

# EXTENDED-SPECTRUM B-LACTAMASE AND PLASMID-MEDIATED AMPC GENES IN SWINE AND GROUND PORK

CARLA SABIA<sup>1</sup>, SARA STEFANI, PATRIZIA MESSI, SIMONA DE NIEDERHÄUSERN, MORENO BONDI, CARLA CONDÒ, RAMONA ISEPPI and IMMACOLATA ANACARSO

Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

<sup>1</sup>Corresponding author.  
TEL.: +39-059-2055748;  
Fax. +39-059-2055483;  
EMAIL: carla.sabia@unimore.it

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## ABSTRACT

We investigated the presence of ESBL and AmpC-producing *Enterobacteriaceae* isolated from 200 rectal swabs of healthy swine and 200 samples of ground pork. Phenotypic testing by using the double synergy differential test (DSDT) for ESBL/AmpC-positive strains was confirmed by PCR and DNA sequence analysis. The localization of beta-lactamase genes was established by conjugation experiments. ESBL and/or AmpC-producing *Enterobacteriaceae* was found in 52.2% (95/182) of the isolates collected from rectal swabs and 3% (3/100) of isolates obtained from ground pork samples. Polymerase chain reaction and sequencing confirmed the presence of *bla*<sub>TEM-20</sub>, *bla*<sub>TEM-34</sub>, *bla*<sub>TEM-52</sub>, *bla*<sub>CTX-M-1</sub>, *bla*<sub>SHV-12</sub>, *bla*<sub>TEM-1+SHV-12</sub>, *bla*<sub>TEM-20+SHV-12</sub>, *bla*<sub>CMY-2</sub>, *bla*<sub>TEM-1+CMY-2</sub>, *bla*<sub>ACC-1</sub> and *bla*<sub>ACC-2</sub>. The conjugation assays yielded positive results, denoting a plasmid localization of the genes.

## PRACTICAL APPLICATIONS

In this study, the prevalence of ESBL and AmpC-producing *Enterobacteriaceae* isolated from rectal swabs of healthy swine and samples of ground pork were determined. ESBL/AmpC-positive strains were confirmed by PCR and DNA sequence analysis. The most frequently isolated species was *E. coli*, among the most common variants detected TEM-type ESBL, TEM-52 was showed. These findings provide new information about the presence of ESBL/AmpC at the farm level and have important implications for assessments of risks of meat contamination during slaughter.

## INTRODUCTION

Antimicrobial agents in veterinary medicine are used to treat bacterial infections, including life-threatening contagious diseases. In food-producing animals, broad-spectrum antibiotics are also used for prophylactic and metaphylactic purpose. Cephalosporins are added to feed or water to prevent and control colibacillosis infections occurring during the post-weaning period in pigs (Garcia-Alvarez *et al.* 2012). In this regard, ceftiofur, cefquinome, cefalonium, cefoperazone, cefovecin and cefuroxime are the most frequently used cephalosporins and are worldwide approved for the treatment of infections in veterinary medicine (Carattoli 2008; Trott *et al.* 2013). The massive and indiscriminate use of these antimi-

crobial agents in animal production has contributed to the selection and spreading of multidrug resistant *Enterobacteriaceae* (Ojer-Usoz *et al.* 2013; Peternel *et al.* 2014).

Use of antibiotics in food-producing animals was widely discussed and subjected to criticism by the scientific community, because contaminations of antibiotic residues in meat and milk were documented in Europe and China (Wu *et al.* 2013).

In the last decade, the spread of *Enterobacteriaceae* resistant to 3<sup>rd</sup>- and 4<sup>th</sup>-generation cephalosporins, related to the production of extended-spectrum  $\beta$ -lactamase (ESBL), plasmid-mediated AmpC and/or carbapenemase enzymes has emerged as a global problem (EFSA 2011; EFSA 2013;

Liebana *et al.* 2013). Animals and foods of animal origin are considered as potential reservoirs of multidrug-resistance Gram-negative bacteria (Carattoli 2008; Seiffert *et al.* 2013). In European Countries, the most common ESBL genes, isolated from food-producing animals and foods, are those coding CTX-M type (i.e., CTX-M-1, -2, -9, -14, -15, -32, and -55), followed by SHV-12 and TEM-52 enzymes (EFSA 2011; Liebana *et al.* 2013). Among the AmpC-type  $\beta$ -lactamases, CMY-2 is the most common while ACC-1 and DHA-1 were scarcely reported (EFSA 2011).

