1   **Resistance to colistin and production of extended-spectrum β-lactamases and/or AmpC**

2   **enzymes in *Salmonella* isolates collected from healthy pigs in Northwest Spain in two**

3   **periods: 2008-2009 and 2018**

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5   **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:-:- 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 *bla*$_{CMY-2}$ 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 *bla*$_{CTX-M-3}$ and *bla*$_{TEM-1b}$ genes in *S.* 4,12:i:- and the *bla*$_{TEM-1b}$ 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 polymyixin, 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

127 were selected based on the producer´s willingness to collaborate. Ten pooled faecal samples

128 were collected from 10 pens from each herd. Bacteriology on pooled faecal samples was

129 performed according to the ISO 6579:2002/A1:2007 method. Complete phenotypic AMR

130 profiles were further assessed from all *Salmonella* isolates obtained by means of the Sensititre

131 Gram Negative MIC plate (Thermo Fisher Scientific, East Grinstead, UK).

132

133 2.2 Colistin resistance detection and characterization

134 All the 625 *Salmonella* strains isolated between 2008 and 2009 were tested for colistin

135 resistance at the Unit of Microbiology and Immunology at the School of Veterinary Medicine,

136 University of Zaragoza, Spain. In this collection the Minimum Inhibitory Concentration (MIC)

137 of colistin was determined by the broth microdilution method (ISO 20776-1:2006). For the

138 *Salmonella* isolates from 2018, MIC for colistin was determined at the Agrifood Research and

139 Technology Centre of Aragón (Zaragoza, Spain), by means of the Sensititre Gram Negative

140 MIC plate (Thermo Fisher Scientific, East Grinstead, UK). In both cases the epidemiological

141 cut-off (ECOFF) value of >2 mg/L was used for considering "microbiological" resistance, as

142 indicated in the Commission Implementing Decision (2013/652/UE) of 12 November 2013 on

143 the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria

144 (document C(2013) 7145) following recommendations from the European Committee on

145 Antimicrobial Susceptibility Testing (EUCAST, 2019). ECOFF values separate the naive,

146 susceptible wild-type bacterial populations from isolates that have developed reduced

147 susceptibility to a given antimicrobial agent (Kahlmeter et al., 2003). *Escherichia coli* ATCC

148 25922 was used as quality control strain.

149

150 In order to characterize the possible chromosomal origin of colistin resistance, the coding

151 sequences of *pmrA* and *pmrB* genes from resistant strains were analysed. DNA was extracted

152 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.

179   Genetic characterization of ESBL/AmpC-producer *Salmonella* strains from the 2008-2009

180   sampling was assessed by multiplex PCR for detection of the *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{CMY}$ and

181   *bla*$_{SHV}$ genes, following conditions described by Dallenne et al. (2010). Sanger sequencing and

182   sequence analysis were used further to identify gene variants.

183

184   For the *Salmonella* isolates from 2018, detection of ESBL/AmpC production was performed as

185   described above, but in this case, whole-genome sequencing (WGS) using the MinION

186   sequencer (Oxford Nanopore Technologies, Oxford, UK) was used for genomic

187   characterization of the positive isolates. DNA genomic extraction was performed with Wizard

188   Genomic DNA Purification kit (Promega, Madison, USA) and DNA quality and concentration

189   were measured by NanoDrop (Thermo Fisher Scientific, Wilmington, USA) and Qubit

190   (Invitrogen, Carlsbad, USA) devices. Genomic library was performed following the 1D Native

191   barcoding genomic DNA protocol, with EXP-NBD104 and SQK-LSK109 kits (Oxford

192   Nanopore Technologies), and sequencing was run in a FLO-MIN106 flow cell. Downstream

193   analyses were performed as follows: sequencing reads were basecalled with MinKNOW

194   software (Oxford Nanopore Technologies), demultiplexing process was carried out with the

195   barcoding pipeline of Epi2Me interface (Metrichor, Oxford, UK) and trimming of adaptors and

196   barcodes from the reads was assessed by Porechop. Long-read assembly was achieved by Canu

197   (Koren et al., 2017), and subsequent genomic assemblies were analysed with Bandage (Wick

198   et al., 2015) and BLAST+ (Camacho et al., 2009), including the ResFinder and PlasmidFinder

199   databases, in order to determine the total resistance gene and plasmid content, respectively, and

200   the location of these genes in the genome.

