Sero- and genotyping of *Salmonella* in slaughter pigs, from farm to cutting plant, with a focus on the slaughter process

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**Abstract**

The objective of this study was to investigate the role of the slaughtering process in *Salmonella* carcass contamination by typing isolates recovered previously in a double study of the following: (1) a tracking survey from the farm to the slaughterhouse and (2) a survey of the slaughterhouse environment (i.e., lairage area, slaughter line, cutting plant and carcasses). The *Salmonella* serotypes identified on the carcasses of the 16 tracked batches were frequently linked to lairage, whereas the serotypes detected at the farm, transport or pig-related samples (i.e., caecum content and lymph nodes) were only occasionally detected at the carcass level. Multi-locus variable-number tandem repeats (MLVA) of 77 *Salmonella enterica* ser. Typhimurium isolates from seven of these batches confirmed the link between the isolates recovered from carcasses and holding pens. Only four of the 16 positive carcasses had profiles previously isolated from lymph nodes or caecal content.

In the second part of the study, a total of 131 *S. enterica* ser. Typhimurium and 74 *S. enterica* ser. Derby isolates were further characterised by MLVA and Pulsed Field Gel Electrophoresis (PFGE), respectively. The MLVA profiles identified in carcasses varied throughout the working day and were frequently linked to those identified in samples from the slaughter line points collected close in time. PFGE and MLVA profiles identified at lairage were also detected in later processing facilities (i.e., slaughter line and cutting plant) as well as in carcasses. Finally, most of the profiles found at the cutting plants were previously identified in the slaughter line or carcass samples. The results from this study show that *Salmonella* contamination in pigs entering the slaughterhouse can be attributed to several sources. Typing of isolates by MLVA and PFGE clarified the sources of carcass contamination and improved the accuracy of cross-contamination attributable values. Without obviating the relevant role of infected pigs entering the slaughterhouse, the present study highlights the lairage and slaughtering as important sources of carcass contamination.

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1. Introduction

During the last two decades, the concern about the role that pork plays in human salmonellosis has gradually increased. During the 1990s and the first years of the present century, it was estimated that 10% of human salmonellosis cases could be attributed to pork (Hald et al., 2004). In the last EU summary report on zoonoses, notic agents and food-borne outbreaks published by the European Food Safety Authority (EFSA), pork was the third most frequently contaminated meat, after fresh chicken and turkey meat (EFSA, 2012). Nevertheless, with the implementation of control programmes implemented, such as Spain, accurate information regarding the status of *Salmonella* infection or *Salmonella* contamination at different stages of pork production is essential to elaborate programmes designed to reduce its prevalence (Arguello et al., 2012; EFSA, 2008a, 2009; García-Feliz et al., 2007, 2009; Gómez-Laguna et al., 2011; Torres et al., 2011).

Several studies have implicated the slaughtering process in the spreading of *Salmonella* in pork (Botteldoorn et al., 2003; Käsbohrer et al., 2000; McDowell et al., 2007). Two studies were performed in Spain to investigate the prevalence and main serovars in four Spanish commercial pig slaughterhouses (Arguello et al., 2012). The relationship reduction in the prevalence of poultry-related salmonellosis, the relative role of pork products has been enhanced (Pires et al., 2011). According to EU regulation 2160/2003, in the near future, control programmes for swine production will also be compulsory in the EU. In those countries where these programmes have not yet been implemented, such as Spain, accurate information regarding the status of *Salmonella* infection or *Salmonella* contamination at different stages of pork production is essential to elaborate programmes designed to reduce its prevalence (Arguello et al., 2012; EFSA, 2008a, 2009; García-Feliz et al., 2007, 2009; Gómez-Laguna et al., 2011; Torres et al., 2011).

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between carcass contamination and contamination in live animals entering the slaughterhouse was specifically evaluated in the first study, whereas a more intensive assessment of slaughterhouse environmental contamination was the goal of the second study. Results from Arguello et al. (2012) described an increase in Salmonella contamination during the processing of live animals into pork carcasses, together with an unsuccessful linkage of Salmonella positive results in pig caecum samples or lymph nodes and positive carcasses. Moreover, cross-contamination was estimated to account for 50% of all contaminated carcasses and several critical points in the slaughter line were identified.

