Antimicrobial resistance, biofilm synthesis and virulence genes in Salmonella isolated from pigs bred on intensive farms

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Abstract

Salmonella is the second cause of foodborne infection in humans in the USA and Europe. Pigs represent the second most important reservoir for the pathogen and the consumption of pork meat is a major risk factor for human salmonellosis. Here, we evaluated the virulence patterns of eleven *Salmonella* isolated from pigs (carcasses and faces) bred in intensive farms in the north of Italy. The two serotypes identified were *S.* Typhimurium and its monophasic variant 1,4,5,12:i:-. None of the isolates was an ESBL producer, as confirmed also by PCR. However, the presence of a multidrug resistant pattern was evident, with all the isolates being resistant to at least to five antimicrobial agents belonging to various classes. Moreover, six out of eleven isolates showed important resistance profiles, such as resistance against colistin and ciprofloxacin, with nine to twelve recorded resistances. The isolates were negative for the biofilm synthesis test, while four different virulotypes were characterized. All the isolates showed the presence of *inv*A, *hil*A, *stn*, *ssr*A, *sip*C. One sample also harbored *ssa*R and *spv*C genes. One strain was positive for all the virulence genes tested and was resistant to 12 antimicrobial agents. The present study contributes new data to the surveillance program for antibiotic resistance. Furthermore, the presence of eleven highly virulent isolates poses concern for human health in relation to their diffusion in the environment.

Introduction

Salmonella is one of the best known foodborne pathogens due to its vast diffusion throughout the world and to its ability to cause both widespread contamination and infection (Pires, 2013). Human salmonellosis ranked second after campylobacteriosis in European Union (EU) with 88715 confirmed cases as reported in the 2015 report on zoonoses and zoonotic agents published by the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) (EFSA and ECDC, 2016).

Salmonella colonizes the intestine of many animals, including mammals, reptiles and birds. Poultry, in particular, serves as an efficient vector for transmission of multiple serovars of *Salmonella* to human people through the consumption of contaminated foods. Besides poultry, pigs are one of the principle species responsible for the transmission of *Salmonella* to humans. *Salmonella* infects pigs following the fecal-oral route and enterocolitis is generally the main clinical manifestation. Asymptomatic carriers are an important reservoir for *Salmonella* on farms. This, together with other factors such as high animal density, poor cleaning and disinfection and poor biosecurity measures, favors the presence of the pathogen. Another critical point is represented by the level of hygiene at the slaughterhouse. Here, cross-contamination of carcasses with faces can occur if relevant food safety regulations are not strictly respected. Control measures aimed at reducing the level of meat contamination, must be applied at all the levels of food chain.

European Regulations (nos.2073/2005 and 217/2014) define the microbiological criteria for several foodborne-pathogens in different foodstuffs, including *Salmonella*. Moreover, each member state of the EU has activated surveillance programs that monitor the prevalence in different animals, foodstuff and environment, together with the characterization of the serovar and antimicrobial- resistance profile of each isolate, as requested by EFSA and ECDC from 2008. In southern Europe, a large part of confirmed cases of human salmonellosis has been linked to the ingestion of contaminated pork products (43.6% of all cases) (Pires *et al*., 2011).

Historically, recommended treatment for cases of blood-stream salmonellosis included antimicrobial agents such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole. Drug resistance, which has spread over the past 20 years, has now limited the use of these antibiotics and quinolone, macrolide, and third-generation cephalosporins are preferred. A further consideration regards multi-drug-resistant (MDR) *Salmonella*, defined as strains with resistance to three or more antimicrobial classes. In Europe, MDR rate is high overall (26.0%), with a peak recorded in France (62.6%), and several serovars exhibited an extremely high rate, including *S.* Kentucky (74.6%), monophasic *S.* Typhimurium 1,4,[5],12:i:- (69.4%) and *S.* Infantis (61.9%) (ECDC/EFSA/EMA, 2017).

