Resistance to colistin and production of extended-spectrum β-lactamases and/or AmpC enzymes in *Salmonella* isolates collected from healthy pigs in Northwest Spain in two periods: 2008-2009 and 2018

**Running title:** Antimicrobial resistance in pig *Salmonella*

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Abstract

Salmonellosis is a common subclinical infection in pigs and therefore apparently healthy animals may represent a reservoir of antibiotic-resistant *Salmonella* for humans. This study estimates and characterizes resistance to two classes of antimicrobials considered of the highest priority within the critically important antimicrobials for humans, i.e. colistin (CR) and 3rd generation cephalosporins (3GC), on a collection of *Salmonella* isolates from pigs from two periods: between 2008-09, when colistin was massively used; and in 2018, after three years under a National Plan against Antibiotic Resistance. Prevalence of CR was low (6 out of 625; 0.96%; 95%CI: 0.44-2.1) in 2008-09 and associated mostly to the *mcr-1* gene, which was detected in four *S.* 4,5,12:i:- isolates. Polymorphisms in the *pmrAB* genes were detected in a *S.* 9,12:i:- isolate. No CR was detected in 2018 out of 59 isolates tested. Among 270 *Salmonella* isolates considered for the assessment of resistance to 3GC in the 2008-2009 sampling, only one *Salmonella* Bredeney (0.37%; 95%CI: 0.07-2.1) showed resistance to 3GC, which was associated with the *blaCMY-2* gene (AmpC producer). In 2018, six isolates out of 59 (10.2%; 95%CI: 4.7-20.5) showed resistance to 3GC, but only two different strains were identified (*S.* 4,12:i:- and *S.* Rissen), both confirmed as extended-spectrum β-lactamases (ESBL) producers. The *blaCTX-M-3* and *blaTEM-1b* genes in *S.* 4,12:i:- and the *blaTEM-1b* gene in *S.* Rissen seemed to be associated with this resistance. Overall, the prevalence of CR in *Salmonella* appeared to be very low in 2008-2009 despite the considerable use of colistin in pigs at that time, and seemed to remain so in 2018. Resistance to 3GC was even lower in 2008-2009 but somewhat higher in 2018. Resistance was mostly coded by genes associated with mobile genetic elements. Most serotypes involved in these antimicrobial resistances displayed a multidrug resistance pattern and were considered zoonotic.

Keywords: antimicrobial resistance, colistin, extended-spectrum cephalosporins, *Salmonella*, swine.
1. Introduction

The use of antimicrobials in primary production has favoured the selection for antimicrobial resistance (AMR) in food-producing animals (Davies and Davies, 2010), thus they become potential reservoirs of antibiotic-resistant genes through the bacteria usually found in these species (Antonelli et al., 2019; Seiffert et al., 2013). *Salmonella* is a bacterium commonly found in the gastrointestinal tract of pigs that can be maintained along the whole meat production chain, from lactating piglets to slaughter pigs, and further be detected on pig carcasses at abattoirs (Bonardi, 2017). Drug resistance has been increasing within this bacterial genus, and is now considered a matter of great concern (EFSA and ECDC, 2019). The presence in the food chain of genes coding for AMR represents a public health risk as it may limit the treatment options for a wide range of infections caused by *Salmonella* in humans, increase their virulence, and thus resulting in higher morbidity and mortality rates (Molbak, 2005).

In the pig industry, the so-called nursery, a period from weaning at 3-4 weeks of age to approximately 10 weeks of age, is a critical production phase in which piglets are susceptible to a variety of enteric infections. At weaning, intestinal dysbiosis is common due to both the significant change in the piglets’ diet (from mostly liquid - milk- to a solid-based diet - feed-) and the piglet stress associated to its separation from the sow and the commingling with other piglets. For many years, antimicrobials have been used as prophylactics during this period to control Gram-negative infections, and the use of aminoglycosides and polymyxins has been a common practice in intensive pig husbandry systems (EMA, 2014, 2016).

Colistin, a type of polymyxin, has been used as an in-feed antimicrobial for years in Spain, mainly during the nursery, due to its high efficacy against Gram-negative bacteria. Until recently, prevalence of colistin resistance was considered low and mostly associated with mutations of the chromosomal genes *pmrA* and *pmrB* (Haeili et al., 2018; Quesada et al., 2015).
However, the recent detection and worldwide spread of new plasmid-mediated genes (mcr-1 to mcr-9) related to resistance to this antibiotic (Carroll et al., 2019; Lima et al., 2019), prompted the World Health Organisation (WHO) to declare colistin as a “Highest Priority Critically Important Antimicrobial” due to its importance against multidrug resistant (MDR) human infections (WHO, 2019). The European Health Authorities have also reconsidered its use for meat production, triggering new EU regulations on the use of this antibiotic in veterinary medicine. Thus, in 2015 oral colistin was banned for its use as prophylactic and the period of administration was reduced to a maximum of 7 days (EMA, 2016). In Spain, the use of colistin has remained high until 2015 (an average of 31.4 mg/PCU from 2010 to 2015). In that year, a voluntary strategic plan called “Programa Reduce Colistina” was established to reduce colistin use in pigs within the Spanish Plan against Antibiotic Resistance (PRAN) (AEMPS, 2019a).

