Evolution of ß-lactams, fluoroquinolones and colistin resistance and genetic profiles in *Salmonella* isolates from pork in northern Italy

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Abstract

The European Food Safety Authority and European Centre of Disease Prevention and Control antimicrobial resistance report published in 2021 shows increasing levels of antimicrobial resistance in *Salmonella* against antibiotics of choice for human salmonellosis (ß-lactams and fluoroquinolones). The aim of the study was to follow the evolution of resistance against some Critical Important Antimicrobials in *Salmonella* isolates from fresh pork collected in Emilia-Romagna region, northern Italy, over two decades. Emilia-Romagna region is characterized by production of well-known pork derived products, as Parma Ham. The samples were collected in three different periods, ranging from 2000 to 2003, 2012 to 2016 and 2018 to 2021. After serotyping, the isolates were phenotypically tested for resistance to three classes of antibiotics: ß-lactams, fluoroquinolones and polymyxins. End-point polymerase chain reaction (PCR) and PCR-Real Time were used for genotypical analyses. The phenotypical resistance to ß-lactams and fluoroquinolones were clearly increasing when comparing the results obtained from isolates collected in the first period (16.7% and 16.7%, respectively) with those of the third period (29.7% and 32.4%, respectively). On the contrary, the resistance to colistin decreased from 33.3% to 5.4%. Genotypically, the 71.4% and 83.3% of the strains harboured ß-lactams and fluoroquinolones genes, respectively, while colistin resistance genes were not detected in the phenotypically resistant strains.

Introduction

Salmonellosis is the second most common foodborne disease in the European Union (EU) after campylobacteriosis. In 2019, the joint European Food Safety Authority (EFSA) and European Centre of Disease Prevention and Control (ECDC) report registered 87,923 human cases of salmonellosis with an EU notification rate of 20.0 cases per 100,000 inhabitants (EFSA and ECDC, 2021).

In 2018, the National Reference Centre for salmonellosis (Istituto Zooprofilattico Sperimentale delle Venezie) reported that chickens and pigs are the most prevalent species for the isolation of *Salmonella* in Italy, thus making the ingestion of their raw and uncooked meat or cross-contamination during food preparation the main routes of infection for consumers (www.izsvenezie.it; OIE, 2019).

Pigs are commonly asymptomatic reservoirs of *Salmonella* and intermittent faecal shedding of the microorganism causes contamination of pens and equipment on farms (Farina and Scatozza, 1998). Faecal cross contamination of carcasses also occurs frequently during slaughter (De Busser *et al*., 2011). However, prevalence of *Salmonella* in pig carcasses set down by Regulation (EU) No 217/2014 must not exceed 6.0% and this result mostly depends on the application of rigorous hygienic standards during the slaughtering process. Concerning pig meat and products thereof placed on the market, zero tolerance is mandatory in the EU, as assessed by Regulation (EC) No 2073/2005.

Following Directive EC 2003/99, all EU countries must implement surveillance plans to monitor *Salmonella* prevalence in animals, feed, food, and humans. In Italy, a National *Salmonella* surveillance network named the "Entervet system" has been active since 2002 and includes the recording of all the *Salmonella* isolates detected from animals, food of animal origin, and farms (Entervet, 2002).

The most recent European data on antimicrobial resistance (AMR) highlight that a high number of *Salmonella* isolates are multi-drug resistant (MDR), *i.e.* are resistant to three or more classes of antibiotics (EFSA and ECDC, 2021).

In order to limit this phenomenon, the World Health Organization (WHO) has created a list of Critical Important Antimicrobials (CIA), which is regularly updated. These molecules need to be used with caution or only in exceptional cases, both in human and veterinary medicine. The antibiotics belonging to the CIA group are 3rd, 4th, and 5th generation cephalosporins, glycopeptides, macrolides, ketolides, polymyxins and quinolones (Scott *et al*., 2019). The veterinary sales of CIA in Europe present a decreasing trend between 2011 and 2018. Sales have been reduced of the 24% for 3rd and 4th generation cephalosporins, 70% for polymyxins, 4% for fluoroquinolones, 74% for other quinolones (ESVAC, 2018).

