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Original Article

Multidrug resistant *Salmonella enterica* isolated from conventional pig farms using antimicrobial agents in preventative medicine programmes

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ABSTRACT

A longitudinal study was conducted to investigate the presence of multidrug antimicrobial resistance (multi-AR) in *Salmonella enterica* in pigs reared under conventional preventative medicine programmes in Spain and the possible association of multi-AR with ceftiofur or tulathromycin treatment during the pre-weaning period. Groups of 7-day-old piglets were treated by intramuscular injection with ceftiofur on four farms ($n = 40$ piglets per farm) and with tulathromycin on another four farms ($n = 40$ piglets per farm). A control group of untreated piglets ($n = 30$ per farm) was present on each farm. Faecal swabs were collected for *S. enterica* culture prior to treatment, at 2, 7 and 180 days post-treatment, and at slaughter. Minimal inhibitory concentrations of 14 antimicrobial agents, pulsed-field gel electrophoresis and detection of resistance genes representing five families of antimicrobial agents were performed. Plasmids carrying cephalosporin resistant (CR) genes were characterised. Sixty-six *S. enterica* isolates were recovered from five of eight farms. Forty-seven isolates were multi-AR and four contained bla_{CTX-M} genes harboured in conjugative plasmids of the IncI1 family; three of these isolates were recovered before treatment with ceftiofur. The most frequent AR genes detected were *tet*(A) (51/66, 77%), *sul1* (17/66, 26%); *tet*(B) (15/66, 23%) and *qnrB* (10/66, 15%). A direct relation between the use of ceftiofur in these conditions and the occurrence of CR *S. enterica* was not established. However, multi-AR was common, especially for ampicillin, streptomycin, sulphonamides and tetracycline. These antibiotics are used frequently in veterinary medicine in Spain and, therefore, should be used sparingly to minimise the spread of multi-AR.

Introduction

Salmonella enterica is a major foodborne pathogen, causing infections in human beings and animals worldwide. In 2014, 89,873 confirmed human cases of salmonellosis were reported in the European Union (EU) (EFSA, 2017). Control programmes for *S. enterica* in eggs and poultry have been effective in reducing the number of salmonellosis cases in human beings (EFSA, 2014). However, this trend is levelling out, owing to the persistence of *S. enterica* in pigs and porcine products (Pires et al., 2011). On the basis of examination of mesenteric lymph nodes of fattening pigs, there is a wide range (0–36.4%) in the frequency of detection of *S. enterica* between countries in the EU (EFSA, 2015). In Spain, a major producer of pigs in the EU, *S. enterica* was detected in 30% of pigs (EFSA, 2015).

Vaccination of pigs against *S. enterica* serovars that are non-pathogenic for pigs is not recommended, since pigs can be asymptomatically infected and can transmit the bacteria to human beings (San Román et al., 2013). *S. enterica* control programmes in pigs are mainly based on hygiene/disinfection, biosecurity measures and farm management practices. Treatment with antimicrobial agents are necessary for control of clinical outbreaks involving bacteria as primary or secondary pathogens, and have been used as a metaphylactic and prophylactic tool when there is a high probability of an outbreak of unknown bacterial aetiology (Barton, 2014). The selective pressure exerted by antimicrobial agents may contribute to the emergence of bacteria with antimicrobial resistance (AR) (Garcia-Migura et al., 2014). Pigs

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Table 1
Salmonella enterica serovars isolated from faeces at different intervals and treatments administered in each farm during the rearing cycle.

