Occurrence of extended spectrum β-lactamase- and AmpC-producing *Escherichia coli* in meat samples

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**Abstract**

The aim of the study was a preliminary determination of occurrence of extended spectrum β-lactamases (ESBL)- and AmpC-producing *Escherichia coli* (*E. coli*) in raw meat samples collected from slaughter-houses located in different regions of Poland. A total of 141 samples were tested, comprising 78 pork samples, 44 beef samples, and 19 chicken meat samples. Isolated and identified *E. coli* strains were examined for the ESBL and/or AmpC β-lactamases production by the use of four disc diffusion and minimum inhibitory concentration tests. All strains positive in one or both tests were examined by PCR for the presence of the *bla*<sub>CTX</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CMY-2</sub> group genes. During the study, 154 *E. coli* strains were isolated from 95 samples. Among these, 18 (11.7%) strains were identified in phenotypic tests as ESBL-producing and seven (4.5%) strains as AmpC-positive. The presence of the genes encoding selected ESBL-s (TEM, CTX, SHV) was identified in 14 of the strains recognised as ESBL-positive in phenotypic tests. All AmpC-positive isolates showed the presence of the CMY-2 group encoding genes. One of these strains had also the CTX-M and TEM genes, and four of them expressed the TEM marker.

**Key words:** extended spectrum β-lactamases, AmpC, *Escherichia coli*, meat.

**Introduction**

Despite that the aetiology of foodborne diseases includes microbial, parasitic, and toxic (chemical) agents, bacteria probably remain the most common cause of this group of illnesses (27). One of significant factors escalating the threat caused by pathogenic bacteria, also these transmitted by food, is an increasing resistance to antimicrobial agents, bacteria probably remain the most common cause of this group of illnesses (27). One of significant factors escalating the threat caused by pathogenic bacteria, also these transmitted by food, is an increasing resistance to antimicrobial agents, revealed in conjunction with implementation new generations of antimicrobials, reflecting a huge adaptable potential of microorganisms. The discovery of bacterial enzymes capable to inactivate antimicrobials allowed to identify one of the most important modes of microorganism’s counteraction against antibiotics. A relatively quick appearance of bacterial resistance after implementation of a new group of antimicrobials, and quick spread of genes encoding such resistance, located often in plasmids, constitute important features, escalating the risk of ineffective treatment.

Extended spectrum β-lactamases (ESBL), discovered in last decades of the 20<sup>th</sup> century, are a group of more than 500 bacterial enzymes able to hydrolyse penicillins, caphalosporines (I<sup>th</sup>–IV<sup>th</sup> generations), and monobactams. The majority of these enzymes are derivatives (mutants) of classical β-lactamases (mainly TEM-1, TEM-2, and SHV-1); however, the origin of some of them is not clear. The AmpC β-lactamases are clinically important cephalosporinases encoded in the chromosome (13). Plasmid-mediated AmpC β-lactamases have arisen as a result of the transfer of genes encoding the inducible AmpC β-lactamase into plasmids (26). In contrast to ESBLs, AmpC β-lactamases are generally not sensitive, or much less sensitive to β-lactamase inhibitors, like clavulanic acid, sulbactam, and tazobactam. Both ESBLs and AmpC β-lactamases are mainly present in bacteria from the *Enterobacteriaceae* family, but also in non-fermentative, Gram-negative microorganisms from the genera of *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, etc. (13, 21).
E. coli is recognised as highly capable of acquiring and transferring antimicrobial resistance genes (14, 22, 24). Bacterial plasmids are often vectors of ESBL and AmpC genes (10). A quite frequent occurrence of ESBL and/or AmpC-producing E. coli in animals, create a real threat of their transfer through food to humans. There is, however, relatively few reports concerning the prevalence of ESBL-producing E. coli in raw meat and meat products, and not much information about a possible transfer of ESBL and/or AmpC-producing E. coli from animals thorough meat to humans.

The aim of the study was a preliminary estimation of the prevalence of ESBL and/or AmpC-producing E. coli in raw meat collected from slaughter-houses located in various regions of Poland.