According to European legislation, antimicrobial resistance must be monitored on a representative number of isolates of *Salmonella enterica* from pigs and poultry and foods derived from these species (ECDC/EFSA/EMA, 2017).

There are several mechanisms responsible for the development of resistant bacteria following the pressure of antimicrobial use. Among these, there is the synthesis of hydrolytic enzymes like β- lactamases, the main cause of antibiotic resistance in Gram negative bacteria. Extended-spectrum-β- lactamases (ESBLs) are one of the major determinants of resistance against oxymino-cephalosporins in *Enterobasteriaceae*. These enzymes are commonly called cephalosporinases, hydrolase penicillins, I-III generation cephalosporins, cephamycins (cefoxitin) and oxyiminocephalosporins (Jacoby et al., 2009). They are encoded by genes (*bla*TEM, *bla*CTX-M, *bla*SHV) located on either plasmids, which can be transferred among bacteria (*i.e.* during bacterial conjugation), or on chromosomes (Philippon *et al*., 2002).

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Antimicrobial Susceptibility Testing (EUCAST, 2015) has defined a series of antibiotic panels to test, comprising molecules belonging to different families and with different mechanisms of action. The existence of *Salmonella* strains with remarkable resistance profiles, together with other pathogenic factors such as biofilm synthesis ability, or particular virulence patterns (defined by the presence of specific virulence associated genes) like epithelial cell adhesion, phagocytes and non-phagocytes cell-invasion, is an increasing phenomenon that must be monitored continuously. *Salmonella* is capable of biofilm formation, which is an important mechanism of resistance to antimicrobials and plays an important role in the virulence of microrganisms (Simm *et al*., 2014). Furthermore, bacteria that are capable of biofilm formation on food processing surfaces can cause food spoilage or transmission of diseases.

The aims of the present study are (i) to determine the presence of antibiotic resistance by applying an EUCAST panel with disc diffusion and minimum inhibitory concentration (MIC), (ii) to determine the ability to produce biofilm and (iii) to define virulotypes by polymerase chain reaction (PCR) marking specific genes.

Materials and Methods

Sampling

The study was conducted on carcasses (sponges) and faces (swabs) collected from 150 pigs in 6 slaughterhouses located in the Emilia Romagna and Lombardy regions from September 2016 to May 2017. All 300 samples (150 faces and 150 carcasses) were collected following the UNI EN ISO 17604:2015 method, as described by European Regulation CE 2073/2005. Pigs came from intensive farms located in different provinces of northern Italy. Rectal samples were collected in the lairage of the slaughterhouses, using sterile swabs. They were immediately put in a transport container with Buffer Peptone Water (BPW, Biolife Italiana) and were then incubated overnight at 37°C in the lab. Four different carcass sites of 100 cm^2 each, as described in annex A of Standard ISO 17604, were swabbed before chilling using a sterile sponge moistened with Buffered Peptone Water (BPW, Biolife Italiana). After sampling, the sponge was placed in a sterile bag and stored at +4°C during transport and was then incubated at 37°C for 18 h ± 2 h in the lab.

Salmonella isolation and serotyping

Isolation of *Salmonella* from collected samples was carried out according to UNI EN ISO 6579:2008. Positive controls ATCC 13076 (*S*. Enteritidis) and ATCC 14028 (*S*. Typhimurium) were used. Biochemical characterization, presence of somatic antigen and oxidase activity were analyzed. Biochemical identification to the genus level was performed by using the API20E® microsubstrate system (bioMérieux, Italia spA).

The complete serological characterization of *Salmonella* was performed by slide agglutination for the determination of somatic antigens, while, for the determination of flagellar antigens, the method of tube agglutination according to the technique of Spicer (1956), modified by Edwards (1962) and Morris *et al*. (1972) was followed. The results of the determinations of the antigens were then used for the final serological characterization according to the scheme of Kauffmann - White - Le Minor (Grimont and Weill, 2007).