Molecular genotyping methods give us the chance to establish the relatedness among Salmonella isolates grouped by serotyping (Foley et al., 2007). Several authors have further investigated by molecular typing techniques the relationships among isolates obtained at slaughterhouses to elucidate the sources of contamination and the contamination cycles of the slaughter process (Botteldoorn et al., 2004; De Busser et al., 2011; Duggan et al., 2010; Giovannacci et al., 2000; Swanenburg et al., 2001c; Van Hoek et al., 2012; Wonderling et al., 2003).

The aim of the present study was to further investigate the sources of Salmonella carcass contamination in four Spanish commercial pig slaughterhouses. For this purpose, serotype results from the tracking study, described in Arguello et al. (2012), were analysed by batch and slaughterhouses to elucidate the sources of contamination and the contamination cycles of the slaughter process (Botteldoorn et al., 2004; De Busser et al., 2011; Duggan et al., 2010; Giovannacci et al., 2000; Swanenburg et al., 2001c; Van Hoek et al., 2012; Wonderling et al., 2003).

2. Materials and methods

2.1. Study design

The Salmonella isolates included in this study were obtained in the course of a previous investigation of the prevalence of Salmonella contamination and the main serovars found in Spanish pig slaughterhouses, including on carcasses, live animals and in the slaughterhouse environment (Arguello et al., 2012). The survey included two studies, a tracking from farm to slaughterhouse study and an environmental contamination study, performed in four Spanish commercial pig slaughterhouses (A, B, C, and D). The tracking study from the farm to the slaughterhouse was performed by following a total of sixteen different batches of slaughtered pigs in slaughterhouses A and B, with eight batches per slaughterhouse. Briefly, 10 selected pigs within each batch were sampled at the farm by collecting faecal samples. After slaughtering, the caecal contents, mesenteric lymph nodes (MLN) and carcass swabs immediately before chilling were collected from the same selected pigs. Moreover, swabs from the surfaces of trucks and holding pens involved in the transport and resting of the selected animals as well as random holding pens, different points along the slaughter line (e.g., scalding tank water, bung dropper, evisceration and carcass splitting) and 50 randomly selected carcasses before chilling were sampled at each sampling day.

A more intensive assessment of the slaughterhouse environment was conducted during the environmental contamination study in slaughterhouses C and D, which were investigated on three different days distributed at the beginning, middle and end of the work week. Holding pens, several points along the slaughter line (e.g., scalding tank water, evisceration, renal extraction, carcass splitting and fat removal) and carcasses before and after chilling were sampled on Monday, Wednesday and Friday in each slaughterhouse. More information about the slaughterhouses' characteristics, the sampling design and the samples collected is available in the previous article (Arguello et al., 2012). Moreover, the adjacent cutting plant of slaughterhouses C and D was evaluated the following working day by collecting samples from machinery, belt surfaces and the hands of the personnel. Despite the fact that the results obtained in cutting plants were not included in the previous publication, the isolates collected from the meat processing activities of this facility (e.g., belt surfaces, personnel hands and their implements) were also included in the present study.

2.2. Salmonella isolation and serotyping

All of the samples were analysed according to the ISO standard methodology (EN-ISO 6579:2002 and EN-ISO 6579:2002/Amd 1:2007). Suspected Salmonella colonies were screened using the indole test and the 4-methylumbelliferyl caprilate fluorescence test (Mucap test, BioLife). A single confirmed Salmonella isolate from each positive sample was serotyped according to the Kaufman–White scheme using commercial antisera (Grimont and Weill, 2007).

2.3. Salmonella isolates selection

Seventy-seven S. enterica ser. Typhimurium isolates (henceforth named S. Typhimurium) were selected for further molecular typing. These isolates were obtained in tracking from farm to slaughterhouse study, during the tracking of three batches of slaughtered pigs (IV, V and VI) in two different visits (3 and 4) to slaughterhouse A and four batches of slaughtered pigs (XI, XII, XIII and XIV) in another two visits (2 and 3) to slaughterhouse B. All S. Typhimurium isolates recovered from samples collected at the farm, trucks, holding pens, caecal content, MLN, carcasses and slaughter environment during each sampling day were included. Batches were selected on the basis of the presence of isolates at several tracking stages to clarify relationships among S. Typhimurium isolates from different production steps.

All of the S. Typhimurium and S. enterica ser. Derby isolates (henceforth named S. Derby) recovered from the samples collected during the three visits to slaughterhouses C (74 S. Typhimurium and 22 S. Derby isolates) and D (57 S. Typhimurium and 52 S. Derby isolates) during the environmental contamination study were also selected for molecular typing.

