# Prevalence and characterization of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in food-producing animals in Northern Italy

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SUMMARY

The aim of this study was to assess the production of extended spectrum beta-lactamases (ESBL) in 56 strains of *Enterobacteriaceae*, obtained from 100 rectal swabs of farm animals, and to evaluate the horizontal transfer capacity of the genetic determinants of resistance. The ESBL-positive strains were confirmed by phenotypic testing, confirmed by PCR and DNA sequence analysis. The localization of beta-lactamase genes was established by conjugation experiments. Of the 56 analyzed strains, 20 (36%) resulted positive for ESBL production by the double-disk synergy test, and belonged to *Escherichia coli* 15 (75%) and *Klebsiella ozaenae* 5 (25%) species. Molecular analysis showed that all ESBL-producing isolates possessed genes encoding for TEM-type enzymes and/or CTX-M. The conjugation assays yielded positive results, thus denoting a plasmidic localization of the genes. This study highlights the high percentage of ESBL-positive *Enterobacteriaceae* and the mobility of the responsible genes. Gene mobility implies highly negative consequences in terms of drug therapy because of the spread of antibiotic resistance.

*KEY WORDS: Enterobacteriaceae*, Antimicrobial resistance, ESBL, Food-producing animals.

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Antibiotic resistance is a worldwide health problem in many fields, such as human and veterinary medicine, livestock holdings, agriculture and environment (Marshall *et al*., 2011). In recent years, the spread of *Enterobacteriaceae* resistant to 3rd and 4th generation cephalosporins in farm animals was highlighted as an emerging problem. Such resistance has been demonstrated to be related to extended-spectrum β-lactamase (ESBL) production. (Li *et al*., 2007). ESBL are plasmid-encoded enzymes, capable of inactivating a large number of β-lactam antibiotics, including extended-spectrum and very-broad-spectrum cephalosporins and monobactams. The ESBL are also commonly inhibited by β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. Furthermore, resistance to broad-spectrum cephalosporins can be due to overexpression of chromosomal or plasmid-mediated AmpC enzymes, encoded by genes such as $bla_{CMY\text{-}2}$. Such enzymes also confer resistance to cephamycins and cannot be inhibited by the β-lactamase inhibitors. In humans, *Enterobacteriaceae* able to produce ESBLs and AmpC broad-spectrum β-lactamases have been studied for more than two decades (Bradford, 2001, Paterson *et al*., 2005). ESBL genes are usually transported by plasmids, facilitating so their spread among Gram-negative bacilli.

The most frequently reported ESBL subtypes in both *Salmonella* and *Escherichia coli* from food-producing animals and foods within the European Union (EU) are CTX-M-1, CTX-M-14, TEM-52 and SHV-12 (Chiaretto *et al*., 2008).

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A wide range of additional CTX-M subtypes (CTX-M-1, -2, -3, -8, -9, -14, -15, -17/18, -20, -32, -53) (EFSA, 2011), TEM (TEM-20, -52, -106, -126) and SHV variants (SHV-2, -5, -12) (Chiaretto *et al*., 2008, Endimiani *et al*., 2012) have been detected in both food-producing animals and in food, in several European countries. In Italy, the prevalence of ESBL-producing Salmonella is 0.5-0.6%, but in this study, isolates of *E. coli* ESBL-carrying were found in all the five poultry farms tested (Bortolaia *et al*., 2010). The massive and indiscriminate use of different classes of antibiotics in the veterinary context has contributed to the selection and spread of multidrug-resistant *Enterobacteriaceae*. Livestock animals are considered important reservoirs of antibiotic-resistant Gram-negative bacteria and their role on human health has drawn considerable global attention (Aiello *et al*., 2003, Seiffert *et al*., 2013). For this purpose, 100 rectal swabs of healthy food-producing animals were analyzed to detect and characterize the production of ESBL by *Enterobacteriaceae* and the ability of horizontal transfer of the genetic determinants of resistance.

