ESβL E. coli isolated in pig's chain: Genetic analysis associated to the phenotype and biofilm synthesis evaluation.

ESβL *E. coli* isolated in pig's chain: Genetic analysis associated to the phenotype and biofilm synthesis evaluation

E. Barilli[1a]

A. Vismarra[1a,*]

alice.vismarra@unipr.it

Z. Villa[1a]

P. Bonilauri[2b]

C. Bacci[1a]

[1a]Department of Veterinary Sciences, Food Safety Unit, University of Parma, Italy

[2b]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna – IZSLER - Sezione di Reggio Emilia, Italy

*Corresponding author at: Dept. of Veterinary Sciences, University of Parma, Via del Taglio 10, 43126, Parma Italy.

Abstract

Resistance to new generation cephalosporins is an important public health problem globally, in terms of economic and social costs, morbidity and mortality. Beta-lactamase enzymes are mainly responsible for the antibiotic resistance of Gram negative bacteria and extended-spectrum-β-lactamases (ESβLs) are one of the major determinants of resistance against oxymino-cephalosporins in *Enterobacteriaceae*. Food-producing animals represent one of the sources of antibiotic resistant bacteria, including pigs.

Here we analysed the presence of *E. coli* resistant to III generation cephalosporins isolated from different matrices collected from intensively bred pigs. A total of 498 *E. coli* were isolated from faeces and carcasses of pigs at slaughterhouse as well as from pork meat and sausages. Among these, 73 were phenotypically confirmed to be ESβL producers. Genetic analysis revealed that all except two harboured at least one of the three selected genes: $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV}. Furthermore, six of the *E. coli* ESβL isolated from faeces and carcasses swabs, were also able to produce biofilm, highlighting the virulence potential of these strains. The presence of Multi-Drug-Resistance patterns (MDR) recorded by the 73 ESβL *E. coli* was significant (60% of the strains were resistant to more than six antibiotics in MIC test).

Results from the present study show that the transmission of resistant bacteria is possible along the food chain, including production of pork, one the most highly consumed meats around the world. Transmission is possible through the ingestion of raw meat products, and following cross-contamination between raw and cooked foods during preparation. The potential risk for human health demonstrated here, associated with the consumption of pork contaminated with bacterial strains characterized by multidrug resistance patterns, and the ability to produce ESβL and biofilm, is cause for concern. It is imperative to study future control strategies to avoid or limit as much as possible the transmission of these highly pathogenic strains through food consumption and/or contact with the environment.

Keywords: *E. coli*; ESβL; pPigs; pPork meat; aAntibiotic resistance; bBiofilm

1.1 Introduction

Antibiotic resistance (AMR, antimicrobial resistance), the ability of various microorganisms to resist the action of an antibiotic, with consequent ineffectiveness of therapeutic treatment and persistence of the infection has become a major risk to world public health (World Health Organisation, 2014). AMR has been shown to cause not only an increase in economic and social costs, but more importantly is responsible for approximately 25,000 deaths a year in the European Union. It is estimated that the problem could exceed 700,000 cases a year (European Food Safety Authority, 2017). In human medicine, antimicrobial resistance is a widespread problem both within the community and in the hospital environment and can involve common (cystitis, urethritis, skin infections) and/or more complex infections (meningitis, pneumonia, pyelonephritis) (Crivaro et al., 2015).

In human and veterinary medicine, the problem is similar and the use of antibiotics for therapeutic purposes has contributed to the selection and spread of bacterial strains resistant to the majority of available antibiotics. The phenomenon is also complicated by the reduced availability of new antibiotics. Bacteria, under the pressure of antimicrobial use, have developed a series of resistance mechanisms. One of these is the synthesis of hydrolytic enzymes

like β-lactamases. β-lactamase enzymes are mainly responsible for antibiotic resistance in Gram negative bacteria and extended-spectrum-β-lactamases (ESβLs) are one of the major determinants of resistance against oxymino-cephalosporins in *Enterobacteriaceae* (Jacoby, 2009). As previously reported, infections due to ESβL-producing pathogens are widely associated with significant morbidity and mortality (Badal et al., 2013). These enzymes are commonly called cephalosporinases, hydrolase penicillins, I–III generation cephalosporins, cephamycins (cefoxitin) and oxyimino-cephalosporins (Jacoby, 2009).