2.4. Pulsed-field gel electrophoresis (PFGE)

PFGE using XbaI (Fermentas, Lifesciences, Spain) for restriction enzyme digestion was used to characterise S. Derby isolates. The technique was performed using the Centers for Disease Control and Prevention (CDC) PulseNet protocol (Ribot et al., 2006) with minor changes. The plugs of DNA were washed twice with double-distilled water and another four times with TE 10:1 buffer (10 mM Tris-1 mM EDTA) at 54 °C. Electrophoresis was performed in a Chef-DR™-III (Bio-Rad Laboratories, S.A., Madrid, Spain) using the following settings: initial switch time 2.2 sec, final switch time 63.8 sec, a gradient of 6 V/cm, 120º angle and 22 h of electrophoresis in 0.5×TBE buffer (Sigma-Aldrich). Two lambda markers (lambda DNA c857 ind 1 Sam7, GelSyringe™, New England Biolabs, Ipswich, UK) and two size standard strains Salmonella Braenderup H9812 were included on each gel.

2.5. Multi-locus variable-number tandem repeats (MLVA)

Four of the VNTR loci described by Lindstedt et al. (2004), STTR5, STTR6, STTR9 and STTR10, as well as two new tandem repeat loci identified as STTR11 and STTR12 were used to characterise the selected S. Typhimurium isolates. The new loci were identified with the Tandem Repeat Finder program (Benson, 1999) available at http://tandem.bu.edu. STTR11 and STTR12 are situated in genes tolA, a membrane spanning protein, and yohM, a nickel-cobalt efflux protein, at positions 814617 and 3184177 of the S. Typhimurium LT2 chromosome, respectively (McClelland et al., 2001). The main characteristics of the VNTR loci used are described in Table 1.

Briefly, one or two colonies of a pure culture of each selected S. Typhimurium isolate were suspended in 100 μl molecular grade water contained in a 1.5 ml screw cap microcentrifuge tube. The
samples were boiled in water at 100 °C and then centrifuged at 14,000 rpm for 8 min. The supernatant was used for PCR amplification. The primers were multiplexed in two solutions using the Maxima™ Hot Start PCR Master Mix (Fermentas, Lifesciences, Spain) in a total volume of 50 µl and a final primer concentration of 0.1 µM. Multiplex 1 was performed with primers STR12 and STR6, whereas multiplex 2 included the STR5, STR9, STR10 and STR11 primers. PCR parameters were set as indicated by Lindstedt et al. (2004). PCR products were pooled as follows: 5 µl of multiplex PCR1 and 2 µl multiplex PCR2 were mixed and diluted with 43 µl of molecular grade water to obtain a total volume of 50 µl. Two µl of the pooled products were combined with 0.25 µl of Rox-labelled MegaBACE ET550-R standard (GE Healthcare, Madrid, Spain) and 7.75 µl of 0.1% Tween 20 for a final volume of 10 µl. The ET size standard was previously diluted 1:20 in MegaBACE loading solution containing 70% formamide, 1 mM EDTA (GE Healthcare, Madrid, Spain). The samples were then denatured at 94 °C for 2 min and immediately subjected to capillary electrophoresis. The electrophoresis was run for 75 min using MegaBACE matrix (GE Healthcare, Madrid, Spain) with an injection voltage of 3 kV for 45 s and a running voltage of 10 kV. The amplification for each locus was identified using the MegaBACE Genetic Profiler software v1.2 (GE Healthcare, Madrid, Spain) taking into account the colour and size range. Internal control strains (SM 492, SM 353, SM 349) were used to corroborate the accuracy and repeatability of the technique. The fragment sizes were transferred to an Excel file. The amplicon sizes were extracted and converted to allele numbers following Lindstedt et al. (2004). The smallest amplicon found was named 01 for each locus and successive allele sizes were named 02, 03, etcetera. When no amplification was found, the allele was named 0. The allele strings were introduced in the following order: STR12-STR9-STR5-STR6-STR10-STR11.

2.6. Bioinformatics tools

The banding patterns from PFGE and repeat units from MLVA analysis were analysed with BioNumerics® version 6.5 (Applied-Maths, Ghent, Belgium). The PFGE results were compared by cluster analysis using the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) with a position tolerance of 1.5% and optimisation of 1.0%. The Hunter-Gaston diversity index was used to calculate the polymorphism of individual loci (Hunter and Gaston, 1988) by the free software provided by the Health Protection Agency’s Bioinformatics Unit (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl).