β-lactam antibiotics, a class of broad-spectrum antibiotics, are licensed for the treatment of systemic bacterial infections in pigs, and have become some of the most used in pig production against Gram-negative bacteria (Cameron-Veas et al., 2015; Van Rennings et al., 2015). On average, 75.5 mg/PCU were used in Spain between the 2010-2016 period (AEMPS, 2018). Resistance to β-lactam antibiotics is mediated by a wide range of genes coding for β-lactamase enzymes and usually associated with mobile genetic elements that are selected through the use of antibiotics (Michael and Schwarz, 2016; Paterson and Bonomo, 2005). It has been found that the use of antibiotics such as amoxicillin or even ceftiofur, a 3rd generation cephalosporin (3GC) resistant to the activity of β-lactamase enzymes, on commercial reared pigs may trigger a transitory development of cephalosporin resistance in E. coli (Cameron-Veas et al., 2015). The emergence of resistance to this class of antibiotics has been put in evidence worldwide for different Salmonella serotypes, including those from Spanish hospitals (de Toro et al., 2011; Elnekave et al., 2019; Michael and Schwarz, 2016; Seiffert et al., 2013).
Thus, the aim of the present study was to estimate and characterize colistin resistance and extended-spectrum β-lactamase (ESBL) and AmpC enzyme production in two collections of *Salmonella* strains, one isolated from slaughtered pigs in Spain between 2008 and 2009, that is, much before the policies of antibiotic reduction for veterinary use in the country were initiated; and the second from fattening pigs from the same geographical region from 2018, when a significant reduction on the use of antibiotics for veterinary use had been observed (AEMPS, 2019b). Thus, we obtained two snapshots of the situation in two periods clearly differentiated with regard to the level of consumption of this type of antibiotics.

2. Material and methods

2.1 *Salmonella* isolates

A large survey on the prevalence of *Salmonella* infection was carried out between 2008 and 2009 on 1,997 slaughtered pigs from 80 farms in the NW of Spain, the largest pig production region of the country (Vico et al., 2011). *Salmonella* was isolated from mesenteric lymph nodes of 625 pigs (31.3%) from 75 herds (93.7%), following the ISO 6579:2002/A1:2007 method. All these isolates were serotyped and composed the *Salmonella* strain collection considered for the study on colistin resistance. On a representative proportion of these isolates (44.5%) phenotypic antimicrobial resistance profiles against 19 antimicrobial agents had been also assessed by the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) recommendations, as described in detail by Vico et al., 2011. However, colistin resistance was not properly tested as the disk diffusion method is not considered a suitable method for this purpose (EUCAST, 2019). Neither ESBL/AmpC production was assessed in that study.

In 2018, and within the context of a survey on AMR in finishing pigs (≈5 months old), a study on 29 pig herds was carried out in the same geographical region as the previous one. Herds
were selected based on the producer’s willingness to collaborate. Ten pooled faecal samples were collected from 10 pens from each herd. Bacteriology on pooled faecal samples was performed according to the ISO 6579:2002/A1:2007 method. Complete phenotypic AMR profiles were further assessed from all *Salmonella* isolates obtained by means of the Sensititre Gram Negative MIC plate (Thermo Fisher Scientific, East Grinstead, UK).

2.2 Colistin resistance detection and characterization

All the 625 *Salmonella* strains isolated between 2008 and 2009 were tested for colistin resistance at the Unit of Microbiology and Immunology at the School of Veterinary Medicine, University of Zaragoza, Spain. In this collection the Minimum Inhibitory Concentration (MIC) of colistin was determined by the broth microdilution method (ISO 20776-1:2006). For the *Salmonella* isolates from 2018, MIC for colistin was determined at the Agrifood Research and Technology Centre of Aragón (Zaragoza, Spain), by means of the Sensititre Gram Negative MIC plate (Thermo Fisher Scientific, East Grinstead, UK). In both cases the epidemiological cut-off (ECOFF) value of >2 mg/L was used for considering “microbiological” resistance, as indicated in the Commission Implementing Decision (2013/652/UE) of 12 November 2013 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (document C(2013) 7145) following recommendations from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). ECOFF values separate the naive, susceptible wild-type bacterial populations from isolates that have developed reduced susceptibility to a given antimicrobial agent (Kahlmeter et al., 2003). *Escherichia coli* ATCC 25922 was used as quality control strain.