These enzymes have been identified most often in *Escherichia coli*. *E. coli* lodges in the intestine of humans and animals, in the environment, and behaves mainly as a commensal. It can however cause various infections complicated by resistance to antibiotics, in particular infections of the urinary or genital tract (Buelow et al., 2017). The importance of *E. coli* in the spread of antibiotic resistance, in particular mediated by ~~E~~β-lactamases, is related to its diffusion in the environment and to the ability to exchange mobile gene carriers of resistance (plasmids and transposons) with other bacteria.

The β-lactamase encoding genes can be intrinsic or acquired, i.e. by transfer between bacteria. Currently, the most widespread ESβL in Europe is the enzyme CTX-M, followed by the enzymes TEM and SHV, which represent the three main families of ESβL. The CTX-M enzymes, encoded by $bla_{\text{CTX-M}}$, were first identified in 1989 and were termed ~~1~~"cefotaximase-Munich~~1~~" due to their high efficiency in hydrolysing cefotaxime, compared to ceftazidime, as opposed to the TEM and SHV enzymes (Mathers et al., 2015; Tal et al., 2015). Particularly, the genes of the bla_{SHV} family are for the most part producers of ESβL active against third generation cephalosporins and also monobactam and carbapenem (Liakopoulos et al., 2016).

The expression of ESβL is generally low in many *Enterobacteriaceae*, but inducible when exposed to β-lactam. Many studies have reported a strong and enhanced production of β-lactamases by mutation induced by treatment with III and IV generation cephalosporins. In many bacteria, ESβL enzymes are inducible and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins.

Administration of the same or similar antibiotics to both animals and humans will select the same resistance genes, significantly reducing the lifespan of an antibiotic (van Breda et al., 2017). Animals in this way can act as reservoirs of resistance genes and these can be transmitted directly or indirectly (through food, water) to human pathogens (Davies and Davies, 2010; Marshall and Levy, 2011).

The persistence and spread of antibiotic resistance by *E. coli* is facilitated not only by its diffusion, but also by its considerable resistance in the environment and the ability of numerous strains to form complex communities of bacteria, even of different species, defined biofilms. The presence of biofilms on numerous surfaces concerns the external environment (soil, water systems), the hospital environment and the food chain, where the use of antibiotics, sanitizing and disinfection treatments have favoured the selection of highly resistant microorganisms.

The objectives of the present study included the phenotypic and genetic evaluation of ESβL *E. coli* from the analysis of faeces, carcasses and food from intensively bred pigs. The selected geographical area where we collected the samples is characterized by the highest density of pig farms, slaughterhouses and typical pork meat products (DOP, IGP) in Italy. Moreover, all the confirmed ESβL were subjected to minimal inhibitory concentration (MIC) panels for the evaluation of antibiotic susceptibility. Lastly, the application of a protocol to define their ability to synthesize biofilm was used to further evaluate the virulence patterns of these strains.

~~2.~~2 Materials and methods

~~2.1.~~2.1 Sample collection

From February 2016 to July 2017, 846 samples from intensive pigs were collected. A total of 200 faeces swabs and 200 carcass sponges were collected from animals coming from five farms located in different provinces of Emilia-Romagna and Lombardy regions (Reggio Emilia, Modena, Parma, Mantova and Cremona). We sampled faeces from different animal groups at the lairage and we follow the same during slaughtering. In this way we randomly collected samples from the same consignment of animals, otherwise a specific relation animal-carcass do not exist. Moreover, 446 pork meat products for human consumption bought at supermarket located in the same geographic zone were part of the sample set.