One of the main mechanisms of resistance in the *Enterobacteriaceae* is the ability to synthetize β-lactamases. These enzymes are able to hydrolyse β-lactams, that are widely used in the treatment of *Salmonella* infections. More than 4,300 different β-lactamases have been described, and due to genetic mutation, these enzymes

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have expanded their range of action to a large number of antibiotics. Mutations have generated the so-called Extended Spectrum Beta Lactamases (ESBLs), which include the β-lactamases TEM, SHV, CTX-M, and AmpC (Tooke *et al*, 2019).

Ciprofloxacin is the antibiotic of choice for *Salmonella* infection in humans. In recent years, many countries have reported a widespread use of fluoroquinolones in livestock for the treatment and control of infectious diseases, causing a dramatic increase of ciprofloxacin resistance in *Salmonella* isolates both from food and clinical cases (Chen *et al*., 2019a; EFSA and ECDC, 2021; Wong *et al*., 2014). A double mutation in the *gyrA* gene and a single mutation in the *parC* gene are mainly responsible for ciprofloxacin resistance in *Salmonella* (Hooper, 2001). A large proportion of ciprofloxacin-resistant *Salmonella* strains often carry plasmid-mediated quinolone resistance (PMQR) genes, including *qnr*, *aac(6')-Ib-cr*, *oqxAB* and *qepA* genes (Gunell *et al*., 2009). Recently, the association between low levels of resistance to nalidixic acid and one or more PMQR genes has been reported. A conjugative plasmid harbouring the *bla* $_{CTX-M}$ and PMQR genes pattern, encoding resistance to cephalosporins and ciprofloxacin, has been reported in *Salmonella* isolates (Chen *et al*., 2019b).

Colistin is considered an antibiotic of last resort in human medicine (Liu *et al*., 2016). This molecule acts by destroying the negative charge on the outer membrane of Gram-negative bacilli, resulting in cell death (Carrol *et al*., 2019). The genes that encode colistin resistance are the *mcr* variants (Sun *et al*., 2018). Nine variants of the *mcr* gene have been detected in *Salmonella* isolates from humans and animals, ranging from *mcr-1* (the most widespread) to *mcr-9* (Borowiak *et al*., 2020; Rebelo *et al*., 2018). In Europe, *mcr-1* is particularly frequent in microorganisms isolated from the microbiome of humans and animals as well as from food of animal origin, suggesting a potential dissemination along the food chain (Lu *et al*., 2019).

The present study was focused on the prevalence of AMR *Salmonella* detected in pig meat during three different periods, namely in 2000-2003, 2012-2016, and 2018-2021, in Emilia-Romagna region, northern Italy. All the isolates were phenotypically and genotypically tested for 3rd generation cephalosporin (cefotaxime and ceftazidime), ciprofloxacin, nalidixic acid and colistin resistance. In addition, the comparison between *Salmonella* serotypes and their AMR profile in the different sampling periods was part of the study.

Materials and methods

Sample collection, *Salmonell*a detection and serotyping

A total of 1,469 pig meat samples were tested in three different periods in Emilia Romagna region, northern Italy. During 2000-2003 (Period A) and 2012-2016 (Period B), 87 and 1067 samples respectively were collected at retail and at slaughter. In the three-year period 2018-2021 (Period C), 315 samples were collected at slaughter. All samples were tested following the ISO 6579 methods (UNI EN ISO 6579:1993; UNI EN ISO 6579:2002; UNI EN ISO 6579:2017). Briefly, meat samples were pre-enriched 1:10 in Buffered Peptone Water (BPW) (Biolife Italiana, Milan, Italy) and incubated at 37°C overnight. Rappaport-Vassiliadis Soya Broth (Biolife Italiana) and Mueller-Kaufmann Tetrationate-Novobiocin Broth (Biolife Italiana) were used for overnight enrichment at 41.5°C and 37°C, respectively. The broth cultures were plated onto XLD agar (Biolife Italiana) and *Salmonella* Chromogenic agar (Biolife Italiana) and incubated at 37°C for 20-24 h. *Salmonella*-suspect colonies were confirmed serologically (Omnivalent, *Salmonella* antisera, 292537 Denka Seiken, Tokyo, Japan) and biochemically (API 20E®, bioMérieux, Marcy l'Etoile, France). The isolates were serotyped by the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, Brescia, Italy and stored at -80°C for further testing.