3. Results

3.1. Tracking from farm to slaughterhouse study results

3.1.1. Serotyping results

Fifteen Salmonella serotypes were recovered during the tracking of sixteen batches of 10 animals each from farm to slaughterhouse in abattoirs A and B (Table 2). Only in batch III, all of the samples yielded negative results. Four batches were positive at the farm and in three of them, at least one of the serotypes identified at the farm was also detected in animal-related samples collected at the slaughterhouse (i.e., caecum content, MLN or carcass). In contrast, Salmonella Derby was identified in faecal samples collected at the farm in one of the batches (batch VIII) but could not be isolated in any of the animal-related samples collected at the slaughterhouse. Salmonella Typhimurium was found in two of the three positive trusts and the same serotype was also found in animal-related samples at slaughter, whereas S. Rissen, which was found on the other slaughter truck, was isolated from the caecal content of the transported pigs but not from their MLN or carcasses (Table 2).

The widest diversity of serotypes was found at lairage, which was highly contaminated both before and after the stay of the tracked pigs. S. Typhimurium was identified on holding pens harbouring pigs from 14 of the 16 batches. This serotype was also identified in the caecal content or MLN samples in 11 of these batches. In contrast, from 11 batches in which serotypes other than S. Typhimurium were detected at the holding pens, only 6 were also contaminated by with the same serotype in the caecal content or MLN samples. Moreover, serotypes found at holding pens were also frequently identified on carcasses. In fact, only the serotype S. Brandenburg was identified in carcasses (batch IX in visit 1 to slaughterhouse B), without being previously isolated from the lairage area during the same working day.

In 11 of the 16 batches, the serotypes detected on the carcasses were also found in caecal contents or MLN samples. All of the sampled carcasses were negative in three of the selected batches. One of them (batch III) was also negative in the other animal-related samples (i.e., MLN and caecum content), whereas the other two were positive in the caecal content samples. Carcass contamination with Salmonella serotypes not previously detected in caecal contents or MLN was observed in 5 batches. In batches IX and XII, the serotypes detected in carcasses were completely different from those recovered from the caecum content and MLN (Table 2).

3.1.2. MLVA results

MLVA typing divided S. Typhimurium isolates into 21 different profiles in total, with five among the Salmonella isolates recovered in visits 3 and 4 to slaughterhouse A and seven and six different profiles detected in visits 2 and 3 to slaughterhouse B, respectively. One profile was shared by isolates from both visits to slaughterhouse A and another one by isolates from visit 4 to slaughterhouse A and visit 2 to slaughterhouse B. The MLVA profiles detected in tracked pigs are shown in Table 3.

The profile 11-12-5-3-0-2 was the most common in animal-related samples from both visits to abattoir A. This profile was identified in 10 of 21 and 16 of 27 S. Typhimurium isolates recovered during visits 3 and 4, respectively and could be detected in the lairage, the slaughter line and the pig-related samples (e.g., caecal content, MLN and carcass) from batches IV, V and VI as well as in the truck involved in the transport of the pigs from batch IV (Table 3). In contrast, there was no predominant molecular type among S. Typhimurium isolates from slaughterhouse B.

### Table 1

<table>
<thead>
<tr>
<th>Loci</th>
<th>Dye and primers sequence (5’-3’)</th>
<th>Repeat (bp)</th>
<th>Smallest product size (bp)</th>
<th>No. alleles</th>
<th>Max (pi)</th>
<th>Missing amplicon (%)</th>
<th>D. Indexb</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR 5</td>
<td>Lindstedt et al., 2004</td>
<td>6</td>
<td>212</td>
<td>12</td>
<td>0.434</td>
<td>0</td>
<td>0.761</td>
</tr>
<tr>
<td>STR 6</td>
<td>Lindstedt et al., 2004</td>
<td>6</td>
<td>300</td>
<td>12</td>
<td>0.332</td>
<td>12.7</td>
<td>0.810</td>
</tr>
<tr>
<td>STR 9</td>
<td>Lindstedt et al., 2004</td>
<td>9</td>
<td>153</td>
<td>5</td>
<td>0.790</td>
<td>7.3</td>
<td>0.362</td>
</tr>
<tr>
<td>STR 10</td>
<td>Lindstedt et al., 2004</td>
<td>6</td>
<td>329</td>
<td>8</td>
<td>0.712</td>
<td>73.2</td>
<td>0.444</td>
</tr>
<tr>
<td>STR 11</td>
<td>HEX-GGCCAAACAGACGAGCAAGCAGC/ GITTCTGGCCAGCATCTACCC</td>
<td>45</td>
<td>434</td>
<td>2</td>
<td>0.956</td>
<td>0</td>
<td>0.085</td>
</tr>
<tr>
<td>STR 12</td>
<td>TET-GGATGCTGCTGCCGGGAAGG/ GGGCAGTCTGCATACGCGTGTTTGGAGC</td>
<td>6</td>
<td>114</td>
<td>10</td>
<td>0.307</td>
<td>0</td>
<td>0.813</td>
</tr>
</tbody>
</table>

a Max (pi). Fraction of samples that have the most frequent repeat number in this locus (range 0.0 to 1.0).

