One health approach under a concept of farm to fork

Epidemiology and control of hazards in pork production chain – SAFEPORK

Evolution of Salmonella excretion by sows during gestation in link with the faecal microbiome.

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Pork meat is estimated to be responsible of 10 to 20% of human salmonellosis cases. Control strategies at the farm could reduce contamination at the slaughterhouse. One of the targeted sector of the production is the maternity, where sows could be Salmonella reservoirs. The aim of this study was to characterize the faecal microbiome of sows excreting or not Salmonella during gestation phases. A total of 76 sows were selected and fecal matters were analysed at the beginning or the end of gestation period. Salmonella detection was conducted using a method including two selective enrichment media (MSRV and TGB). Nine (9) isolates per positive samples were collected. Among the 76 sows tested, 31 were shedding Salmonella. The sows in the first third of their gestation shed significantly more frequently Salmonella (22/29) than those in the last third (9/47) (χ² 0.05). The shedding status of 19 of the sows that were previously sampled in the first third of their gestation was followed, this time in the last third, confirming reduction of the shedding. Association between changes in the intestinal microbiome and this evolution of Salmonella shedding will be explored. MiSeq sequencing is currently being conducted on the feces to identify shifts in the composition or diversity in the microbial community that could be associated to these variations. A large number of Salmonella isolates that were collected were genotyped by a high resolution melt (HRM) technique. These results showed the presence of a major HRM profile (136 isolates / 169) and two minor profiles (24 and 9 /169).

Introduction

In Canada, Salmonella is estimated to cause 269.26 infection per 100 000 inhabitants each year making this pathogen a priority in public health [1]. Pork meat was estimated to be responsible of 10 to 20% of reported human salmonellosis cases in Europe and therefore is an important source of this pathogen [2]. Better control strategies at the farm could help to reduce the contamination of the carcass at the slaughterhouse. Indeed it has been shown that the influx of contaminated pigs at the slaughterhouse was a risk factor for the contamination of the carcass during slaughtering and hence of the products that reaches the consumers [3]. In the porcine production, sows can be an important Salmonella reservoir with contamination levels usually higher than the pigs in the fattening phase [4]. These carrier sows could be an important contamination source for the piglets [2, 5]. The dynamic of the excretion of Salmonella by the sows during gestation is still not very well known. It seems important to better understand the different factors that could influence this excretion to better understand the transmission of this microorganism to piglets. The aim of this study was to characterize the variation of the excretion of Salmonella by the sows in the gestation phase in relation with the faecal microbiome.

Material and method

Sampling: A total of 76 sows at various gestation stages were selected randomly in a breeding farm known for its frequent Salmonella contamination. For each selected sow 100 gr of fresh fecal matter were collected and analysed. After the first sampling 19 of the 76 sows that were sampled at the beginning of their gestation (first 50 days) were sampled a second time at the end of this period (last 50 days). For each sampled sow 1.5 ml of feces was also collected and frozen in liquid nitrogen for 16s rRNA amplicon MiSeq sequencing: A total of 76 sows at various gestation stages were selected randomly in a breeding farm known for its frequent Salmonella contamination. For each selected sow 100 gr of fresh fecal matter were collected and analysed. After the first sampling 19 of the 76 sows that were sampled at the beginning of their gestation (first 50 days) were sampled a second time at the end of this period (last 50 days). For each sampled sow 1.5 ml of feces was also collected and frozen in liquid nitrogen for 16s rRNA amplicon MiSeq sequencing.

References


Table 1. List of zoonotic agents that produce disease or infection in pigs (adapted from Khan et al., 2013). Pathogens with asterisk indicate those with potential foodborne transmission.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Zoonotic agents that produce disease or infection in pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yersinia enterocolitica*</td>
<td>Leptospira interrogans</td>
</tr>
<tr>
<td>Balantidium coli</td>
<td>Listeria monocytogenes*</td>
</tr>
<tr>
<td>Brucella suis</td>
<td>Menangle virus</td>
</tr>
<tr>
<td>Bungowannah virus</td>
<td>Methicillin-resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>Burkholderia pseudomallei*</td>
<td>Microsporum spp/Trichopyton spp.</td>
</tr>
<tr>
<td>Campylobacter spp.*</td>
<td>Mycobacterium spp.</td>
</tr>
<tr>
<td>Crystosporidium suis</td>
<td>Nipah virus</td>
</tr>
<tr>
<td>Cysticercus cellulosae*</td>
<td>Norwalk virus</td>
</tr>
<tr>
<td>Ebola reston virus</td>
<td>Pasteurella multocida</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>Salmonella spp.</td>
</tr>
<tr>
<td>Erysipelothrix rhusiopathiae</td>
<td>Sarcocystis suihominis*</td>
</tr>
<tr>
<td>Escherichia coli*</td>
<td>Streptococcus suis</td>
</tr>
<tr>
<td>Extended-spectrum beta-lactamases (ESBL) producing bacteria*</td>
<td>Swine influenza viruses</td>
</tr>
<tr>
<td>Francisella tularensis*</td>
<td>Toxoplasm gondii*</td>
</tr>
<tr>
<td>Giardia spp.*</td>
<td>Trichinella spiralis*</td>
</tr>
<tr>
<td>Hepatitis E virus*</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>Japanese encephalitis virus</td>
<td>Yersinia enterocolitica*</td>
</tr>
</tbody>
</table>

