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Contamination of pig carcass with *Salmonella enterica* serovar Typhimurium monophasic variant 1,4 [5], 12: i:- originates mainly in live animals

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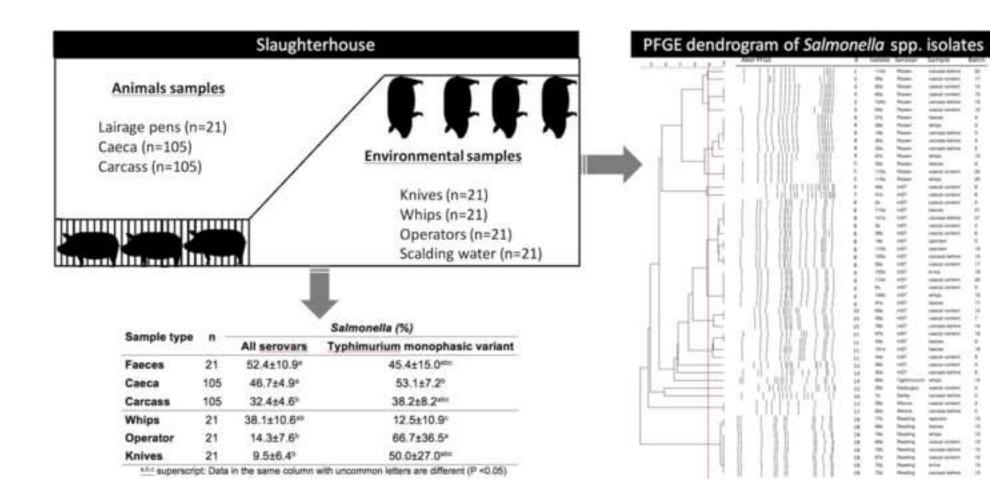
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*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

Pork meat is considered one of the major sources of Salmonella food infection in humans and distribution of virulent serotypes such as monophasic variants of S. Typhimurium have emerged as a public health threat.

A high proportion of pigs were infected Salmonella spp. being the monophasic variant the most prevalent.

Necessity of a mandatory European programme to control the bacteria during pork production.

1 Contamination of pig carcass with Salmonella enterica serovar Typhimurium monophasic variant 1,4 [5], 12: i:- originates mainly in live animals 2 3 4 Clara Marín^{a*}, Ma Carmen Chinillac^a, Marta Cerdà-Cuéllar^b, Laura Montoro-Dasi^{c,d}, Sandra Sevilla-5 Navarro^{a,c}, Teresa Ayats^b, Francisco Marco-Jimenez^d, Santiago Vega^a 6 7 8 9 ^aInstituto de Ciencias Biomédicas. Departamento de Producción Animal, Sanidad Animal, Salud 10 Pública Veterinaria y Ciencia y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad 11 CEU-Cardenal Herrera, 46115 Alfara del Patriarca, Valencia, Spain 12 ^bCentre de Recerca en Sanitat Animal (CReSA)-Institut de Recerca i Tecnología Agroalimentàries 13 (IRTA), Campus de la Universitat Autònoma de Barcelona, 08193, Bellaterra, Barcelona, Spain 14 ^cCentro de Calidad Avícola y Alimientación Animal de la Comunidad Valenciana, C/Nules, 16, 15 12539, Alguerias del NP, Castellón, Spain. ^dInstituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, 46022, Valencia, 16 17 Spain 18 19 20 *To whom correspondence should be sent: Instituto de Ciencias Biomédicas. Departamento de 21 Producción Animal, Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología de los 22 Alimentos, Facultad de Veterinaria, Universidad CEU-Cardenal Herrera, 46115 Alfara del

