

## PRELIMINARY RESULTS REGARDING THE PREVALENCE OF CTX-M GENES IDENTIFIED IN *E. COLI* STRAINS ISOLATED FROM SLAUGHTERED PIGS

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### Abstract

Extended spectrum beta-lactamase (ESBL)-producing enterobacteriaceae and AmpC cephalosporinases are of major importance for public health because these bacteria have low sensitivity to antibiotics such as extended spectrum cephalosporins, which are antimicrobials widely used both in human and in veterinary medicine. Such strains, especially *Escherichia coli* (*E. coli*), have been frequently isolated from pigs too, production animals being considered carriers with major implications in the transmission chain of these strains in humans. The aim of this study was to characterise the molecular substrate of ESBL-positive *E. coli* strains isolated from slaughtered pigs from 3 slaughter houses from the Moldova area by identifying the CTX-M genes. After collection, the samples were primarily processed for phenotypical identification and confirmation of ESBL-positive *E. coli* strains. Bacterial DNA extraction for the target strains was carried out using the “boiled preps” method. Identification of the *bla*<sub>CTX-M</sub> (*bla*<sub>CTX-M-9</sub>; *bla*<sub>CTX-M-1</sub>) genes was carried out by PCR using the specific protocol. Molecular investigations revealed that out of the 118 analysed samples, the *bla*<sub>CTX-M-V</sub> gene was identified in 61% (72/118). Characterisation of the CTX-M groups signalled the presence of the CTX-M-I group in 44/72 (61.11%) of the analysed strains, and the presence of the CTX-M-9 group in 18/72 (25%) of the strains. This study emphasised a high prevalence of CTX-M enzyme-producing *E. coli* strains isolated from the caecum of slaughtered pigs.

**Key words:** *E. coli*, CTX-M, slaughtered pigs

Antimicrobial resistance (AMR) is a subject of global interest, with special implications for public health, especially in terms of complicating the treatment of bacterial infections. The occurrence and spread of AMR was assigned to wrong or excessive use of antibiotics both in human and in veterinary medicine.

Along with intensive use of antibiotics in veterinary medicine, the degree of antimicrobial resistance in production animals followed an ascending trend since the first reported cases of AMR. Also, it has been proven that animal farms and wastewater treatment plants of slaughter houses contain a more diverse set of plasmids and genetic cassettes, compared to the wastewater from hospitals, which means that the formers could be considered a hot point for horizontal transfer of antimicrobial resistance genes (Yuan W. *et al.*, 2020).

Production animals, such as poultry or pigs, which are carriers of ESBL-positive Enterobacteriaceae, even without showing clinical signs of disease, are possible reservoirs of ESBL enzymes that can be transferred to humans either through the food chain, or by improper handling and improper cooking of meat (Carattoli A., 2008). The pig slaughtering process includes several stages; some of them ensure decrease of microbial contaminants, but others, such as the evisceration stage, increase the risk of contamination (Warriner K. *et al.*, 2002 Wu S. *et al.*, 2009). Consequently, faecal carriage with ESBL-producing *E. coli* strains in pigs is of special importance through the transfer of these strains from the intestines of animals to meat during slaughtering.

The number of reported ESBL enzymes is continuously increasing (Ur Rahman S. *et al.*, 2018); however, the most common remain those that belong to the CTX-M group (Peirano G. *et al.*, 2019). In production animals, in the 1990s, the most frequently identified ESBL genes were *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>, but nowadays the most common are *bla*<sub>CTX-M</sub> (Hawkey P.M. *et al.*, 2009; Irrgang

A. *et al.*, 2018). There are hundreds of versions of the *bla*<sub>CTX-M</sub> genes identified, and they have a more intense activity against cephalosporins, such as cefotaxime, than against other oximino- $\beta$ -lactams (Blair J.M.A *et al.*, 2015).

The aim of our study was to characterise the molecular substrate and to determine the prevalence of CTX-M genes in *E. coli* strains isolated from the caecum of pigs slaughtered in three slaughter houses from the Moldova region.

## Material and method

The work protocol for the caecum samples and stool cultures was first aimed at opening the caecum in an aseptic manner and collecting  $1 \pm 0.1$  g from the content. After this stage, the sample of caecal content was pre-enriched by using buffered peptone water (Oxoid, Basingstoke, United Kingdom). The screening of ESBL-producing *E. coli* strains was carried out by cultivation on the MacConkey medium (Oxoid, Basingstoke, United Kingdom) with an addition of cefotaxime (CTX) (Sigma-Aldrich). The colonies that showed morphology characteristic of *E. coli* strains were confirmed based on the chemical properties using the media TSI, MIU and API 20E. Phenotypical confirmation of the production of ESBL enzymes was carried out through the microdilution in broth method, using the EUVSEC2 plates that contain cefoxitine, cefepime, cefotaxime, ceftazidime and clavulanic acid combined with cefotaxime or ceftazidime. The results were interpreted based on the synergy tests between the clavulanic acid and cefotaxime or ceftazidime. After phenotypical processing of the obtained isolates, bacterial DNA extraction was carried out through the “boiled preps” technique. Molecular characterisation of strains first aimed at identifying the *bla*<sub>CTX-M</sub> genes and then at identifying the genes that encode two of the most common groups of CTX-M enzymes: CTX-M-1 and CTX-M-9. The molecular investigations were carried out by PCR while observing the protocol recommended by Wedley *et al.* The patterns of nucleotide sequences, length and alignment temperature for each target gene are indicated in *table 1*.