201

202   2.4 Pulsed-Field Gel Electrophoresis (PFGE) analysis

203   PFGE was carried out on those resistant *Salmonella* isolates coming from the same pig herd to

204   assess their potential clonal origin, according to the Pulse-Net protocol (Ribot et al., 2006).

205   Briefly, *Salmonella* isolates were embedded in agarose plugs (Lonza, Rockland, ME, USA) and

206   lysed afterwards using Sarcosyl (Sigma-Aldrich Co., St. Louis, MO, USA) and Proteinase K

207   (Ambion Inc., Austin, TX, USA). Digestion of DNA was performed with the restriction enzyme

208   *Xba*I (Roche Diagnostics, Mannheim, Germany). Fragments were then separated by

209   electrophoresis using the CHEF-DR III system (BioRad, Hercules,CA, USA) under the

210   following conditions: an initial switch time of 2.2 s to a final switch time of 64 s for 17 h at 6

211   V/cm. *Salmonella* Braenderup H9812 (Culture Collection, University of Göteborg, Sweden)

212   was used in the analysis as a molecular size marker.

213

214   PFGE pattern analysis was further performed with the BIONUMERICS software (version 6;

215   Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient and unweighted pair

216   group method with arithmetic averages (UPGMA dendrogram type) with a position tolerance

217   of 1.5% and optimization of 2.0%.

218

219   <u>2.5 Statistical analyses</u>

220   Descriptive prevalence estimates with their 95% Confidence Intervals (95% CI) were calculated

221   for each period. Analyses were performed using MedCalc v. 18.10 (MedCalc, Ostend,

222   Belgium).

223

224   **3. Results**

225   <u>3.1 Colistin resistance</u>

226   Six (0.96%; 95%CI: 0.44-2.1) *Salmonella* isolates showed colistin resistance in the 2008-2009

227   sampling. They came from 4 different pig herds located far apart from each other (an average

228   of 200 km). Resistant isolates belonged to the following serotypes: *S.* 4,5,12:i:- (4), *S.*

229   Enteritidis (1), and *S.* 9,12:-:- (1). Three of the resistant *S.* 4,5,12:i:- isolates belonged to the

230   same herd (herd number 2), and were therefore analysed by PFGE to assess their genetic

231 relatedness. A perfect match was observed among them (Figure 1). Therefore, only four

232 different *Salmonella* strains were actually found resistant to colistin. The other three resistant

233 isolates came from three different herds (nos. 7, 18 and 21) (Table 1). The proportion of pig

234 herds presenting *Salmonella* isolates phenotypically resistant to colistin among the *Salmonella*-

235 positive herds was 5.3% (95%CI: 2.1-12.9).

236

237 The *mcr-1* gene was detected in the four *S.* 4,5,12:i:-. In one isolate (*S.* 9,12:-:-) polymorphisms

238 that produced protein variants, one in *pmrA* gene (T89S) and 5 in *pmrB* gene (M15T, G73S,

239 V74I, I83V, A111T) were identified. None of the colistin resistant isolates presented any of the

240 other three *mcr* genes tested.

241

242 The resistance detected in *S.* Enteritidis was neither associated with polymorphisms in *pmrA* or

243 *pmrB* genes nor with *mcr* genes. Thus, further WGS was carried out on this isolate by MinION

244 sequencer (Oxford Nanopore Technologies, Oxford, UK) as described above, but no genes that

245 may be associated with this resistance were detected.

246

247 In the 2018 sampling, *Salmonella* was isolated from 17 (58.6%) of the pig herds. A total of 59

248 isolates were recovered from the corresponding 59 pen pooled faecal samples (20.3% of the

249 total samples collected), an average of 3.5 positive faecal samples per herd. No *Salmonella*

250 isolates were found resistant to colistin among them (0%; 95%CI: 0-6.1).

251

252 3.2 Resistance to 3rd generation cephalosporins

253 Regarding ESBL/AmpC-producing *Salmonella*, only one (0.37%; 95%CI: 0.07-2.1)

254 *Salmonella* isolate (*S.* Bredeney) showed AmpC production in the 2008-2009 sampling

255 according to the Total ESBL + AmpC Confirm kit (Rosco Diagnostica, Taastrup Denmark).