Antimicrobial susceptibility test

Confirmed colonies of *Salmonella* were then subjected to antimicrobial susceptibility test for III and IV generation cephalosporins. The disc diffusion test on agar was used in the first part of the study following the protocol defined by EUCAST (2015). Briefly, confirmed *Salmonella* colonies were seeded onto a nutritive agar (Tryptic Soy Agar-TSA) and incubated at 37°C for 24 h. One single colony was put in Tryptic Soy Broth (TSB) then incubated for 4-6 h at 37°C. The culture-broth was seeded uniformly on Mueller Hinton Agar plates, antimicrobial discs were added and incubated at 37°C for 18±2 h. For the disc diffusion agar test, these antimicrobial agents were selected: Cefotaxime (30 µg), Cefotaxime (30 µg) + Clavulanate (10 µg), Ceftazidime (30 µg), Ceftazidime (30 µg) + Clavulanate (10 µg), Cefepime (30 µg), Cefepime (30 µg) + Clavulanate (10 µg), Amoxicillin (20 µg) + Clavulanate (10 µg), Amoxicillin (20 µg). All isolates were tested for the antimicrobial susceptibility with the minimum inhibitory concentration (MIC). A panel of antibiotic agents was chosen from those proposed by EUCAST (2015), as listed in Table 1 Sulfamethoxazole, Trimethoprim, Ciprofloxacin, Tetracycline,

Table 1. *Salmonella* serotypes isolated from faces and carcasses in the North of Italy and MIC results.

Sample	*Salmonella* serovar	SMX >4	TMP >4	CIP >0,5	TET >2	MERO >8	AZI >16	NAL >32	FOT >2	CHL >8	TGC >2	TAZ >4	COL >2	AMP >8	GEN >4
71 FS	S. Typhimurium	512	1	0,25	32	0,12	4	16	0,25	8	0,25	2	4	32	32
77 CS	S. Typhimurium monophasic variant 1,4,5,12:i:-	1024	32	8	32	0,5	64	128	0,25	64	1	2	4	64	32
86 CS	S. Typhimurium monophasic variant 1,4,5,12:i:-	1024	32	1	64	0,25	16	64	0,25	8	4	0,5	8	64	32
88 CS	S. Typhimurium monophasic variant 1,4,5,12:i:-	1024	32	0,25	64	0,25	16	32	1	8	4	0,5	16	64	32
90 CS	S. Typhimurium	512	8	1	2	0,12	4	64	0,25	16	1	2	1	32	32
97 CS	S. Typhimurium	1024	32	0,25	64	0,12	16	128	0,5	128	4	0,5	4	64	32
100 CS	S. Typhimurium	1024	16	4	64	0,5	64	128	4	128	1	2	16	64	32
104 CS	S. Typhimurium	1024	8	0,12	64	0,25	16	16	1	8	4	2	16	64	32
105 CS	S. Typhimurium	1024	32	2	32	0,25	16	128	0,25	128	1	0,5	1	64	32
109 CS	S. Typhimurium monophasic variant 1,4,5,12:i:-	1024	32	8	64	0,25	64	128	4	128	8	0,5	16	64	32
118 CS	S. Typhimurium	1024	32	4	32	0,12	2	128	1	16	8	2	16	64	32

FS: faeces swab CS: carcasses sponge. SMX: Sulphamethoxazole; TMP: Trimethoprim; CIP: Ciprofloxacin; TET: Tetracycline; MERO: Meropenem; AZI: Azithromycin; NAL: Nalixidic Acid; FOT: Cefotaxime; CHL: Chloramphenicol; TGC: Tigecycline; TAZ: Ceftazidime; COL: Colistin; AMP: Ampicillin; GEN: Gentamicin.

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Meropenem, Azithromycin, Nalixidic Acid, Cefotaxime, Chloramphenicol, Tigecycline, Ceftazidime, Colistin, Ampicillin, Gentamicin. A suspension made with one *Salmonella* colony diluted in sterile water was prepared and incubated at 37°C for 4-6 h. Then, 10 µL of each sample were added to 11 mL of Mueller-Hinton broth and 50 µL of this preparation were added to the SENSITRE micro-plates containing the antimicrobial agents at different concentrations (Thermofisher, USA). The reaction can be read as sensitive-intermediate or resistant.

