

## Evolution of $\beta$ -lactams, fluoroquinolones and colistin resistance and genetic profiles in *Salmonella* isolates from pork in northern Italy

Ilaria Carosino,<sup>1</sup> Silvia Bonardi,<sup>1</sup> Martina Rega,<sup>1</sup> Andrea Luppi,<sup>2</sup> Luca Lamperti,<sup>1</sup> Maria Cristina Ossiprandi,<sup>3</sup> Cristina Bacchi<sup>1</sup>

<sup>1</sup>Food Hygiene Unit, Department of Veterinary Science, University of Parma; <sup>2</sup>Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia-Romagna, Parma; <sup>3</sup>Microbiology Unit, Department of Veterinary Science, University of Parma, Italy

### 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 ( $\beta$ -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:  $\beta$ -lactams, fluoroquinolones and polymyxins. End-point polymerase chain reaction (PCR) and PCR-Real Time were used for genotypical analyses. The phenotypical resistance to  $\beta$ -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  $\beta$ -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 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> generation cephalosporins,

Correspondence: Martina Rega, Department of Veterinary Sciences, University of Parma, Via del Taglio 10, 43126 Parma, Italy. Tel.: +39.0521.032743. E-mail: martinarega92@gmail.com

Key words: *Salmonella*, pork,  $\beta$ -lactams, ciprofloxacin, colistin.

Acknowledgments: The authors would like to thank Dr. Andrea Luppi of Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna (IZSLER), Parma and IZSLER of Brescia for the priceless advice.

Contributions: The authors contributed equally.

Conflict of interest: The authors declare no potential conflict of interest.

Funding: This work was supported by Cariparma Foundation (Grant number 2019.0065), Parma, Italy.

Availability of data and material: Data and materials are available by the authors.

Received for publication: 14 July 2021.

Accepted for publication: 15 February 2022.

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

©Copyright: the Author(s), 2022

Licensee PAGEPress, Italy

Italian Journal of Food Safety 2022; 11:9972

doi:10.4081/ijfs.2022.9972

*Publisher's note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.*

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 3<sup>rd</sup> and 4<sup>th</sup> 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 synthesize  $\beta$ -lactamases. These enzymes are able to hydrolyse  $\beta$ -lactams, that are widely used in the treatment of *Salmonella* infections. More than 4,300 different  $\beta$ -lactamases have been described, and due to genetic mutation, these enzymes