Farm	Antimicrobial agents used at different post-birth intervals			Sampling at different post-birth intervals			
				Number of isolates/number of samples analysed (%) Number of serovars			
	Intramuscular (day 7)	Pre-starter/starter 1 (days 21–49)	Starter 2 (days 50–79)	Day 7	Day 9	Day 14	Day 187
1	Untreated	Amoxicillin Colistin sulphate	NA	2/30 (7%) 2 Rissen	3/30 (10%) 3 Rissen	3/28 (11%) 3 Rissen	2/28 (7%) 2 Rissen
	Tulathromycin			0/40	0/40	0/39	2/39 (5%) 2 Derby
	Sows			0/7			
3	Untreated	Amoxicillin Apramycin	Tiamulin Oxytetracycline	1/30 (3%) 1 Panama	3/30 (10%) 3 Typhimurium	0/30	2/24 (8.3%) 2 Panama
	Tulathromycin	Tiamulin Oxytetracycline		2/40 (5%) 2 Panama	1/40 (2.5%) 1 Panama	0/40	3/36 (8.3%) 1 Panama 2 Typhimurium
	Sows			1/7 (14.2%) 1 Typhimurium			
6	Untreated			0/30	0/30	0/30	0/24
	Tulathromycin			0/40	0/40	0/40	2/34 (5.8%) 1 Rissen 1 Typhimurium
	Sows			0/7			
7	Untreated			0/30	0/30	0/30	0/16
	Tulathromycin			0/40	0/40	0/40	0/30
	Sows			0/7			
2	Untreated	Amoxicillin Apramycin Tiamulin	Tiamulin Oxytetracycline	13/30 (43%) 2 Brandenburg 11 Rissen[a]	4/30 (13%) 4 Rissen[a]	1/30 (3.3%) 1 Rissen	1/22 (4.5%) 1 Typhimurium
	Ceftiofur	Oxytetracycline		7/40 (18%) 3 Anatum[a] 3 Rissen[a] 1 Brandenburg	5/40 (13%) 3 Brandenburg 2 Rissen	1/38 1 Rissen	1/38 (2.6%) 1 Typhimurium
	Sows			1/7 (14.2%) 1 Brandenburg			
4	Untreated			0/30	0/30	0/30	0/18
	Ceftiofur			0/40	0/40	0/40	0/35
	Sows			0/7			
5	Untreated			0/30	0/30	0/26	4/17 (24%) 4 Rissen
	Ceftiofur			0/40	0/40	0/37	1/33 (3%) 1 Rissen
	Sows			0/7			
8	Untreated			0/29	0/29	0/21	0/21
	Ceftiofur			0/37	0/36	0/34	0/0
	Sows			0/7			
Total strains 66				27/614 (4.4%)	16/555 (0.9%)	5/533 (0.9%)	18/415 (4.3%)

[a] A total of four cephalosporin resistant (CR) *Salmonella enterica* serovars were found. One *S.* Anatum isolates were obtained at day 7 in the cephalosporin treated group. Three *S.* Rissen isolates were obtained in the untreated group (2 strains at day 7 and 1 strain at day 9). NA, not applicable.

carrying AR bacteria may have implications for public health, since there is a risk of foodborne transmission to consumers through the food chain.

Third and fourth generation cephalosporins, such as ceftiofur and cefquinome, are licensed for the treatment of systemic bacterial infections in pigs (Cameron-Veas et al., 2015). These β-lactam antimicrobial agents are some of the most important compounds used in human medicine, constituting the main therapeutic choice for treatment of infections caused by *Enterobacteriaceae* (Collignon et al., 2009). The possible selection of cephalosporin resistant (CR) *S. enterica*, together with concerns relating to their entry into the food chain, has raised questions regarding the use of these antimicrobial agents in pigs. In Spain, multi-AR was detected in 55% of *S. enterica* isolates in 2015 (EFSA, 2017).

This longitudinal study was undertaken to evaluate the presence of multi-AR *S. enterica* on conventional pig farms in Spain that use antimicrobial agents in their preventative medicine programmes. β-Lactam antimicrobial agents (penicillins and cephalosporins) and macrolides (tulathromycin and tildipirosin) are the most commonly prescribed drugs in pigs during the suckling period in Spain (Moreno, 2014). The aim of this study was to assess the effect of ceftiofur and tulathromycin treatment on the emergence of CR *S. enterica* during the pre-weaning period, and in the *S. enterica* population in pigs from day 7 until slaughter. The genotypic and phenotypic diversity of the *S. enterica* serovars obtained from each of the farms were analysed. Since