The production chain of swine is one of the most important and productive strength of Modena area, as demonstrated by the number of industries involved in the production and transformation. Food safety, through the quality of the product, must be the objectives for a growing competition at national and international level. The use of antibiotics in farms not only for therapeutic, but also for preventive and auxin purpose, is in large part responsible for the selection and spread of antibiotic-resistant microorganisms in the environment, which can reach humans through the food chain.

Thus, considering the adverse effects that  $\beta$ -lactam resistant microbes can have on public and animal health, this study focused on assessing the prevalence phenotypes and genotypes of ESBL and AmpC-producing *Enterobacteriaceae* isolated from rectal swabs of healthy pigs in some farms and ground pork bought in different food markets. The farms and food markets were all in province of Modena, Italy.

## MATERIAL AND METHODS

### Bacterial Strains

*Enterobacteriaceae* were isolated from 200 swine rectal swabs (from 20 farms in Modena, Italy) and 200 samples of ground pork (from 20 food markets in Modena, Italy) in the period December 2013–December 2014. All farms and food markets from which the samples were collected were randomly chosen.

Rectal samples were seeded on MacConkey agar (bioMerieux, Florence, Italy), supplemented with cefotaxime (1  $\mu$ g/mL) and incubated for 24 h at 37C.

Meat samples (25 g) were placed in sterile plastic bags with 225 mL buffered peptone water (Oxoid, Milan, Italy) and then homogenised for 2 min in Stomacher (Lab Blender, Seward, London, UK). One hundred microliters from the appropriate dilutions were inoculated on MacConkey agar (bioMerieux, Florence, Italy), supplemented with cefotaxime (1  $\mu$ g/mL) and incubated for 24 h at 37C. Up to three colonies with typical *Enterobacteriaceae* morphology from each sample were select and sub-cultured onto Agar MacConkey at 37C for 24 h. The isolates were confirmed using Vitek-2 (bioMerieux, Florence, Italy).

### Phenotypic Identification of ESBLs and AmpC

Isolates were tested for ESBL/AmpC producing using the Double Synergy Differential Test (DSDT) (Sabia *et al.* 2012) including cefotaxime (CTX, 30 $\mu$ g; Beckton, Dickinson and Company, Breda, Netherlands), cefotaxime with clavulanic acid (CTX-CLA, 30/10  $\mu$ g), ceftazidime (CAZ, 30  $\mu$ g), ceftazidime with clavulanic acid (CAZ-CLA, 30/10  $\mu$ g), cefotaxime plus boronic acid (CTX-BA) (30 $\mu$ g-10  $\mu$ L of a 60 mg/mL solution of benzo(b)thiophene-2-boronic acid in Dimethyl Sulfoxide: DMSO; Sigma, Milan, Italy) and ceftazidime plus boronic acid (CAZ-BA) (30  $\mu$ g- plus 10  $\mu$ L of a 60 mg/mL solution of benzo(b)thiophene-2-boronic acid in DMSO).

For ESBL detection, results were interpreted as recommended by the CLSI (2008). ESBL production was confirmed when the diameter of the inhibition zone around the CTX-CLA and/or the CAZ-CLA disk was 5 mm or more larger than that around the CTX and CLA disks, respectively. For AmpC  $\beta$ -lactamase detection, results were interpreted based on increased susceptibility to cefotaxime and/or ceftazidime in the presence or absence of boronate.

### Polymerase Chain Reaction and Sequencing of Extended Spectrum $\beta$ -Lactamase and AmpC Genes

Regarding the molecular tests, DNA was extracted using a standard heat lysis protocol (Pérez-Pérez and Hanso 2002). For the detection of blaTEM, blaSHV and blaCTX-M genes, the multiplex-PCR described by Kim *et al.* (2009) was used. Furthermore, primers sets described by Pérez-Pérez and Hanson (2002) to detect AmpC products were used.