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sequencing. Detection: Salmonella detection was conducted using a method adapted from the method described by De Busser et al. in 2013 [6]. This method uses multiple selective enrichments to obtain the highest description of eventual variety of strains in the samples. First the samples were pre-enriched in buffered peptone water for 18h at 37°C. Modified Semi-Solid Rappaport-Vassiliadis Agar (MSRV) (24-48h 42°C) and Tetrathionate Brilliant green Bile broth (TBG) (24h 42°C) selective enrichment media were then used in parallel and further inoculated on Brilliant Green Sulfa agar (BGS). Two BGS were inoculated from the MSRV from two different region of the migration zone (far and near the inoculation spot) and one from the TBG. A maximum of nine (3 per BGS) isolates per positive samples were collected. Genotypic characterization: Each isolate was genotyped using a high resolution melt (HRM) technique adapted from the method described by Bratchikov et al. in 2011 [7]. The melting curves of three regions (two CRISPRs and one VNTR) were analysed using a LC-96 real-time PCR. The combination of the curves for these three regions was considered as defining a new type. Representative isolates of each of the different types were serotyped.

MiSeq : Total sample DNA was extracted using a bead beating method (MP FastPrep-25) followed by a phenol / chloroform purification. 16S-RNA gene amplicon sequencing libraries were prepared following the Illumina MiSeq protocol [8]. The V3-v4 region of the 16S rRNA gene was amplified using primers targeting the total bacterial and archaeal population (Table no.1). Each sample was then indexed using the Nextera index kit (Illumina). For each PCR steps, PCR product purification was conducted using Agencourt AMPure XP beads. The purified and pooled PCR products were sequenced by the Illumina MiSeq sequencing system. The composition of the fecal microbiota will be compared between sow at the beginning and at the end of their gestation but also between sows shedding Salmonella or not.

Table no.1: Primer used for MiSeq sequencing.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target region</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1SF_III</td>
<td>V3-V4 regions</td>
<td>Caporaso JG et al, ISME J. (2012) [9]</td>
</tr>
<tr>
<td>806R_III</td>
<td>V3-V4 regions</td>
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</tr>
</tbody>
</table>

Results

Among the 76 the sampled sows, 31 were shedding Salmonella at the time of the sampling. The sows in the first third of their gestation shed significantly more frequently Salmonella (22/29) than those in the last third (9/47) ($\chi^2 P < 0.05$) (Figure 1A). Of the 19 sows that were previously sampled in the first third of their gestation and were selected for a second sampling, this time in the last third, 13/19 were shedding at beginning of the gestation and only 2 were still positive at the end. This confirms reduction of the shedding during the gestation (McNemar’s $P < 0.001$) (Figure 1B).

Figure no.1: Salmonella shedding by sows at the beginning and the end of gestation. A) All sampled sows. B) Sows sampled twice.

Discussion

These results show that sows can be important reservoirs of Salmonella in the swine production with a total of 40 % of the sampled sows shedding when all the gestation period was considered. These levels are similar to what was previously described in Canada by Wilkins et al. in 2010 [4]. In the farm that was sampled here most of the strains were from the same HRM profile, corresponding to the S. Infantis serovar. This strain could be a resident strain in the environment of the farm. When only the beginning of the gestation was considered, the level of shedding was much higher with 76 % of positive sows. At the opposite, levels where lower at the end of the gestation with 17 % of the sampled sows shedding Salmonella. Variation in the levels of excretion during the gestation cycle of the sow had already been described with low levels in the late gestation and lactation phase and an excretion peak after weaning [10]. In addition with the variation of the levels of excretion we showed variation on the strains excreted by the two sows that were positive both at the beginning and the end of the gestation. In these two cases the use of a modified Salmonella isolation protocol proved to be beneficial since it permitted to detect strains of two different profiles in the same sample. It has been hypothesized that factors such as hormonal changes or in feeding activities during the gestation could be responsible of modifications of the fecal microbiota and linked to these variations in Salmonella excretion. Therefore, in this study, the association of the changes in the intestinal microbiome during the gestation and the evolution of Salmonella shedding will be explored. MiSeq sequencing is currently being conducted on the feces to identify shifts in the composition and diversity of the microbial communities that could be associated with these variations.
A total of 169 isolates of Salmonella that were collected from 21 positive samples were genotyped by a high resolution melt technique (HRM) [7]. The results show the presence of one major HRM profile (136 isolates / 169) and of two minor profiles (21 and 9 isolates /169). Serotyping showed that the strains from the profile 1 were of S. Infantis serovar and the strains from the profiles 2 and 3 belonged to the S. Derby serovar.