In order to characterize the possible chromosomal origin of colistin resistance, the coding sequences of *pmrA* and *pmrB* genes from resistant strains were analysed. DNA was extracted from pure culture by boiling (at 100°C for 10 minutes) and subjected to conventional PCR using
published primers (Sun et al., 2009). The purified PCR products were Sanger sequenced (GenBank accession no. MK534439 to MK534444). DNA sequences of pmrA and pmrB genes were then compared to the reference Salmonella strain LT2 using BLAST. In addition, the presence of the plasmid-mediated colistin resistance genes mcr-1, mcr-2, mcr-3 and mcr-4 was tested by conventional PCR (Lima et al., 2019) on all those strains with MIC >1 mg/L. The purified PCR products of positive samples were sequenced in order to confirm the identity (GenBank accession no. MK506810 to MK506813).

### 2.3 Detection of extended-spectrum β-lactamase (ESBL) and AmpC production

A subset of isolates (n=270) from the original 2008-2009 collection was considered. The selection of these isolates was based on the following criteria:

1. All strains showing phenotypic resistance to colistin were included.
2. From each Salmonella-positive herd, at least one isolate of each serotype found in that herd was included.
3. In addition, when isolates belonging to the same serotype showed different resistance profiles within a given herd, one isolate per profile was then included.

For phenotypic detection of ESBL/AmpC production the Total ESBL + AmpC Confirm kit (Rosco Diagnostica, Taastrup Denmark) was used. This kit consists of 6 tablets containing cefotaxime and ceftazidime alone or combined with β-lactamase inhibitors (i.e. clavulanate and/or cloxacillin). As defined by manufacturer’s instructions, if a difference of ≥5 mm was observed between the inhibition zones of the tablets containing a cephalosporin plus cloxacillin with and without clavulanate, the tested isolate was considered ESBL positive. If a difference of ≥5 mm was detected between the inhibition zones of the tablets containing a cephalosporin plus clavulanate with and without cloxacillin, the tested isolate possessed AmpC.
Genetic characterization of ESBL/AmpC-producer *Salmonella* strains from the 2008-2009 sampling was assessed by multiplex PCR for detection of the *bla*\textsubscript{TEM}, *bla*\textsubscript{CTX-M}, *bla*\textsubscript{CMY} and *bla*\textsubscript{SHV} genes, following conditions described by Dallenne et al. (2010). Sanger sequencing and sequence analysis were used further to identify gene variants.

For the *Salmonella* isolates from 2018, detection of ESBL/AmpC production was performed as described above, but in this case, whole-genome sequencing (WGS) using the MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) was used for genomic characterization of the positive isolates. DNA genomic extraction was performed with Wizard Genomic DNA Purification kit (Promega, Madison, USA) and DNA quality and concentration were measured by NanoDrop (Thermo Fisher Scientific, Wilmington, USA) and Qubit (Invitrogen, Carlsbad, USA) devices. Genomic library was performed following the 1D Native barcoding genomic DNA protocol, with EXP-NBD104 and SQK-LSK109 kits (Oxford Nanopore Technologies), and sequencing was run in a FLO-MIN106 flow cell. Downstream analyses were performed as follows: sequencing reads were basecalled with MinKNOW software (Oxford Nanopore Technologies), demultiplexing process was carried out with the barcoding pipeline of Epi2Me interface (Metrichor, Oxford, UK) and trimming of adaptors and barcodes from the reads was assessed by Porechop. Long-read assembly was achieved by Canu (Koren et al., 2017), and subsequent genomic assemblies were analysed with Bandage (Wick et al., 2015) and BLAST+ (Camacho et al., 2009), including the ResFinder and PlasmidFinder databases, in order to determine the total resistance gene and plasmid content, respectively, and the location of these genes in the genome.

### 2.4 Pulsed-Field Gel Electrophoresis (PFGE) analysis

PFGE was carried out on those resistant *Salmonella* isolates coming from the same pig herd to assess their potential clonal origin, according to the Pulse-Net protocol (Ribot et al., 2006).
Briefly, *Salmonella* isolates were embedded in agarose plugs (Lonza, Rockland, ME, USA) and lysed afterwards using Sarcosyl (Sigma-Aldrich Co., St. Louis, MO, USA) and Proteinase K (Ambion Inc., Austin, TX, USA). Digestion of DNA was performed with the restriction enzyme *XbaI* (Roche Diagnostics, Mannheim, Germany). Fragments were then separated by electrophoresis using the CHEF-DR III system (BioRad, Hercules, CA, USA) under the following conditions: an initial switch time of 2.2 s to a final switch time of 64 s for 17 h at 6 V/cm. *Salmonella* Braenderup H9812 (Culture Collection, University of Göteborg, Sweden) was used in the analysis as a molecular size marker.

PFGE pattern analysis was further performed with the BIONUMERICS software (version 6; Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient and unweighted pair group method with arithmetic averages (UPGMA dendrogram type) with a position tolerance of 1.5% and optimization of 2.0%.