**Table 1**

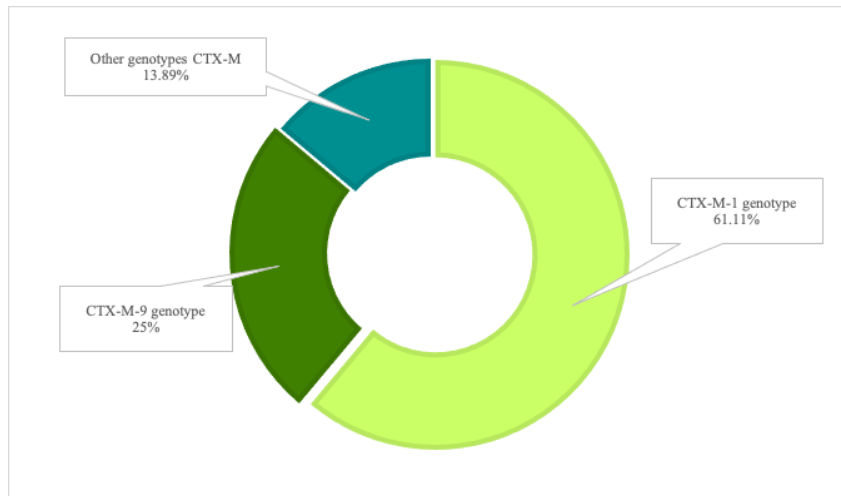
Primers used for the identification of genes that encode the CTX-M enzymes

Primers	Nucleotide sequences (5'-3')	Length (bp)	Alignment temperature	Reference
<b>CTX-Mu</b>	Fw: 5'-ATGTGCAGYACCAGTAARGTKATGGC-3' Rev: 5'-TGGGTRAARTARGTSACCAGAAAYCAGCGG-3'	593	58°C	Wedley A.L. et al., 2011
<b>CTX-M-1</b>	Fw: 5'-ATGGTTAAAAATCACTGCG-3' Rev: 5'-TTACAAACCGTCGGTGAC-3'	876		
<b>CTX-M-9</b>	Fw: 5'-ATGGTGACAAAGAGAGTGCAAC-3' Rev: 5'-TTACAGCCCTTCGGCGATG-3'	876		

## Results and discussions

118 strains of presumptive ESBL enzyme-producing *Escherichia coli* strains were analysed from a molecular point of view. The conducted investigations highlighted the presence of the *bla*<sub>CTX-M-U</sub> gene in 72/118 (61%) of the strains. Characterisation of the CTX-M groups in the strains taken under study has signalled the presence of the CTX-M-1 group in 44/72 (61.11%) of the isolates, which was the dominating group, while the CTX-M-9 group was identified in 18/72

(25%) of the strains. Furthermore, 10/72 (13.89%) strains of animal origin with positive signal for the *bla*<sub>CTX-M-U</sub> gene did not belong to any of the two aforementioned groups (figure 1).



**Figure 1** Prevalence of CTX-M genotypes identified in the analysed *E. coli* strains

According to the Commission Implementing Decision 2013/652/EU, in the period 2015 and 2017, within the routine AMR monitoring program, the EFSA collected information about the prevalence of *E. coli* strains resistant to antibiotics isolated from the caecum of pigs and from pork (European Food Safety Authority; European Centre for Disease Prevention and Control, 2019). In 2017, in the European Union (EU), the prevalence of ESBL-producing *E. coli* strains isolated from the caecum of pigs was 30.62%, lower than the one reported in 2015 (30.2%) (European Food Safety Authority; European Centre for Disease Prevention and Control, 2019). In regard to East European countries, in 2017, Hungary had the highest prevalence rate (56.2%), while in 2015, Bulgaria was the country with the highest prevalence (49.8%) (European Food Safety Authority; European Centre for Disease Prevention and Control, 2017; European Food Safety Authority; European Centre for Disease Prevention and Control, 2019). In addition, within the same routine AMR monitoring program, Romania reported in 2015 a prevalence of 46.6% of ESBL-positive *E. coli* strains isolated from the caecum of pigs, and in 2017, a prevalence of 53.7%, being the second country, immediately after Hungary, with a high prevalence in East Europe. The high prevalence of ESBL-producing *E. coli* strains isolated from pigs obtained in this study can be correlated with a high consumption of antibiotics of the type of penicillin or third-generation or fourth-generation cephalosporins in the industry of production animals; the highest sales for the aforementioned antibiotics were registered in East and South Europe (European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption, 2019; Ieva B *et al.*, 2020). Moreover, the fact that screening was carried out on samples collected from the caecal content, which is a natural habitat for the *E. coli* strains, should be taken into consideration.

The prevalence of ESBL-positive *E. coli* strains in fattening pigs (studies conducted on samples of caecal content) varies significantly in the EU depending on the countries. In general, the prevalence of ESBL-producing *E. coli* strains isolated from pigs has seen an ascending trend along the years.

At a worldwide level, strategies to solve the global problem of AMR have been discussed and will basically also be relevant in the battle against the spread of ESBL enzyme-producing

bacteria in the industry of production animals. For example, the information in the specialty literature recommends special attention to the principles of use of antibiotics, closer monitoring both of the consumption of antibiotics and of the antimicrobial resistance, as well as establishing preventive measures to diminish the risk of introduction or spread of AMR bacteria at the farm level. Moreover, faecal carriage with ESBL-positive enterobacteria represents a risk for the staff working in farms or in slaughter houses by possible intestinal colonisation with AMR pathogenic bacteria. In addition, pork can be cross-contaminated either during the slaughtering process, or by the staff.

### Conclusions

This study emphasised a high prevalence of CTX-M enzyme-producing *E. coli* strains isolated from the caecum of slaughtered pigs. Furthermore, monitoring and rational use measures of antibiotics in farms are necessary.

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