Faeces samples were harvested in the lairage of the slaughterhouses using sterile swabs and were conserved at 8 °C +/- 2 °C until arrival at the Food Inspection Laboratory of Parma University. Carcass sampling was carried following the UNI EN ISO 17604:2015 method, as described by Commission Regulation EC 2073/2005 (Anonymous, n.d.). Carcasses were selected and sampled with sterile sponges (Sanisponge, VWR chemicals, USA) after slaughter and before the cooling process. Each sponge was placed into a sterile bag and 25 mL of the pre-enrichment broth (Buffer peptone Water, BPW) were added before transport at 8 °C +/- 2 °C to the lab. Pork meat samples (sausages, meat slices, loin, salami dough, cotechino, thighs for ham production) were collected in collaboration with the "Istituto Zooprofilattico Sperimentale della Lombardia e dell~~'~~Emilia Romagna".

~~2.2.~~2.2 *Escherichia coli* isolation

To each faecal swab, 9 mL of Tryptic Soy Broth (TSB, Biolife, Italia) were added and then incubated at 37 °C for 4 h. Ten microliters of the culture broth were seeded onto MacConkey Agar plates (Please, remove "MacConkey Agar plates" and replace with Trypton Bile X-Gluc Agar Plates (TBX Agar, Biolife, Italia).) (Biolife, Italia) and incubated for 18 +/- 2 h at 37 °C (please, replace this sentence with this new one: 24 h at 42°C).

Two hundred-twenty-five mL of BPW were added to each sterile bag containing carcass sponges and incubated overnight at 37 °C. Ten microliters of the culture broth were seeded on Tryptone Bile X-Gluc Agar plates (TBX Agar, Biolife, Italia), according to UNI EN ISO 16649-2:2001, and incubated at 42 °C for 24 h.

E. coli isolation from food samples was done by the Istituto Zooprofilattico Sperimentale of Lombardy and Emilia-Romagna regions following the UNI EN ISO 16649-2:2001.

For the three types of samples, from each plate, a single typical colony, characterized by a blue-green colour, was selected and seeded onto Triptic Soy Agar (TSA, Biolife, Italia). After 24 h of incubation at 37 °C the definitive biochemical identification to the genus level was performed by using the API20E® microsubstrate system (bioMérieux, France).

Positive control *E. coli* ATCC 25922 was used in all the identification phases.

~~2.3.~~2.3 ESβL *E. coli* phenotypic evaluation

All *E. coli* isolates were tested for III generation cephalosporin susceptibility through disc diffusion test as defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2015). Cefotaxime (5 µg) and ceftazidime (10 µg) were the antibiotic agents selected. Briefly, confirmed colonies of *E. coli* seeded on TSA plate were used to prepare the inoculum to reach the value of 0.5 McFarland (1.5×10^8 cells/mL), as described by EUCAST (Matuschek et al., 2014). The culture-broth was then seeded uniformly on Mueller Hinton Agar using a sterile swab, antimicrobial discs were added on the plates, and incubated at 37 °C for 18 ± 2 h. To define the resistance (R) or susceptibility (S), we used the zone diameter breakpoints proposed by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2016): cut-offs for cefotaxime (5 µg) were *R* <17 mm and S ≥20 mm, while for ceftazidime (10 µg) were *R* <19 mm and S ≥22 mm.

The isolates with resistance to both cephalosporins tested were phenotypically confirmed with the combination disk test (CDT), as described in European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2015). The CDT was done applying a disc diffusion test using a series of antibiotic agents, including cefotaxime 30 µg, ceftazidime 30 µg, cefotaxime 30 µg with clavulanate 10 µg and ceftazidime 30 µg with clavulanate 10 µg. A MIC was performed on all the strains identified as probable producers of ESßL for the evaluation of antibiotic susceptibility, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2016) panel.

~~2.4.~~2.4 ESβL *E. coli* genetic evaluation

DNA extraction was performed on confirmed isolated colonies of *E. coli*. One to three colonies seeded 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 *E. coli* were tested for the presence of $bla_{\text{CTX-M}}$, 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} and *E. coli* NTCT 13353 for $bla_{\text{CTX-M}}$) and negative controls were added and the presence of a specific (here we intended non specific products, please change specific in non-specific. Thank you) 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.