Material and Methods

Sampling. A total of 141 meat samples were collected, consisting of 78 pork samples, 44 beef samples, and 19 poultry (Gallus gallus) meat samples. The samples were randomly collected between September 2012 and February 2013 in abattoirs located in various regions of Poland.

Bacteriological examinations. For bacterial isolation, 25 g of tissue was taken from the sample, added to 225 mL of buffered peptone water, and homogenised in stomacher for 2 min (1). Thereafter, 1 mL of homogenate was transferred into a tube containing 9 mL of lauryl sulfate tryptose (LST) broth with inverted Durham tube and incubated at 37°C for 48 h. From the tubes with positive gas production (considered as E. coli-positive), a loopful was streaked onto MacConkey agar (MCA) and simultaneously onto MCA supplemented with 2 mg/L of cefotaxime (MCA/CTX) (29, 30). The inoculated plates were incubated at 37°C for 24 h, and the colonies showing typical E. coli morphology were inoculated onto agar with 5% of sheep blood. After 24 h of incubation at 37°C, E. coli were identified by the use of biochemical test API® Rapid ID 32 E (BioMerieux, France) according to the producer’s instruction.

Detection of ESBL-producing E. coli. The presence of ESBL and/or AmpC cephalosporinases (included recently into the ESBL group) in all identified E. coli was determined by the use of disk test D68C (Mast Diagnostica GmBH, Germany). ESBL and/or AmpC production was indicated by comparison of inhibitory zone diameters of cefpodoxime (CPD) disk, CPD + ESBL-inhibitor disk, CPD + AmpC-inhibitor disk, and CPD + ESBL-inhibitor + AmpC-inhibitor disk. These disks were put on Mueller-Hinton agar inoculated with suspension (0.5 McFarland turbidity) of the investigated E. coli and incubated at 37°C for 24 h. The test results were interpreted according to the manufacturer’s directions.

Minimum inhibitory concentration (MIC). Identified E. coli recognised in the disk test as ESBL and/or AmpC-positive were examined by the use of the confirmatory MIC test Sensititier® ESBL (Trek, USA). The test plate contained 96 wells divided into groups coated with various antimicrobial agents. Sixteen antimicrobials were tested: ampicillin, cefazolin, cefepime, cefotaxime, cefotaxime/clavulanic acid, cefoxitin, cefpodoxime, cefazidime, ceftazidime/clavulanic acid, ceftriaxone, cefalotin, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin/tazobactam. Two or three colonies of the tested strain were suspended in sterile water to obtain 0.5 McFarland turbidity. One loopful (10 µL) of the suspension was transferred into the 11 mL tube containing cation adjusted Mueller-Hinton broth with TES buffer. Subsequently, 50 µL of the broth suspension was transferred into each well of the test plate. The plate was covered with the adhesive seal and incubated under aerobic condition at 34–36°C for 18–24 h. After the incubation, the results of the test were read in AutoReader® (Trek, USA). The examined strain was considered as ESBL-positive when its MIC for antimicrobial agent tested in combination with clavulanic acid was three or more (twofold) concentrations lower than in the case when the MIC is tested for the antimicrobial agent without clavulanic acid. The strain was recognised as AmpC-positive when MIC for cefoxitin was ≥32 mg/L.

Detection of blaCTX, blaTEM, blaSHV, and blacMY2 group genes. All E. coli strains identified in the phenotypic tests as ESBL and/or AmpC-positive were investigated by PCR to detect the presence of resistance genes. Total DNA was obtained using the heat-shock technique. Two colonies of the investigated strain were suspended in 200 µL of PBS, boiled for 10 min and, after cooling, centrifuged at 10 000 rpm for 20 s. The supernatant was diluted 1:10 in deionised water and used for the PCR. Four sets of primers, described before (12, 16, 19), were used (Table 1). The amplification was carried out in a 25 µL volume containing: 12.5 µL of Maxima Master Mix (Thermo Scientific); 0.1 µL of each primer (50 nM); 11.3 µL of water, and 1 µL of template DNA (30). The PCR was initiated by 5 min of denaturation at 95°C followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at temperature appropriate for the respective primers (Table 1) for 30 s, and extension for 30 s at 72°C; the last extension step was prolonged to 10 min. The PCR products were analysed by electrophoresis in 1.5% agarose gel containing ethidium bromide.