PCR-positive amplicons were purified by the QIAquick PCR Purification Kit (Qiagen, Milan, Italy) and directly sequenced using amplification primers on the 3130 Genetic Analyzer (Applied Biosystems, Milan, Italy). Purification and sequencing were carried out by Genex CZ, s.r.o. Sequence alignment and analysis were performed online using the BLAST program of the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

### Conjugation Assay

Transferability of ESBL/AmpC genes from the isolates was tested by conjugation to *Escherichia coli* J53-2 (met-, pro-, rif R). The donor and recipient strains were inoculated in trypticase soy broth (TSB) and incubated at 37C overnight. After incubation, the donor and recipient were mixed (with a ratio of 1:9) and incubated at 37C for 24 h. Transconjugants were selected on MacConkey agar containing cefotaxime (2 mg/L, Sigma, Milan, Italy) plus rifampicin (100 mg/L Sigma, Milan, Italy). Conjugation frequency per recipient was expressed by division of the number of transconjugants by the initial number of recipients. Plasmid DNA extraction

**TABLE 1.** PHENOTYPE AND GENOTYPE OF ESBL/AMPC B-LACTAMASES PRODUCERS ISOLATED FROM RECTAL SWABS AND GROUND PORK

N° of isolates	Genotypes	SYN-CLA	SYN-BA
Rectal swabs (182)	ESBL (82) (45,1)	82 (100)	
	AmpC (13) (7,1)		13 (100)
Ground pork (100)	AmpC (3) (3)		3 (100)

ESBL, extended-spectrum  $\beta$ -lactamase; SYN-CLA, synergy with clavulanic acid; SYN-BOR, synergy with boronic acid.

from donors and transconjugants was performed using a plasmid midi prep kit (Qiagen, Italia S.p.A) according to manufacturer's instructions. The sizes of plasmids were estimated by electrophoresis on 0.7% agarose gels using plasmids from *Escherichia coli* V517 as the standard markers (Macrina *et al.* 1978) and the presence of beta-lactamase genes was confirmed by PCR and sequencing, as described above.

## RESULTS AND DISCUSSION

A total of 282 *Enterobacteriaceae* strains were isolated and identified by means of their biochemical properties and subsequently they were confirmed with VITEK-2 (bioMérieux, Florence, Italy).

One hundred eighty two isolates from pig rectal swabs were recovered, with a high prevalence of *E. coli* (90.7%). The other strains were identified as *Citrobacter freundii* (4.4%), *Enterobacter cloacae* (1.09%), *Hafnia alvei* (1/182, 0.5%), *Klebsiella ozaenae* (1.6%) and *Salmonella spp. subsp. arizonae* (1.6%).

One hundred isolates from meat samples were recovered and *E. coli* remains the prevalent specie (42%), with *Pantoea agglomerans* (27%) in second place. The other strains were identified as *Citrobacter freundii* (9%), *Enterobacter*

*cloacae* (8%), *Klebsiella ozaenae* (3%), *Serratia liquefaciens* (10%) and *Salmonella spp. subsp. arizonae* (1%).

All the isolated strains, were tested for both ESBL and AmpC beta-lactamase production by DSDT. About the 182 isolated from rectal swabs, eighty-two (45%) strains, showed an increase ( $\geq 5$  mm) in the inhibition zone diameter for cefotaxime and ceftazidime in the presence of amoxicillin/clavulanic acid (AMC) compared to when these antibiotics were tested alone: these isolates were classified as ESBL producers (Table 1). Thirteen isolates (7.1%) showed the enlargement of the inhibition zone in the presence of boronate and were classified as AmpC producers.

About the 100 isolated from ground meat, only three strains (3%), exhibited an increase  $\geq 5$  mm of inhibition zone diameter around CTX and/or CAZ disks for synergy with boronate, indicating AmpC production (Table 1).

Molecular analysis of ESBL and AmpC determinants was performed for all isolates which result positive by the DSDT (Table 2). A clear prevalence of TEM-type ESBL was found in *E. coli* isolated from rectal swabs. TEM-52 was the most detected ESBL enzyme (48 isolates), followed by TEM-34 (16 isolates) and TEM-20 (four isolates). Gene from other ESBL families, SHV-12, was found in combination with TEM-20 in two isolates and with TEM-1 in one isolate. In addition CTX-M-1 gene was detected in five isolates. Considering AmpC-type  $\beta$ -lactamases, CMY-2 was identified in combination with TEM-1 in four isolates and in five alone. Other species, TEM-type ESBL were found in two *K. ozaenae*, while CTX-M-1 was detected in four *C. freundii*. Finally, CMY-2 was found in combination with TEM-1 in three *C. freundii* and in one alone (Table 2).

No ESBL-producing *Enterobacteriaceae* was found in ground pork; finally AmpC-type determinants ACC-1 and ACC-2 were detected in only three ground pork isolates (*E. cloacae*, *Salmonella subsp. arizoane* and *S. liquefaciens*), respectively (Table 2).