~~2.5.~~2.5 Biofilm synthesis

For examining the formation of bacterial biofilm, the protocol described by Christensen et al. (1985) was applied using a 96-well plate. After biochemical confirmation, a single *E. coli* colony was chosen and put in TSB+ 1% glucose. 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 1~~x~~x 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. To confirm or not the presence of biofilm synthesis, the plates were read at 620 nm with a spectrophotometer. The cut-off value (ODc) was defined as five standard deviations (SD) above the mean OD of the negative control. 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 the positive control (*Pseudomonas aeruginosa* PAO1).

~~3.~~3 Results

~~3.1.~~3.1 ESβL *E. coli* isolated from faecal swabs

All the rectal swabs were positive for the presence of *E. coli*. Fifty-six were resistant to III generation cephalosporins, in particular 31 (15.5%) showed resistance only to cefotaxime 5 µg, six strains (3%) only to ceftazidime 10 µg

and 19 (9.5%) to both antibiotics. The combination disk test was applied to the 56 positive strains and 44 out of 200 were phenotypically confirmed as ESβL.

The percentage of strains resistant to the antibiotics tested in MIC are reported in ~~table~~Table 1.

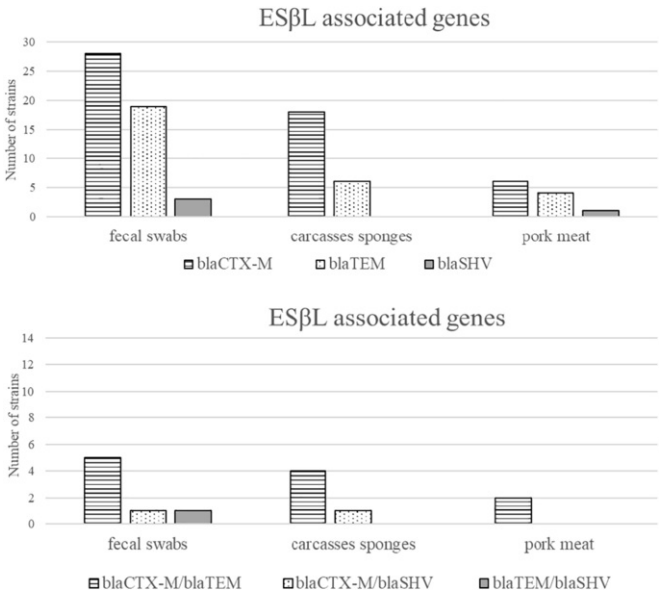
Table 1 ESβL *E. coli* tested in MIC. Percentage of resistance (R, resistance; S, susceptibility) strains are reported for each antibiotic agents tested. The antibiotics used were: meropenem (**MERO**), amikacin (**AMI**), gentamicin (**GEN**), azithromycin (**AZT**), ciprofloxacin (**CIP**), piperacillin/tazobactam (**P/T4**), amoxicillin/clavulanic acid (**AUGC**); ceftolozane/tazobactam (**C/T4**); colistin (**COL**); tigecycline (**TGC**); sulphamethoxazole/trimethoprim (**SXT**); tobramicin (**TOB**); ceftazidime/tazobactam (**CZA)**; imipenem (**IMI**); ertapenem (**ETP**).

alt-text: Table 1

Antibiotic agents (MIC breakpoint mg/L)	Faecal strains (tot. 44)	Carcasses strains (tot. 20)	Food strains (tot. 9)
MERO S ≤ 0.2–R > 8	2.3%	10%	22.2%
AMI S ≤ 8–*R* > 16	45.5%	60%	44.4%
GEN S ≤ 2–R > 4	56.8%	75%	66.6%
AZT S ≤ 1–R > 4	90.9%	90%	77.7%
CIP S ≤ 0.5–R > 1	77.2%	85%	77.7%
P/T4 S ≤ 8–R > 16	25%	20%	22.2%
AUGC S ≤ 8–R > 8	34%	30%	11.1%
C/T4 S ≤ 1–R > 1	43.1%	75%	33.3%
COL S ≤ 2–*R* > 2	36.3%	65%	44.4%
TGC S ≤ 1–R > 2	47.7%	60%	55.5%
STX S ≤ 2–R > 4	90.9%	80%	77.7%
TOB S ≤ 2–*R* > 4	31.8%	40%	33.3%
CZA S ≤ 8–R > 8	31.8%	20%	22.2%
IMI S ≤ 2–*R* > 8	2.3%	5%	0%
ETP S ≤ 0.5–R > 1	9%	5%	11.1%