b Diversity Index based on Hunter-Gaston index.
The MLVA profile identified at the farm (Batch XII, visit 2) was also detected at the holding pens but not in isolates recovered from other samples. It is noteworthy that at visit 2 to slaughterhouse B, none of the three profiles found in monitored or randomly sampled carcasses were related to those identified in any of the other samples collected. In visit 3 to slaughterhouse B, the S. Typhimurium profile 8-9-0-3-7-2 was identified in the samples from the truck, in holding pens and in caecal contents but not on carcasses. In slaughterhouse C, the MLVA profile was restricted to isolates found on each sampling day, whereas 3 of them (20%) presented a single PFGE profile different times had different profiles detected. PFGE using XbaI yielded 24 different profiles (Fig. 1). The number of profiles identified ranged from 2 (visit 3 to slaughterhouse D) to 15 (visit 1 to slaughterhouse D). Profile XD23 was the most prevalent, comprising 38 isolates identified on carcasses and in environmental samples in slaughterhouse D as well as on carcasses in slaughterhouse C. Profile XD21 was only detected on two of the sampling days in slaughterhouse C and was identified in holding pens, along the slaughter line and in cutting plant, although it was not detected on carcasses. The other profiles were represented by a lower number of isolates and 18 of them were identified in a single isolate.

3.2. Environmental contamination study

3.2.1. Serotyping results

Nine serotypes were found among 546 Salmonella isolates recovered during the environmental contamination study, considering also the cutting plant isolates (not included in the previous publication by Arguello et al., 2012). S. Typhimurium, S. Rissen and S. Derby were detected on different sampling days within each slaughterhouse. In total, they constituted more than 80% of the isolates recovered from caecal contents or MLN isolates, whereas the other 12 matched isolates recovered from the slaughterhouse environment or different facilities. Moreover, samples collected together in time (at the same sampling round) from different slaughter line activities and from carcasses frequently shared the same MLVA profile, whereas profiles from samples of the same slaughter line activity but collected at different times had different profiles.

In slaughterhouse C, the MLVA profiles identified in 19 S. Typhimurium-contaminated carcasses (57.6%) did not match those identified in any of the isolates from holding pens or the slaughter line. In slaughterhouse D, 14 of the S. Typhimurium-positive carcasses (70%) shared profiles with the slaughterhouse environment, whereas only six of them (30%) were not linked to other samples. S. Typhimurium MLVA profiles identified among isolates recovered from the slaughter line were frequently linked to those detected on carcasses in both slaughterhouses and all of the MLVA profiles except for one detected at the cutting plant facilities were previously detected on carcasses or at earlier points from the slaughter line or lairage.

3.2.2. MLVA characterisation of S. Typhimurium isolates

Seventy-four S. Typhimurium isolates obtained from slaughterhouse C and 57 S. Typhimurium isolates from slaughterhouse D were further typed with MLVA. The profiles detected are shown in Table 4. The number of profiles identified on each sampling day ranged from 3 (visit 2 to slaughterhouse C) to 10 (visit 1 to slaughterhouse C). Three MLVA profiles were shared among isolates from different visits to slaughterhouse C and another three were also shared by isolates from visits 2 and 3 in slaughterhouse D. At least one of the profiles detected at each visit could be tracked in different facilities. Moreover, samples collected together in time (at the same sampling round) from different slaughter line activities and from carcasses frequently shared the same MLVA profile, whereas profiles from samples of the same slaughter line activity but collected at different times had different profiles.

3.2.3. PFGE characterisation of S. Derby isolates

A total of 74 isolates of S. Derby recovered from slaughterhouses C and D were further analysed. PFGE using XbaI yielded 24 different profiles (Fig. 1). The number of profiles identified ranged from 2 (visit 3 to slaughterhouse D) to 15 (visit 1 to slaughterhouse D). Profile XD23 was the most prevalent, comprising 38 isolates identified on carcasses and in environmental samples in slaughterhouse D as well as on carcasses in slaughterhouse C. Profile XD21 was only detected on two of the sampling days in slaughterhouse C and was identified in holding pens, along the slaughter line and in cutting plant, although it was not detected on carcasses. The other profiles were represented by a lower number of isolates and 18 of them were identified in a single isolate.