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  $\beta$ -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*<sub>CTX-M</sub> 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 3<sup>rd</sup> 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, *Salmonella* 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-Kauffmann Tetrionate-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 30 µg 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*<sub>CTX-M1</sub>, *bla*<sub>CTX-M2</sub>, *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>), as described by Roschansky *et al.* (2014). Preliminary tests were performed to define the correct annealing temperature for each primer and the presence of a specific product was avoided by the melting curve analysis. In each reaction the following positive controls were used: *K. pneumoniae* NCTC 13368 for *bla*<sub>SHV</sub>, *E. coli* NCTC 13351 for *bla*<sub>TEM</sub> and *Salmonella* NCTC 13353 for *bla*<sub>CTX-M</sub>. 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*<sub>FOX</sub>, *bla*<sub>MOX</sub>, *bla*<sub>ACC</sub>, *bla*<sub>EBC</sub> and *bla*<sub>DHA</sub>) genes was verified using the multiplex PCR protocol described by Pérez-Pérez and Hanson (2002), with the only exception of the MgCl<sub>2</sub> 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  $\mu$ L. Each reaction contained: 5X GoTaq G2 Flexi DNA polymerase (Promega Italia, Milano, Italy) at a final concentration of 1X; 2.5 mM of MgCl<sub>2</sub>; 0.2 mM of dNTPs; 0.5 mM of each primer. Template DNA (2  $\mu$ L) was added to 48  $\mu$ L 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  $\mu$ L 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  $\beta$ -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  $\beta$ -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  $\beta$ -lactams (18.3%) and fluoroquinolones (4.8%). Among serotypes, *S. Derby* and *S. Typhimurium* were mostly resistant to  $\beta$ -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  $\beta$ -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  $\beta$ -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*<sub>CTX-M1</sub>, *bla*<sub>SHV</sub>, *gyrA* mutations in Ser83 and Asp87, and *parC* mutation in Ser80. In Period B,  $\beta$ -lactams (*bla*<sub>CTX-M1</sub>, *bla*<sub>TEM</sub>) and fluoroquinolones resistance genes were found (*gyrA* mutations in Ser83/Asp87, *parC* mutations in Ser80/Glu84, and *qnr*). In Period C,  $\beta$ -lactams (*bla*<sub>CTX-M1</sub>, *bla*<sub>TEM</sub>) 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*<sub>CTX-M1</sub> 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*<sub>CTX-M1</sub>, *bla*<sub>TEM</sub> 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	$\beta$ -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 food-borne 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 <i>S. Derby</i>	AMR genotypic pattern	
A	3	1	ESBL+AmpC	<i>bla</i> <sub>CTX-MI</sub>	
		1	NAL	<i>parC80</i>	
		B	1	ESBL	<i>bla</i> <sub>CTX-MI</sub>
			1	ESBL	<i>bla</i> <sub>CTX-MI</sub> + <i>bla</i> <sub>TEM</sub>
			2	ESBL	-
			3	ESBL+COL	<i>bla</i> <sub>CTX-MI</sub>
1	NAL	<i>gyrA83+gyrA87+parC80+parC84</i>			
1	NAL+COL	<i>gyrA83+gyrA87+parC80</i>			
1	COL	-			
C	8	2	ESBL	-	
<b><i>S. Typhimurium</i></b>					
B	29	2	ESBL	-	
		7	COL	-	
		1	ESBL+COL	<i>bla</i> <sub>CTX-MI</sub>	
		1	ESBL+COL	<i>bla</i> <sub>CTX-MI</sub> + <i>bla</i> <sub>TEM</sub>	
C	22	2	COL	-	
		1	ESBL+CIPRO	<i>bla</i> <sub>CTX-MI</sub> + <i>gyrA87+parC80</i>	
		1	ESBL+CIPRO	<i>bla</i> <sub>TEM</sub> + <i>gyrA87</i>	
		1	ESBL+CIPRO	<i>bla</i> <sub>CTX-MI</sub> + <i>bla</i> <sub>TEM</sub>	
		1	ESBL+CIPRO	<i>bla</i> <sub>CTX-MI</sub> + <i>bla</i> <sub>TEM</sub>	
		2	ESBL+CIPRO	<i>bla</i> <sub>CTX-MI</sub> + <i>gyrA87+parC80+qnr</i>	
		1	ESBL+CIPRO	<i>bla</i> <sub>CTX-MI</sub> + <i>gyrA87</i>	
		2	CIPRO+NAL	<i>gyrA87+parC80</i>	
		2	CIPRO+NAL	<i>gyrA87+parC80</i>	

**Table 4. Antibiotic resistance pattern in several serovars.**

Sampling period	<i>Salmonella</i> serovars	No. of isolates	No. of resistant isolates	AMR phenotypic pattern	AMR genotypic pattern	
A	Enteritidis	2	2	COL	-	
	Tennessee	3	1	ESBL + NAL	<i>bla</i> <sub>S<sub>IV</sub></sub> + <i>gyrA83+gyrA87+parC80</i>	
	B	Blockey	1	1	COL	-
		Dublin	2	0	-	-
		Thompson	1	0	-	-
		Anatum	4	1	ESBL	-
B	London	7	3	COL	-	
		7	3	COL	-	
		8	2	ESBL	<i>bla</i> <sub>CTX-MI</sub>	
	Bredeney	8	2	ESBL	<i>bla</i> <sub>CTX-MI</sub>	
		3	3	COL	-	
		1	1	ESBL+COL	-	
		4	4	NAL	<i>gyrA83+gyrA87+parC80</i>	
	1	1	CIPRO+NAL	<i>gyrA83+gyrA87+parC80+qnr</i>		
Typhimurium Monophasic	4	-	-	-		
Agona	2	-	-	-		
C	Rissen	5	1	ESBL	<i>bla</i> <sub>CTX-MI</sub>	
	Infantis	2	1	ESBL+CIPRO+NAL	<i>bla</i> <sub>CTX-MI</sub> + <i>bla</i> <sub>TEM</sub>	