Looking at the genomic profile, 28 strains out of 44 (63.6%) were positive for $bla_{\text{CTX-M}}$ gene with Cq values ranging from 22.05 to 27.49. Bla_{TEM} was highlighted in 19 ESβL *E. coli* (43.2%), with recorded Cq values ranging between 21.53 and 28.36, while only three strains showed the presence of bla_{SHV} target (6.8%) (Cq values from 25.41 to 27.45). In five strains $bla_{\text{CTX-M}}$ and bla_{TEM} were both present. The simultaneous presence of $bla_{\text{CTX-M}}$ and bla_{SHV} was

highlighted in a single sample, as well as the combination of bla_{TEM} and bla_{SHV}. None of *E. coli* isolated here was positive for all the three genes analysed and more than that two strains were negative for the three genes specific for ESβL (see Figs. 1 and 2).



Figs. 1 and 2 ESßL genes identified by Real-time PCR in strains of *E. coli* phenotypically confirmed.

alt-text: Figs. 1 and 2

Biofilm synthesis by ESβL strains was observed in five isolates. Three strains showed a weak ability in biofilm formation (OD: 0.082–0.089–0.090), one was a moderate biofilm synthesizer (OD: 0.153) and one was a strong biofilm producer (OD: 0.302). In ~~table~~Table 2 are reported the ESβL genes harboured by biofilm producer strains.

Table 2 ESBL genes harboured by *E. coli* strains positive for biofilms. FS: faecal sample; CS: carcass sponge.

alt-text: Table 2

Sample	Province	Biofilm production	ESβL genes		
			bla_{TEM}	bla_{SHV}	bla_{CTX-M}
FS 3	Mantova	Weak	+	-	-
FS 17	Mantova	Weak	-	-	+
FS 54	Mantova	Moderate	+	-	+
FS 55	Mantova	Weak	-	-	+
FS 63	Cremona	Strong	-	-	+
CS 160	Mantova	Weak	+	-	+

~~3.2.~~3.2 ESβL *E. coli* isolated from carcasses sponges

A total of 200 *E. coli* were isolated from the carcass sponges. Resistance to cefotaxime 5 µg was recorded for 22 isolates (11%), while 12 (6%) strains were resistant both to ceftazidime 10 µg and to cefotaxime 5 µg. A total of 34

samples were subjected to the combination disk test and 20, out of the 200 tested, were phenotypically confirmed to be ESβL.

The percentage of strains resistant to the antibiotics tested in MIC are reported in ~~table~~Table 1. Here, a single strain was resistant to all the antibiotics tested.

Real-time PCR highlighted that 18 ESβL isolates harboured the *bla*$_{CTX-M}$ gene with Cq values ranging from 21.34 to 27.89. Seven strains were positive for *bla*$_{TEM}$ target and in four isolates were both present the genes *bla*$_{CTX-M}$ and *bla*$_{TEM}$ as reported in Fig~~figure~~ 1. The simultaneous presence of *bla*$_{SHV}$ and *bla*$_{CTX-M}$ was recorded only in one strain.

For biofilm formation, only one isolate resulted to be a weak producer with a recorded OD of 0.093, and this strain showed the presence of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes, as shown in ~~table~~Table 2.

~~3.~~3.3 ESβL *E. coli* isolated from food samples

From 446 pork meat samples analysed, 98 *E. coli* were isolated, with forty-eight from pork meat products and 50 from pork sausages. Resistance against cephalosporins was recorded in 12 strains for cefotaxime 5 µg and in ten strains for ceftazidime 10 µg. Resistance to both molecules was found in six isolates (6/98). CDT confirmed the presence of nine ESβL, five from pork meat and four isolated from pork sausages.

