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

Sara Stefani, Ilaria Giovanelli, Immacolata Anacarso, Carla Condò, Patrizia Messi, Simona de Niederhäusern, Moreno Bondi, Ramona Iseppi, Carla Sabia
Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

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.
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 cefotaxime (1 μg/mL) or ceftazidime (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 (bioMérieux, Florence, Italy), according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2012. The following antimicrobials were tested: ampicillin (AM), amoxicillin/clavulanic acid (AMC), cefalothin (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 Commi
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 blaCTX-M-1 and 2 isolates as blaCTXM-15, in 2 isolates the blaCTX-M-1 gene was found in combination with the blaTEM-201 gene, in other 2 isolates the blaTEM-1 gene was found in combination with the blaCTX-M-1 and finally in 5 isolates the blaTEM-52 gene was found.

In the 5 K. ozaenae strains, the blaTEM-1 gene, was found in 3 isolates in combination with the blaCTX-M-15 and in combination with the blaCTX-M-1 in 1 isolate. In addition, the blaTEM-24 gene was found, in combination with the blaCTX-M-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 (Barđoń 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.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Species</th>
<th>Origin</th>
<th>Antimicrobial agent/MIC value (mg/l)</th>
<th>ESBL test result</th>
<th>Plasmide β-lactamase genes detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-52</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-52</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-52</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-52</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-201 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-1 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalCTX-M-15</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalCTX-M-15</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalCTX-M-15</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-52</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-52</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-52</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-201 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-1 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>E. coli</td>
<td>Pig</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalCTX-M-15</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>K. ozaenae</td>
<td>Cattle</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-1 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>K. ozaenae</td>
<td>Cattle</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-1 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>K. ozaenae</td>
<td>Cattle</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-1 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>K. ozaenae</td>
<td>Cattle</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-1 + blalCTX-M-1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>K. ozaenae</td>
<td>Cattle</td>
<td>AM &gt;32 AMC &gt;32 CAZ &gt;32 CTX &gt;32 FEP &gt;32 CF &gt;32 CXM &gt;32 CPD &gt;32 FOX &gt;32 IMP</td>
<td>+ blalTEM-1 + blalCTX-M-1</td>
<td></td>
</tr>
</tbody>
</table>

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 beta-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 organisms endowed with antibiotic resistance of farm animals in the dissemination of microorganisms.

**Acknowledgments**

We thank Dr Enrico Stefani, Department of Public Health, Veterinary Service of Modena and Sassuolo, for supporting this work.

**References**


Prevalence and characterization of extended-spectrum β-lactamase-producing Enterobacteriaceae


38. Switzerland.