Biofilm synthesis

For examining the formation of bacterial biofilm, the protocol described by Christensen (1985) was applied using a 96-well plate. After biochemical confirmation, a single *Salmonella* colony was chosen and put in TSB. After 2 h incubation at 37°C, 200 µL of broth were seeded in five wells for each sample. The test for each strain was repeated three times. The plates were then incubated at 37°C for 24 h, washed for three times with 300 µL of sterile PBS 1X and subsequently dried for 30 min at 42°C upside down. Two hundred microliters of Crystal Violet stain were added to each well and incubated at room temperature for 15 min in the dark. Plates were then washed as before, and incubated overnight at room temperature away from direct light. The day after 200 µL of 95% ethanol were added to fix the biofilm formation and the plates were read at 620 nm with a spectrophotometer. The cut-off value (ODc) is defined as five standard deviations (SD) above the mean OD of the negative controls. Biofilm formation is classified according to Stepanovic *et al.* (2000) into categories based upon the ODs obtained: OD ≤ODc, non-adherent; ODc<OD≤2xODc, weak biofilm formation; 2xODc<OD≤4xODc, moderate biofilm formation; and 4xODc<OD, strong biofilm formation. Controls included a well with only medium (negative) and one with *Pseudomonas aeruginosa* PAO1 (positive).

Genes related to antimicrobial resistance and to virulence factors

One to three colonies of *Salmonella* on TSA plates were selected and incubated overnight at 37°C in 4 mL of BPW. One millilitre of the culture broth was used for DNA extraction using a commercial kit (Invitrogen, USA).

The isolates of *Salmonella* were tested for the presence of *bla*CTX-M, *bla*TEM, and *bla*SHV genes with a Real-time PCR using oligonucleotides described by Roschansky *et al.* (2014) with the SsoAdvanced SYBR Green Supermix (Bio-Rad, USA). Preliminary tests to define the correct annealing temperature for each primer were done. In each reaction positive (*K. pneumoniae* NCTC 13368 for *bla*SHV, *E. coli* NTCT 13351 for *bla*TEM, *E. coli* NTCT 13353 for *bla*CTX-M) and negative controls were added and the presence of aspecific products was avoided through melting curve analysis. The amplification protocol was characterized by a denaturation step (95°C for 30 s) and 35 repeated cycles (95°C for 10 s; 60°C for 10 s; 72°C for 30 s; 40°C for 30 s). Fluorescence signals were collected in every cycle and each sample was tested in fourfold.

The identification of virulence genes (*inv*A, *hil*A, *Spv*C, *Sip*A, *Stn, pef*A, *rck*, *sip*C, *ssa*R, *ssr*A, *sop*B) was done using a series of primers reported in Table 2. A classic PCR end-point protocol was applied, adapting the annealing temperatures and the elongation time (at 72°C for each cycle) in accordance with oligonucleotides single properties.

Table 2. Oligonucleotide sequences with annealing temperatures and molecular weight used for ESBL-genes and virulence associated-genes.