2.5 Statistical analyses

Descriptive prevalence estimates with their 95% Confidence Intervals (95% CI) were calculated for each period. Analyses were performed using MedCalc v. 18.10 (MedCalc, Ostend, Belgium).

3. Results

3.1 Colistin resistance

Six (0.96%; 95%CI: 0.44-2.1) *Salmonella* isolates showed colistin resistance in the 2008-2009 sampling. They came from 4 different pig herds located far apart from each other (an average of 200 km). Resistant isolates belonged to the following serotypes: *S. 4,5,12:i:-* (4), *S. Enteritidis* (1), and *S. 9,12:i:-* (1). Three of the resistant *S. 4,5,12:i:-* isolates belonged to the same herd (herd number 2), and were therefore analysed by PFGE to assess their genetic
relatedness. A perfect match was observed among them (Figure 1). Therefore, only four different *Salmonella* strains were actually found resistant to colistin. The other three resistant isolates came from three different herds (nos. 7, 18 and 21) (Table 1). The proportion of pig herds presenting *Salmonella* isolates phenotypically resistant to colistin among the *Salmonella*-positive herds was 5.3% (95%CI: 2.1-12.9).

The *mcr*-1 gene was detected in the four *S*. 4,5,12:i:- isolates. In one isolate (*S*. 9,12::i::) polymorphisms that produced protein variants, one in *pmrA* gene (T89S) and 5 in *pmrB* gene (M15T, G73S, V74I, I83V, A111T) were identified. None of the colistin resistant isolates presented any of the other three *mcr* genes tested.

The resistance detected in *S*. Enteritidis was neither associated with polymorphisms in *pmrA* or *pmrB* genes nor with *mcr* genes. Thus, further WGS was carried out on this isolate by MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) as described above, but no genes that may be associated with this resistance were detected.

In the 2018 sampling, *Salmonella* was isolated from 17 (58.6%) of the pig herds. A total of 59 isolates were recovered from the corresponding 59 pen pooled faecal samples (20.3% of the total samples collected), an average of 3.5 positive faecal samples per herd. No *Salmonella* isolates were found resistant to colistin among them (0%; 95%CI: 0-6.1).

### 3.2 Resistance to 3rd generation cephalosporins

Regarding ESBL/AmpC-producing *Salmonella*, only one (0.37%; 95%CI: 0.07-2.1) *Salmonella* isolate (*S*. Bredeney) showed AmpC production in the 2008-2009 sampling according to the Total ESBL + AmpC Confirm kit (Rosco Diagnostica, Taastrup Denmark). The genetic analysis showed the presence of the *bla*CMY-2 gene in this isolate. However, six
isolates (10.2%; 95%CI: 4.7-20.5) were confirmed as ESBL producers in 2018. These isolates came from only two different pig herds as five of them (nos. 101, 102, 103, 106 and 107) belonged to the same pig herd. PFGE analysis showed that they were clustered within the same group (100% homology), suggesting clonality (Figure 1). Therefore, only two different Salmonella strains were actually found resistant to 3GC in 2018 (3.4%; 95%CI: 0.93-11.5). One of these five isolates (no. 103) was serotyped to identify the clone, being classified as the monophasic variant of serotype Typhimurium (S. 4,12:i:-). The sixth Salmonella isolate belonged to serotype Rissen (6,7:f,g:-) (Table 2). Serotyping was performed at VISAVET Health Surveillance Centre (Madrid, Spain) following the White–Kauffmann–Le Minor scheme (Grimont and Weill, 2007).

The S. 4,12:i:- isolate harboured two genes related to this type of resistance, the $\text{bla}_{\text{CTX-M-3}}$ gene within a IncHI2A plasmid and the $\text{bla}_{\text{TEM-1b}}$ gene detected on both the IncHI2A plasmid and the chromosome. In addition to these two genes, it also harboured genes encoding for resistance to aminoglycosides, tetracyclines, trimethoprim and sulphonamides (Table 3). Regarding the S. Rissen isolate, it harboured the chromosomal $\text{bla}_{\text{TEM-1b}}$ gene as the most likely responsible for this resistance. It also harboured some other genes encoding for resistance to aminoglycosides, phenicols, trimethoprim, sulphonamides and tetracyclines (Table 3).

The herd-prevalence of Salmonella isolates resistant to 3GC in Salmonella-positive pig herds in 2008-09 was 1.3% (95%CI: 0.24-7.2), while in 2018 it was 11.1% (95%CI: 3.1-32.8).