256 The genetic analysis showed the presence of the *bla*$_{CMY-2}$ gene in this isolate. However, six

257 isolates (10.2%; 95%CI: 4.7-20.5) were confirmed as ESBL producers in 2018. These isolates

258 came from only two different pig herds as five of them (nos. 101, 102, 103, 106 and 107)

259 belonged to the same pig herd. PFGE analysis showed that they were clustered within the same

260 group (100% homology), suggesting clonality (Figure 1). Therefore, only two different

261 *Salmonella* strains were actually found resistant to 3GC in 2018 (3.4%; 95%CI: 0.93-11.5). One

262 of these five isolates (no. 103) was serotyped to identify the clone, being classified as the

263 monophasic variant of serotype Typhimurium (*S*. 4,12:i:-). The sixth *Salmonella* isolate

264 belonged to serotype Rissen (6,7:f,g:-) (Table 2). Serotyping was performed at VISAVET

265 Health Surveillance Centre (Madrid, Spain) following the White–Kauffmann–Le Minor

266 scheme (Grimont and Weill, 2007).

267

268 The *S*. 4,12:i:- isolate harboured two genes related to this type of resistance, the $bla_{CTX-M-3}$ gene

269 within a IncHI2A plasmid and the bla_{TEM-1b} gene detected on both the IncHI2A plasmid and

270 the chromosome. In addition to these two genes, it also harboured genes encoding for resistance

271 to aminoglycosides, tetracyclines, trimethoprim and sulphonamides (Table 3). Regarding the *S*.

272 Rissen isolate, it harboured the chromosomal bla_{TEM-1b} gene as the most likely responsible for

273 this resistance. It also harboured some other genes encoding for resistance to aminoglycosides,

274 phenicols, trimethoprim, sulphonamides and tetracyclines (Table 3).

275

276 The herd-prevalence of *Salmonella* isolates resistant to 3GC in *Salmonella*-positive pig herds

277 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).

278

279 **4. Discussion**

280 This study takes advantage of two *Salmonella* surveys, the first one (2008-2009) carried out

281 much before the onset of the voluntary strategic plan to reduce colistin use in pigs and the

282 implementation of national policies to reduce the overall use of antibiotics in animals, and the

283 second one (2018) three years after the implementation of those plans. However, although the

284 surveys were carried out within the same geographical area, their results cannot be directly

285 comparable as they differed in the study design and the type of samples considered (lymph

286 nodes *vs*. pooled faecal samples). Still, they resulted useful to provide a snapshot of the situation

287 regarding resistance to these two important antimicrobial classes in these two periods.

288

289 Overall, the prevalence of colistin resistance in *Salmonella* isolates in Spain in 2008-2009

290 appeared to be low (<1%), despite of having been isolated when colistin was extensively used

291 in Spanish pig herds. This situation seemed to be somewhat better than that in other European

292 countries such as Portugal and Italy, where colistin was also commonly used in pigs (Carnevali

293 et al., 2016; Figueiredo et al., 2015). Most of the colistin resistant isolates (3 out of 4) belonged

294 to important zoonotic serotypes (*S*. 4,5,12:i:- and *S*. Enteritidis), suggesting their potential

295 transmission to humans through contaminated food.

296

297 Colistin resistance in this period was mostly associated with the presence of the *mcr-1* gene, as

298 4 (66.6%) out of the 6 *Salmonella* resistant isolates harboured it. The *mcr-1* gene was detected

299 exclusively in *S*. 4,5,12:i:-, which supported the idea that *S*. Typhimurium and its monophasic

300 variant are the most common serotypes harbouring *mcr* genes (Lima et al., 2019). The earliest

301 *mcr-1* gene was detected on a *Salmonella* isolated in February 2008, which implies that this

302 plasmid gene was circulating at least one year earlier than the first report of an *Enterobacterium*

303 bearing this gene in Spain (Quesada et al., 2016), and at the same time it was detected in

304 *Salmonella* isolates from Germany (Borowiak et al., 2017), indicating that it was already

305 widespread in Europe at that time. All *mcr-1* positive *Salmonella* isolates found in this study

306 had been previously characterized as MDR (to aminopenicillins, phenicols, aminoglycosides,

307 sulphonamides and tetracyclines) by Vico et al. (2011), which may have had some implications

308 in the maintenance of colistin resistance (Lima et al., 2019).

309

310   Three out of the four *Salmonella* isolates harbouring the *mcr-1* gene belonged to the same pig

311   herd, and the PFGE analysis showed their more than likely clonal origin (Figure 1). Considering

312   the low prevalence of *mcr-1* in this collection of *Salmonella* isolates, it seems that clonality

313   prevailed over horizontal gene transmission in the spread of *mcr-1* in *Salmonella* in this pig

314   population. In any case, the transferability of this resistance mechanism via plasmid

315   dissemination to other *Salmonella* and other bacterial species is of concern and should be

316   closely monitored.

