***Salmonella* infection in mesenteric lymph nodes of breeding sows**

Running title: *Salmonella* infection and serology in sows

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ABSTRACT (248 words)

Salmonellosis is one of the main foodborne diseases worldwide. Breeding sows asymptomatically infected with *Salmonella* can transmit the pathogen to piglets and humans. *Salmonella* isolation in mesenteric lymph nodes (MLN) is considered the most reliable way to demonstrate asymptomatic infection in swine. Since breeding sows studies have been performed in feces so far, the aim of this work was to study the occurrence of *Salmonella* infection in MLN of these animals, in comparison to their serological status. *Salmonella* fecal shedding was determined at farm level to establish the framework. Additionally, risk factors associated with *Salmonella* MLN infection were analyzed. The pathogen was detected in MLN from 6.1% sows belonging to 40% of farms studied. Typhimurium was the most frequent serovar isolated in sows MLN; Enteritidis and Derby were also found in distributed throughout different farms. Interestingly, 43.8% (7/16) of the MLN isolates were pansusceptible and distributed throughout all the farms sampled. Notably, one emerging DT195 clone resistant to ampicillin, streptomycin, sulfonamide, tetracycline, nalidixic acid and cefotaxime was also detected. These *Salmonella* serovars differed from those found in fecal samples taken at farm, where London was the most frequent. Seroprevalence in sows was higher than MLN infections, showing no-concordance (k=0.15) between blood and MLN paired samples. Administration of dry food and allocation in unreformed premises were the main risk factors associated to *Salmonella* infection in sows MLN. Strategies focused on correct these risk factors most likely will help to reduce *Salmonella* in breeding sows and in the food chain.

Key words: *Salmonella*, prevalence, sows, lymph nodes, shedding, antimicrobial-resistance, risk factors.

INTRODUCTION

Non-typhoidal salmonellosis is a worldwide-distributed zoonosis caused by *Salmonella*, a pathogen of public health relevance. After an initial control in fowl, pig products are emerging as an important source of *Salmonella* for humans in the European Union (EU) (EFSA-ECDC, 2015). To protect the health of the consumers, the EU is legislating for the reduction of *Salmonella* in pigs and derivate food, including breeding sows as a vertical source of infection of the pathogen to the litter (DOUE, 2003).

Spain is the fourth largest country in pig production, after China, USA and Germany, showing an increase of 228.5% from 2008 to 2017 (MAPA, 2017). Results of the salmonellosis EU baseline studies indicated that Spain was on the top of *Salmonella* prevalence at pig herd level, detecting the bacteria in pooled fecal samples (PFS) from 64% of breeding holdings (EFSA, 2008, EFSA, 2009). However, there is no data available reporting the prevalence of *Salmonella* in sow offal (as raw material, mixed or not with meat for food processing), probably due to practical limitations for sampling sows in the abattoir.

Salmonella can be present in feces after an active infection of enterocytes and lymphatic system and/or by passive transmission of the bacteria through the gut after ingestion. Reliable diagnostics of *Salmonella* infection can only be assessed by the pathogen isolation in the gut wall or lymphatic system, being mesenteric lymph nodes (MLN) the sample of choice (DOUE, 2003; EFSA, 2006). Accordingly, sows infected asymptomatically in MLN can be both, a source of human infections through offal, and intermittent shedders of *Salmonella* transmitting the pathogen to piglets, particularly under certain stress circumstances such as parturition.

In some EU countries, serological diagnosis is considered an alternative in *Salmonella* control programs in fattening pigs (Merle *et al.*, 2011, Meroc *et al.*, 2012, Mousing *et al.*, 1997). Nevertheless, no correlation was observed between serology and the presence of *Salmonella*

in MLN or fecal samples of these animals (San Román *et al.*, 2018). One of the objectives of this work was to determine the correlation between the serological status and MLN infection in sows, assessing the usefulness of ELISA as a tool to determine *Salmonella* infection in a sow or in a herd.

This study was designed to determine: *i*) prevalence and type of *Salmonella* present in sows' MLN; *ii*) seroprevalence and ELISA performance in sows with respect to MLN infection; and *iii*) main risk factors associated to MLN infection.