	Oligonucleotide	Primer sequence 5'- 3'	Annealing temperature (°C)	Amplicon size (bp)	Reference
Class A β- lactamase genes (multiplex real-time PCR)	*blaCTX-M*	F-CGATGTGCAGTACCAGTAA R-TTAGTGACCAGAATCAGCGG	50	192	Roschanski *et al*., 2014
	blaTEM	F- GCATCTTACGGATGGCATGA R- GTCCTCCGATCGTTGTCAGAA	50	100	Roschanski *et al*., 2014
	blaSHV	F- TCCCATGATGAGCACCTTTAAA R- TCCTGCTGGCGATAGTGGAT	50	104	Roschanski *et al*., 2014
Virulence genes (End-point PCR)	*invA*	F-ACAGTGCTCGTTTACGACCTGAAT R-AGACGACTGGTACTGATCGATAAT	60	243	Chiu *et al*., 2006
	HilA	F-CGTGAAGGGATTATCGCAGT R-GTCCGGGAATACATCTGAGC	56	96	Wang *et al*., 2009
	SpvC	F-ACTCCTTGCACAACCAAATGCGGA R-TGTCTCTGCATTTCGCCACCATCA	56	571	Chiu *et al*., 2006
	SipA	F-CCATTCGACTAACAGCAGCA R-CGGTCGTACCGGCTTTATTA	56	449	Wang *et al*., 2009
	Stn	F-TTGTCTCGCTATCACTGGCAACC R-ATTCGTAACCCGCTCTCGTCC	59	617	Prager *et al*., 1995
	pefA	F-TTGCACTGGGTGGTTCTGG R-TGTAACCCACTGCGAAAG	56	485	Heithoof *et al*., 2008
	rck	F-AACGGACGGAACACAGAGTC R-TGTCCTGACGAAAGTGCATC	59	189	Capuano *et al*., 2013
	sipC	F-AGACAGCTTCGCAATCCGTT R-ATTCATCCCTTCGCGCATCA	60	446	Fardsanei *et al*., 2017
	ssaR	F-GTTCGGATTTGCTTCGG R-TCTCCAGTGACTAACCCTAACCAA	59	1628	Hu *et al*., 2008
	ssrA	F-CTTACGATTACGCCATTTACGG R-ATTTGGTGGAGCTGGCGGGAGT	58	706	Kutsukake *et al*., 2006
	sopB	F-CCTCAAGACTCAAGATG R-TACGCAGGAGTAAATCGGTG	56	1987	Raffatellu *et al*., 2005

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Results

Eleven strains of *Salmonella* were isolated from intensively-bred pigs at slaughter, one from faces (0.66%; CI 95%=0.12-3.68) and ten from carcasses (6.6%; CI 95%=3.6-11.8). Two serovars were identified: *S.* Typhimurium and *S.* Typhimurium monophasic variant 1,4,5,12:i:- , as reported in Table 1.

The disc diffusion test performed for the identification of resistance patterns against III and IV generation cephalosporins gave negative results. None of the *Salmonella* isolated produced ESBLs, data confirmed also through the Real-time PCR reactions for *bla*CTX-M, *bla*TEM, and *bla*SHV genes.

The results of the MIC test showed that all the *Salmonella* isolated on swine carcasses showed resistance towards sulfamethoxazole (SMX), ampicillin (AMP) and gentamicin (GEN) (Table 1). The *S.* Typhimurium isolated from faeces showed resistance to the same antibiotics and to tetracycline (TET) and colistine (COL). All the *Salmonella* isolated from carcasses were resistant to trimethoprim (TMP) while meropenem (MERO) and ceftazidime (TAZ) were still effective against the isolates tested. Resistance against quinolones was recorded in seven and eight isolates from carcasses for ciprofloxacin (CIP) and nalixidic acid (NAL), respectively, with high MIC values. Seven and six isolates were also strongly resistant to chloramphenicol (CHL) and to the macrolide tigecycline (TGC), with MIC values ranging from 2 to 16 times higher than the cut-off for CHL and 2 to 4 times higher than the limit for TGC. Only three out of eleven isolates were resistant to azithromycin (AZI). All the isolates were resistant at least to five antimicrobial agents, belonging to different classes. Moreover, six out of eleven isolates showed important resistance profiles, as reported in Table 1. None of the isolates synthesized biofilm. Data for virulence genes pattern are reported in Table 3. All the isolates were positive for *inv*A, *hil*A, *stn*, *ssr*A, *sip*C. All but one strain also harbored *ssa*R and *spv*C genes. A total of four virulotypes were defined, as shown in Table 4. The majority of the strains (6/11) were in the first group with negative signal for genes *pef*A and *rck*. Three isolates showed another specific pattern (virulotype 2) characterized by the absence of *sip*A gene. Two strains of *S.* Typhimurium had two singular genetic profiles. One strain harbored all the genetic *loci* investigated, while in the second strain genes *pef*A, *rck*, *ssa*R, *spv*C and *sop*B were all negative in PCR.