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Abstract

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Pork is considered, after eggs, the major source of infection in humans in the EU, with Salmonella Typhimurium, including monophasic strains. Widespread distribution of virulent serotypes such as monophasic variants of S. Typhimurium (mST, 1,4,[5],12:i- and 1,4,12:i-) have emerged as a public health threat. mST constitutes a high proportion of the multi-drug-resistant isolates and has been increasing in pigs since 2010. Despite the current situation, within the EU there is no mandatory programme for the control of Salmonella at pork production level. In this context, the aim of this study was to investigate the relationship between Salmonella strains isolated from animals at the slaughterhouse and those isolated from carcass before chilling. During the study, a total of 21 pig herds were intensively sampled during processing at the slaughterhouse. ERIC-PCR was performed among isolates recovered at the different steps in the slaughterhouse to assess the genetic relationship. Then, PFGE was done to study the pulsotypes among the different Salmonella serovars isolated. The results showed a high level of Salmonella pork batch contamination upon arrival at the slaughterhouse (71.4%) and at the end of the slaughtering process (66.7%), with mST the main serovar isolated from both origins (53.1% and 38.2%. respectively). The slaughter environment poses a potential risk for carcass contamination and it is considered an important source of Salmonella spp. Similarly, this study shows that 14.3% of the strains isolated from carcasses have the same Xbal-PFGE profile as those previously recovered in the slaughterhouse environment, but not in the live animals from that same batch. Moreover, this study demonstrates a strong association between the Salmonella status of the batch on arrival at the slaughterhouse and pork carcass contamination. These results highlight the importance of Salmonella control during pork production despite the lack of a mandatory European programme to control the bacteria.

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Keywords: Pork, mST, PFGE, ERIC-PCR, Slaughterhouse

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1. Introduction

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According to the 2018 EFSA summary report on zoonoses, zoonotic agents and food-borne outbreaks, Salmonella was responsible for 24.4% (91,662) of food-borne outbreaks in the European Union (EU) (EFSA, 2018). It is estimated that 4.5% of outbreaks are associated with pig meat and products thereof (EFSA, 2016). Pork is considered, after eggs, the major source of infection in humans in the EU, with S. Typhimurium, including monophasic strains (S. 1,4,[5],12:iand S. 1,4,12:i-) being frequently implicated (Andres and Davies, 2015; Davies et al., 2016). Nonetheless, no outbreak data have been reported by Spain, as the notification of non-typhoidal salmonellosis in humans is voluntary (EFSA, 2016). This is striking, as Spain is the second largest swine producer in the EU and fourth worldwide (Marquer et al., 2014). In fact, Spain is among the countries with the highest Salmonella prevalence, 36.2% at slaughterhouse, with 31.3% prevalence of monophasic strains of S. Typhimurium (EFSA, 2016). Widespread distribution of virulent serotypes such as monophasic variants of S. Typhimurium (1,4,[5],12:i- and 1,4,12:i-) have emerged as a public health threat, as it is the third most frequently isolated serovar from human cases of salmonellosis in Europe, representing 8.3% of confirmed human cases in 2015 (EFSA, 2016). Monophasic S. Typhimurium constitutes a high proportion of the multi-drug-resistant isolates and has been increasing in pigs since 2010 (EFSA, 2016). Despite the current situation, there is no mandatory programme within the EU for the control of Salmonella at pork production level. In fact, each member state has to consider whether interventions should be set at farm and/or slaughterhouse level (De Busser et al., 2013).

The control of *Salmonella* carriage and shedding in swine remains a challenge (Davies et al., 2016). The risk of *Salmonella* contamination is known to increase across the production chain, at farm level and transport from the farm to the slaughterhouse, reaching its maximum at the slaughterhouse and in subsequent processing (Arguello et al., 2013a,b; Duggan et al., 2010; Visscher et al., 2011;). At the moment, the slaughterhouse remains the most appropriate stage of the food chain for evaluation of the carriage of *Salmonella* and other zoonotic agents by farm animals, particularly in swine (Bonardi et al., 2013). When animals and the carcass are processed,