Antibiotic susceptibility

The antibiotic susceptibility was determined according to EUCAST recommendations (2018) by the disk diffusion technique using Mueller Hinton agar plates (Biolife Italiana). All the isolates were tested for the ability to produce ESBLs and AmpC using cefotaxime (CTX) and ceftazidime (CAZ). First, a screening test using two disks added with CTX (5 µg) and CAZ (10 µg) was performed (Rosco, Taastrup, Denmark). Resistant (CTX < 17 mm, CAZ < 19 mm) and intermediate resistant isolates (17 mm < CTX < 20 mm, 19 mm < CAZ < 22 mm) were tested by the combination disk test (CDT) with cefotaxime 30 µg (CTX30) in combination with a disk containing CTX30 and clavulanic acid (CTX30+C) and in combination with cloxacillin (CTX30+CX). ESBL and AmpC resistance pattern was evaluated by comparing the inhibition diameter around the CTX30 and the CTX30+C and CTX30+CX, respectively. The CDT assay was also performed for ceftazidime (CAZ).

The isolates were tested for resistance to fluoroquinolones using a 5 µg ciprofloxacin disk (CIPR05) and one of 30µg nalidixic acid (NAL30). The inhibition diameter was evaluated following EUCAST guidelines for CIPR (S ≥ 25 mm and R < 22 mm) and CLSI guidelines for NAL (S ≥ 19 mm and R ≤ 13 mm).

Sensititre plates™ (Thermofisher Scientific,Waltham, MA, USA) were used for the detection of resistance to colistin, defining the Minimal Inhibitory Concentration (MIC) of *Salmonella* isolates. Plates were prepared following the manufacturer's instructions, and resistance to colistin was set at MIC > 2 µg/mL.

DNA isolation and PCR for gene identification

The phenotypically AMR *Salmonella* isolates were tested by PCR. Three colonies of each strain were inoculated from Tryptic Soy Agar (TSA) (Biolife Italiana) into 5 mL of sterile water and incubated at 37°C for 24 h. Cells from 1.5 mL of the overnight culture were lysed by heating at 95°C for 10 min and then centrifuged at 15000 x g for 5 min; the supernatant was used for amplification.

A Real-time Polymerase Chain Reaction with Sybr Green (SsoAdvanced SYBR Green Supermix Bio-Rad, Hercules, CA, USA) was applied to verify the presence of ESBL-associated genes (bla_{CTX-M1}, bla_{CTX-M2}, bla_{TEM} and bla_{SHV}), as described by Roschansky *et al*. (2014). Preliminary tests were performed to define the correct annealing temperature for each primer and the presence of aspecific product was avoided by the melting curve analysis. In each reaction the following positive controls were used: *K. pneumoniae* NCTC 13368 for bla_{SHV}, *E. coli* NCTC 13351 for bla_{TEM} and *Salmonella* NCTC 13353 for bla_{CTX-M}. Negative controls were represented by nuclease free water. The amplification protocol included a denaturation step (95°C for 3 min) and 39 repeated cycles (95°C for 15 s; 50°C for 15 s; 72°C for 20 s). Fluorescence signals were collected in every cycle and each sample was tested twice for each primer.

The presence of AmpC (bla_{FOX}, bla_{MOX}, bla_{ACC}, bla_{EBC}, and bla_{DHA}) genes was verified using the multiplex PCR protocol described by Pérez-Pérez and Hanson (2002), with the only exception of the $MgCl_2$ concentration (2 mM instead of 1.5 mM).

Fluoroquinolone resistance is due to *gyrA* and *parC* genes mutation, as described by Onseedaeng and Ratthawongjirankul (2016). In this study *gyrA* Ser83 mutation (*gyrA83*), *gyrA* Asp87 mutation (*gyrA87*),

parC Ser80 mutation (*parC80*) and *parC* Glu84 mutation (*parC84*) were evaluated. The gene amplification was carried out with GoTaq G2 Flexi DNA polymerase kit (Promega Italia, Milano, Italy), and with 2X PCR Taq MasterMix (Applied Biological Material Inc) for *gyrA* and *parC* mutations, respectively.