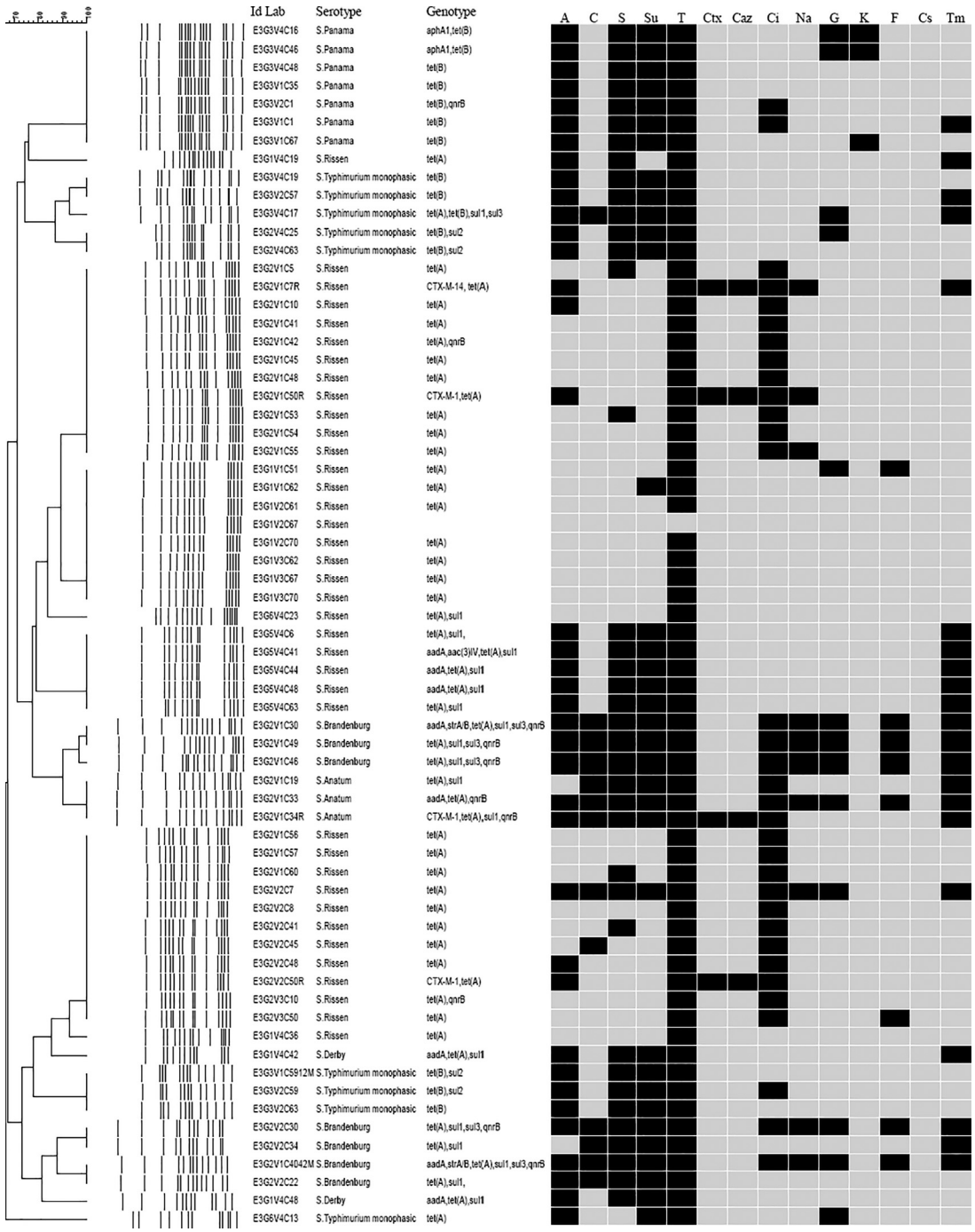

Fig. 1. Dendrogram illustrating *Xba*I-pulse field gel electrophoresis (PFGE) profiles and antimicrobial resistance phenotype of *Salmonella enterica* ($n = 66$) isolated from faeces of fattening pigs in Catalonia, Spain. Black squares represent resistance. A, Ampicillin (wild type, WT, $\leq$8 mg/L); C, chloramphenicol (WT $\leq$ 16 mg/L); S, streptomycin (WT $\leq$ 16 mg/L); Su, sulphamethoxazole (WT $\leq$ 64 mg/L); T, tetracycline (WT $\leq$ 8 mg/L); Ctx, cefotaxime (WT $\leq$ 0.25 mg/L); Caz, ceftazidime (WT $\leq$ 0.5 mg/L); Ci, ciprofloxacin (WT $\leq$ 0.064 mg/L); Na, nalidixic acid (WT $\leq$ 16 mg/L); G, gentamicin (WT $\leq$ 2 mg/L); K, kanamycin (WT $\leq$ 8 mg/L); F, florfenicol (WT $\leq$ 16 mg/L); Cs, colistin (WT $\leq$ 2 mg/L); Tm, trimethoprim (WT $\leq$ 2 mg/L). Codes of isolates (Id Lab) were based on the numbers assigned to the farm (G), visit number (V) and pig number (C).

cephalosporins and fluoroquinolones are the antimicrobial agents of choice for treatment of human salmonellosis, the presence of genes coding for these antimicrobial agents was assessed.