contamination of pig carcass can result from the skin or intestinal contents from the pig itself, but also due to cross-contamination from other carcasses or surfaces at the slaughterhouse (Botteldoorn et al., 2003). Salmonella serovars present on pig carcass can be different from those detected in the same batches from the farm (Bonardi et al., 2017). However, many studies have shown that good hygienic practices at slaughter are more effective in reducing the prevalence of Salmonella than on-farm interventions (Baptista et al., 2010a). Despite all the efforts made during the last 20 years in the control of Salmonella in pig production (Andres and Davies, 2015), our driving hypothesis was that the vast majority of Salmonella serovars present on pig carcass ready for commercialisation have their origin in the same batches on the farm, so that Salmonella enters the slaughterhouse mainly along with the live animals. Thus, a longitudinal study was conducted to investigate the possible relationship between Salmonella strains isolated from animals at the slaughterhouse and those isolated from carcass before chilling.

2. Material and methods

All the procedures used in this study were performed in accordance with Directive 2010/63/EU EEC for animal experiments.

2.1 Study design

This study was conducted from September 2015 to September 2016 in 8 slaughterhouses from the Valencian Region, Eastern Spain. The processing plants selected slaughters 90% of the pork production in the Valencia Region (MAGRAMA, 2016). Samples were collected during 21 sampling visits from 21 batches of pigs. The batch definition used was a group of pigs coming from a single farm in a given day. All farms were finishing farms, with minimum nine-month old pigs at an average live weight of 160 kg.

2.2 Sample collection

At each sampling visit, pooled faecal material was collected from lairage pens at the

slaughterhouse. Faeces samples (≥500 g) were taken aseptically into a sterile jar from five different points distributed all over the pen. Pens were washed and disinfected between batches; the faeces collected were thus linked to an individual batch. Overall, 21 batches were studied. From each batch, five animals were randomly selected and followed along the processing line. Then, the caecum from each individual animal was aseptically collected and placed into a sterile bag. Caeca were incised with a sterile scalpel blade and approximately 50 mL of the contents were placed in a 500 mL sterile jar. Finally, carcass swabs from individual animals were collected at the end of the processing line by swabbing a 100 cm² area at each of the four sampling sites (ham, belly, rump and jowl) rubbing the sterile swab (bioMerieux, Madrid, Spain) 10 times vertically and horizontally (Mannion et al., 2012).

At the same time, immediately after each individual was processed, environmental swabs of the slaughtering staff were collected from three sites (knives, whips and operators) by vigorous swabbing of the surface, using sterile wet swabs (bioMerieux, Madrid, Spain). Moreover, 1 L of

2.3 Salmonella isolation

scalding water was collected directly into a sterile jar.

Samples were collected directly into sterile sample jars and analysed according to ISO 6579:2002 (Annex D). Firstly, samples were pre-enriched in 1:10 vol/vol Buffered Peptone Water 2.5% (BPW, Scharlau®, Barcelona, Spain) and then incubated at 37±1 °C for 18±2 h. The pre-enriched samples were transferred onto Semi-Solid Modified Rappaport Vassiliadis (MSRV, Difco®, Valencia, Spain) agar plates and incubated at 41.5±1 °C for 24-48 h. Plates showing the typical haze around the inoculation spot on the MSRV plates were subcultured onto Xylose–Lysine–Deoxycholate (XLD, Liofilchem®, Valencia, Spain) and ASAP (Chromogenic *Salmonella* spp. agar plate, bioMerieux, Madrid, Spain) and incubated at 37±1 °C for 24-48 h. After incubation, five presumptive *Salmonella* colonies were streaked onto nutrient agar plates (Scharlab®, Barcelona, Spain) and incubated at 37±1 °C for 24±3 h. Then, a biochemical test (API-20®, bioMerieux, Madrid, Spain) was performed to confirm *Salmonella* spp. Confirmed *Salmonella* strains were serotyped in accordance with the Kauffman–White–Le–Minor technique (Grimont and

Weill, 2007) at the Laboratori Agroalimentari (Cabrils, Spain) of the Departament d'Agricultura, Ramaderia, Pesca i Alimentació.