The multiple plasmid-mediated quinolone resistance (PMQR) genes (*qnrA, qnrB, qnrS*) were detected using the protocol described by Doma *et al*. (2020). PCR was performed with a final volume of 50 µL. Each reaction contained: 5X GoTaq G2 Flexi DNA polymerase (Promega Italia, Milano, Italy) at a final concentration of 1X; 2.5 mM of $MgCl_2$; 0.2 mM of dNTPs; 0.5 mM of each primer. Template DNA (2 µL) was added to 48 µL of the MasterMix and the PCR protocol consisted of an initial denaturation step at 92°C for 5 min, followed by 35 cycles of DNA denaturation at 95°C for 45 s, primer annealing at 53°C for 45 s and primer extension at 72°C for 1 min. A final extension step at 72°C for 10 min was added. A 13 µL aliquot of PCR product was analysed by gel electrophoresis with 1.5% agarose (Thermofisher Scientific).

The isolates resistant to colistin were tested for the presence of the *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4, mcr-5* genes following the multiplex PCR protocol described by Rebelo *et al*. (2018). *E. coli* strain NCTC 13846 was used as positive control.

Statistical analysis

Salmonella strains showing more than one phenotypic and genotypic AMR profile were subjected to statistical analysis, to evaluate a possible correlation between the different antimicrobial patterns. The Odds Ratio (OR) were considered as follows: OR > 1 positive relation; OR = 1 no relation; OR < 1 negative relation. A chi-squared test was applied to verify the statistical significance of the data ($P<0.05$).

Results

Phenotypical and genotypical AMR

In the three sampling periods, numbers and prevalence of *Salmonella* positive pig meat samples were the following: in Period A, 12 *Salmonella* were isolated (13.8%; CI 95% = 8.1-19.5); in Period B, 82 *Salmonella* were isolated (7.7%; CI 95% = 6.1-9.3); in Period C, 37 *Salmonella* were isolated (11.7%; CI 95% = 8.9-14.5) (Table 1). During the two decades (2000-2021), resistance to β-lactams (21.4%) and colistin (22.9%) were the most common (Table 2). The most frequently detected serovars varied over the years; in Period A, *S.* Derby (25%) and *S.* Tennessee (25%), and in Periods B and C, *S.* Typhimurium (35.3% and 59.5%) and *S.* Derby (29.3% and 21.6%). Since *S.* Typhimurium and *S.* Derby were isolated in more than one sampling period, it was possible to compare their AMR profiles over time (Tables 3 and 4).

A total of 60 *Salmonella* isolates (45.8%) were phenotypically resistant to one or more antimicrobials. Phenotypical AMR in *Salmonella* was higher in Period A (58.3%), compared to Period B (43.9%) and C (45.9%) (Table 1).

As shown in Table 2, during Period A, the highest number of AMR was recorded for colistin (33.3%), followed by fluoroquinolones (16.7%) and ß-lactams (16.7%). Analysing serovar prevalence, one *S.* Derby harboured ESBL and AmpC enzymes and one was resistant to fluoroquinolones (NAL). In Period B, colistin-resistance was the most common (29.3%), followed by resistance to ß-lactams (18.3%) and fluoroquinolones (4.8%). Among serotypes, *S.* Derby and *S.* Typhimurium were mostly resistant to ß-lactams (29.2% and 13.8%, respectively) and colistin (20.8% and 31%, respectively) (Table 3). In Period C, the most common resistance in *Salmonella* isolates was against fluoroquinolones (32.4%), followed by resistance to ß-lactams (29.7%) and colistin (5.4%). Fifty per cent of *S.* Typhimurium isolates were resistant to ciprofloxacin (Table 2). On the contrary, 25% of *S.* Derby isolates showed resistance to ß-lactams with ESBL profiles (Table 3).