317

318   Although *mcr-1* gene was the only colistin resistance gene detected in this study among the

319   four *mcr* variants tested by PCR in colistin resistant isolates, another five *mcr* genes (*mcr-5* to

320   *mcr-9*) have been described so far (Carroll et al., 2019; Lima et al., 2019). In regard to these

321   newer gene variants, to the author´s knowledge only *mcr-5* has been identified in Spain,

322   particularly in *E. coli* isolates (García et al., 2018), but its prevalence seems to be low worldwide

323   (Wise et al., 2018). Since the presence of different *mcr* gene variants appears to be emerging,

324   it should be advisable to include them in future surveillance programs.

325

326   Chromosomal mutations on *pmrA* and *pmrB* genes were found in the *S.* 9,12:-:- strain. These

327   mutations may play a role in its phenotypic resistance to colistin, but the type of polymorphisms

328   detected did not match any of the colistin resistance-related polymorphisms described in

329   *Salmonella* until now (Olaitan et al., 2015; Quesada et al., 2015; Sun et al., 2009). Further

330   studies will be required to determine whether they are truly related to colistin resistance or not.

331   It is of worth to note that this isolate belongs to serogroup D1, as *S.* Enteritidis and *S.* Dublin.

332   A previous study on MIC distributions for colistin for different *Salmonella* serotypes suggested

333   that *S.* Enteritidis and *S.* Dublin were less susceptible to this drug than other *Salmonella*

334   serotypes (Agersø et al., 2012). Therefore, some sort of intrinsic resistance may be expected for

335   isolates from this serogroup. Indeed, we could not detect any genetic mechanism of colistin

336   resistance, among those we studied, for the colistin-resistant *S*. Enteritidis isolate, which would

337   support this hypothesis, or even the possible existence of a novel, and yet undetected, colistin

338   resistance mechanism.

339

340   Regarding the 2018 survey, no colistin-resistant *Salmonella* were detected among the 17

341   *Salmonella*-positive pig herds, suggesting that this type of resistance was low at that time. In

342   that year, the consumption of colistin was extremely low compared to before 2016 (a 98%

343   drop). The low prevalence of this type of resistance may be related to the establishment of this

344   successful national program for colistin reduction in Spain (AEMPS, 2018). Indeed, *in vitro*

345   studies on *Pseudomonas aeruginosa* have shown that colistin-resistant phenotypes may become

346   susceptible to colistin after a series of passages in colistin-free medium (Lee et al., 2016), which

347   may also occur for *E. coli* and *Salmonella*. In addition, some field epidemiological studies also

348   suggest that cessation of colistin use may help over time to reduce the frequency of detectable

349   colistin resistance, and of *mcr-1* gene, carried by *Enterobacteriaceae* in pigs (Randall et al.,

350   2018; Wang et al., 2020), likely because its presence would be associated with a significant

351   biological fitness cost (Nang et al., 2018). However, considering that the *mcr-1* gene has been

352   also detected in colistin-susceptible *Enterobacteriaceae* (Ovejero et al., 2017; Pham Thanh et

353   al., 2016), and that we only tested the presence of *mcr* genes on those isolates with MIC >1

354   mg/L, it is possible that we may have somewhat overlooked the presence of dormant *mcr-1*

355   genes in some of the susceptible isolates.

356

357   Prevalence of resistance to 3GC was even lower (0.37%) than colistin resistance in the 2008-

358   2009 survey, but it was within that observed in Europe for those years (Seiffert et al., 2013).

359   This result may be expected as this type of antimicrobials were more recently introduced for

360   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 bla_{CMY-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).

387

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).

394

The *S.* Rissen isolate harboured the chromosomal $bla_{\text{TEM-1b}}$ 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 $bla_{\text{TEM-1b}}$ 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.

404

In addition to the $bla_{\text{TEM-1b}}$ 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

413　1 mg/L (EUCAST, 2006), which may be interpreted as a certain decrease in susceptibility to

414　this antibiotic. Resistance to tigecycline along with resistance to 3GC would become this strain

415　susceptible to only other critically important antibiotics such as carbapenems or colistin,

416　increasing the risk of treatment failure in case of human infection.