Results

During the study, 154 isolates of E. coli were recovered from 95 (67.4%) of 141 examined samples.
The strains were detected in 46 pork, 33 beef, and in 16 poultry meat samples. Among *E. coli* isolates, 18 (11.7%) were identified in phenotypic tests as ESBL-producing and 7 (4.5%) strains as AmpC-positive. All of ESBL and/or AmpC-positive strains demonstrated growth on MCA/CTX medium.

Among the ESBL-positive *E. coli*, seven were isolated from chicken meat, seven from beef, and four from pork. In the group of AmpC-positive strains, five were isolated from poultry meat and two from pork. No AmpC-positive *E. coli* strains were isolated from beef.

The results of PCR confirmed the presence of the genes encoding selected ESBL-s (TEM, CTX, SHV) in 14 strains recognised as ESBL-positive. The mentioned genes were not found in four of phenotypically ESBL-positive strains. All strains recognised as ESBL-positive showed the presence of CMY-2 gene. Furthermore, one of AmpC-positive strains had additional genes encoding CTX-M and TEM, and in four of the mentioned strains the gene *bla* _TEM_ marker was indicated. None of the phenotypically ESBL-positive isolates had *bla* _CMY-2 group_ genes. The characteristics of all identified ESBL and/or AmpC-positive *E. coli* strains is shown in Table 2.

**Table 1. Characteristics of the primers used in PCR**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence 5’–3’</th>
<th>Annealing temp. (°C)</th>
<th>Amplicon size (bp)</th>
<th>References</th>
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<tbody>
<tr>
<td><em>bla</em> <em>CTX</em></td>
<td>ATGTGCAGYACCAGTAARGTKATGGC TGGGTARAARTARGSACAGAAYACAGCGG</td>
<td>60</td>
<td>593</td>
<td>19</td>
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<tr>
<td><em>bla</em> <em>TEM</em></td>
<td>TGGATTTCAACATTCCGTGT TTACCAATGCTTAATCAGTGA</td>
<td>53</td>
<td>861</td>
<td>16</td>
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<tr>
<td><em>bla</em> <em>SHV</em></td>
<td>CAAAACGCCGGGTTATTTC</td>
<td>53</td>
<td>937</td>
<td>16</td>
</tr>
<tr>
<td><em>bla</em> <em>CMY-2 group</em></td>
<td>GCACTTAGCCACCTATACGGCAG GCTTTTCAAGAATGCGCCAGG</td>
<td>60</td>
<td>758</td>
<td>12</td>
</tr>
</tbody>
</table>

**Table 2. Characteristics of ESBL- or AmpC-producing *E. coli* strains tested**

<table>
<thead>
<tr>
<th>No. of strain</th>
<th>Source</th>
<th>Results of disk test</th>
<th>MIC</th>
<th>Genes detected in PCR</th>
<th>Genes detected in PCR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td><em><em>bla</em> <em>CTX</em></em>*</td>
<td><em><em>bla</em> <em>TEM</em></em>*</td>
<td><em><em>bla</em> <em>SHV</em></em>*</td>
<td><em><em>bla</em> <em>CMY-2 group</em></em>*</td>
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<td>001.</td>
<td>chicken</td>
<td>ESBL</td>
<td>ESBL</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>002.</td>
<td>chicken</td>
<td>ESBL</td>
<td>ESBL</td>
<td>-</td>
<td>+</td>
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<tr>
<td>003.</td>
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<td>ESBL</td>
<td>ESBL</td>
<td>+</td>
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<tr>
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<td>chicken</td>
<td>ESBL</td>
<td>ESBL</td>
<td>+</td>
<td>-</td>
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<tr>
<td>008.</td>
<td>chicken</td>
<td>ESBL</td>
<td>ESBL</td>
<td>+</td>
<td>-</td>
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<tr>
<td>010.</td>
<td>chicken</td>
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<td>ESBL</td>
<td>+</td>
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<td>011.</td>
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<tr>
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<td>pork</td>
<td>ESBL</td>
<td>ESBL</td>
<td>+</td>
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</tr>
<tr>
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<td>pork</td>
<td>ESBL</td>
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<td>-</td>
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<tr>
<td>025.</td>
<td>pork</td>
<td>ESBL</td>
<td>ESBL</td>
<td>+</td>
<td>-</td>
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Discussion