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  $\beta$ -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  $\beta$ -lactams (3<sup>rd</sup>, 4<sup>th</sup> 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  $\beta$ -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*<sub>CTX-M1</sub> and *bla*<sub>TEM</sub>, 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*) (Cuyper *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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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.

## References

- Barilli E, Bacci C, StellaVilla Z, Merialdi G, D’Incau M, Brindani F, Vismarra A, 2018. Antimicrobial resistance, biofilm synthesis and virulence genes in *Salmonella* isolated from pigs bred on intensive farms. *Ital J Food Saf* 7:7223.
- Beloeil PA, Chauvin C, Proux K, Madec F, Fravallo P, Alioum A, 2004. Impact of the *Salmonella* status of market-age

- pigs and the pre-slaughter process on *Salmonella* caecal contamination at slaughter. *Vet Res* 35:513-30.
- Bonardi S, 2017. *Salmonella* in the pork production chain and its impact on human health in the European Union. *Epidemiol Infect* 145:1513-26.
- Borowiak M, Baumann B, Fischer J, Thomas K, Deneke C, Hammerl JA, Szabo I, Malorny B, 2020. Development of a novel *mcr-6* to *mcr-9* multiplex PCR and assessment of *mcr-1* to *mcr-9* occurrence in colistin-resistant *Salmonella enterica* isolates from environment, feed, animals and food (2011-2018) in Germany. *Front Microbiol* 11:80.
- Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ, 2005. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 63:219-28.
- Carroll LM, Gaballa A, Guldemann C, Sullivan G, Henderson LO, Wiedmann M, 2019. Identification of novel mobilized colistin resistance gene *mcr-9* in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate. *Mbio* 10:e00853-19.
- Chen K, Dong N, Chan EW, Chen S, 2019a. Transmission of ciprofloxacin resistance in *Salmonella* mediated by a novel type of conjugative helper plasmids. *Emerg Microbes Infect* 8:857-65.
- Chen K, Chan EWC, Chen S, 2019b. Evolution and transmission of a conjugative plasmid encoding both ciprofloxacin and ceftriaxone resistance in *Salmonella*. *Emerg Microbes Infect* 8:396-403.
- Cuypers WL, Jacobs J, Wong V, Klemm EJ, Deborggraeve S, Van Puyvelde S, 2018. Fluoroquinolone resistance in *Salmonella*: insights by whole-genome sequencing. *Microb Genom* 4:e000195.
- De Busser EV, Maes D, Houf K, Dewulf J, Imberechts H, Bertrand S & De Zutter L, 2011. Detection and characterization of *Salmonella* in lairage, on pig carcasses and intestines in five slaughterhouses. *Ital J Food Saf* 145:279-86.
- Directive 2003/99/EC of the European parliament and of the council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC.
- Doma AO, Popescu R, Mitulețu M, Muntean D, Dégi J, Boldea MV, Radulov I, Dumitrescu E, Muselin F, Puvăca N, Cristina RT, 2020. Comparative evaluation of *qnrA*, *qnrB*, and *qnrS* genes in Enterobacteriaceae ciprofloxacin-resistant cases, in swine units and a hospital from western Romania. *Antibiotics (Basel)* 9:698.
- Elzbieta M, Stefaniuk ST, 2019. Colistin resistance in Enterobacteriales strains-A current view. *Pol. J. Microbiol* 68:417-27.
- European Medicine Agency (EMA), Committee for Medicinal Products for Veterinary Use (CVMP), Committee for Medicinal Products for Human Use (CHMP), 2016. Updated advice on the use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health; EMA: London, UK.