Twenty-nine out of 60 (48.3%) phenotypically resistant *Salmonella* isolates were found to harbour AMR genes. The only antimicrobial resistance genes found in Period A were bla_{CTX-M1}, bla_{SHV}, *gyrA* mutations in Ser83 and Asp87, and *parC* mutation in Ser80. In Period B, ß-lactams (bla_{CTX-M1}, bla_{TEM}) and fluoroquinolones resistance genes were found (*gyrA* mutations in Ser83/Asp87, *parC* mutations in Ser80/Glu84, and *qnr*). In Period C, ß-lactams (bla_{CTX-M1}, bla_{TEM}) and fluoroquinolones (*gyrA* mutation in asp87, *parC* mutation in ser80 and *qnr*) resistant genes were found in 81.8% and 75% isolates, respectively (Table 2). In period A and B, bla_{CTX-M1} and *parC* mutation in Ser80 were the only genes found in the most common *Salmonella* serotypes, *i.e. S.* Derby and *S.* Typhimurium. In Period C, only *S.* Typhimurium isolates carried resistant genes, *i.e.* bla_{CTX-M1}, bla_{TEM} and *gyrA* mutation in Asp87, *parC* mutation in Ser80 and *qnr* genes.

Table 1. Antimicrobial resistance in *Salmonella* isolates from pork samples.

Sampling period	No. of food samples	No. of isolates (%)	No. phenotypically resistant isolates (%)	No. genotypically resistant isolates (%)
A	87	12 (13.8)	7/12 (58.3)	3/7 (42.8)
B	1067	82 (7.7)	36/82 (43.9)	13/36 (36.1)
C	315	37 (11.7)	17/37 (45.9)	13/17 (76.5)
Total	1469	131 (8.9)	60/131 (45.8)	29/60 (48.3)

Table 2. Phenotypically and genotypically resistant *Salmonella* isolates to different antibiotics.

Sampling period	β-lactams		Fluoroquinolones		Colistin	
	Phenotypic Profile (%)	Genotypic Profile (%)	Phenotypic Profile (%)	Genotypic Profile (%)	Phenotypic Profile (%)	Genotypic Profile (%)
A	2/12 (16.7)	2/2 (100)	2/12 (16.7)	2/2 (100)	4/12 (33.3)	-
B	15/82 (18.3)	9/15 (60)	4/82 (4.8)	4/4 (100)	24/82 (29.3)	-
C	11/37 (29.7)	9/11 (81.8)	12/37 (32.4)	9/12 (75)	2/37 (5.4)	-
Total	28/131 (21.4)	20/28 (71.4)	18/131 (13.7)	15/18 (83.3)	30/131 (22.9)	-

Phenotypic and genotypic co-resistance statistical analysis

The simultaneous presence of ESBL genes and PMQR was detected only in two S. Typhimurium isolated in period C. A negative statistically significant correlation between the two genes simultaneous presence was observed (OR=0.0238, P=0.0001).

Discussion

Salmonella is a major cause of foodborne diseases in humans and is particularly associated with the consumption of food of animal origin. Antimicrobial resistance in *Salmonella* is increasing, likely due to the abuse/misuse of antibiotics in farmed animals, including pigs. AMR compromises the efficacy of antibiotics used to treat human infections, thus suggesting a reducing in antibiotic use in livestock (Pelyuntha *et al*., 2021).

The meat chain process, including on-farm management, transportation and lairage at slaughter and slaughtering operations are potential risk factors for *Salmonella* contamination of carcasses. Different studies have shown that the prevalence of *Salmonella*-positive pigs at farm is

Table 3. Antibiotic resistance pattern in *S.* Derby and *S.* Typhimurium.

Sampling period	No. isolates	No. resistant strains	AMR phenotypic pattern	AMR genotypic pattern
S. Derby				
A	3	1	ESBL+AmpC	bla_{CTX-M1}
		1	NAL	*parC80*
B	24	1	ESBL	bla_{CTX-M1}
		1	ESBL	bla_{CTX-M1}+ bla_{TEM}
		2	ESBL	-
		3	ESBL +COL	bla_{CTX-M1}
		1	NAL	*gyrA83+gyrA87+parC80+parC84*
		1	NAL+COL	*gyrA83+gyrA87+parC80*
		1	COL	-
C	8	2	ESBL	-
S. Typhimurium				
B	29	2	ESBL	-
		7	COL	-
		1	ESBL+COL	bla_{CTX-M1}
		1	ESBL+COL	bla_{CTX-M1}+ bla_{TEM}
C	22	2	COL	-
		1	ESBL+CIPRO	bla_{CTX-M1}+*gyrA87+parC80*
		1	ESBL+CIPRO	bla_{TEM}+*gyrA87*
		1	ESBL+CIPRO	bla_{CTX-M1}+ bla_{TEM}
		1	ESBL+CIPRO	bla_{CTX-M1}+ bla_{TEM}
		2	ESBL+CIPRO	bla_{CTX-M1}+ *gyrA87+parC80+ qnr*
		1	ESBL+CIPRO	bla_{CTX-M1}+*gyrA87*
		2	CIPRO+NAL	*gyrA87+parC80*
		2	CIPRO+NAL	*gyrA87+parC80*