MATERIAL AND METHODS

Sampling design

This study was performed in Navarra, a Northern Spain region with moderate-low prevalence of *Salmonella* in fattening-pigs (San Román *et al*., 2018). In this context, a total of 65,308 breeding sows belonging to 763 farms were registered in 2014 (INTIA, personal communication). Most of these animals (37,964 sows) belonged to 16 large farms, which contained more than 1,200 sows each and were managed by 7 integrator companies. These large herds were based on a closed replacement system using gilts born in the farm, with a replacement rate of ≈50%.

To know the prevalence of *Salmonella* shedding at herd level, in comparison to that observed in the EU baseline study (EFSA, 2009), fecal samples were obtained in 12 out of the 16 large farms and processed in pools as detailed below. The study was designed to obtain fecal samples proportionally to: (*i*) the number of rooms of each reproductive unit (i.e. gestation, farrow, replacement and confirmed gestation unit); and (*ii*) the number of sows kept in each room, representing the different reproductive cycle stages. Accordingly, gestation (n=340) and farrowing (n=205) sows were sampled in 12 farms, but those allocated in replacement (n=30) and confirmed gestation unit (n=25) facilities could only be analyzed in 4 and 3 of

these farms, respectively. Thus, a total of 600 individual fecal samples from 5 or 10 rooms/farm were collected from the rectum by using the double-glove method and individual sterile containers in order to avoid cross contamination.

Within this framework, 15 out of the 16 large holdings containing a total of 33,545 sows were included in the study. The sows sampled were selected from those regularly sent by the farmer to the abattoir for breeding replacement, thus all sows were multi-parity but the parity number was unknown. Due to the lack of previous information on *Salmonella* MLN infection in sows, 15-20 animals/farm (depending on the herd size) were considered representative to detect the presence of *Salmonella* infection, assuming an estimated minimum prevalence of 20% by farm and a 95% of confidence interval. The pooling was not taken into account for sample size calculations, since pooling was highly efficient compared to individual sampling in previous studies (Arnold et al, 2009). Accordingly, a total of 264 sows were sampled for MLN and 237 of them (one farm failed) were also bled at the abattoir. All samples were identified and processed individually.

Salmonella isolation and characterization

Both MLN and fecal samples were processed by the ISO 6579:2002/Amd 1:2007 (hereafter, ISO 6579) (ISO, 2007) as previously detailed (San Román *et al*., 2018). Presumptive *Salmonella* isolates were stored at -20ºC in 10% skimmed milk (Pronadisa, Spain) and sent to the National Centre for Animal Salmonellosis (Algete, Madrid, Spain) for confirmation and serotyping by the Kauffman-White Scheme (Grimont & Weill, 2007). Thereafter, *S.* Typhimurium isolates were phage-typed by standardized protocols in the National Centre of Microbiology at the Instituto de Salud Carlos III (Madrid, Spain) according to standard protocols (Echeita *et al.*, 2005).

All *Salmonella* isolates were analyzed by the Kirby-Bauer disk diffusion test (CLSI, 2013) in cation-adjusted Mueller-Hinton plates against 12 antimicrobials (all from BD, Spain) of 7

different families, as previously detailed (San Román *et al*., 2018). The antimicrobials tested were: ampicillin and amoxicillin-clavulanic acid (aminopenicillins; A); chloramphenicol (phenicols; C); streptomycin and gentamicin (aminoglycosides; S); sulfisoxazole, trimethoprim and trimethoprim-sulfametoxazole (sulfonamides; Su); tetracycline (tetracyclines; T); nalidixic acid (quinolones; Nx); ciprofloxacin (fluoroquinolones; Cip); cefotaxime (third generation cephalosporins; Cfx). *E. coli* ATCC 25922, *S*. Typhimurium ATCC 14028 and DT104 were used as controls in each experiment. Clinical breakpoints to classify isolates as susceptible or resistant were defined by the Clinical and Laboratory Standards Institute (CLSI, 2013). Isolates were considered multidrug-resistant when exhibiting resistance to at least three antimicrobial families.