- Entervet. 2002. <http://entervet.izsvenezie.it>
- European Food Safety Authority (EFSA); European Centre for Disease Prevention and Control (ECDC), 2021. The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. EFSA J 19:e06490.
- European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), 2018. Sales of veterinary antimicrobial agents in 31 European countries in 2018. Trends from 2010 to 2018. Tenth ESVAC Report. EMA 10:1-101
- Farina, R. and Scatozza, F, 1998. Trattato di malattie infettive degli animali, 2<sup>a</sup> Edizione, UTET, Torino.
- Gunell M, Webber MA, Kotilainen P, Lilly AJ, Caddick JM, Jalava J, Huovinen P, Siitonen A, Hakanen AJ, Piddock LJ, 2009. Mechanisms of resistance in non-typhoidal *Salmonella enterica* strains exhibiting a nonclassical quinolone resistance phenotype. *Antimicrob Agents Chemother* 53:3832-6.
- Hooper DC, 2001. Emerging mechanisms of fluoroquinolone resistance. *Emerg Infect Dis* 7:337-41.
- ISO, 1993. Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Salmonella* spp. ISO Norm 6579:1993. International Organization for Standardization, Geneva, Switzerland.
- ISO, 2002. Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Salmonella* spp. ISO Norm 6579:2002. International Organization for Standardization, Geneva, Switzerland.
- ISO, 2017. Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Salmonella* spp. ISO Norm 6579:2017. International Organization for Standardization, Geneva, Switzerland.
- Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe). <https://www.izsvenezie.it/>.
- Jeon HY, Kim YB, Lim SK, Lee YJ, Seo KW, 2019. Characteristics of cephalosporin-resistant *Salmonella* isolates from poultry in Korea, 2010-2017. *Poult Sci* 98:957-65.
- Jiang HX, Song L, Liu J, Zhang XH, Ren YN, Zhang WH, Zhang JY, Liu YH, Webber MA, Ogbolu DO, Zeng ZL, Piddock LJ, 2014. Multiple transmissible genes encoding fluoroquinolone and third-generation cephalosporin resistance co-located in non-typhoidal *Salmonella* isolated from food-producing animals in China. *Int J Antimicrob Agents* 43:242-7.
- Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *Lancet Infect Dis* 16:161-8.
- Lu X, Zeng M, Xu J, Zhou H, Gu B, Li Z, Jin H, Wang X, Zhang W, Hu Y, Xiao W, Zhu B, Xu X, Kan B, 2019. Epidemiologic and genomic insights on *mcr-1*-harbouring *Salmonella* from diarrhoeal outpatients in Shanghai, China, 2006-2016. *EbioMedicine* 42:133-44.
- Mąka Ł, Popowska M, 2016. Antimicrobial resistance of *Salmonella* spp. Isolated from food. *Rocz Panstw Zakl Hig* 67:343-58.
- Min KL, Son ES, Kim JS, Kim SH, Jung SM, Chang MJ, 2018. Risk factors of colistin safety according to administration routes: intravenous and aerosolized colistin. *PLoS ONE* 13:e020758.
- Onseadaeng S, Rathawongjirakul P, 2016. Rapid Detection of genomic mutations in *gyrA* and *parC* genes of *Escherichia coli* by Multiplex allele specific Polymerase Chain Reaction. *J Clin Lab Anal* 306:947-55.
- Pelyuntha W, Ngasaman R, Yingkajorn M, Chukiatsiri K, Benjakul S, Vongkamjan K, 2021. Isolation and characterization of potential *Salmonella* phages targeting multidrug-resistant and major serovars of *Salmonella* derived from broiler production chain in Thailand. *Front Microbiol* 12:662461.
- Pérez-Pérez FJ, Hanson ND, 2002. Detection of plasmid-mediated Amp<sup>C</sup> - Lactamase genes in clinical isolates by using Multiplex PCR. *J. Clin. Microbiol* 40:2153-62.