12 of 15 S. Derby-contaminated carcasses (80%) shared their PFGE profile with those detected at the slaughterhouse on the same sampling day, whereas 3 of them (20%) presented a single PFGE profile unique among the S. Derby isolates.

3.3. MLVA loci results

Loci STTR5, STTR11 and STTR12 were amplified in the 205 isolates of S. Typhimurium, whereas locus STTR10, located on a plasmid, was...
only present in 16.8% of these isolates. The highest diversity index was found for STTR12, followed by STTR6 and STTR5 (Table 2). In contrast, only two different profiles were found for the most stable locus, STTR11.

### 4. Discussion

Several studies have investigated *Salmonella* contamination of pigs at the time of slaughter and the role that the harvest plays in the final carcass contamination (Berends et al., 1997; Botteldoorn et al., 2003; McDowell et al., 2007; Swanenburg et al., 2001a). Consistently, the environment is contaminated to a greater or lesser extent in commercial pig slaughterhouses and these authors have stressed the importance of the slaughter process in the final carcass contamination by *Salmonella*. In accordance with these assertions, a previous study in four Spanish pig slaughterhouses demonstrated an increase in *Salmonella* contamination during the processing of live animals into pork as well as the importance of slaughtering practices; cross-contamination accounted for an estimated 50% of all of the contaminated carcasses (Arguello et al., 2012).

Salmonella contamination was reported in most of the evaluated points along the slaughter line and *Salmonella* was frequently present in the lairage area, even after cleaning procedures. Although phenotypic typing methods such as serotyping or phage typing are useful as preliminary tools for *Salmonella* classification, molecular methods such as PFGE or MLVA are needed to clarify the relationship among isolates from the same serotype (Larsson et al., 2009). The accuracy, simplicity and lower cost of MLVA compared to other typing methods enhances its usefulness for *S.* Typhimurium analysis (Lindstedt et al., 2004) and it has been successfully used with isolates recovered from the slaughterhouse (Prendergast et al., 2011; Van Hoek et al., 2012). In contrast, PFGE remains the gold standard for *Salmonella* typing and *Salmonella* Derby isolates from the slaughterhouse environment have been successfully restricted using *Xba*I (Botteldoorn et al., 2004; De Busser et al., 2011; Giovannacci et al., 2000; Gomes-Neves et al., 2012; Piras et al., 2011; Wonderling et al., 2003). The present study is a further investigation of contamination sources and pathways in these Spanish pig slaughterhouses by using serotyping and molecular typing methods in *Salmonella* isolates obtained in the course of the previous survey.

Two hundred and eight *S.* Typhimurium and 74 *S.* Derby isolates could be further separated into profiles by MLVA and PFGE, respectively. Loci STTR5 and STTR6 showed frequent polymorphism according to previous studies (Dyet et al., 2010; Lindstedt et al., 2004; Prendergast et al., 2011). One of the two new loci included in this study, STTR12, also had high variability. On the other hand, the other three loci used, STTR10, STTR9 and STTR11, were less polymorphic. Only two different alleles were found in STTR11, which seems to be highly preserved among *S.* Typhimurium isolates. In our study, the *S.* Derby isolates were divided

### Table 3

MLVA profiles detected in S. Typhimurium isolates recovered in seven batches tracked from farm to slaughterhouse during the farm to slaughterhouse tracking study.

<table>
<thead>
<tr>
<th>Abattoir &amp; Visit</th>
<th>Batch</th>
<th>Profile</th>
<th>Samples from tracked batches</th>
<th>Randomly selected samples</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Farm</td>
<td>Transport</td>
</tr>
<tr>
<td>A-3</td>
<td>IV</td>
<td>11-12-5-3-0-2†</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td></td>
<td>NPR†</td>
<td>1-2-5-3-0-2</td>
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<td>[ ]</td>
</tr>
<tr>
<td>A-4</td>
<td>V</td>
<td>11-12-5-3-0-2†</td>
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</tr>
<tr>
<td></td>
<td>NPR†</td>
<td>8-9-0-0-7-2</td>
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<td>[ ]</td>
</tr>
<tr>
<td>B-2</td>
<td>XI</td>
<td>5-6-0-4-8-2</td>
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<td>4-5-8-4-9-2</td>
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<td>7-8-0-1-0-2</td>
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<td>NPR†</td>
<td>3-4-4-3-0-2</td>
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1 NPR Non pig related-profile.