Discussion

Salmonella continues to be the second foodborne pathogen cause of illnesses in US and in Europe (Scallan *et al*., 2011; EFSA and ECDC, 2016). Therefore, continuous monitoring of isolates is increasingly necessary, in order to study not only the prevalence of *Salmonella* in the environment, but more importantly its virulence patterns.

In the present study, *Salmonella* was isolated from 10 pig carcasses and one feces swab out of 300 samples analyzed. Higher percentages (49.3%) were reported by Bonardi *et al.* (2016) in a study conducted from June to October from faces collected in the lairage and not directly from the rectum, as in the present study. This could justify the low prevalence we reported, together with the fact that the majority of the sampling was done during the winter months. A recent study published by van Damme *et al.* (2018) showed that 31% of rectal samples was positive for *Salmonella*, but most of them were only slightly contaminated, in the range between -1 and 0 Log CFU/g. Two serotypes have been identified: *S.* Typhimurium (63.6%) and *S.* Typhimurium monophasic variant 1,4,5,12:i:- (36.4%). Of the 2401 serotyped

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Table 3. Virulence genes identified by PCR in the isolates.

	Sample	*inv*A	*Hil*A	*pef*A	*rck*	*Stn*	*ssr*A	*ssa*R	*spv*C	*sip*A	*sip*C	*sop*B
S. Typhimurium	71 FS	+	+	-	-	+	+	+	+	+	+	+
S. Typhimurium monophasic variant 1,4,5,12:i:-	77 CS	+	+	+	+	+	+	+	+	-	+	+
S. Typhimurium monophasic variant 1,4,5,12:i:-	86 CS	+	+	-	-	+	+	+	+	+	+	+
S. Typhimurium monophasic variant 1,4,5,12:i:-	88 CS	+	+	-	-	+	+	+	+	+	+	+
S. Typhimurium	90 CS	+	+	-	-	+	+	+	+	+	+	+
S. Typhimurium	97 CS	+	+	+	+	+	+	+	+	+	+	+
S. Typhimurium	100 CS	+	+	-	-	+	+	+	+	+	+	+
S. Typhimurium	104 CS	+	+	-	-	+	+	+	+	+	+	+
S. Typhimurium	105 CS	+	+	+	+	+	+	+	+	-	+	+
S. Typhimurium monophasic variant 1,4,5,12:i:-	109 CS	+	+	+	+	+	+	+	+	-	+	+
S. Typhimurium	118 CS	+	+	-	-	+	+	-	-	+	+	-

Table 4. Most commonly detected haplotype in the isolates.

	*inv*A	*Hil*A	*pef*A	*rck*	*Stn*	*ssr*A	*ssa*R	*spv*C	*sip*A	*sip*C	*sop*B	Number of isolates/tot
Virulotype 1	+	+	-	-+	+	+	+	+	+	+	6/11	
Virulotype 2	+	+	+	+	+	+	+	+	-	+	+	3/11
Virulotype 3	+	+	+	+	+	+	+	+	+	+	+	1/11
Virulotype 4	+	+	-	-+	+	-	-	+	+	-	1/11	

Salmonella isolated from pigs in Europe (EU) in 2015, 56.9% were *S.* Typhimurium and this percentage is congruent with our data. *S.* Derby was the second most common serovar identified, followed by the group of monophasic strains of *S.* Typhimurium (EFSA and ECDC, 2016). Results from pork meat samples and carcasses analyzed in the EU suggest that the percentage of *S.* Typhimurium isolates is decreasing year by year (from 39.3% of serotyped isolates in 2011 to 23.0% in 2014). This contrasts with the percentage of reported monophasic strains of *S.* Typhimurium, which has increased from 2.6% to 22.3% in 2015, and also with our data. Considering human cases of salmonellosis, the main serovars isolated in 2015 were *S.* Enteritidis (44.4%), *S.* Typhimurium (17.4%) and its monophasic variant (7.8%). Similar data were presented by the WHO (Anonymous: SalmSurv-Salmonella Surveillance network www.who.int) from 2006 to 2010 with *S.* Enteritidis as the most common serotype worldwide (65%), followed by *S.* Typhimurium (12%).