Serological study

Individual blood samples (n=237) were obtained at the abattoir and sera were extracted after incubation (RT, 4h) by centrifugation (4ºC, 10 min, 1,500 x*g*) in a Multifuge 3L-R (Sorvall, Heraeus) and stored at -20ºC until use. The Herd-Check® Swine *Salmonella* indirect ELISA test (IDEXX™ Laboratories, Switzerland) was used. This test had shown an optimal sensitivity and specificity in fattening pig sera (88% and 74%, respectively) compared with other commercial kits (Vico *et al.*, 2010). Optical density (O.D.) values were normalized and analyzed at different cut-off values (i.e. 10%, 20% and 40%), according to the manufacturer`s instructions.

Concordance test

Considering the 100% specificity of bacteriology, a farm was considered positive when *Salmonella* was confirmed in at least one MLN sample. Concordance analysis between infection and serology was performed by Kappa test (*k*) using MLN and blood paired samples (n=237). Descriptive statistics and prevalence were estimated with a 95% confidence interval ($CI_{95\%}$). Statistical comparison of percentages was performed by a *Chi*-square test with

Fisher's correction ($p \leq 0.05$) when required, using the SPSS 15.0.1 statistical software (SPSS Inc., Chicago, IL).

Questionnaire data and statistical analysis

A questionnaire consisting of 70 variables was used to assess possible risk factors associated to *Salmonella* MLN infection. Questions were divided into five main sections in the farm survey: *i*) farm general characteristics related to herd size, number of gestation units, and number of full-time workers; *ii*) biosecurity aspects such as existence and maintenance of outside fence and footbath, use of specific clothes, entrance restrictions, rodent control programs, and presence of cats, dogs and wild birds,; *iii*) feeding: type of feed, number of diets, and water supplier; *iv*) use of antimicrobial agents: type, number and length of treatments; and *v*) farmer´s personal information: age, educational level, and additional training on pig production. In order to provide reliable information, all the surveys were filled out with the assistance of the farm veterinarian.

For statistical analysis, a screening of possible risk factors was carried out by a univariable *Chi*-square test. Significant variables ($p \leq 0.05$) were further considered in a multivariable random-effect logistic regression model in which the outcome variable was the "culture positive"; the explanatory variables included in the model as fixed effect were those from the questionnaire; and the random effect was the farm. Multivariable analysis was performed by the STATA software (StataCorp, L.P., College Station, TX). An odds ratio (OR) >1 indicated that animal exposure to the factor increases the risk of *Salmonella* positivity, whereas an OR<1 indicates a reduced risk of animal positivity due to exposure to the factor.

RESULTS

***Salmonella* prevalence at farm level in fecal samples**

The framework of study before starting the study was defined by the presence of *Salmonella* in 13/120 (10.8%) PFS that belonged to 50% (6/12) farms studied (Table 1). Farms with at least one PFS positive showed a 21.6% of mean prevalence, but most (58.3%) of them exhibited ≤10% positive PFS. Farms from integrators B and F showed higher proportion of positive PFS. A wide variety of serovars were identified in these samples, with isolates pansusceptible (6/11) or resistant to streptomycin (5/11) alone or combined with tetracycline; *S*. Typhimurium was not found in PFS (Table 2).

***Salmonella* MLN infection in sows**

As shown in Table 1, *Salmonella* spp. was found in 16 out of 264 (6.1%) MLN of sows that belonged to 6 out of 15 (40%) breeding farms. The mean prevalence of the pathogen within positive farms was 14.5%. However, most (80%) of the farms showed ≤10% animals infected, displaying a marked left-biased distribution of the infection (Figure 1).

A total of 6 serovars from 4 different serogroups were detected in sows' MLN. The most common serovar was Typhimurium (43.7%) followed by Derby (18.7%), Enteritidis (12.5%) and Montevideo (12.5%) (Table 2).