ESβL strains were tested also for the MIC evaluation according to EUCAST and the percentage of strains resistant to different antibiotic agents are reported in ~~table~~Table 1.

Bla$_{CTX-M}$ gene was present in six strains with recorded Cq values ranging between 22.27 and 25.75, four were positive for *bla*$_{TEM}$ marker (Cq values 20–22.35) and only one highlighted the presence of *bla*$_{SHV}$. *Bla*$_{CTX-M}$ and *bla*$_{TEM}$ were simultaneously identified in two isolates (see Fig~~figure~~ 1).

None of the ESβL strains isolated from pork products was biofilm producer.

~~4.~~4 Discussion

Antibiotic resistance is an increasing and evolving phenomenon. Among food-producing animals, pigs, cattle and poultry are the main animal species involved (Carattoli, 2008; ECDC/EFSA/EMA, 2017). In intensive pigs farming, antibiotics are used for long periods either for treatment or prevention of infections or as performance enhancers. This widespread use has favoured the selection of the resistant strains of several important bacterial pathogens. In pigs, cephalosporins are used to treat respiratory disease in particular, and this has induced the selection and development of bacteria resistant to molecules such as cefotaxime or ceftazidime (III generation cephalosporins) (Jorgensen et al., 2007). The most recent report published by EFSA stated that 31.9% of *E. coli* isolated from pigs in Europe were resistant to cephalosporins (through the activation of β–lactamases), with a prevalence of 40% in Italy (European Food Safety Authority, 2017). In the only one study, published until today, on this topic and conducted in Emilia-Romagna region, the authors showed that ESβL profiles were present in 36% (20/56) of the isolated strains (from rectal swabs collected from intensive pigs), and 75% of these were represented by *E. coli* (Stefani et al., 2014). In our study, among the 200 *E. coli* strains isolated from faeces of pigs bred in intensive farms, 22% were ESβL producers and the analysis of carcasses (please, replace carcasses with carcass) sponges taken from slaughterhouses revealed the presence of *E. coli* that are resistant to new generation cephalosporins. In particular, 34 strains resulted to be resistant to cefotaxime (17%) and ceftazidime (6%), even if only 20 were confirmed by CDT (10%).

The transmission of resistant strains related with the ingestion of contaminated foods consumed raw or undercooked has been highlighted in several studies (Chen et al., 2017; Randall et al., 2017). The transmission of ESβL bacteria through food is also due to cross-contamination among different kind of foods, for example contamination of vegetables by meat during preparation, and in this perspective the risk for the consumer increases exponentially. Poultry meat, for instance, represents a high risk for the consumer in terms of isolation percentage of pathogens resistant to several antibiotic agents (Kawamura et al., 2014; Ojer-Usoz et al., 2013). In Denmark, a monitoring program on poultry, pork and beef showed a prevalence of isolation of ESβL *E. coli* producers in 83.8%, 12.5% and 3.7%, respectively (Carmo et al., 2014). Looking at pork meat, the most recent EFSA document on zoonoses reported a mean value of 7% of isolation for ESβL *E. coli* in Europe, with the highest percentages recorded in Portugal (21.6%) and Bulgaria (20.8%). In Italy, a total of 279 *E. coli* have been isolated from pork meat and 22 were ESβLs (7.9%) (ECDC/EFSA/EMA, 2017). In Germany, a recent work conducted at processing level showed that 27 batches of pork meat out of 63 analysed were positive for ESβL isolates. From these samples a total of 36 ESβL *Enterobacteriaceae* were isolated, of which 50% were *E. coli*, characterized by multidrug resistance profiles (Schill et al., 2017). In the present study, 98 *E. coli* were isolated from 446 food samples, of which 22 were resistant to cephalosporin agents with an increased resistance to cefotaxime. However, only nine were confirmed as β-lactamases producers with CDT (9.2%). The increasing resistance to cefotaxime in *E. coli* has been reported by several authors. In Belgium, for example, a recent study reported a percentage of *E. coli* resistant to cefotaxime of 70% isolated from food producing-animal (Lambrecht et al., 2017).