**TABLE 2.** GENOTYPE OF THE STRAINS PRODUCING ESBL AND AMPC-TYPE B-LACTAMASES, ISOLATED FROM RECTAL SWABS AND GROUND PORK

Species	Source	Genotypes	
		ESBL <sub>s</sub>	AmpC
<i>E. coli</i>	Rectal swabs	TEM-1+SHV-12 (1)	TEM-1+CMY-2 (4)
		TEM-20 (4)	CMY-2 (5)
		TEM-20+SHV-12 (2)	
		TEM-34 (16)	
		TEM-52 (48)	
<i>C. freundii</i>	Rectal swabs	CTX-M-1 (5)	
		CTX-M-1 (4)	TEM-1+CMY-2 (3) CMY-2 (1)
<i>K. ozaenae</i>	Rectal swabs	TEM-20 (1)	
<i>E. cloacae</i>	Ground pork	TEM-34 (1)	ACC-1 (1)
<i>Salmonella subsp. arizoane</i>	Ground pork		ACC-1 (1)
<i>Serratia liquefaciens</i>	Ground pork		ACC-2 (1)

All 98 isolates were able to transfer genes encoding ESBL or AmpC by conjugation. The transfer frequency for these strains was  $1.8 \times 10^{-6}$  for recipient cell. In all the original strains the plasmids with different molecular weights were present: one large plasmid with different-sized from 50 to 55 kb and other plasmids of low molecular weight plasmids (from 4.5 to 17 kb). The transconjugants presented only the large plasmid unlike the parental strains; PCR and sequencing confirmed that the transconjugants carried the TEM-20, TEM-34, TEM-54, CTX-M-1, ACC-1, ACC-2 and CMY-2 gene. The conjugation assays yielded positive results, thus denoting a plasmidic localization of the genes (Carattoli *et al.* 2008).

Recently, ESBL/AmpC-producing organisms have been detected in food-producing animals and food of animal origin in different EU countries, this seems the proposed manner to explain the dissemination of resistance to  $\beta$ -lactams in the community (EFSA 2011; Ojer-Usoz *et al.* 2013). The most frequently identified species as ESBL/AmpC producer have been *E. coli* and non-typhoidal salmonellae and to a lesser extent, *Klebsiella pneumoniae*, *Citrobacter freundii* or *Enterobacter* spp. (EFSA 2013). The global spread of multi-resistant ESBL and AmpC-producing *Enterobacteriaceae* can be partially explain by mobility of the resistance bearing genetic elements e.g. plasmids, transposons and insertion sequence elements (Carattoli *et al.* 2008) This fact is a major concern in terms of epidemiology and infection control. Transmission pathways between humans and animals are currently a subject of active discussion (Rodriguez *et al.* 2009; Wieler *et al.* 2011).

The use of broad-spectrum veterinary cephalosporins (especially “third-generation” and “fourth-generation” cephalosporins) has been proposed as an important reason for the occurrence of these resistant bacteria among food-producing animals and meat products (Agersø *et al.* 2012; Hansen *et al.* 2013). Relevant are also measures to control dissemination, for example, by implementing increased farm biosecurity and controls on animal trade (of ESBL/AmpC carriers), by improving hygiene throughout the food chain, and by implementing other general postharvest controls for foodborne pathogens. The effectiveness of any control measures should be monitored on a regular basis by targeted surveys of food animals and foods for cephalosporin-resistant bacteria, using selective isolation methods and pre-enrichment of samples as necessary.

## CONCLUSION

This study found ESBL and/or AmpC-producing *Enterobacteriaceae* in 52.2% (95/182) isolates collected from rectal swabs and 3% (3/100) of isolates obtained from ground pork samples. Considering the swine isolates, our results showed that *E. coli* was the prevalent ESBL producer and

TEM-52 was the most common detected TEM-type ESBL. On the other hand, no ESBL-producing *Enterobacteriaceae* were found in ground pork; only AmpC-type  $\beta$ -lactamase was found in three meat samples. In Italy, few information concerning antimicrobial susceptibility in food-borne pathogens and commensal bacteria isolated from food-producing animals are available (Carattoli 2008; Stefani *et al.* 2014). Further studies are required to monitor the spread of  $\beta$ -lactams resistant bacteria in farm animals and foodstuffs, in order to improve the consumer's knowledge about exposure to these bacteria and to elucidate the mechanisms of transmission of resistant genes through the food chain.

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