2.4 Molecular typing of Salmonella isolates

Two different subtyping methods were carried out for genotyping *Salmonella* isolates. All isolates were first genotyped by enterobacterial repetitive intergenic consensus (ERIC)-PCR, as previously described (Moré et al., 2017). Representative isolates from the different *Salmonella* ERIC-PCR patterns identified per sample were further analysed by pulsed-field gel electrophoresis (PFGE).

PFGE was performed according to the PulseNet standardised protocol "Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*" (www.pulsenetinternational.org). Restriction endonuclease digestion was carried out using Xbal (Roche Applied Science, Indianapolis, IN, USA).

ERIC and PFGE band patterns were analysed using Fingerprinting II software, v3.0 (Bio-Rad, Hercules, CA, USA). Similarity matrices were calculated with the Dice coefficient and cluster analysis was performed by the unweighted-pair group method with arithmetic mean (UPGMA). The isolates with a minimum level of similarity of 90% were considered genetically similar or identical.

2.5 Statistical analysis

A generalised linear model (GLM), which assumed a binomial distribution for *Salmonella* presence, was fitted to the data to determine whether there was an association between sample type collected (faeces, caeca, carcass, whips, operator and knives) and *Salmonella* status of the batch. A batch was considered infected upon arrival at the slaughterhouse, if at least one of the five samples collected from caeca was positive. A batch was considered positive at the end of the processing, if at least one of the five samples collected from the carcasses was positive. For this analysis, the error was designated as having a binomial distribution, and the probit link function

was used. Binomial data for each sample were assigned a one if they had *Salmonella* or a zero if they did not. A P-value of less than 0.05 was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard error of the least squares means. All statistical analyses were carried out using a commercially available software program (SPSS 21.0; SPSS Inc., Chicago, IL).

3. Results

During this study, a total of 315 samples were collected from different points of the slaughterhouse (Fig. 1). Samples were collected from the lairage pens (faeces, n=21), scalding water (n=21), whip surfaces (n=21), operators (n=21), working knives (n=21), caecal content (n=105) and carcasses after processing (n=105).

According to the different batches sampled (n=21), 71.4% (n=15) arrived at the slaughterhouse colonised by *Salmonella* spp. (caecal content) and 66.7% (14/21) of carcasses were also contaminated with *Salmonella* spp. at the end of processing.

The frequency of *Salmonella* contamination throughout the different slaughter steps according to the samples collected is summarised in Table 1. From all samples collected at the slaughterhouse, 34.0% (107/315) were positive for *Salmonella* spp. The higher prevalence was found in faeces from lairage pens and caecal content (52.4±10.9% and 46.7±4.9%, respectively), followed by whips (38.1±10.6%), carcass (32.4±4.6%), operator (14.3±7.6%) and knives (9.5±6.4%). None of water samples collected were positives to *Salmonella* spp.

Salmonella Typhimurium monophasic variant (mST) was the serovar more frequently isolated in that kind of samples, most frequently being contaminated with Salmonella (faeces and caeca), (45.4±15.0% and 53.1±7.2%, respectively) (Table 1). Carcass samples showed significantly reduced frequency of positives (32.4±4.6%, P=0.000), but a similar rate of mST serovar (38.2±8.2%, P=0.523), compared with faeces and caecal samples. For environmental samples, no significant differences were observed for operator and knife samples, which showed a low proportion of positives (14.3±7.6%, P=0.523 and 9.5±6.4%, P=0.523, respectively). However,

a high percentage of mST was found in both samples (66.7±6.5% and 50.0±27.0%, respectively). On the contrary, a relatively high proportion of *Salmonella*-positive samples was observed in whips (38.1±10.6%), but mST frequency was lower (12.5±10.9%).