Along with prevalence evaluation, the present study included the analysis of genes related to the ESβL phenotype. Looking at the genomic profile a series of genes related to ESβL producing bacteria have been described. *Bla*$_{CTX-M}$, for instance, encodes for enzymes involved in the hydrolysis of cefotaxime, while *bla*$_{TEM}$ and *bla*$_{SHV}$ are responsible for the hydrolysis of ceftazidime. Among these, the most common gene in Europe and Italy is *bla*$_{CTX-M}$ (Carattoli, 2008; Fischer et al., 2014). The monitoring programme conducted in Italy showed that *bla*$_{CTX-M}$ was present in all the ESβL *E. coli* isolated from pork meat. The same report highlighted that of the 195 *E. coli* able to synthesize β-lactamase enzymes isolated from intensive pigs, 189 harboured the *bla*$_{CTX-M}$ gene, while only six were positive for *bla*$_{SHV}$ target (ECDC/EFSA/EMA, 2017). Our study confirmed all the data reported by ECDC/EFSA/EMA. In

fact, the incidence of *bla*$_{CTX-M}$ was higher than the other two genomic targets analysed in all the three types of samples considered. Of the 73 confirmed *E. coli* ESβL, 52 strains harboured *bla*$_{CTX-M,}$ 30 were positive for *bla*$_{TEM}$ and four for *bla*$_{SHV.}$ Zhao et al. (2015) reported similar data in a study conducted on a total of 120 ESβL-producing UPEC strains isolated from long-term hospitalized patients. On the other hand, a recent work published by Australian authors referred a percentage of *bla*$_{CTX-M}$ gene in *E. coli* isolated from piglets of 3.4% (Van Van Breda et al., 2017). In our study, the spread of ESBL-associated genes should be evident only in group 1 (Cremona), where gene *bla*$_{CTX-M}$ was highlighted in 11 ESBL *E. coli* isolated from faeces and in 12 isolated from carcass sponges. It is well known that in *E. coli bla*$_{CTX-M}$ lactamase is mainly located on plasmids; in this way the transfer among bacteria is facilitated and this could explain its wide diffusion. In our research, two ESBL *Escherichia coli* isolated from faeces resulted to be negative for all the three markers, and here we can assume the presence of different β-lactamases encoding genes, as also reported by Polsfuss et al. (2012).

The spreading of resistance genes in the environment is also favoured by the ability to form biofilm on surfaces. The presence of biofilm allows bacteria to exchange genetic elements with each other, including genes associated with antibiotic resistance (Maheshwari et al., 2016; Vogeleer et al., 2014). In the present study, six strains were able to synthesize biofilm. Two in particular were moderate and strong biofilm producers, and were both isolated from faecal swabs. This is consistent with the presence of enteroaggregative *E. coli* strains within the intestine that are able to synthesize complex structures on the mucosa, favouring intestinal colonization (García-Heredia et al., 2016; Vijay et al., 2015). *E. coli* strains that are able to synthesize biofilm, together with other bacteria such as *Klebsiella* spp. and *P. aeruginosa*, are responsible for chronic urinary infections, pyelonephritis and septicaemia (Park et al., 2014; Tofte et al., 2017).

Biofilm formation is closely related to resistance of *E. coli* towards antimicrobial drugs and to increased chronicity of urinary tract infection. In a recent study it was demonstrated that over 60% of *E. coli* isolated from the urinary tract of hospitalized patients were able to make biofilm and that strong biofilm producers were less susceptible to antimicrobial agents than the non-biofilm producers (Tajbakhsh et al., 2016). In the present study, all biofilm producers were MDR and one, in particular, was resistant to all the antibiotic agents tested by MIC. This underlines how biofilm synthesis is an important virulence factor, including its ability to favour the transmission of plasmids and genetic elements harbouring antibiotic resistance genes.