Materials and methods

Study design

This study was approved by the Animal Care and Ethics Committee of the University of Lleida, Spain (approval number DAAM7684; date of approval 12 October 2012). This was a longitudinal study on *S. enterica* prevalence carried out on a cohort of eight farms located in Catalonia, Spain. Sampling was carried out at the same time as a study on *Escherichia coli* published by Cameron-Veas et al. (2016). None of the farms had a history of cephalosporin use for at least 2 years prior to the study. The sampling period started in November 2012 and finished in May 2014. Seventy 7-day-old piglets (seven separate litters per farm) were ear tagged in both ears for identification throughout the study period. This sample size was based on an estimated prevalence of *S. enterica* shedders of at least 40% (Casanova-Higes et al., 2017). An increment of at least 30% of animals excreting AR bacteria after treatment was considered to be substantive. On the basis of these numbers, the sample size selected for the study was 32 animals in each of the

control and antimicrobial treated groups, with a confidence level of 95% and a power of 80%.[1]

Farms were randomly divided into two groups of four farms each, according to the antimicrobial treatment administered for experimental purposes, i.e. ceftiofur or tulathromycin. Within each farm, one group of 7-day-old pigs ($n = 40$) was treated with either ceftiofur (Naxcel, Zoetis; 5 mg/kg of body weight) or tulathromycin (Draxxin, Zoetis; 2.5 mg/kg of body weight), administered by a single intramuscular injection (Cameron-Veas et al., 2016). The remaining selected pigs from each farm ($n = 30$) were kept as an untreated control group. In most cases, the groups remained separated in distant pens over the study period, including during transportation to the finishing farm; the exception was farm 2, which was managed under a farrow-to-finish cycle system. Most farms belonged to the same large integration system; the exception was farm 1, which was owned by an independent farmer. During the rearing period, all pigs were fed under a standard nutritional programme (see Appendix: Supplementary Table 1) set by the companies, including the prophylactic use of antimicrobial agents (Table 1), from days 21 (weaning) to 49 (pre-starter and starter 1 feeds), as well as from days 50 to 70 (starter 2 feed). Of the 560 animals selected for inclusion in the study, 164 were not sampled at some point due to either death or loss of ear tags (Table 1).

Faecal samples were collected from 7-day-old piglets (day 7) before treatment with tulathromycin or ceftiofur, as detailed in Table 1, at days 9 and 14 of life, and immediately before the animals were sent for slaughter (day 187 ± 2 days). On day 7, faecal samples were also collected from the respective sows ($n = 7$ per farm).

Salmonella enterica *culture and characterisation*

Faecal samples were taken from the rectum of each pig and transported to the laboratory at 4 °C on the same day of sampling. On receipt, 5 g of faeces from each animal were homogenised in 25 mL buffered peptone water and incubated at 37 °C for 24 h. After incubation, 0.1 mL homogenate were inoculated onto Rappaport-Vassiliadis semisolid medium and incubated at 42 °C for 24 and 48 h (the latter if negative at 24 h), followed by streaking 1 μL on XLT4 medium with and without ceftriaxone (1 mg/L). Culture media were from Merck and antibiotics were from Sigma–Aldrich. One *S. enterica* isolate from each positive sample was selected for confirmation and serotyping at the Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural, Cabrils, Spain, by the reference slide agglutination method, for specific somatic flagella and capsular antigens, using the Kauffmann–White scheme (Popoff et al., 2004).

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of 14 antimicrobial agents were determined using the broth microdilution method (VetMIC GN-mo, Swedish National Veterinary Institute) using ampicillin, chloramphenicol, streptomycin, sulphamethoxazole, tetracycline, cefotaxime, ceftazidime, ciprofloxacin, nalidixic acid, gentamicin, kanamycin, florfenicol, colistin and trimethoprim (Fig. 1) (Sola-Gines et al., 2015). Epidemiological cut-off values were determined according to recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[2] Multidrug resistance was defined as resistance to antibiotics of at least three different families (Schwarz et al., 2010).