From February to July 2013, 100 rectal swab samples were collected from healthy food-producing animals (50 breeding pigs and 50 dairy cattle) from 4 farms in Modena (Italy). Rectal samples were seeded on MacConkey agar (bioMérieux, Florence, Italy), with and without ceftazidime (1 μg/mL) or cefotaxime (1 μg/mL) and incubated for 24 h at 37°C. Up to 3 colonies with typical *Enterobacteriaceae* morphology from each sample were selected and subcultured onto MacConkey agar for 24 hours at 37°C. The identified isolates were confirmed using API ID 32 E (bioMérieux) and by conventional biochemical methods.

Antibiotic susceptibility was determined by the broth microdilution method on Mueller–Hinton broth (bioMerieux, Florence, Italy), according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2012. The following antimicrobials were tested: ampicillin (AM), amoxicillin/clavulanic acid (AMC), cephalothin (CF), cefuroxime (CXM), cefpodoxime (CPD), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), cefoxitin (FOX), imipenem (IMP); (all the antimicrobials were purchased from Sigma Aldrich, Milan, Italy). The European Committee on Antimicrobial Susceptibility Testing guidelines for the susceptibility categorization (EUCAST, 2013) were used and *E. coli* ATCC 25922 was employed as quality control strain. ESBL-producing isolates were screened by the phenotypic confirmatory test, using both cefotaxime and ceftazidime alone and in combination with clavulanic acid, according to CLSI recommendations.

Bacterial isolates confirmed for their capacity to produce ESBLs were further analysed by PCR. DNA was extracted using a standard heat lysis protocol (Pèrez-Pèrez *et al*., 2002). Thereafter, specific primers were used to search for $bla_{\text{TEM}}$, $bla_{\text{SHV}}$ e $bla_{\text{CTX-M}}$ (Kim *et al*., 2009).

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

Conjugal transfer of resistance determinants was performed in liquid medium using the *E. coli* J53-2 (*met*-, *pro*-, $rif^R$) as recipient. Transconjugants were selected on MacConkey agar containing cefotaxime (2 mg/L) plus rifampicin (100 mg/L). For plasmid analysis, plasmid DNA was obtained from the transconjugants using the QIAGEN plasmid Midi kit, (Qiagen, Milan, Italy) following the manufacturer's instructions. Transconjugants harboring a single plasmid, originated from the ESBL-producing isolates, were subsequently selected and investigated. *E. coli* V517 (53.7, 5.6, 5.1, 3.9, 3.0, 2.7, et 2.1 kb) was used as the standard marker for plasmid size analysis (Macrina *et al*., 1978) and the presence of beta-lactamase genes was confirmed by PCR, as described above.

A total of 56 *Enterobacteriaceae* strains were isolated and identified by means of their biochemical properties and confirmed with API ID 32 E. Among the identified strains, the isolated species were: *Escherichia coli* (n=34), *Pseudomonas aeruginosa* (n=11), *Pseudomonas fluorescens* (n=1), *Klebsiella ozaenae* (n=7), *Acinetobacter lwoffi* (n=2) and *Proteus mirabilis* (n=1). Broad-spectrum cephalosporin-resistant

enzymes were found in 25 isolates (44%) using the double-disk synergy test and the strains containing ESBL were detected in 20 (36%) of them, of which 15 *E. coli* and 5 *K. ozaenae* (Table 1).

The PCR products from the ESBL-producing *E. coli* strains were directly sequenced, analyzed, and of the 15 *E. coli*, 4 isolates were identified as $bla_{CTX\text{-}M\text{-}1}$ and 2 isolates as $bla_{CTXM\text{-}15,}$ in 2 isolates the $bla_{CTX\text{-}M\text{-}1}$ gene was found in combination with the $bla_{TEM\text{-}201}$ gene, in other 2 isolates the $bla_{TEM\text{-}1}$ gene was found in combination with the $bla_{CTX\text{-}M\text{-}1}$ and finally in 5 isolates the $bla_{TEM\text{-}52}$ gene was found.