β-lactamases are encoded by genes located either on plasmids, which can be transferred between bacteria, or on chromosomes. These plasmids may also harbour multiple, additional co-resistance genes, including genes for resistance to sulfamethoxazole/trimethoprim, aminoglycosides, and fluoroquinolones, as well as genes for other β-lactamases, making these infections challenging to treat (Hsu and Tamma, 2014). The MIC panel applied to ESβL *E. coli* showed high resistance percentages towards antibiotic agents commonly used in pigs, in particular, to sulfamethoxazole/trimethoprim (77.7–90%) and to gentamicin (56.8–75%). EFSA has reported similar data for sulfamethoxazole (72%), while for gentamicin, only a 4% prevalence of resistance has been reported for *E. coli* isolated from pigs' faeces in the EU (European Food Safety Authority, 2017). A recent study conducted in Australia on weaned piglets reported a 44.9% of resistance for sulfamethoxazole/trimethoprim and an estimated 7.4% for gentamicin (van Van Breda et al., 2017). Several studies demonstrated that the use of these kind of molecules could induce the production of resistant clones with percentages ranging from 69% to 100% (Gibbons et al., 2016; Mazurek et al., 2015). Gentamicin is also widely used in human medicine, and results from our study confirm once again the importance of pigs and food of animal origin as possible reservoir in transmitting resistance to humans via food consumption.

In relation to carbapenems, a low resistance level was recorded for *E. coli* isolated from pigs and pork in our study (meropenem: 2.3–22.2%; imipenem: 0–5%; ertapenem: 5–11.1%). Currently the use of this antibiotic class is forbidden in farm animals and resistance towards them should be due to the circulation of genes coding for carbapenemase. A recent study reported, for the first time, the presence of the *oxa*-181 gene in swine (Pulss et al., 2017), but reports of *E. coli* isolated from animals with marked resistance to carbapenem agents are generally rare. Ciprofloxacin and colistin, two molecules widely used as preventives on pigs' farms, and as life-saving drugs in human medicine, were shown to possess a high percentage of resistance in this study. Resistance to ciprofloxacin, above all, was detected in 77.2% of faecal isolates, in 85% of the carcasses isolates, and 77.7% in strains isolated from food. Resistance to colistin ranged from 36.3 to 65%. EFSA reported for Italy extremely lower percentages in pigs for both the molecules, 15% for ciprofloxacin and 0.6% for colistin (European Food Safety Authority, 2017). The spread of resistance against colistin, mediated by the *mcr*-1 plasmid-gene, has also been reported in China (Liu et al., 2016) and is currently of serious concern. The inclusion of this genetic marker in a surveillance programme is increasingly important, as is constant and continuous monitoring of antibiotic resistance at all levels, "from farm to fork".

In conclusion, we have reported new data on the presence of ESβL *E. coli* in pigs and pork meat. In particular, the overall prevalence was of 14.6%, with a recorded 22%, 10% and 9.2% of isolation for faeces, carcasses and pork meat, respectively. All but two strains harboured genes associated to lactamases with a predominance of *bla*$_{CTX-M}$. Six ESβL *E. coli* isolated from faeces and carcasses were also biofilm producers, highlighting the virulence potential of these strains. Furthermore, all the ESβL strains showed in MIC marked multidrug resistance profiles. In particular, resistance to sulfamethoxazole/trimethoprim and to gentamicin was high and also for ciprofloxacin and colistin the recorded resistance prevalence was important. Results suggest that transmission of multidrug ESβL *E. coli* along the food chain is possible. The potential risk for human health associated to the consumption of pork meat contaminated with strains characterized by multidrug resistance patterns, ESβL producing skill and biofilm synthesis ability should be a cause of concern.

Conflict of interests

The authors declare no potential conflict of interests.

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Highlights

- *E. coli* ESßL producers were isolated from faeces, carcasses and meat of intensively farmed pigs.
- Multi-drug resistance pattern was highlighted in ESßL strains.
- All but two harboured at least one gene related to ESßL
- Eight percent of the isolated *E. coli* ESßL make biofilm.

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