The frequency of *Salmonella* serovar isolated during the slaughter processing is summarised in Table 2. As reported above, from 107 isolates recovered, the most prevalent *Salmonella* serovar isolated during the slaughter processing was mST (44.9%), followed by serovars Rissen (21.5%), Reading (11.2%), Albona (4.7%), Derby (1.9%), Kedougou and Typhimurium (0.9%). From all strains isolated, 14.0% (15/107) could not be revived and, consequently, were not serotyped; the results were expressed as *Salmonella* spp. The results obtained from different serovars related to the sample collected are represented in Table 2.

To assess the genetic relationship among isolates recovered at the different steps of the slaughterhouse, 107 isolates were typed by ERIC-PCR. Next, 57 different ERIC-PCR profiles were further analysed by PGFE. The PFGE analysis showed a total of 18 different PFGE pulsotypes among the different serovars (Fig. 2). No PFGE pattern could be obtained from six isolates. mST and S. Rissen, the two most abundant serovars, also showed the highest genetic diversity, with 8 and 5 different pulsotypes, respectively (Fig. 2). In contrast, Reading, the third most frequent serovar, showed a low diversity, with all isolates grouped in a single cluster with the same pulsotype. The remaining serovars (Albona, Derby, Kedougou, Typhimurium) were represented by one or two pulsotypes, each including only one or two isolates.

Isolates of carcass origin were distributed among 9 different pulsotypes, 3 for S. Rissen isolates, 3 for mST, 1 for each of the serovars Albona, Derby and Reading. Isolates of faeces were allocated in 5 different pulsotypes associated with three serovars: mST with 3 pulsotypes, Rissen with 2 and Reading with 1.

Ten pulsotypes (X3, X4, X5, X8, X9, X10, X11, X16, X17, X18) included isolates of faeces, caecal content and/or carcass (Fig. 2). Notably, some of them (X4-batch 3, X8-batch 21, X17-batch 2, X18-batch 13) showed carcass strains to have the same Xbal-PFGE pattern as their own animal batch upon arrival at the slaughterhouse (faeces or caecal content isolates). Also, the same

strain (pulsotype) was isolated from carcasses and slaughterhouse environment (knives, whips and operator) during processing (same batch), represented by pulsotypes X4, X8, X18 (batches 3, 19, 13, respectively). Similarly, the same pulsotype was found among caecal isolates and the slaughterhouse environment (whips, operator) from the same batch (X5-batch 20, X8-batch 2, X18-batch13). Finally, the same pulsotype was found in carcass isolates and the slaughterhouse environment, but different from their own animal batch. On the contrary, several PFGE patterns obtained from caecal content and animal faeces isolates show several strains not to be disseminated during the carcass processing, as they were not found in carcasses or in environmental samples.

4. Discussion

This study demonstrated a high level of *Salmonella* pork batch contamination upon arrival at the slaughterhouse (71.4%) and at the end of the slaughtering process (66.7%), mST being the main serovar isolated from both sources (53.1% and 38.2%, respectively). The high level of *Salmonella* spp. detected can be explained by the lack of a *Salmonella* control programme in pork in Spain (Arguello et al., 2012). Moreover, the results obtained correlate with the previously reported high prevalence of *Salmonella* infection in Spanish pig farms (EFSA, 2018). Pork is considered the second source of *Salmonella* human infection in the EU, with *S.* Typhimurium, including monophasic variants (1,4,[5],12:i- and 1,4,12:i-), being frequently implicated (EFSA, 2018). Notably, mST strains were the most frequent in this study. Currently, monophasic variants of *S.* Typhimurium (1,4,[5],12:i- and 1,4,12:i) have emerged as a public health threat, as it is the third most frequently isolated serovar from human cases of salmonellosis in Europe, representing 7.9% of confirmed food-borne outbreaks. It also constitutes a high proportion of the multi-drugresistant isolates and has been increasing in pigs since 2010. The international dissemination of 1,4,[5],12:i- mST in swine populations is likely to be related to the selective advantage offered by multi-drug-resistant strains associated with stable genetic elements, also carrying virulence

determinants within bacterial lineages that are well adapted to the porcine host and are prevalent in human infections as a result of contaminated pig meat (EFSA, 2018).