Nine out of the 16 MLN isolates (56.25%) belonged to serovars Typhimurium or Derby and showed resistance to three (streptomycin, sulfonamide and tetracycline) or more (ACSSuT and ASSuT-Nx-Cfx) antimicrobials leading to three different multidrug-resistant patterns (Table 2). Interestingly, 43.7% (7/16) of the isolates of MLN origin were pansusceptible and distributed in all farms but one (farm 4), while the three multidrug-resistant profiles were distributed in three different breeding farms (Table 2). The multidrug-resistant *S*. Derby SSuT (three isolates) was common to Farms 2 and 4, and the two multidrug-resistant *S*. Typhimurium were restricted to one origin each. In fact, the five Typhimurium isolates found in Farm 1 showed the typical penta-resistant profile (ACSSuT) associated to DT104 phage-type, and the DT195 isolate obtained in Farm 4 showed a particular ASSuT-Nx-Cfx

multidrug-resistant profile. Overall, the distribution of the *Salmonella* phenotypes suggested a different origin of infection for each farm.

Seroprevalence

To determine the usefulness of this serological tool, ELISA results at 40% O.D. cut-off were compared with *Salmonella* infection by the ISO 6579 method in MLN samples from the same sows, as "gold standard" technique. Accordingly, the ELISA results indicated that 100% of farms had at least one seropositive sow with a 41.8% of mean seroprevalence vs. 40% of farms with a mean of 6.1% of sows found infected in MLN (Table 1). The percentage of seropositive sows varied from 15% to 80% (Table 2) depending on farm. Furthermore, all the farms presented a seroprevalence >10% (Figure 1), indicating a much higher *Salmonella* seroprevalence than bacteriological results and large discrepancy between both diagnostic techniques. For instance, the two farms showing 80% of seropositive sows exhibited either uninfected or 30% of infected sows within the farm (Table 2). Furthermore, the discrepancy between techniques was revealed by the high proportion of sows negative by bacteriology (86/99) but positive in ELISA (Table 3). Accordingly, the absence of concordance between serology and microbiology was statistically confirmed by a Kappa index (k=0.15).

Risk factor analysis of *Salmonella* MLN infection in sows

Most (13 out of 15) of the farms accurately completed the questionnaire, except two farms that showed absence of *Salmonella* in all its analyzed animals. A total of 12 out of 70 variables were initially associated with *Salmonella* MLN infection in the univariate scrutiny. From them, only two variables remained significant in the multivariable logistic regression model, indicating that administration of dry food (as compared with food mixed with water) and a lack of shed/barn/building renovations in the last 5 years were significant risk factors (Table 4).

DISCUSSION

The presence of *Salmonella* in feces could be due not only to excretion from MLN but also to cross-contamination or passive ingestion and survival of the pathogen in the intestinal tract of the animal without causing infection. Thus, *Salmonella* isolation in MLN is the most reliable way to demonstrate asymptomatic infection in swine (DOUE, 2006). Also, MLN infection is considered a reservoir and source of intermittent excretion and dissemination of the pathogen to piglets (EFSA, 2011). However, studies on sows MLN infection are lacking, due to intrinsic limitations of sampling sows in the abattoir and the availability of representative number of animals for each sampling. To our knowledge, only one previous study has been published about *Salmonella* MLN infection in breeding sows (Keteran *et al.*, 1982). The prevalence found there were (58.2%) higher than in our conditions (6.1%) but results are not exactly comparable, since in the former study the animals were maintained for 10 days in lairage, while in our study sows were slaughtered within 2 hours of arrival. In our study, the 6.1% MLN infection found in sows was similar to that observed in MLN of fattening pigs (7.2%) of the same framework (San Román *et al*., 2018).

Herein, we present a novel study of *Salmonella* infection in sows MLN and its concordance with serology in paired samples taken from 237 sows. Overall, large disagreement between *Salmonella* infection and seropositivity (41.7%) was observed, being even higher than the disagreement observed previously for fattening pigs (San Román *et al*., 2018). This discrepancy could be attributed to endemicity of infection in breeding holdings, higher possibility of re-infections or antigenic contacts in sows than in young pigs (Vico et al., 2010; Vico *et al*., 2011; Meroc *et al*., 2012). Additionally, a longer persistence of humoral immune response than infection itself could also be an explanation (Scherer *et al.*, 2008). Other hypotheses such as lack of ISO 6579 sensitivity (Mainar-Jaime *et al.*, 2013), lack of ELISA specificity (Vico *et al*., 2011) or absence of exotic serogroups antigens in the ELISA plates

coating (Van Winsen *et al.*, 2001) could also contribute. However, serology has been applied in large studies salmonellosis control in Denmark, Germany and Belgium, where infection and serology are considered highly correlated in finishing pigs (Mousing *et al*., 1997; Merle *et al*., 2011; Meroc *et al*., 2012). Our results indicate that the serological diagnosis is of very limited interest (if any) to control salmonellosis in sows.