4. Discussion

This study takes advantage of two Salmonella surveys, the first one (2008-2009) carried out much before the onset of the voluntary strategic plan to reduce colistin use in pigs and the implementation of national policies to reduce the overall use of antibiotics in animals, and the
second one (2018) three years after the implementation of those plans. However, although the surveys were carried out within the same geographical area, their results cannot be directly comparable as they differed in the study design and the type of samples considered (lymph nodes vs. pooled faecal samples). Still, they resulted useful to provide a snapshot of the situation regarding resistance to these two important antimicrobial classes in these two periods.

Overall, the prevalence of colistin resistance in *Salmonella* isolates in Spain in 2008-2009 appeared to be low (<1%), despite of having been isolated when colistin was extensively used in Spanish pig herds. This situation seemed to be somewhat better than that in other European countries such as Portugal and Italy, where colistin was also commonly used in pigs (Carnevali et al., 2016; Figueiredo et al., 2015). Most of the colistin resistant isolates (3 out of 4) belonged to important zoonotic serotypes (*S*. 4,5,12:i:- and *S*. Enteritidis), suggesting their potential transmission to humans through contaminated food.

Colistin resistance in this period was mostly associated with the presence of the *mcr-1* gene, as 4 (66.6%) out of the 6 *Salmonella* resistant isolates harboured it. The *mcr-1* gene was detected exclusively in *S*. 4,5,12:i:-, which supported the idea that *S*. Typhimurium and its monophasic variant are the most common serotypes harbouring *mcr* genes (Lima et al., 2019). The earliest *mcr-1* gene was detected on a *Salmonella* isolated in February 2008, which implies that this plasmid gene was circulating at least one year earlier than the first report of an *Enterobacterium* bearing this gene in Spain (Quesada et al., 2016), and at the same time it was detected in *Salmonella* isolates from Germany (Borowiak et al., 2017), indicating that it was already widespread in Europe at that time. All *mcr-1* positive *Salmonella* isolates found in this study had been previously characterized as MDR (to aminopenicillins, phenicols, aminoglycosides, sulphonamides and tetracyclines) by Vico et al. (2011), which may have had some implications in the maintenance of colistin resistance (Lima et al., 2019).
Three out of the four *Salmonella* isolates harbouring the *mcr-1* gene belonged to the same pig herd, and the PFGE analysis showed their more than likely clonal origin (Figure 1). Considering the low prevalence of *mcr-1* in this collection of *Salmonella* isolates, it seems that clonality prevailed over horizontal gene transmission in the spread of *mcr-1* in *Salmonella* in this pig population. In any case, the transferability of this resistance mechanism via plasmid dissemination to other *Salmonella* and other bacterial species is of concern and should be closely monitored.

Although *mcr-1* gene was the only colistin resistance gene detected in this study among the four *mcr* variants tested by PCR in colistin resistant isolates, another five *mcr* genes (*mcr-5* to *mcr-9*) have been described so far (Carroll et al., 2019; Lima et al., 2019). In regard to these newer gene variants, to the author’s knowledge only *mcr-5* has been identified in Spain, particularly in *E. coli* isolates (García et al., 2018), but its prevalence seems to be low worldwide (Wise et al., 2018). Since the presence of different *mcr* gene variants appears to be emerging, it should be advisable to include them in future surveillance programs.

Chromosomal mutations on *pmrA* and *pmrB* genes were found in the *S. 9,12:* strain. These mutations may play a role in its phenotypic resistance to colistin, but the type of polymorphisms detected did not match any of the colistin resistance-related polymorphisms described in *Salmonella* until now (Olaitan et al., 2015; Quesada et al., 2015; Sun et al., 2009). Further studies will be required to determine whether they are truly related to colistin resistance or not. It is of worth to note that this isolate belongs to serogroup D1, as *S. Enteritidis* and *S. Dublin*. A previous study on MIC distributions for colistin for different *Salmonella* serotypes suggested that *S. Enteritidis* and *S. Dublin* were less susceptible to this drug than other *Salmonella* serotypes (Agersø et al., 2012). Therefore, some sort of intrinsic resistance may be expected for
isolates from this serogroup. Indeed, we could not detect any genetic mechanism of colistin resistance, among those we studied, for the colistin-resistant S. Enteritidis isolate, which would support this hypothesis, or even the possible existence of a novel, and yet undetected, colistin resistance mechanism.

Regarding the 2018 survey, no colistin-resistant Salmonella were detected among the 17 Salmonella-positive pig herds, suggesting that this type of resistance was low at that time. In that year, the consumption of colistin was extremely low compared to before 2016 (a 98% drop). The low prevalence of this type of resistance may be related to the establishment of this successful national program for colistin reduction in Spain (AEMP S, 2018). Indeed, in vitro studies on Pseudomonas aeruginosa have shown that colistin-resistant phenotypes may become susceptible to colistin after a series of passages in colistin-free medium (Lee et al., 2016), which may also occur for E. coli and Salmonella. In addition, some field epidemiological studies also suggest that cessation of colistin use may help over time to reduce the frequency of detectable colistin resistance, and of mcr-1 gene, carried by Enterobacteriaceae in pigs (Randall et al., 2018; Wang et al., 2020), likely because its presence would be associated with a significant biological fitness cost (Nang et al., 2018). However, considering that the mcr-1 gene has been also detected in colistin-susceptible Enterobacteriaceae (Ovejero et al., 2017; Pham Thanh et al., 2016), and that we only tested the presence of mcr genes on those isolates with MIC >1 mg/L, it is possible that we may have somewhat overlooked the presence of dormant mcr-1 genes in some of the susceptible isolates.