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The slaughter environment poses a potential risk for carcass contamination and is considered an important source of Salmonella spp. by several authors (Arguello et al., 2012; Gomes-Neves et al., 2012; Mannion et al., 2012; De Busser et al., 2013). Similarly, this study shows that 14.3% of the strains isolated from carcasses have the same Xbal-PFGE profile as those previously recovered in the slaughterhouse environment, but not in the live animals from that same batch (caecal content or lairage pens faeces). This could be explained because Salmonella could remain on contaminated equipment and be transferred to other carcasses that are subsequently slaughtered. Moreover, Salmonella can also be spread by workers, as the hands and tools of meat handlers can frequently be contaminated. However, cross-contamination at slaughterhouse is easy to control with the implementation of proper measures of hygiene and staff protocols that reduce the impact of the slaughterhouse environment on carcass contamination. According to the current legislation, these control measures should be registered in the Slaughterhouse Hazard Analysis and Critical Control Points (HACCP) (Hernández et al., 2012). On the other hand, this study shows that there is a strong association between the Salmonella status of the batch upon arrival at the slaughterhouse and pork carcass contamination, as previously reported (Baptista et al., 2010b; Andres and Davies, 2015). In fact, the same strains were isolated from carcasses and from their corresponding animal batch upon their arrival at the slaughterhouse, with a high frequency. Thus, control measures applied in pre-harvest stage (mainly at farm level) would reduce the burden on subsequent steps of the production chain. consequently leading to less-contaminated pork carcasses (Andres and Davies, 2015). Salmonella status of the batch at farm can vary depending on several factors, such as feeding practices, including the degree to which the feed is ground, and the pH and type of feed, the management procedures, such as continuous or all-in/all-out production systems, different types of herds (farrow-to-finish herds or fattening herds), size of the herds and the level of hygiene and general health status of the pigs (Bonardi, 2017). However, despite all the investments made at farm level over the last 20 years to control *Salmonella* spp. in pig production, no reduction of the on-farm *Salmonella* prevalence has been shown (EFSA, 2016). This is mainly because, within the EU, there is no mandatory programme for the control of *Salmonella* at primary swine production level, as indicated above. For this reason, more studies are needed to develop measures for *Salmonella* control at farm level.

Moreover, the importance of transport and the stay in the lairage pens must be studied in depth, as these stages play a double role. In one way, some authors demonstrate the animal transport to the processing plant or long stays in lairage pens increases *Salmonella* prevalence in faeces (Bonardi, 2017). This fact could be explained because a stressful situation could induce the carrier batch to shed *Salmonella* at higher rates due to a disturbance in intestinal functions that may increase the spread of intestinal bacteria in livestock (Mulder, 1995; Marin and Lainez, 2009). Thus, the assessment of *Salmonella* status of the pig batch at the slaughterhouse could be the best option to detect the bacteria and to avoid underestimating the prevalence obtained when samples are collected at farm level (EFSA, 2008; Arguello et al., 2012; EFSA 2016).