Although in the last years there has been a dynamic increase of the studies concerning antimicrobial resistance caused by ESBL and AmpC beta-lactamases (10), relatively few reports regarding the presence of these microorganisms in raw meat have been published until now. These data concerns more often the occurrence of the mentioned microorganisms in animals at the farm (3, 4) or the slaughter-house (5, 9), and in less or more processed meat or meat products (8, 20, 25). The investigations concerning raw meat were performed most often with poultry meat (2, 6, 28), and more rarely with meat of other animal species (23, 32).

According to the data mentioned in the EFSA Scientific Opinion (10), percentages of samples from food-producing animals or food samples, in which ESBL-carrying E. coli were detected, varied from 0.2% to 40.1%, depending on the country. Occurrence in the range of 10%–40% was found when healthy poultry or pigs were analysed for the presence of ESBL-positive E. coli isolates in Portugal (17), the Netherlands (7), France (11), and slightly lower percentages were identified in the Czech Republic (15). The recent data from Turkey shows that in 13.4% of E. coli isolated from meat (poultry and beef) ESBL production was confirmed by the phenotypic tests (1).

The results of our study indicated the highest prevalence of ESBL- and AmpC-producing E. coli (36.8% and 26.3%, respectively) in poultry meat samples. This is consistent with the results presented by other investigators (2, 23, 32), and seems to be a consequence of an intensive use of antimicrobial agents in poultry farms. However, in some countries, e.g. in Sweden, where cephalosporins are not used in broilers, the occurrence of E. coli carrying CTX-M-1 and CMY-2 genes is not associated with a selection pressure caused by the use of the mentioned antimicrobials, but is explained rather as a possible consequence of import of birds carrying these strains. E. coli blaCTX-M-1-positive were found in intestinal content of a day-old chickens imported into Sweden as breeding-stock (parent animals) (10).

In the present study, two E. coli isolates from poultry meat and two from beef were ESBL-positive in phenotypic tests, but were negative for the blaCTX_M, blaTEM, and blasIV genes. The most probable reason of these results is that identified β-lactamases belonged to the one of the subgroups encoded by genes that were not detectable in PCR used during the investigation.

A relatively high percentage of ESBL-expressing strains (21.2%) were isolated from beef as compared to pork where only 8.7% of such E. coli was identified. It is an interesting observation considering the fact that in the study performed some years ago in Poland on rectal/cloacal swabs collected from food-producing animals (pigs, cattle, broilers, layers, and turkeys), ESBL-positive E. coli were not found in cattle (30).

However, in the present investigation, the percentage of beef samples contaminated with ESBL-producing E. coli was 13.5%. This divergence is difficult to explain, and needs additional research. All isolates recognised in phenotypic tests as AmpC-positive, showed the presence of the bla_CMY_2 group genes, which are the most common among AmpC β-lactamases (10).

The prevalence of ESBL and/or AmpC-producing E. coli identified in poultry, beef, and pork samples during the present study seems to be comparable with the data reported in the studies from other countries (10). However, this study was performed with a relatively low number of samples and requires further confirmation. Nevertheless, the obtained results confirm the presence of ESBL and/or AmpC-producing E. coli in raw meat and support the data concerning possible transmission of antimicrobial-resistant strains from animals through food chain to humans. This phenomenon may significantly increase the risk connected with the recently observed growth of antimicrobial-resistance among bacteria pathogenic for humans.

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