In the 5 *K. ozaenae* strains, the $bla_{TEM\text{-}1}$ gene, was found in 3 isolates in combination with the $bla_{CTX\text{-}M\text{-}1}$, and in combination with the $bla_{CTX\text{-}M\text{-}15}$ in 1 isolate. In addition, the $bla_{TEM\text{-}24}$ gene was found, in combination with the $bla_{CTX\text{-}M\text{-}1}$ in 1 isolate only.

Seven out of 15 *E. coli* and all the 5 *K. ozaenae* isolates were able to transfer the ESBL phenotype to a recipient. The presence of the respective β-lactamase genes was confirmed in all transconjugants by PCR analysis, with primers encoding CTX-M or TEM-type ESBLs. The conjugation assays yielded positive results, thus denoting a plasmidic localization of the genes.

Recently, there has been an increase in studies in several countries describing the prevalence and characteristics of ESBL-producing *Enterobacteriaceae* in hospitalized companion animals (Sidjabat *et al*., 2007), in cattle (Bardoň *et al*., 2013, Pitout *et al*., 2012, Reist *et al*., 2013, Prescott *et al*., 2008) and pigs and chicken (Guardabassi *et al*., 2004, Hasman *et al*., 2005, Hansen *et al*., 2013).

The results shown in the present investigation emphasize the role of livestock animals in the spread of ESBL-producing *Enterobacteriaceae*, and the risk that these microorganisms can reach humans through the food chain. In fact, 36% (20/56) of the analyzed bacterial strains

TABLE 1 - *Identification, minimum inhibitory concentrations (MICs) and further characterization of the 20 ESBL producers isolated from 100 rectal swabs of livestock animals.*

| *Sample number* | *Species* | *Origin* | *Antimicrobial agent/MIC value (mg/l)* | | | | | | | | | | *ESBL test result* | *Plasmide β-lactamase genes detected* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *AM* | *AMC* | *CAZ* | *CTX* | *FEP* | *CF* | *CXM* | *CPD* | *FOX* | *IMP* | | |
| 13 | *E. coli* | Pig | >32 | >32 | 1 | 4 | 2 | 8 | >32 | 2 | 4 | <0.25 | + | $bla_{TEM\text{-}52}$ |
| 39 | *E. coli* | Pig | >32 | >32 | 2 | >64 | <4 | 4 | >32 | 4 | 32 | <0.25 | + | $bla_{TEM\text{-}52}$ |
| 40 | *E. coli* | Pig | >32 | >32 | 2 | >64 | 4 | 8 | >32 | 14 | 64 | <0.25 | + | $bla_{TEM\text{-}52}$ |
| 41 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 2 | 8 | >32 | 8 | 8 | <0.25 | + | $bla_{CTX\text{-}M\text{-}1}$ |
| 42 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 4 | 4 | >32 | >16 | 8 | <0.25 | + | $bla_{TEM\text{-}201}$ + $bla_{CTX\text{-}M\text{-}1}$ |
| 43 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 4 | 8 | >32 | >16 | 8 | <0.25 | + | $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M\text{-}1}$ |
| 44 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 8 | >16 | >32 | >16 | 32 | <0.25 | + | $bla_{CTX\text{-}M\text{-}15}$ |
| 45 | *E. coli* | Pig | >32 | >32 | 4 | >16 | >16 | 4 | >32 | >16 | 8 | 0.5 | + | $bla_{CTX\text{-}M\text{-}1}$ |
| 46 | *E. coli* | Pig | >32 | 16 | 4 | >16 | >16 | 8 | 16 | 8 | 8 | 0.5 | + | $bla_{CTX\text{-}M\text{-}1}$ |
| 47 | *E. coli* | Pig | >32 | >32 | 2 | >64 | 4 | 8 | >32 | 8 | 8 | <0.25 | + | $bla_{TEM\text{-}52}$ |
| 48 | *E. coli* | Pig | >32 | >32 | 2 | >64 | 4 | 8 | >32 | 8 | 4 | <0.25 | + | $bla_{TEM\text{-}52}$ |
| 49 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 2 | 8 | >32 | 8 | 4 | <0.25 | + | $bla_{CTX\text{-}M\text{-}1}$ |
| 51 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 16 | 8 | >32 | 4 | 4 | <0.25 | + | $bla_{TEM\text{-}201}$ + $bla_{CTX\text{-}M\text{-}1}$ |
| 54 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 2 | 8 | >32 | 4 | 8 | <0.25 | + | $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M\text{-}1}$ |
| 56 | *E. coli* | Pig | >32 | >32 | 4 | >64 | 2 | 4 | >32 | 4 | 32 | <0.25 | + | $bla_{CTX\text{-}M\text{-}15}$ |
| 6 | *K. ozaenae* | Cattle | >32 | >32 | <1 | 8 | 2 | 8 | 16 | 4 | 8 | <0.25 | + | $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M\text{-}1}$ |
| 7 | *K. ozaenae* | Cattle | >32 | >32 | <1 | 8 | 2 | 4 | 16 | 4 | 8 | <0.25 | + | $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M\text{-}1}$ |
| 8 | *K. ozaenae* | Cattle | >32 | >32 | 2 | 16 | 2 | 8 | >32 | 4 | 8 | <0.25 | + | $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M\text{-}15}$ |
| 9 | *K. ozaenae* | Cattle | >32 | >32 | 2 | 8 | 2 | 8 | >32 | 4 | 128 | <0.25 | + | $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M\text{-}1}$ |
| 12 | *K. ozaenae* | Cattle | >32 | >32 | 2 | 8 | 2 | 8 | >32 | 2 | 8 | <0.25 | + | $bla_{TEM\text{-}24}$ + $bla_{CTX\text{-}M\text{-}1}$ |