Two double disc combinations (cefotaxime with cefotaxime/clavulanic acid and ceftazidime with ceftazidime/clavulanic acid) were used to confirm the extended spectrum β-lactamase (ESBL) phenotype. Synergy was defined as an increase in zone diameter ≥ 5 mm. Cefoxitin was used for the detection of *ampC*-type β-lactamase (CLSI, 2008).

Antimicrobial resistant genetic determinants

Resistance to cephalosporins was assessed by PCR for bla_{TEM}, bla_{CTXM}, bla_{CMY-1}, bla_{CMY-2} and bla_{SHV} (Hasman et al., 2005). Sequence analysis was performed using Vector NTI advance 11 (InforMax). The amplified nucleotide sequences were compared to those in the National Center for Biotechnology Information (NCBI) data base using BLAST.[3]

Isolates exhibiting low susceptibility to ciprofloxacin were tested for the plasmid-mediated quinolone resistance genes (PMQRs) *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* (Wasyl, 2014). The presence of resistance genes for the aminoglycosides *aadA*, *strA*/*strB*, *aac*(3)IV, *aadB*, *aphA1* and *aphA2*, the tetracyclines *tet*(A), *tet*(B) and *tetC*), and the sulphonamides *sul1*, *sul2* and *sul3* were also tested by PCR (Kozak et al., 2009).

Pulsed field gel electrophoresis and plasmid characterisation

To assess the clonality of the strains and the epidemiological relatedness within farms, *Xba*I pulsed field gel electrophoresis (PFGE) macro-restriction was performed using a Chef-DR II System (Biorad) (Ribot et al., 2006). PFGE profiles were compared using Fingerprinting II Informatixe software (Applied Maths). Isolates were considered to have a unique pattern when at least one band difference was detected (Liebana et al., 2002). Bands were analysed using the Dice coefficient and unweighted pair group method with arithmetic averages (optimisation 1.5% and position tolerance 1.5%).

Replicons from plasmids with an ESBL phenotype were characterised by PCR-based replicon typing (Carattoli et al., 2005). Filter mating experiments using the rifampicin resistant *E. coli* strain HB101, together with plasmid DNA extraction and electroporation, were performed as described by Cameron-Veas et al. (2015). Transconjugants were selected on Luria–Bertani (LB) agar plates containing rifampicin (100 mg/L) and ceftriaxone (1 mg/L). The presence of a unique plasmid in each transconjugant harbouring the cephalosporin resistant gene was confirmed and their sizes were determined using S1-nuclease digestion, followed by PFGE (Barton et al., 1995).

Statistical analysis

McNemar's test (paired samples) was used to evaluate whether the prevalence of *S. enterica* and CR *S. enterica* in the different herds differed between consecutive sampling times for a given treatment (ceftiofur or tulathromycin) (Kirkwood and Sterne, 2003). The χ^2 test was used to assess whether the prevalence of *S. enterica* differed among herds or treatments at a given sampling time. When the number of observations for one of the categories was small (i.e. <5), Fisher's exact test was used (Kirkwood and Sterne, 2003). Statistical analyses were carried out using SAS version 9.1.3 (IBM) and the significance level was set at $P < 0.05$.

Results

Occurrence of Salmonella enterica

A total of 2117 faecal samples were collected during the course of the study. Sixty-six (3.1%) samples were positive for *S. enterica*; these were obtained from five out of the eight farms sampled (Table 1). On the five positive farms, 27 isolates were obtained from day 7, 16 from day 9, five from day 14 and 18 from day 187 ± 2. CR *S. enterica* was detected on one farm (farm 2; Table 1), prior to ceftiofur administration (3/21 isolates) and at day 9 (1/9 isolates). No significant differences were observed in the prevalence of CR *S. enterica* between consecutive sampling times after treatment with ceftiofur.