Prevalence of resistance to 3GC was even lower (0.37%) than colistin resistance in the 2008-2009 survey, but it was within that observed in Europe for those years (Seiffert et al., 2013). This result may be expected as this type of antimicrobials were more recently introduced for animals (i.e. ceftiofur), and are used only on an individual basis and through parenteral
(intramuscular or subcutaneous) administration, which would reduce the risk of selection pressure as compared to the in-feed antimicrobials. Resistance was associated with the production of AmpC enzymes, which seemed to be encoded by the blaCMY-2 gene present in a S. Bredeney. This gene was firstly detected in Spain in 1999 (Navarro et al., 2001) and, although is usually associated with mobile genetic elements (Seiffert et al., 2013), has been scarcely found in Enterobacteriaceae from pigs in Spain (Dandachi et al., 2018). Indeed, to the authors’ knowledge, this is the first time this gene is detected in a S. Bredeney isolated from pigs in the country. However, it has been previously detected in S. Bredeney isolates associated with human cases (de Toro et al., 2013; González-Sanz et al., 2009), suggesting its zoonotic potential. This isolate also displayed a MDR pattern, which could contribute to the maintenance of the resistance to 3GC in animals through the co-selective pressure exerted by the over usage of non-β-lactams antibiotics (Dandachi et al., 2018).

The prevalence of resistance to 3GC in Salmonella isolates from the 2018 was 10.2% (95%CI: 4.7-20.5), as six isolates were found resistant among the 59 analysed. However, five of them belonged to the same herd and were genetically identical, thus only two different strains could be considered resistant to this class of antimicrobials in this collection. Therefore, a 3.4% prevalence of resistance or higher (no more isolates were compared by PFGE to detect potential clones among the susceptible ones) was expected. This prevalence was much higher than the current prevalence in the EU (0.5%) (EFSA and ECDC, 2019) but within the interval defined as “low resistance (<10%)”, according to EFSA criteria. Overall, resistance to 3GC was still much lower than that for other antimicrobial classes. This may be associated with the lower use of cephalosporins in animal production during the last years in Spain (an average of 0.35 mg/PCU from 2011 to 2016) compared to other antimicrobials classes (β-lactams >70 mg/PCU; tetracyclines >125 mg/PCU; macrolides >18 mg/PCU; fluoroquinolones >10 mg/PCU; etc.) (AEMPS, 2018).
Both strains resistant to 3GC in 2018 belonged to serotypes usually found in pigs, i.e. the S. 4,12:i:- and S. Rissen, and both were confirmed as ESBL producers. Thus, the mechanism of resistance differed from that observed in the resistant isolate from the 2008-2009 period. Although results from these two periods should not be directly comparable, this difference supports the highly changing epidemiology of resistance to 3GC along the years (Hawkey and Jones, 2009).

The S. Rissen isolate harboured the chromosomal blaTEM-1b gene, which has been usually associated with resistance to ampicillin rather than to ESBL production. In fact, in Spain, this gene has been circulating in ampicillin-resistant S. Enteritidis isolates from Spanish hospitals, and usually linked to a transferable plasmid (García et al., 2019). It would be the overexpression of the blaTEM-1b gene which may be behind the production of ESBL in this isolate (Devanga Ragupathi et al., 2016). Bearing in mind that S. Rissen is considered an emerging serotype in pigs and pork, and although has been less involved in human infections is still capable of causing sporadic outbreaks (Campos et al., 2019), it could be a potential vector for the transmission of this resistance to humans.

In addition to the blaTEM-1b gene, this isolate harboured other chromosomal and plasmid genes encoding for resistance to aminoglycosides (ant(3')-Ia and aadA2), phenicols (cmlA1 and floR2), tetracyclines (tetA4), trimethoprim (dfrA1) and sulphonamides (sul1) (Table 3). Of particular interest was the presence of the chromosomal tetA4 gene, which may confer resistance to tigecycline (Akiyama et al., 2013). Tigecycline is a tetracycline-derivative antibiotic recently marketed and considered a last-resort antimicrobial for the treatment of ESBL-producing MDR Salmonella (Capoor et al., 2009). Interestingly, the phenotypic analysis of resistance of this isolate showed a MIC of 2 mg/L for tigecycline, above its ECOFF value of
1 mg/L (EUCAST, 2006), which may be interpreted as a certain decrease in susceptibility to this antibiotic. Resistance to tigecycline along with resistance to 3GC would become this strain susceptible to only other critically important antibiotics such as carbapenems or colistin, increasing the risk of treatment failure in case of human infection.