More than 50 different *Salmonella* serovars were found in feces of breeding sows of the baseline EU study, being Derby (23.9%) and Typhimurium (17.9%) the most frequent (EFSA, 2009). Contrarily, in our study, *S.* Typhimurium was not detected in feces but was the serovar most frequently identified in MLN. Since this serovar is the most commonly reported in human infections in 2014 (EFSA-ECDC, 2016), sow offal could contribute to this public health concern.

Besides the foodborne hazard, the inappropriate use of antimicrobial agents in humans (ECDC, 2018) and animals (EMA, 2018) has led to a quick emergence of multidrug-resistant *Salmonella* of special epidemiological surveillance (DOUE, 2003). A 48.7% of multidrug-resistant isolates has been found in sows feces at the EU country level (EFSA-ECDC, 2019). In our work, only 18.2% of multidrug resistant *Salmonella* were found in sows feces at farm, whereas (56.2% were found invading the MLN of the sows analyzed at abattoir highlighting the differences in profiles between *Salmonella* isolates of the different origins.

Notably, we isolated one *S.* Typhimurium phage-type DT195 resistant to six different families of antimicrobials including third generation cephalosporins. This finding represents a proportion of cefotaxime-resistant isolates (0.4%) a bit lower to that observed in EU (1.2%) and Spain (1.1%) (EFSA-ECDC, 2019). However, it should be particularly monitored, since this antimicrobial resistance has emerged during last years and represents the treatment of choice in humans, particularly for children.

Previous information indicated that a main risk factor associated to *Salmonella* fecal shedding in sows was a high replacement rate by external gilts (Davies *et al.*, 2000). In Navarra, the intensive production of breeding sows was based on a closed self-replacement with gilts born in the herd, which could contribute positively to the moderate-low prevalence observed in our study. Here, the main risk factors associated to MLN infection were both administration of dry food (instead of food mixed with water) and maintenance of sows in non-renovated farms. The former could be associated to a lower persistence of *Salmonella* in feed water after acidic fermentation (Van Winsen *et al*., 2000; Missotten *et al*., 2015); and the latter, to an inefficient disinfection of old materials and/or the presence of *Salmonella* vectors, such as lizards, birds or rodents (Andrés-Barranco *et al*., 2014). Besides strategies directed to correct these risk factors, other measures could be implemented to reduce MLN infections in sows, such as nutritional programs including the addition of organic acids or prebiotics (Andrés-Barranco *et al*., 2015).

Since some studies suggest a vertical dissemination of *Salmonella* from breeding to fattening pigs (EFSA, 2011), correction of risk factors associated to salmonellosis in breeding sows would contribute to improve the epidemiological status and, thus, to minimize the risk of pork food contamination from farm to fork.

Overall, moderate-low prevalence of *Salmonella* was observed in sows MLN of Navarra vertically-integrated production system, in agreement with the moderate-low prevalence observed in fattening pigs of the same framework. These results indicated that Navarra is a swine production area of Spain offering safe pork products for humans. However, correction of the risk factors identified here would contribute to improve the control of this important zoonosis at farm level and, thus, the competitiveness of this economical sector.

Acknowledgments

The work was financed by Departamento de Industria, Energía e Innovación of the Navarra Government [reference IIQ14064.RI1] and Instituto Navarro de Tecnología e Infraestructuras Agroalimentarias S.A. -INTIA- [reference CAM2011030054].

VG, SS, BSR and LMG contracts were funded by UPNA postdoctoral fellowship, Erasmus Mundus EMUNDUS18 Program, CSIC JAE-Doc Program, and INIA-European Social Fund, respectively. We are grateful to farmers, veterinaries and slaughterhouse workers as well as to the temporal students Naroa Remondegui, June Landa (JAE-Intro CSIC-FEDER fellowship), Ruth Erro and Mirian Samblas. We thank John Wild for the English revision.

Author disclosure

Authors have not conflict of interest.

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