2 MLN. Mesenteric Lymph Nodes

† Profiles detected in isolates from this location.
into 24 profiles, including 18 single profiles. S. Derby is a highly variable serovar which complicates the establishment of relationships among isolates (Hauser et al., 2011; Valdezate et al., 2005). Although Wonderling et al. (2003) tried to overcome this problem by grouping related profiles, in the present study a decision was made to avoid grouping similar profiles based on the assumption that those S. Derby isolates which share a common PFGE profile would definitely have a common origin.

Several profiles were found at each visit to the four monitored slaughterhouses. This result is in agreement with previous surveys, which by slaughter isolates genotyping, have indicated that the slaughter environment is contaminated by several strains at the same time (Botteldoorn et al., 2004; Piras et al., 2011; Prendergast et al., 2011; van Hoek et al., 2012; Wonderling et al., 2003). Furthermore, most profiles differed among visits to the same slaughterhouse, a fact previously reported (Giovannacci et al., 2000; Letellier et al., 2009; Piras et al., 2011; Wonderling et al., 2003). The fact that a few MLVA and PFGE profiles were shared at different visits to the same slaughterhouse could be ascribed to permanent contamination of the slaughter environment, as has been asserted by other authors (Baptista et al., 2010; Piras et al., 2011; Swanenburg et al., 2001c; van Hoek et al., 2012). Another reasonable hypothesis is that pigs entering the slaughterhouse might share the same or very similar profiles. The same farms or the same suppliers usually slaughter their pigs at the same slaughterhouses and furthermore, even without common farm of origin, S. Typhimurium or S. Derby isolates could share the same MLVA or PFGE profiles.

The role that transport plays in carcass contamination has been previously highlighted by Magistrali et al. (2008). In our tracking study, some of the carcasses from batch IV in slaughterhouse A were contaminated with the same S. Typhimurium MLVA profile found on the truck used for the transport of the pigs, thus confirming that Salmonella isolates present at transport can reach the slaughtering process and contaminate carcasses. In contrast, the MLVA profile detected in the truck of batch XIII at slaughterhouse B was also detected in holding pens and caecal contents but not in the monitored carcasses. Likewise, S. Rissen was detected in the truck and caecal content of pigs from batch XII but not in other samples from the same batch.

Serotypes recovered from carcasses were related to those identified in holding pens in almost all of the positive batches. The lairage has been linked to carcass contamination in several studies and it is fairly clear that this constitutes an important source of contamination for the incoming pigs (Hurd et al., 2002; Swanenburg et al., 2001b). Molecular typing of S. Typhimurium isolates confirmed the link among isolates detected on carcasses and in the holding pens where the pigs were kept in the three batches from slaughterhouse A and in some of the batches from slaughterhouse B. The link between isolates from carcasses and lairage has been previously demonstrated (De Busser et al., 2011; Duggan et al., 2010). In the environmental study (abattoirs C and D), MLVA profiles detected at holding pens were frequently identified among isolates from the slaughter line or cutting plant facilities and from carcasses. Interestingly, MLVA and PFGE profiles found in holding pens before the entrance of the pigs were afterwards found at other sampling points on the same day such as the slaughter line, carcasses and even the cutting plant. This result confirms that the lairage is an important risk point for incoming Salmonella-free pigs, as has been proposed previously (Arguello et al., 2012; Letellier et al., 2009) and that it constitutes a source of contamination for further processing at harvest. Another interesting finding was that one S. Derby PFGE profile, X023, was detected at several holding pens after cleaning procedures. This fact could be a consequence of spreading by the staff in charge of the lairage cleaning by their boots or by washing activities. Moreover, this profile was detected in holding pens at visits 1 and 3 to slaughterhouse D, performed over a six-week period. Although it is reasonable to think that the origin of the isolate detected at visit 3 was different from the one detected at visit 1, the possibility that it persisted during this time in the environment, as previously proposed (Duggan et al., 2010; Hald et al., 2003), cannot be excluded. These results highlight, once more, the importance of proper cleaning and disinfection procedures in the control of Salmonella within the slaughterhouse.

