PRELIMINARY INVESTIGATIONS ON PREVALENCE OF ESBL-PRODUCTION *ESCHERICHIA COLI* STRAINS IN SWINE FROM BOTOȘANI COUNTY

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Abstract

Administration of antimicrobials to food-producing animals increases the risk of higher antimicrobial resistance in normal intestinal flora. The present preliminary study was conducted to investigate the presence of extended spectrum beta-lactamase (ESBL)-producing Escherichia coli strains in healthy swine from Botoșani County. During 2016-2018, a total of 87 samples of luminal contents of gut sections (cecal) were collected and tested. Fifty-one (51,72%) E. coli isolates were identified as ESBL-producing strains. These preliminary results reflect the selective pressure, caused by intense and less prudent use of the antimicrobials in swine production in our country. Moreover, commensal E. coli can be a reservoir for antimicrobial resistance genes, which can be transferred to pathogenic bacteria. Therefore, resistance genes transferring from farm to fork represent a public health emerging danger by the potential of producing difficult-to-treat pathogens.

Keywords: *Escherichia coli*, ESBL-producing strains, swine

Introduction

Antibiotic resistance in animals becomes a public health issue when there is transmission of antibiotic resistant bacteria, or their resistance genes, from animals to humans. The extensive use of antimicrobials in food-producing animals has potential to raise antimicrobial-resistance in enteric commensal bacteria. These bacteria may constitute a significant reservoir of antibiotic resistance determinants, which can be transferred to pathogenic bacteria for humans and animals. Food contamination with antimicrobial-resistant bacteria is a public health concern because the resistant organisms can be transferred to humans through the consumption of contaminated food and can thus compromise human health (EFSA/ECDC, 2017).

The antimicrobial agents can be divided into several groups, three of which are used in veterinary medicine, namely, penicillin, first to fourth-generation cephalosporins and β-lactamase inhibitors (Geser et al, 2011). Beta-lactam antimicrobials are one of the important antimicrobial agents in veterinary medicine respectively in swine production. β-lactamases are enzymes that break down β-lactam antibiotics, which constitute the biggest class of antibiotics. Resistance to this class of antibiotics, mediated by extended-spectrum β-lactamases (ESBL) has been increasingly reported (van Damme et al, 2017). Excessive use of extended-spectrum β-lactam antibiotics, mainly the 3rd generation cephalosporins, leads to the production of ESBLs amongst Gram negative rods of the *Enterobacteriaceae* family, such as *Escherichia coli.* ESBL enzymes break down penicillin, amino-, ureido-, and carboxypenicillins, 1st, 2nd, and 3rd generation cephalosporins. Carbapenems are the only β-lactam antibiotics that have an effective functioning on ESBL-producing bacteria.

Escherichia coli are highly adapted organisms that live in the gastrointestinal tract of both animals and humans and survive in environment (water, soil or fecal matter). The presence of *E. coli* can indicate fecal contamination in the environment and can play an integral part in monitoring the transmission of antimicrobial resistance genes within bacterial populations (Hansen et al., 2013). The horizontal transfer of resistance genes, especially mobile genetic elements, has led to

the rapid emergence of multidrug-resistant *E. coli* in the swine production, which may be delivered to humans (Collignon et al, 2007).