### Table no.2: HRM profiles of the Salmonella isolated from sows at the beginning and end of their gestation.

<table>
<thead>
<tr>
<th>CRISPR 1</th>
<th>CRISPR 2</th>
<th>Yohm</th>
<th>HRM types</th>
<th>Beginning of gestation</th>
<th>End of gestation</th>
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</thead>
<tbody>
<tr>
<td>Isolates</td>
<td>Samples</td>
<td>Isolates</td>
<td>Samples</td>
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<td>Samples</td>
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<td>1</td>
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<td>112</td>
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<tr>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

**Discussion**

These results show that sows can be important reservoirs of Salmonella in the swine production with a total of 40% of the sampled sows shedding when all the gestation period was considered. These levels are similar to what was previously described in Canada by Wilkins et al. in 2010 [4]. In the farm that was sampled here most of the strains were from the same HRM profile, corresponding to the S. Infantis serovar. This strain could be a resident strain in the environment of the farm. When only the beginning of the gestation was considered, the level of shedding was much higher with 76 % of positive sows. At the opposite, levels where lower at the end of the gestation with 17 % of the sampled sows shedding Salmonella. Variation in the levels of excretion during the gestation cycle of the sow had already been described with low levels in the late gestation and lactation phase and an excretion peak after weaning [10]. In addition with the variation of the levels of excretion we showed variation on the strains excreted by the two sows that were positive both at the beginning and the end of the gestation. In these two cases the use of a modified Salmonella isolation protocol proved to be beneficial since it permitted to detect strains of two different profiles in the same sample. It has been hypothesized that factors such as hormonal changes or in feeding activities during the gestation could be responsible of modifications of the fecal microbiota and linked to these variations in Salmonella excretion. Therefore, in this study, the association of the changes in the intestinal microbiome during the gestation and the evolution of Salmonella shedding will be explored. MiSeq sequencing is currently being conducted on the feces to identify shifts in the composition and diversity of the microbial communities that could be associated with these variations.
in Salmonella shedding. Identification of bacterial or archaelal population that are linked with the excretion could help to better understand this phenomenon and lead to the development of new methods of control.

**Conclusion**

In the conditions of this study we confirmed that the sows can be an important reservoir of Salmonella. These sows were shedding a major strain at the beginning of the gestation phase and the shedding was reduced in the late gestation. Some variations in the types of strains that were excreted were also observed. We are currently evaluating the impact of the microbiota on this variation.

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**References:**


**Acknowledgment:**

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**Salmonellosis in pigs: what does disease surveillance data from England Wales tell us?**

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The Animal and Plant Health Agency (APHA) Farmfile database allows incidents of pig disease diagnosed in the network of APHA laboratories across England and Wales to be collated and analysed. Between 2005 and 2014, there were 884 disease incidents in pigs involving salmonellosis with the annual diagnostic ratio ranging from 7.2 to 14.6% of submissions tested. Nearly half of the diagnoses were made in submissions of pigs for post-mortem examination, the remainder were from submissions of samples. Seventy percent of the diagnoses involved Salmonella Typhimurium which was the most serotype diagnosed each year. However, since 2010, the numbers of incidents due to monophasic Salmonella variants 4,12:i:- and 4,5,12:i:- recorded in the database have increased each year, and in the first quarter of 2015, were responsible for the same number of disease incidents as S. Typhimurium for the first time, reflecting the increasing prevalence of these variants in the pig population. Significantly, 53 different non-Salmonella diagnoses were made in salmonellosis incidents between 2005 and 2014, with 60% of salmonellosis incidents from post-mortem examinations being diagnosed with at least one other disease. Most other diagnoses group into enteric, systemic, or respiratory syndromes and indicate that salmonellosis in post-weaned pigs is frequently part of more complex disease. The most prevalent concurrent viral diseases were due to porcine circovirus2, porcine reproductive and respiratory syndrome virus and swine influenza virus. The most prevalent concurrent bacterial diseases were due to Pasteurella multocida, Streptococcus suis, Haemophilus parasuis and Escherichia coli (mainly causing enteric colibacillosis). These disease combinations, clinical signs and other epidemiological features of salmonellosis incidents will be described and exemplify the importance of comprehensive diagnostic investigations of disease outbreaks to assist identification of factors which may influence the occurrence of disease due to Salmonellosa infection in pigs. Case descriptions will illustrate potential scenarios predisposing to salmonellosis.

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