Table 4. Antibiotic resistance pattern in several serovars.

Sampling period	*Salmonella* serovars	No. of isolates	No. of resistant isolates	AMR phenotypic pattern	AMR genotypic pattern
A	Enteritidis	2	2	COL	-
	Tennessee	3	1	ESBL + NAL	$blaS_{HV}$+*gyrA83+gyrA87+parC80*
			1	COL	-
	Blockey	1	1	COL	-
	Dublin	2	0	-	-
	Thompson	1	0	-	-
B	Anatum	4	1	ESBL	-
			3	COL	-
	London	7	3	COL	-
	Bredeney	8	2	ESBL	bla_{CTX-M1}
			3	COL	
			1	ESBL+COL	-
	Virchow	4	1	NAL	*gyrA83+gyrA87+parC80*
			1	CIPRO+NAL	*gyrA83+gyrA87+parC80+qnr*
	Typhimurium Monophasic	4	-	-	-
	Agona	2	-	-	-
C	Rissen	5	1	ESBL	bla_{CTX-M1}
	Infantis	2	1	ESBL+CIPRO+NAL	bla_{CTX-M1}+bla_{TEM}

lower than the prevalence on animal samples and carcasses at the abattoir (Barilli *et al*., 2018; Beloeil *et al*., 2004; Bonardi, 2017). Several studies have investigated the prevalence of AMR enzymes in *Salmonella* isolates from humans, while fewer studies have been performed on food-derived isolates (Mąka and Popowska, 2016). The aim of the present study was to compare the prevalence of AMR *Salmonella* isolates detected in pork during three different periods covering two decades (2000-2021). To the authors knowledge, this is one of the first study comparing long-term resistances against ß-lactams, fluoroquinolones and colistin in *Salmonella* isolates from pig meat in Europe.

For many years, resistance to older antibiotics (*e.g*., ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole) has increased, leading to new generation treatment options for salmonellosis in farmed animals. The most commonly used antimicrobials have thus become β-lactams (3^{rd}, 4^{th} generation cephalosporins) and fluoroquinolones (ciprofloxacin) (Pelyuntha *et al*., 2021).

Cephalosporins are frequently used to treat human infections, particularly in children affected by salmonellosis (Shigemura *et al*., 2020). Over the years, ESBL – and AmpC β-lactamases have supported the spread of resistant bacteria, both in humans and animals (Jeon *et al*., 2019).

Our study shows that the prevalence of ESBL *Salmonella* isolates detected from pork has almost doubled over two decades (Table 2). In parallel, an increase in the number of strains harbouring ESBL resistance genes, predominantly $bla_{CTX\text{-}M1}$ and $bla_{TEM,}$ was observed. Concerning AmpC, no genes were found in the three periods, suggesting the existence of intrinsic resistance mechanisms (Table 3). These data are encouraging because AmpC genes are frequently located on plasmids leading a major rate of AMR transmissibility (Mąka and Popowska, 2016).