A clonal spread of resistance to 3GC was detected within a herd for a S. 4,12:i:- isolate. In recent years, the monophasic variant of S. Typhimurium has positioned as one of the most frequent serotypes in finishing pigs and an emerging cause of human salmonellosis (EFSA and ECDC, 2018). This serotype is usually associated with high levels of AMR, which would be an important factor for its survival (Sun et al., 2020). In fact, as for S. Rissen, this serotype displayed phenotypical resistance to cefotaxime and ceftazidime, and also to ampicillin, chloramphenicol, gentamicin, sulfamethoxazol, tetracycline and tigecycline.

Similarly to S. Rissen, resistance against 3GC in S. 4,12:i:- would be driven by the blaTEM-1b gene (present both in the chromosome and the IncHI2A plasmid), but also by the blacTX-M-3. The CTX-M-type enzymes have been one of the most common β-lactamase families found in Salmonella isolates of human and animal origin worldwide (Seiffert et al., 2013), and seem to be originated from environmental bacteria (Hawkey and Jones, 2009). These genes are usually within conjugative plasmids co-carrying genes conferring resistance to other antibiotics such as aminoglycosides and quinolones (Hawkey and Jones, 2009). Indeed, we found four genes within the same IncHI2A plasmid encoding for resistance to aminoglycosides, along with genes encoding for resistance to trimethoprim and sulphonamides, but no quinolone resistance genes were detected (Table 3).

This S. 4,12:i:- strain also exhibited resistance to tigecycline, showing a MIC value of 16 mg/L, that is, much greater than the ECOFF value for this antibiotic for Salmonella (EUCAST, 2006).
No genes that could be related to this type of resistance were detected. It might have happened that the acquisition of mutations in the \textit{tet}(B) gene would have favoured tigecycline resistance, as it has been found when mutations occurred on other \textit{tet} genes (Linkevicius et al., 2016).

Since \textit{S}. \textit{4,12:i:}- is one of the major serotypes associated with human infections, the monitoring of this type of resistance in this serotype is highly advisable.

Among the genes detected in this isolate it was of particular interest the presence of the \textit{armA} gene within the IncHI2A plasmid, which codifies for a 16S rRNA methyltransferase. This kind of enzymes confers resistance to all clinically relevant aminoglycosides by blocking the attachment of these compounds to its intracellular target, the ribosome (Granier et al., 2011).

As aminoglycosides are considered critically important antimicrobials for human medicine (WHO, 2019), the surveillance and control of this resistance mechanism, especially when encoded in a conjugative plasmid along with other risky resistance genes, are crucial to preserve the therapeutic options against MDR \textit{Salmonella}.

\section{5. Conclusions}

Between 2008 and 2009 the prevalence of resistance to colistin appeared to be low in \textit{Salmonella} isolates from pigs from NW Spain despite the massive use of colistin in the pig herds. Resistance to 3GC was even lower in that population of \textit{Salmonella} isolates. In both cases, resistance was mostly coded by genes associated with mobile genetic elements. While colistin resistance seemed to remain low 10 years later, resistance to 3GC may be slowly increasing slowly as they are of more common use in animals now. In any case, most of the serotypes involved in both types of antimicrobial resistance displayed also a MDR pattern and were considered zoonotic, which may have important implications to human health.

\section*{Declaration of Competing Interest}
The authors declare that there is no conflict of interest.

Acknowledgements

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Table 1. Description of the 4 *Salmonella* strains resistant to colistin in the 2008-2009 sampling.

Table 2. Description of the 3 *Salmonella* strains resistant to 3rd generation cephalosporins.

Table 3. Resistance genes detected on the two *Salmonella* strains analysed by whole-genome sequencing and the corresponding gene location.

Figure 1. *XbaI* banding pattern and corresponding dendrogram showing 100% homology among the three colistin-resistant *S. 4,5,12:i:-* isolates collected in farm 2 in the 2008-2009 sampling, and among the five ESBL-producers *S. 4,5,12:i:-* isolates collected from farm 11 in the 2018 sampling.
Table 1.