417

418　A clonal spread of resistance to 3GC was detected within a herd for a *S.* 4,12:i:- isolate. In

419　recent years, the monophasic variant of *S.* Typhimurium has positioned as one of the most

420　frequent serotypes in finishing pigs and an emerging cause of human salmonellosis (EFSA and

421　ECDC, 2018). This serotype is usually associated with high levels of AMR, which would be an

422　important factor for its survival (Sun et al., 2020). In fact, as for *S.* Rissen, this serotype

423　displayed phenotypical resistance to cefotaxime and ceftazidime, and also to ampicillin,

424　chloramphenicol, gentamicin, sulfamethoxazol, tetracycline and tigecycline.

425

426　Similarly to *S.* Rissen, resistance against 3GC in *S.* 4,12:i:- would be driven by the bla_{TEM-1b}

427　gene (present both in the chromosome and the IncHI2A plasmid), but also by the $bla_{CTX-M-3}$.

428　The CTX-M-type enzymes have been one of the most common β-lactamase families found in

429　*Salmonella* isolates of human and animal origin worldwide (Seiffert et al., 2013), and seem to

430　be originated from environmental bacteria (Hawkey and Jones, 2009). These genes are usually

431　within conjugative plasmids co-carrying genes conferring resistance to other antibiotics such

432　as aminoglycosides and quinolones (Hawkey and Jones, 2009). Indeed, we found four genes

433　within the same IncHI2A plasmid encoding for resistance to aminoglycosides, along with genes

434　encoding for resistance to trimethoprim and sulphonamides, but no quinolone resistance genes

435　were detected (Table 3).

436

437　This *S.* 4,12:i:- strain also exhibited resistance to tigecycline, showing a MIC value of 16 mg/L,

438　that is, much greater than the ECOFF value for this antibiotic for *Salmonella* (EUCAST, 2006).

439 No genes that could be related to this type of resistance were detected. It might have happened

440 that the acquisition of mutations in the *tet*(B) gene would have favoured tigecycline resistance,

441 as it has been found when mutations occurred on other *tet* genes (Linkevicius et al., 2016).

442 Since *S.* 4,12:i:- is one of the major serotypes associated with human infections, the monitoring

443 of this type of resistance in this serotype is highly advisable.

444

445 Among the genes detected in this isolate it was of particular interest the presence of the *armA*

446 gene within the IncHI2A plasmid, which codifies for a 16S rRNA methyltransferase. This kind

447 of enzymes confers resistance to all clinically relevant aminoglycosides by blocking the

448 attachment of these compounds to its intracellular target, the ribosome (Granier et al., 2011).

449 As aminoglycosides are considered critically important antimicrobials for human medicine

450 (WHO, 2019), the surveillance and control of this resistance mechanism, especially when

451 encoded in a conjugative plasmid along with other risky resistance genes, are crucial to preserve

452 the therapeutic options against MDR *Salmonella*.

453

454 **5. Conclusions**

455 Between 2008 and 2009 the prevalence of resistance to colistin appeared to be low in

456 *Salmonella* isolates from pigs from NW Spain despite the massive use of colistin in the pig

457 herds. Resistance to 3GC was even lower in that population of *Salmonella* isolates. In both

458 cases, resistance was mostly coded by genes associated with mobile genetic elements. While

459 colistin resistance seemed to remain low 10 years later, resistance to 3GC may be slowly

460 increasing slowly as they are of more common use in animals now. In any case, most of the

461 serotypes involved in both types of antimicrobial resistance displayed also a MDR pattern and

462 were considered zoonotic, which may have important implications to human health.

463

464 **Declaration of Competing Interest**

465   The authors declare that there is no conflict of interest.

466

472

473   **References**

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706

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

708

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

710

711   **Table 3.** Resistance genes detected on the two *Salmonella* strains analysed by whole-genome

712   sequencing and the corresponding gene location.

713

714   **Figure 1.** *XbaI* banding pattern and corresponding dendrogram showing 100% homology

715   among the three colistin-resistant *S.* 4,5,12:i:- isolates collected in farm 2 in the 2008-2009

716   sampling, and among the five ESBL-producers *S.* 4,5,12:i:- isolates collected from farm 11 in

717   the 2018 sampling.