The prevalence of *S. enterica* at day 7 was higher ($P < 0.01$) on farm 2 (20/70, 28.6%) than on the remaining farms (2/70, 2.8%). On farm 2, prior to antimicrobial treatment, the untreated control group had a higher prevalence of *S. enterica* (13/30, 43%) than that of the ceftiofur treated group (7/40, 17.5%; $P = 0.018$). Thereafter, the prevalence of *S. enterica* in the untreated group reduced significantly ($P < 0.01$) to 13% of shedders at day 9 in both untreated and treated groups (4/30 and 5/40, respectively) (Table 1). A significant reduction ($P < 0.05$) in *S. enterica* shedding was observed from days 7 to 14 for both the untreated and treated groups. On farm 5, significant differences ($P = 0.022$) in *S. enterica* prevalence were found, at day 187, between pigs treated with ceftiofur (1/33, 3.0%) and untreated pigs (4/17, 24%) (Table 1). *S. enterica* was isolated from sows on 2/8 farms, as well as in their offspring; serovars were the same between sows and piglets on farm 2, but different on farm 3.

Among the 66 isolates of *S. enterica*, the most prevalent serovar was Rissen (38/66, 58%), followed by the monophasic variant of Typhimurium (9/66, 14%), then Panama and Brandenburg (7/66, 11% each) (Table 1). The first two were the most widely distributed serovars, since they were present in four and three of the positive farms, respectively.

[1] See: http://epitools.ausvet.com.au/content.php?page=home (accessed 18 January 2017).
[2] See: http://www.eucast.org/ (accessed 18 January 2017).
[3] See: www.ncbi.nlm.nih.gov (accessed 18 January 2017).

Phenotypic and genotypic resistance

As shown in Fig. 1, *S. enterica* isolates exhibited resistance to tetracycline (98%), ampicillin (58%), sulphamethoxazole (53%), streptomycin (58%), ciprofloxacin (50%), trimethoprim (32%), gentamicin (20%), chloramphenicol (20%) and nalidixic acid (15%). Twelve percent of the isolates were resistant to florfenicol and 4% were resistant to kanamicin. None of the isolates exhibited resistance to colistin. Forty-seven isolates were multidrug resistant, being resistant to at least 3/14 antimicrobial agents tested, and exhibiting 19 different antimicrobial resistance profiles. Among these isolates, 38/66 (57.6%) were resistant to at least four antimicrobial classes, with 31/38 (81.6%) isolates resistant simultaneously to ampicillin (A), streptomycin (S), sulphonamides (Su) and tetracycline (T) alone or in combination with other antimicrobial agents, thus exhibiting the ASSuT multidrug resistant profile (Table 2). In the case of the *S.* Typhimurium monophasic stain, ASSuT is associated with a resistance region localised on the bacterial chromosome. Furthermore, 16/18 (89%) isolates recovered before the animals were slaughtered were multi-resistant. Only one isolate (serovar Rissen) was pan-susceptible (Fig. 1). Four isolates (three *S.* Rissen and one *S.* Anatum from farm 2) were resistant to cephalosporins (Table 2).

The most prevalent antimicrobial genotypes were *tet*(A) (77%), *sul1* (27%) and *tet*(B) (23%) (Table 2). The *qnrB* gene was detected in 10 (15%) isolates among four serovars, namely Brandenburg ($n = 5$), Anatum and Rissen ($n = 2$ each), and Panama ($n = 1$) (Table 2); nine of these isolates were recovered from the same farm (farm 2), while *S.* Panama was recovered from Farm 3 (Fig. 1).

Resistance to cephalosporin in isolates from farm 2 was associated with the presence of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-14}}$ genes in two different *S.* Rissen isolates, and $bla_{\text{CTX-M-1}}$ in one isolate of *S.* Anatum recovered from three 7-day old piglets. An additional *S.* Rissen $bla_{\text{CTX-M-1}}$ was isolated from the same animal in the second visit (at day 9); this animal belonged to the control group (Table 1). CR *S. enterica* was not detected during further visits to this farm. All isolates harbouring CTX-M transferred the genes to the recipient strain by conjugation and transformation. In all cases, CTX-M genes were harboured in a 95 kilobase plasmid belonging to the IncI1 incompatibility group.

Macro-restriction analysis using *Xba*I produced 12–16 bands and distributed the 66 isolates into eight major clusters consisting of isolates with related PFGE profiles (80% identity) and three unique PFGE patterns represented by a single isolate each (Fig. 1). PFGE analysis demonstrated high clonality between *S. enterica* isolates of the same serovar within farms. Indistinguishable

Table 2
Phenotypic and genotypic characterisation of antimicrobial resistance profiles of the *Salmonella enterica* isolates (numbers in curved brackets) obtained in this study, grouped by serovars.