Data recorded here confirm that antibiotic resistance is still growing and evolving. All the isolates were resistant to at least five antibiotic agents, belonging to different classes, in particular 81% of the strains were resistant to colistin, a life-saving drug, and 63% were resistant to ciprofloxacin. Resistance towards colistin in *Salmonella* isolates in EU was recorded by several member states in 2016. The Netherlands and Italy also reported colistin-resistant *Salmonella* isolates in the 5.9% and 21.5% respectively of the isolates causing infection in humans (ECDC/EFSA/EMA, 2017). In pigs, resistance to colistin was also found in *Salmonella* and *E. coli* isolated in UK (Anjum *et al*., 2016). Quinolone resistant bacteria, spread through the ingestion of contaminated food, have been shown to have an impact on the management of human infections (Fabrega *et al*., 2008). As reported in the last ECDC/EFSA/EMA document, the proportions of *Salmonella* isolates in human salmonellosis resistant to ciprofloxacin (quinolone) and cefotaxime (third generation cephalosporin) was overall relatively low (8.8% resistant to ciprofloxacin and 1.1% to cefotaxime). However, the report highlighted that ciprofloxacin resistance more than doubled compared to 2013 (when it was 3.8%). In the present study, resistance to quinolones was recorded for ciprofloxacin (CIP) and nalixidic acid (NAL), also with high values of MIC. Considering third generation cephalosporins, the last ECDC/EFSA/EMA report showed that Italy and Netherlands referred a percentage of resistance for *Salmonella* higher than all other EU countries for 2016. However, in the present study ceftazidime was still effective. Acquired resistance to cephalosporins and other life-saving antibiotic agents are cause of concern due to their elective use for the treatment of children and immunocompromised subjects (ECDC/EFSA/EMA 2017).

None of the isolates was ESBL producers and this was confirmed also by the absence of specific genes tested by Real-time PCR. Diffusion of ESBL-bacteria is widespread in Europe and throughout the world and continuous monitoring is still essential, even if our data are reassuring.

In order to evaluate the potential factors that may contribute to the pathogenicity of *S.* Typhimurium and the monophasic variant 1,4,5,12:i:-, 11 virulence genes were investigated. As mentioned before most of the virulence related genes are located on plasmids (pSTV), such as *spv* operon, or on chromosomal pathogenicity islands (SPIs). Genes such as *inv*A, *sip*A, *sop*B and *sop*E are involved in epithelial cell adhesion, phagocytes and non-phagocytes cell-invasion and are encoded by the SPI-1. SPI-2 encodes for genes such as *ssa*R and *ssr*A that favour the survival and replication within macrophages and subsequent systemic spread (Zhang *et al*., 2003; Yoon *et al*., 2009).

In this study, all the isolates were positive for at least five virulence-related-genes (*inv*A, *hil*A, *stn, ssr*A, *sip*C). The same phenomenon has been highlighted in *S.* Typhimurium isolated in swine, where all the strains analysed were positive for *sip*A, *sip*D, flgK, flgL,fljB, *inv*A, *sop*B and *sop*E2 genes (Almeida *et al*., 2016).

*Inv*A is a putative inner membrane component of SPI-1, essential for entry into epithelial cells, while *hilA* is a transcriptional activator essential for the regulation of the invasion process *(Galin et al*., 1991). All the *Salmonella* tested were positive for both *inv*A and *hil*A genes, highlighting the virulence potential of the strains in relation to their ability to be invasive after the attachment to the intestinal epithelium.