- Rebello AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, Guerra B, Malorny B, Borowiak M, Hammerl JA, 2018. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes. *Eurosurveillance* 23:e17-00672.
- Regulation (EC) No 2073/2005 of the Commission of 15 November 2005 on microbiological criteria for foodstuff. Official J EU L 338:1-26.
- Regulation (EU) No 217/2014 of 7 March 2014 amending Regulation (EC) No 2073/2005 as regards Salmonella in pig carcasses. Official J EU L 69/93
- Roschansky N, Fischer J, Guerra B, Roesler U, 2014. Development of a Multiplex Real-Time PCR for the rapid detection of the predominant Beta-Lactamase genes CTX-M, SHV, TEM and CIT type AmpCs in Enterobacteriaceae. *PLoS ONE* 9:e100956.
- Scott HM, Acuff G, Bergeron G, Bourassa MW, Simjee S, Singer RS, 2019. Critically important antibiotics: criteria and approaches for measuring and reducing their use in food animal agriculture. *Ann N Y Acad Sci* 1441:8-16.
- Shigemura H, Sakatsume E, Sekizuka T, Yokoyama H, Hamada K, Etoh Y, Carle Y, Mizumoto S, Hirai S, Matsui M, Kimura H, Suzuki M, Onozuka D, Kuroda M, Inoshima Y, Murakami K, 2020. Food workers as a reservoir of Extended-spectrum-cephalosporin-resistant Salmonella strains in Japan. *Appl Environ Microbiol* 86:e00072-20.
- Sridhar S, Forrest S, Pickard D, Cormie C, Lees EA, Thomson NR, Dougan G, Baker S, 2021. Inhibitory concentrations of ciprofloxacin induce an adaptive response promoting the intracellular survival of Salmonella enterica serovar Typhimurium. *mBio* 22:e0109321
- Sun S, Negrea A, Rhen M, Andersson DI. Genetic analysis of colistin resistance in Salmonella enterica serovar Typhimurium. *Antimicrob Agents Chemother*. 2009 Jun;53(6):2298-305
- Sun J, Zhang H, Liu YH, Feng Y, 2018. Towards understanding MCR-like colistin resistance. *Trends Microbiol* 26:794-808.
- The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2018. Breakpoint Tables For Interpretation Of Mics And Zone Diameters, Version 8.1. Available online: <http://www.eucast.org>
- Tooke CL, Hinchliffe P, Bragginton EC, Colenso CK, Hirvonen VHA, Takebayashi Y, Spencer J, 2019. B-Lactamases and  $\beta$ -Lactamase Inhibitors in the 21<sup>st</sup> Century, *Journal of Molecular Biology* 431:3472-500.
- Wang J, Stephan R, Karczmarczyk M, Yan Q, Hächler H, Fanning S, 2013. Molecular characterization of bla ESBL-harboring conjugative plasmids identified in multi-drug resistant Escherichia coli isolated from food-producing animals and healthy humans. *Front Microbiol* 4:188.
- Wong MH, Yan M, Chan EW, Biao K, Chen S, 2014. Emergence of clinical Salmonella enterica serovar Typhimurium isolates with concurrent resistance to ciprofloxacin, ceftriaxone, and azithromycin. *Antimicrob Agents Chemother* 58:3752-6.
- World Organisation for Animal Health (OIE), 2019. Terrestrial Animal Health Code, 20<sup>th</sup> ed. World organisation for Animal Health. Paris, France 6:1-6.
- Xu Z, Song Q, Li C, Zhan Y, 2019. Characterization of ciprofloxacin-resistant and ESBL-producing Salmonella enteric serotype Derby in Eastern China. *BMC Microbiol* 19:61