ESBL, extended-spectrum β-lactamase; MIC, minimum inhibitory concentration; AM, ampicillin; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; cephalothin CF; cefuroxime CXM; cefpodoxime CPD; FOX, cefoxitin; IMP, imipenem.

were positive for ESBL production; of these 75% were *E. coli* and 25% were *K. ozaenae*. In accordance with previous studies (Blanc *et al.*, 2006, Smet *et al.*, 2008, Endimiani *et al.*, 2012, Genser *et al.,* 2012, Rodrigues *et al.*, 2013), this molecular analysis demonstrates that the most dominant types of enzymes were CTX-M-1 and TEM-52. While CTX-M-14 is among the most prevalent beta lactamase types in companion animals and poultry in Asia (30-33%), and to a lesser extent in cattle and pigs (14%), it is less prevalent in livestock (4-7%) in Europe, and is even absent in companion animals (Ewers *et al.*, 2012). All of the TEM enzymes, co-expressed with CTX-M ESBLs, were broad-spectrum β-lactamases whereas most TEM-1 enzymes did not match with the ESBL phenotype. In contrast, 3 strains expressing TEM enzymes featured TEM-ESBLs, TEM-24 and TEM-201 (Table 1). In some countries, TEM-ESBLs are much more frequently found in animals, especially in chickens (Costa *et al.*, 2009, Smet *et al.*, 2011, Dierikx *et al.*, 2012). Since the role of farm animals in the dissemination of microorganisms endowed with antibiotic resistance characteristics is now widely recognized, our work represents a contribution to the identification, characterization and distribution of ESBL-producing *Enterobacteriaceae* in the field of veterinary microbiology. Another major issue is the difficulty in monitoring the use of antibiotics in animals and their off-label implementation for non-authorized indications. Lastly, mobile drug resistance genes capable of crossing bacterial species are likely to accelerate dissemination of drug resistance from animals to humans through the consumption of chicken, beef and pork.

Notwithstanding the consistency of other European studies, very little can be found in the literature showing a countrywide prevalence in Italy.

Additional nationwide surveys are warranted to identify the possible spread of ESBL-producing *Enterobacteriaceae*, their dynamic of transmission and the risk factors. It is important to monitor the spread of expanded-spectrum cephalosporin resistance, and further genetic studies are necessary for the control of drug-resistant bacteria in animals, humans and the environment.

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