Production of another virulence factor like *Salmonella* enterotoxin is controlled by the expression of a series of genes such as *stn*. The presence of the gene is not related to a specific serotype (Moore and Feist, 2007) and it was demonstrated that *stn* is also involved in the maintenance of bacterial membrane composition and integrity. *Stn* was detected in all the isolates; the same data was found also in *Salmonella* of different serotypes isolated from both human and animal samples (Murugkar *et al*., 2003).

*Ssr*A/B is a regulatory system that induces the expression of SPI-2 genes. *Ssr*A, together with *ssr*B are important virulence factors involved in systemic salmonellosis after intestinal colonization (Boyen *et al*., 2008). The SPI-2 associated proteins as *ssa*R, in fact, promote the intracellular replication and systemic spread of the infection in mice. In the present study, all the isolates harbored the two genes (*Ssr*A *and ssa*R) and almost the same percentage (90%) was reported by Hur and colleagues (2011) on *S.* Typhimurium isolated from piglets with diarrhea.

*Sip*C is a gene involved in the process of cell adhesion, in the cell invasion process and it also induces a proinflammatory response (Lara-tejero and Galan, 2009). Here, all the isolates were positive for *sip*C, highlighting one time again the virulence potential of these strains.

The virulence factor *sop*B is essential for *Salmonella* enteropathogenicity, causing an acute inflammatory cell influx, intestinal fluid secretion and enteritis that correlate with clinical diarrhea (Zhang *et al*., 2002). It has been demonstrated that strains carrying mutation of *sop*B and *sop*E2 are not able to invade epithelial cell lines (Mirold *et al*., 2001). *Sop*B was identified in all *S.* Typhimurium and in the monophasic variant samples isolated in this study, except one. Similar data were carried out also in *S.* Typhimurium isolated from piglets with diarrhea in Korea, where 96% harbored *sop*B (Hur *et al*., 2011).

*Spv*C is associated with the systemic spread of the infection, favoring the replication in sites as spleen and liver (Foley *et al*., 2008). In particular, the enzymatic activity of this marker reduces the expression of proinflammatory TH1 cytokines (IFN-γ and IL-12) and neutrophil infiltration in the first stages of infection, leading immune response trends to a TH2 shift (Guiney and Feier, 2011; Wu *et al*., 2017). The presence of the *spv*C marker in all the isolates except one, makes these potentially highly virulent because the *spv*B/*spv*C genes can help the bacteria to evade host immune defenses, leading to the aggravation of infection. In *S.* Typhimurium isolated in Spanish broilers, 73.92% were positive for both *spv*B and *spv*C (Lamas *et al*., 2016) while in piglets with acute salmonellosis *spv*C was detected only in 14% of the isolates (Hur *et al*., 2011). The isolates identified here came from animals slaughtered for human consumption and the presence of *spv* operon should be concerning if we consider that some strains of *S. enterica* pathogenic for humans that carry the same factor are

associated with severe disseminated salmonellosis (Foley *et al*., 2008).

Conclusions

In the present study we highlighted the presence of *Salmonella* isolated from pigs characterized by a high virulence potential. In fact, considering the high degree of resistance towards antibiotic agents of various classes and with different mechanisms of action, together with numerous virulence genes identified by PCR, *Salmonella* strains analyzed here are potentially hazardous for human health. The concomitant presence of phenotypic resistance patterns and genes coding for proteins responsible for virulence has to be considered for future evaluation on the circulating strains of *Salmonella* due to the potential of these strains to pose a serious health risk. Here, for instance, one *S.* Typhimurium monophasic variant 1,4,5,12:i:- was positive for almost all the genetic markers coding for virulence factors and it was resistant to 12 antimicrobial agents, colistin and cefotaxime included. To conclude, we have reported the presence of strains of *Salmonella* Typhimurium and its monophasic variant 1,4,5,12:i:- in our territory characterized by a high virulence potential, mainly related to MDR pattern and to the marked presence of virulence-genes.

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