Fluoroquinolones such as ciprofloxacin and nalidixic acid are antibiotics that act on the bacterial type II topoisomerases proteins encoded by the *gyrA* and *parC* genes causing disrupted chromosome replication and rapid bacterial death. Bacteria can acquire mutations in the quinolone-resistance-determining regions (QRDRs) of the chromosomal *gyr* and *par* genes resulting in a lower quinolone-binding affinity of the topoisomerase enzymes. The resistance can be carried by plasmid-mediated quinolone resistance (PMQR) as well, in particular *qnr* genes (*qnrA*, *qnrB*, *qnrS*) (Cuypers *et al*., 2018). In the present study, phenotypically fluoroquinolone resistance doubled throughout the entire period 2000-2021 (16.7% in Period A *vs.* 32.4% in Period C). The genotypic analysis showed an increase in fluoroquinolones resistance genes and the most common were *gyrA* Asp87 and *parC* Ser80 genes mutations (Tables 3 and 4). Sridhar *et al*. (2021) reported that the mechanisms of genetic development of fluoroquinolones resistance are still not clear. In Europe, the level of ciprofloxacin resistance recorded in 2018-2019 in *Salmonella* strains isolated from pig carcasses was 8.1% (EFSA and ECDC, 2021), thus lower than the prevalence of phenotypically ciprofloxacin-resistant *Salmonella* (13.3%) of our study. This difference can be attributed to several contamination steps occurring during meat processing and distribution at retail.

The PMQR are apparently related with the presence of both ESBLs or AmpC genes and their distribution could be driven by other mobile genetic elements located on plasmids (Carattoli *et al*., 2005; Wang *et al*., 2013; Jiang *et al*., 2014). In our study, this relation was found only in two *S.* Typhimurium.

For decades colistin has been the treatment of choice for the intestinal infections in pigs (Elzbieta and Stefaniuk, 2019). Due to increasing resistance (Min *et al*., 2018), its use has been strongly reduced in human medicine since 1970s, and in veterinary medicine since 2016 in mass treatments (EMA/CVMP/CHMP, 2016). Our results are encouraging because of the decreasing number of colistin-resistant isolates from Period B to the present. The phenotypic resistance to colistin in *Salmonella* isolates was never confirmed by the detection of *mcr* genes, thus suggesting the possible presence of mutate genes conferring alternative resistance mechanisms (Sun *et al*, 2009).

S. Typhimurium, together with *S.* Derby, has been identified as an important food-borne pathogen associated with pork products in many parts of the world (Xu *et al*., 2019). In Europe, *Salmonella* serovars isolated from human cases not always correspond to the ones isolated in food producing animals suggesting other transmission route (EFSA and ECDC, 2021). Among the top-20 serovars reported during 2018-2019, *S.* Typhimurium ranked second, and *S.* Derby ranked sixth in humans. Data from pig carcasses assessed that *S.* Derby was the second and *S.* Typhimurium was the third most reported serovars (EFSA and ECDC, 2021), thus confirming our findings. Interestingly, *S.* Typhimurium showed a major number of resistances and carried more resistant genes than *S.* Derby, confirming once again the EU trend highlighted by the EFSA and ECDC report 2021.

Level of ß-lactams resistance is low all over Europe. In particular, *S.* Typhimurium and *S.* Derby EU isolates did not show any resistance to this antibiotic class (EFSA and ECDC, 2021). On the contrary, in the present study, the abovementioned serovars showed high ß-lactams resistance values.

In European countries in 2018-2019, the prevalence of fluoroquinolones resistant *S.* Typhimurium isolates from pig carcasses was 14.5% while *S.* Derby resistance was risible (EFSA and ECDC, 2021). This trend was confirmed in our study, especially for *S.* Derby.

Concerning phenotypical co-resistances, the most frequent in *S.* Typhimurium and *S.* Derby were to ß-lactams and colistin during 2012-2016. However, a decrease in colistin resistance was observed in both serovars during the most recent sampling period (2018-2021).

Conclusions

Actually, the transmission of AMR bacteria and antibiotic residues by ingestion of food is perceived as a menace by the consumers. This threat is even worse when AMR zoonotic bacteria are involved. Our study highlights the increase in *Salmonella* AMR to ß-lactams and fluoroquinolones, which are widely used as first choice treatment in human cases of salmonellosis. Since the isolates were detected from pig meat over two decades (2000-2021), transmission of resistant strains to the consumers cannot be excluded. Only the resistance to colistin showed a decreasing trend, suggesting that the prudent use of this antibiotic in farmed animals can lead to a reduction of AMR level.

Salmonellosis is often transmitted by ingestion of raw or undercooked pork, together with fermented pork products (sausages and salami). To avoid human infections, hygiene during meat handling and proper cooking are the most important tools to protect human health.

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