgess, C.M., O'Regan, E., McGuinness, S., Barry, T., Fanning, S., Duffy, G., 2011. Development and evaluation of DNA and RNA real-time assays for food analysis using the *hilA* gene of *Salmonella enterica* subspecies *enterica*. *Food Microbiology* 28, 447-456.
- Molla, B., Sterman, A., Mathews, J., Artuso-Ponte, V., Abley, M., Farmer, W., Rajala-Schultz, P., Morrow, W.E.M., Gebreyes, W.A., 2010. *Salmonella enterica* in Commercial Swine Feed and Subsequent Isolation of Phenotypically and Genotypically Related Strains from Fecal Samples. *Applied and Environmental Microbiology* 76, 7188-7193.
- Prendergast, D.M., Grady, D.O., McCann, A., McCabe, E., Fanning, S., Egan, J., Fanning, J., Gutierrez, M., 2012. Application of PCR for rapid detection and serotyping of *Salmonella* spp. from porcine carcass swabs following enrichment in semi-solid agar. *Food Research International* 45, 993-999.
- van der Wolf, P.J., Wolbers, W.B., Elbers, A.R.W., van der Heijden, H., Koppen, J., Hunneman, W.A., van Schie, F.W., Tielen, M.J.M., 2001. Herd level husbandry factors associated with the serological *Salmonella* prevalence in finishing pig herds in The Netherlands. *Veterinary Microbiology* 78, 205-219.
- White, A.P., Gibson, D.L., Kim, W., Kay, W.W., Surette, M.G., 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. *Journal of Bacteriology* 188, 3219-3227.