Serovars	Phenotype[a] (number of isolates)	Genotype (number of isolates)
Rissen (38)	ACSSuTCiNaGTm (1)	*tet*(A) (1)
	ASSuTTm (5)	*tet*(A)/*sul1* (2); *aadA*/*tet*(A)/*sul1* (2); *aadA*/*aac(3)IV*/*tet*(A)/*sul1*(1)
	ASTTm (1)	*tet*(A) (1)
	ATCtxCazCiNa (1)	*tet*(A)/$bla_{\text{CTX-M}}$ (1)
	ATCtxCazCi (1)	*tet*(A)/$bla_{\text{CTX-M}}$ (1)
	ATCi (2)	*tet*(A) (2)
	ATCtxCazCiNaTm (1)	*tet*(A)/$bla_{\text{CTX-M}}$ (1)
	TCiNa (1)	*tet*(A) (1)
	STCi (4)	*tet*(A) (4)
	TCi (9)	*tet*(A)/*qnrB* (2); *tet*(A) (7)
	TCCi (1)	*tet*(A) (1)
	TCiF (1)	*tet*(A) (1)
	TGF (1)	*tet*(A) (1)
	T (7)	*tet*(A) (6); *tet*(A)/*sul1*(1)
	SuT (1)	*tet*(A) (1)
	Pan-susceptible (1)	Not found
Typhimurium monophasic (9)	ASSuTCi (1)	*tet*(B)/*sul2* (1)
	ASSuTG (1)	*tet*(B)/*sul2* (1)
	ACSSuTGTm (1)	*tet*(B)/*su1*/*sul2l* (1)
	ASuTG (1)	*tet*(A) (1)
	ASSuTTm (1)	*tet*(B) (1)
	ASSuT (4)	*tet*(B)/*sul2* (2); *tet*(B)(2)
Brandenburg (7)	ACSSuTCiNaGFTm (5)	*aadA*/*strA/B*/*tet*(A)/*sul1*/*qnrB* (1); *tet*(A)/*sul1*/*sul3*/*qnrB* (3); *aadA*/*strA/B*/*tet*(A)/*sul1*/*sul2*/*qnrB* (1)
	ACSSuT (1)	*tet*(A)/*sul1* (1)
	CSSuTTm (1)	*tet*(A)/*sul1* (1)
Panama (7)	ASSuTCi (1)	*tet*(B) (1)
	ASSuTCiTm (1)	*tet*(B)/*qnrB* (1)
	ASSuTGK (2)	*aphA1*/*tet*(B) (2)
	ASSuT (2)	*tet*(B) (2)
	ASSuTK (1)	*tet*(B) (1)
Anatum (3)	CSSuTCiTm (1)	*tet*(A)/*sul1* (1)
	ACSSuTCiNaGFTm (1)	*aadA*/*strA/B*/*tet*(A)/*sul1*/*qnrB* (1)
	ACSSuTCtxCazCiTm (1)	*tet*(A)/*sul1*/*qnrB*/$bla_{\text{CTX-M}}$ (1)
Derby (2)	ASSuTTm (1)	*aadA*/*tet*(A)/*sul1* (1)
	ASSuT (1)	*aadA*/*tet*(A)/*sul1* (1)

Ctx, cefotaxime and ceftazidime; aminoglycosides: aadA, strA/strB, aac(3)IV, aadB, aphA1, aphA2; tetracycline: tet(A), tet(B), tet(C); sulphonamides: sul1, sul2, sul3; quinolones: qnrA, qnrB, qnrB, qnrD, qnrS; β-lactams: $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{SHV}.

[a] See Fig. 1 for antimicrobial nomenclature.

fingerprints were present in isolates from different animals and from isolates obtained at different sampling times, indicating the persistence of clones over time. Two of the *S.* Rissen isolates resistant to cephalosporin recovered from the farm 2 had identical PFGE patterns (E3G2V1C7R and E3G2V1C50R; Fig. 1).