In some individuals, the serotype found on the carcass differed from the serotypes previously detected in caecum contents or MLN. Moreover, several MLVA profiles detected at the farm, transport and in caecal content or MLN from monitored pigs did not match the MLVA profiles from carcasses. These interesting results have been reported previously (Botteldoorn et al., 2004; De Busser et al., 2011) and yield two lessons: on the one hand, they show that carcass contamination can be minimised by good slaughtering practice, even when contaminated pigs enter the slaughter line. On the other hand, they reveal that the Salmonella isolates carried by pigs are not always the source of contamination for their carcasses. The same authors (Botteldoorn et al., 2004) reported significant differences in the fraction attributable to cross-contamination between slaughterhouses. Likewise, in the EFSA baseline study on Salmonella prevalence in slaughter pigs, the proportion of contaminated carcasses differed significantly among slaughterhouses supplied with similar proportions of initially contaminated pigs (EFS, 2008b). Both studies highlight the importance of slaughtering practices. In the present study, the MLVA results also revealed clear differences in the sources of carcass contamination between slaughterhouses C and D. While most of the profiles from carcasses were linked to those detected at dressing
activities from the slaughter line in slaughterhouse D, only some of them matched the profiles found at the slaughter line in slaughterhouse C, mainly among isolates obtained during visit 1 to this abattoir. Despite the small number of carcasses that were contaminated with *S. Derby*, these isolates usually shared a PFGE profile with those recovered from the environment, although a few of them displayed single profiles. Both slaughterhouses had similar slaughter line structure, slaughtered similar number of pigs per hour and shared pig suppliers (Arguello et al., 2012). Therefore, this result demonstrates that part of the contamination can be attributed to internal factors of the slaughtering process, such as handling and hygienic standards.

In the preliminary analysis based on prevalence results, it was estimated that half of the positive carcasses were cross-contaminated in slaughterhouses A and B (Arguello et al., 2012). Further analysis using MLVA for the characterisation of a selection of *S. Typhimurium* isolates from these slaughterhouses showed that only 25% of the isolates obtained from carcasses matched those recovered from the caecal contents or MLN of the same animals. Therefore, the proportion of cross-contaminated carcasses should be higher than what was previously assumed. As has been formerly suggested, to have an accurate value of cross-contamination, studies should include molecular typing methods to corroborate the relatedness of isolates of the same serotype (Botteldoorn et al., 2004; Wonderling et al., 2003).

At slaughterhouses C and D, the same MLVA profile was detected in carcasses sampled close in time, a fact which allows us to suggest a common source of contamination for these carcasses. In addition, some of the profiles from dressing activities isolates matched those recovered from carcasses sampled near the same time. In relatively few cases, MLVA profiles were shared by isolates recovered from carcasses and the slaughter line at different sampling rounds on the same sampling day. Giovannacci et al. (2000) also reported a high concordance among profiles identified among isolates recovered from different samples collected at the same sampling series and De Busser et al. (2011) grouped samples by herd-dependent genotypes. According to these results, it seems that there are continuous *Salmonella* inputs, most likely from infected pigs, into the slaughter line and that the strains involved in contamination vary through the working day. Taking into account that a new batch of pigs was slaughtered every half hour at both slaughterhouses, this variability in *Salmonella* profiles can be explained by the introduction of new profiles by infected pigs. As described by Botteldoorn et al. (2004), these profiles can, in some cases,
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Acknowledgements

In summary, the results from this study show that Salmonella contamination in pigs entering the slaughterhouse can be attributed to several sources, including the farm, transport and especially the lairage. These contaminated pigs are constantly introducing Salmonella into the slaughter process and together with inappropriate slaughter practices, contribute significantly to carcass contamination. The molecular typing methods MLVA and PFGE have proven to be useful tools that should be considered in slaughter studies to investigate the sources of carcass contamination. Both methods have been able to distinguish among isolates of the same serotype and have demonstrated a continuous flow of Salmonella within the slaughterhouse, from the farm level, transport or lairage to the slaughter line, carcasses and finally to the cutting plant. They have also been used to increase the accuracy of cross-contamination attributable values. According to the results obtained in four Spanish commercial pig slaughterhouses, carcass contamination can be related to several sources. Without obviating the relevant role of infected pigs entering the slaughterhouse, the lairage and slaughter are indicated as important sources of the final carcass contamination.

References


Wonderling, L., Pearce, R., Wallace, F.M., Call, J.E., Feder, I., Tamplin, M., Luchansky, J.B., 2003. Use of pulsed-field gel electrophoresis to characterize the heterogeneity and clonality of \textit{Salmonella} isolates obtained from the carcasses and feces of swine at slaughter. Applied and Environmental Microbiology 69, 4177–4182.