<table>
<thead>
<tr>
<th>Id</th>
<th>Herd</th>
<th>Serotype</th>
<th>MIC (mg/L)</th>
<th>mcr genes</th>
<th>Polymorphisms in pmrAB</th>
<th>Multidrug resistant pattern*</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>2</td>
<td>S. 4,5,12:i-</td>
<td>4</td>
<td>mcr-1</td>
<td>-</td>
<td>ACSSuT</td>
</tr>
<tr>
<td>432</td>
<td>18</td>
<td>S. 4,5,12:i-</td>
<td>&gt;4</td>
<td>mcr-1</td>
<td>-</td>
<td>ACSSuT</td>
</tr>
<tr>
<td>162</td>
<td>7</td>
<td>S. Enteritidis</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>SuNa</td>
</tr>
<tr>
<td>522</td>
<td>21</td>
<td>S. 9,12:i:-</td>
<td>4</td>
<td>-</td>
<td>+#</td>
<td>-</td>
</tr>
</tbody>
</table>

*A: aminopenicillins; C, phenicols; S, aminoglycosides; Su, sulphonamides and dihydrofolate reductase inhibitors; T, tetracyclines; Na: quinolones.

# pmrA: T89S; pmrB: M15T, G73S, V74I, I83V, A111T
Table 2.

<table>
<thead>
<tr>
<th>Sampling period</th>
<th>Id</th>
<th>Herd</th>
<th>Serotype</th>
<th>Cefotaxime (MIC, mg/L)</th>
<th>Ceftazidime (MIC, mg/L)</th>
<th>Resistance type (genes)</th>
<th>MDR pattern*</th>
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</thead>
<tbody>
<tr>
<td>2008-09</td>
<td>464</td>
<td>19</td>
<td>S. Bredeney</td>
<td>8</td>
<td>16</td>
<td>AmpC (bla&lt;sub&gt;CMY-2&lt;/sub&gt;)</td>
<td>ACSSuT</td>
</tr>
<tr>
<td>2018</td>
<td>3</td>
<td>1</td>
<td>S. Rissen</td>
<td>8</td>
<td>4</td>
<td>ESBL (bla&lt;sub&gt;TEM-1b&lt;/sub&gt;)</td>
<td>ACSSuT</td>
</tr>
<tr>
<td></td>
<td>103</td>
<td>11</td>
<td>S. 4,12:i:-</td>
<td>8</td>
<td>8</td>
<td>ESBL (bla&lt;sub&gt;CTX-M-3, TEM-1b&lt;/sub&gt;)</td>
<td>ACSSuTTi</td>
</tr>
</tbody>
</table>

*A: aminopenicillins; C, phenicols; S, aminoglycosides; Su, sulphonamides and dihydrofolate reductase inhibitors; T, tetracyclines; Na: quinolones; Ti: Tigecycline.
<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Serotype</th>
<th>Antimicrobial class</th>
<th>Genes</th>
<th>Location</th>
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</thead>
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<tr>
<td>3</td>
<td><em>S. Rissen</em></td>
<td>β-lactams</td>
<td><em>bla</em>&lt;sub&gt;TEM-1b&lt;/sub&gt;</td>
<td>Chromosome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aminoglycosides</td>
<td><em>ant(3')-Ia</em></td>
<td>Chromosome</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>aadA2</em></td>
<td>IncHI2 plasmid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenicols</td>
<td><em>cmlA1</em></td>
<td>Chromosome</td>
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<td></td>
<td></td>
<td></td>
<td><em>fioR2</em></td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracyclines</td>
<td><em>tetA4</em></td>
<td>Chromosome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trimethoprim</td>
<td><em>dfrA1</em></td>
<td>Chromosome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulphonamides</td>
<td><em>Sul1-5</em></td>
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</tr>
<tr>
<td>103</td>
<td><em>S. 4,12:i-:</em></td>
<td>β-lactams</td>
<td><em>bla</em>&lt;sub&gt;CTX-M-3&lt;/sub&gt;</td>
<td>IncHI2A plasmid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;TEM-1b&lt;/sub&gt;</td>
<td>Chromosome, IncHI2A plasmid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aminoglycosides</td>
<td><em>armA</em></td>
<td>IncHI2A plasmid</td>
</tr>
<tr>
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<td></td>
<td></td>
<td><em>aadA5</em></td>
<td>IncHI2A plasmid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>aph(3')-Ia</em></td>
<td>IncHI2A plasmid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>aac(6')-Ib</em></td>
<td>IncHI2A plasmid</td>
</tr>
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<td><em>aac(6')-Iaa</em></td>
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<tr>
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</tr>
<tr>
<td></td>
<td></td>
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<td><em>tetB</em></td>
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<td>IncHI2A plasmid</td>
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<td><em>sul1</em></td>
<td>IncHI2A plasmid</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>sul2</em></td>
<td>Chromosome</td>
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Figure 1.

<table>
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<th>Cluster</th>
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<th>Farm ID</th>
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<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>2</td>
<td>S. 4,5,12:i-</td>
</tr>
<tr>
<td>1</td>
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<td>S. 4,5,12:i-</td>
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<td>S. 4,5,12:i-</td>
</tr>
<tr>
<td>2</td>
<td>101</td>
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<tr>
<td>2</td>
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<td>11</td>
<td>S. 4,5,12:i-</td>
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</tbody>
</table>