Moreover, some authors highlight that transport to the slaughterhouse in contaminated trucks or long stays in lairage contaminated pens are of great concern, as *Salmonella* may be introduced into a *Salmonella*-free batch (Hurd et al., 2002; Bonardi, 2017). Although it is difficult to avoid animal stress in pig production during transport and lairage stay, the role of contaminated trucks and lairage pens can easily be controlled. This can be achieved with proper cleansing and disinfection of the truck and the pens between batches, according to the current standard implemented in European slaughterhouses (HAAPC), as reported above. The controls set out by slaughterhouses that took part in this study certified that the cleaning and disinfection of the trucks and lairage pens were accurate and sufficient to remove the bacteria between different batches. It has been argued that biosecurity plays a very important role in avoiding the introduction of *Salmonella* and other pathogens and also in limiting its spread once it has entered the production chain (Andres and Davies, 2015). However, there is no universal biosecurity protocol that all farms can put into place to minimise the risk of disease introduction. Each farm is unique in terms of

location, facilities, management, host susceptibility and other influential factors (Andres and Davies, 2015). Therefore, biosecurity should be a continuous process which assesses the risks, implements protocols according to needs and costs, evaluates the effectiveness and modifies the procedures as critical areas of risk change (Amass, 2005ab). To this end, it is important to follow the example applied in *Salmonella* control in poultry, which has obtained excellent results at primary production stage, and subsequently in poultry meat. It is important to emphasise that, unlike poultry production, which is much more homogeneous and integrated in few companies, the swine production system is not generally integrated and each farm has its own particularities, making it more difficult to apply proper and standardised biosecurity plans to control the bacteria.

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Figure Legends Fig 1. Samples taken during the study. Fig 2. PFGE dendrogram of Kpnl profiles of Salmonella spp. isolates. The similarity matrices were calculated using the Dice coefficient and UPGMA clustering method. Profiles with a similarity ≥ 90% were considered same pulsotype. X: pulsotypes.

Table 1
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Table 1.Salmonella spp. isolated according to the sample type collected and the relationship with monophasic Salmonella Typhimurium, the most prevalent serovar isolated. Data are presented as least squares means ± standard error of the least squares means.

Sample type			All Salmonella serovars (%)	mST (%)	
	Faeces	21	52.4±10.9 ^a	45.4±15.0 ^{abc}	
Animal samples	Caeca	105	46.7±4.9 ^a	53.1±7.2 ^b	
	Carcass	105	32.4±4.6 ^b	38.2±8.2 ^{abc}	
	Whips	21	38.1±10.6 ^{ab}	12.5±10.9 ^c	
Environmental samples	Operator	21	14.3±7.6 ^b	66.7±6.5 ^a	
	Knives	21	9.5±6.4 ^b	50.0±27.0 ^{abc}	

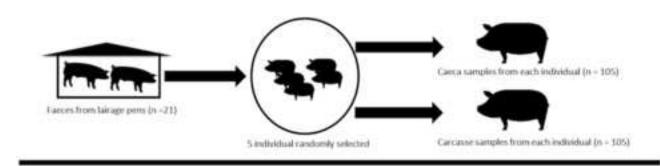
n: total samples collected, mST: *Salmonella* Typhimurium monophasic variant. ^{a,b,c} superscript: Data in the same column with uncommon letters are different (P <0.05).

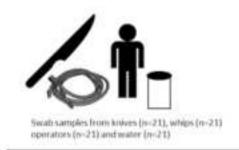
Table 2.Percentage of each *Salmonella* serovar isolated by sample type (excluding mST).

	n	Total (%)	Sample type (%)					
<i>Salmonella</i> serovars			Animal samples			Environmental samples		
30107413			Faeces	Caeca	Carcass	Whips	Operator	Knives
Rissen	23	21.5	8.7	39.1	39.1	13.0	-	-
Reading	12	11.2	8.3	41.7	25.0	8.3	8.3	8.3
Albona	5	4.7	-	40	60	-	-	-
Derby	2	1.9	-	-	100	-	-	-
Kedougou	1	0.9	-	100	-	-	-	-
Typhimurium	1	0.9	-	-	-	100	-	-
NA	15	14.0	20.0	40.0	26.7	13.3	-	-

n= number of isolates from each serovar. NA: isolates not serotyped.

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PIG SAMPLES ENVIRONMENTAL SAMPLES