718

719   Table 1.

Id	Herd	Serotype	MIC (mg/L)	*mcr* genes	Polymorphisms in *pmrAB*	Multidrug resistant pattern*
28	2	*S.* 4,5,12:i:-	4	*mcr-1*	-	ACSSuT
432	18	*S.* 4,5,12:i:-	>4	*mcr-1*	-	ACSSuT
162	7	*S.* Enteritidis	4	-	-	SuNa
522	21	*S.* 9,12:-:-	4	-	+[#]	-

720

721   *A: aminopenicillins; C, phenicols; S, aminoglycosides; Su, sulphonamides and dihydrofolate

722   reductase inhibitors; T, tetracyclines; Na: quinolones.

723   [#] *pmrA*: T89S; *pmrB*: M15T, G73S, V74I, I83V, A111T

724

725   Table 2.

Sampling period	Id	Herd	Serotype	Cefotaxime (MIC, mg/L)	Ceftazidime (MIC, mg/L)	Resistance type (genes)	MDR pattern*
2008-09	464	19	*S*. Bredeney	8	16	AmpC (bla_{CMY-2})	ACSSuT
2018	3	1	*S*. Rissen	8	4	ESBL (bla_{TEM-1b})	ACSSuT
	103	11	*S*. 4,12:i:-	8	8	ESBL ($bla_{CTX-M-3}$, bla_{TEM-1b})	ACSSuTTi

726

727   *A: aminopenicillins; C, phenicols; S, aminoglycosides; Su, sulphonamides and dihydrofolate

728   reductase inhibitors; T, tetracyclines; Na: quinolones; Ti: Tigecycline.
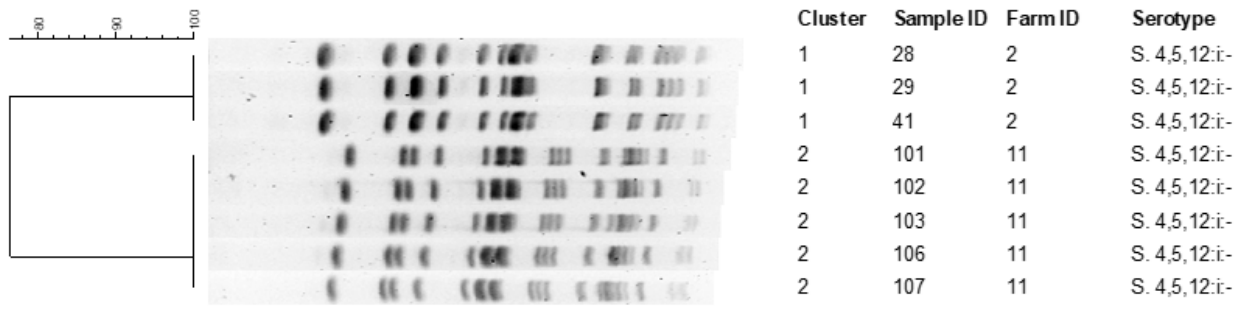
729

730   Table 3.

Isolate no.	Serotype	Antimicrobial class	Genes	Location
3	*S. Rissen*	β-lactams	*bla*TEM-1b	Chromosome
		Aminoglycosides	*ant(3")-Ia*	Chromosome
			aadA2	IncHI2 plasmid
		Phenicols	*cmlA1*	Chromosome
			floR2	Unknown
		Tetracyclines	*tetA4*	Chromosome
		Trimethoprim	*dfrA1*	Chromosome
		Sulphonamides	*Sul1-5*	IncHI2 plasmid
103	*S.* 4,12:i:-	β-lactams	*bla*CTX-M-3	IncHI2A plasmid
			*bla*TEM-1b	Chromosome, IncHI2A plasmid
		Aminoglycosides	*armA*	IncHI2A plasmid
			aadA5	IncHI2A plasmid
			aph(3')-Ia	IncHI2A plasmid
			aac(6')-Ib	IncHI2A plasmid
			aac(6')-Iaa	Chromosome
			aph(3'')-Ib	Chromosome
			aph(6)-Id	Chromosome
		Tetracyclines	*tetB*	Chromosome
		Trimethoprim	*dfrA17*	IncHI2A plasmid
		Sulphonamides	*sul1*	IncHI2A plasmid
			sul2	Chromosome

731

732

733　**Figure 1.**

Cluster	Sample ID	Farm ID	Serotype
1	28	2	S. 4,5,12:i:-
1	29	2	S. 4,5,12:i:-
1	41	2	S. 4,5,12:i:-
2	101	11	S. 4,5,12:i:-
2	102	11	S. 4,5,12:i:-
2	103	11	S. 4,5,12:i:-
2	106	11	S. 4,5,12:i:-
2	107	11	S. 4,5,12:i:-

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