Discussion

A common practice in preventative medicine programmes among large pig producers of Spain is to inject 7-day old piglets with one intramuscular dose of ceftiofur during the lactation period. In the longitudinal study carried out on conventional farms, we found no evidence of a direct effect from this practice on the emergence of CR *S. enterica* strains. This conclusion could be influenced by the low prevalence of *S. enterica* and the low number of CR strains found in the farms studied, as well as by the wide variety of serovars isolated, since different serovars show different abilities to acquire resistance genes (Aarestrup, 2004).

In the four CR isolates recovered from farm 2, CTX-M genes were harboured on a conjugative plasmid of 95 kb, belonging to the IncI1 family of replicons (Rodriguez et al., 2009; Zurfluh et al., 2014). Farm 2 also had a high prevalence of CR *E. coli* during the study period associated with the same group of plasmids (Cameron-Veas et al., 2016). The presence of these CR strains in both treated and untreated cephalosporin groups suggests an exchange of mobile genetic elements between the different *Enterobacteriaceae* could have occurred in this farm. Moreover, we found that most of *S. enterica* strains (especially those recovered on the last visit to the farm and before the animals were sent) exhibited multi-AR and belonged to *S. enterica* serovars frequently associated with human infections (EFSA, 2011). This finding highlights the importance of human infections from pig products if these multi-AR strains enter the food chain.

Although gastroenteritis caused by non-typhoidal *S. enterica* is mostly self-limiting and treatment is not required, ~5% of the individuals will develop bacteraemia, which is potentially fatal and requires antibiotic treatment (Anjum et al., 2011). In these cases, the recommended drugs of choice are fluoroquinolones and cephalosporins. Nowadays, the treatment of many infectious diseases, in both humans and animals, relies upon just one or two drugs (Woolhouse and Farrar, 2014). A restrictive policy of antibiotic usage should be implemented in livestock in the EU, in order to protect human health.

The quinolone resistance gene *qnrB* was detected in different *S. enterica* serotypes coexisting in the same farm. Resistance to tetracycline was frequent and was correlated with the presence of *tet*(A) and *tet*(B) genes. Additionally, resistance to aminoglycosides encoded by *aphA1*, *aadA* and *aac(3)*IV was also present. As demonstrated in Table 1, at least four families of antimicrobial agents (β-lactams, polymyxins, aminoglycosides and tetracyclines) were administered during the rearing cycle on all of the conventional farms included in this study, as usually occurs on intensive pig farms (Moreno, 2014; EMA, 2015[4]). In this study, a high number of isolates exhibited a multi-AR phenotype to streptomycin, ampicillin and sulphonamides, which are antimicrobial agents commonly use in veterinary medicine. Unfortunately, details of the types, frequencies and doses/routes of antimicrobial agents used on the farms studied was not available to determine the possible relationship between antibiotic administration and the presence of the multi-AR resistant strains. To address this concern, it would be desirable to carry out further studies, including farms where antimicrobial agents have never been or are infrequently used.

The most frequent serovar obtained in this study was Rissen, followed by Typhimurium monophasic (4,(5),12:i:-). In Europe, the prevalence of *S.* Typhimurium monophasic (4,(5),12:i:-) causing foodborne outbreaks and its presence in pigs and pork products have been increasing (Hopkins et al., 2010; EFSA, 2011). Additionally, the two major clones (labelled as Spanish and European clones) circulating in Europe show multi-AR resistance to four different antimicrobial families, i.e. ampicillin, streptomycin, sulphonamide and tetracycline (ASSuT family profile) (Garcia et al., 2014). In this study, the nine *S.* Typhimurium monophasic strains exhibited the ASSuT phenotype (Table 2), and five (i.e. E3G3V4C17, E3G3V4C19, E3G2V4C25, E3G2V4C63 and E3G6V4C13; Fig. 1) were recovered just before the animals were slaughtered. Continuous surveillance should be implemented to understand the molecular mechanisms and the environmental forces driving the emergence and spread of these clonal lines.

Conclusions

Multi-AR resistant *S. enterica* was present on pig farms in Spain using preventative veterinary programmes for the treatment of infectious diseases, but a direct effect between the emergence of these multi-AR strains and antimicrobial treatments regularly used on these farms was not observed. The presence of AR genes in *S. enterica* from pigs was confirmed as being associated to transferrable plasmids.

Conflict of interest statement

None of the authors of this paper has any financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tvjl.2018.02.002.

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