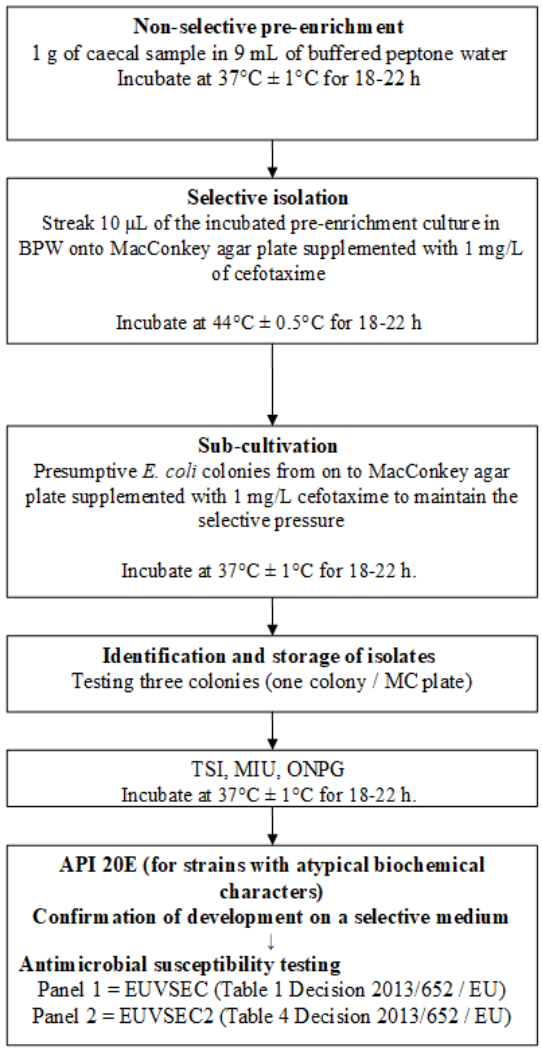
Material and methods

During 2016-2018, 87 samples of luminal contents of gut sections (cecal) were collected from slaughtered swine. All pigs originated from farms in Botoșani County (table 1). The laboratory investigations were carried out at the Sanitary Veterinary and Food Safety Laboratory Iaşi and at Microbiology Laboratory from University of Agricultural Sciences and Veterinary Medicine Iași.

Table 1

Sampling distribution during research period

Year	*2016*	*2017*	*2018*
No. of tested samples	35	23	29



The samples were collected randomly from slaughtered pigs. Sampling was made in sterile, intact bags, without being exposed to extreme temperatures, then transported to the lab. All samples were submitted to laboratory testing in the shortest time, respectively in maximum 48 hours after sampling.

Isolation of ESBL -producing *E. coli* strains from swine cecal samples was performed according to the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) protocol (Fig. 1).

The isolation protocol for samples of luminal contents of gut sections (cecal) consists in use of a pre-enrichment step. Thus, following aseptic opening of the cecum, the content was aspirated with a sterile pipette and then released in a sterile Falcon tube, to which was added 9 ml buffered peptone water (Oxoid, Basingstoke, UK).

Fig. 1 Schematic representation of protocol used for ESBL producing *E. coli* detection and taxonomic classification of the isolates (EURL-AR)

The tubes were incubated with lightly threaded plugs under aerobic conditions at 37 ± 1 ° C. After 20 ± 2 hours of incubation, samples were transplanted into MacConkey agar (Oxoid, Basingstoke, UK), supplemented with cefotaxime (CTX) (Sigma-Aldrich, US), a selective medium recommended for the detection of ESBL-producing *Escherichia coli* strains. After 24 hours of incubation at 44°C, the plates were examined and three presumptive cephalosporinase-producing colonies were transplanted into Petri dishes with cepotaxime-supplemented MacConkey (CTX) medium (Sigma-Aldrich, US) (fig.2). After another 24 hours of incubation at 37 ± 1 ° C, *Escherichia coli*-type colonies were examined and identified. For bacterial species confirmation, the isolated strains were cultured on MIU (mobility, indole, urea) / TSI (glucose, lactose, hydrogen sulfide, gas) polytropic media (Oxoid, Basingstoke, UK) (fig.3).

Fig. 2 *Escherichia coli* colonies presumably synthesizing ESBL on MacConkey agar (Oxoid, UK) supplemented with cefotaxime (Sigma-Aldrich, US)

Fig. 3 *Escherichia coli* confirmation on MIU/TSI media (Oxoid, UK)

Broth microdilution was performed forward for antimicrobial susceptibility testing of *Escherichia coli* isolates. For this purpose, in the first step was used *Sensititre E. coli* EUVSEC plate (TrekDiagnostic System, Thermofisher) and the protocol recommended by the producer. Minimum inhibitory concentrations (MIC) (mg/l) were determined for the following antibiotics: sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), tetracycline (TET), meropenem (MERO), azithromycin (AZI), nalidixic acid (NAL), cefotaxime (FOT), chloramphenicol (CHL), tigecycline (TGC), ceftazidime (TAZ), colistin (COL), ampicillin (AMP) and gentamicin (GEN). For each plate was used a positive control.

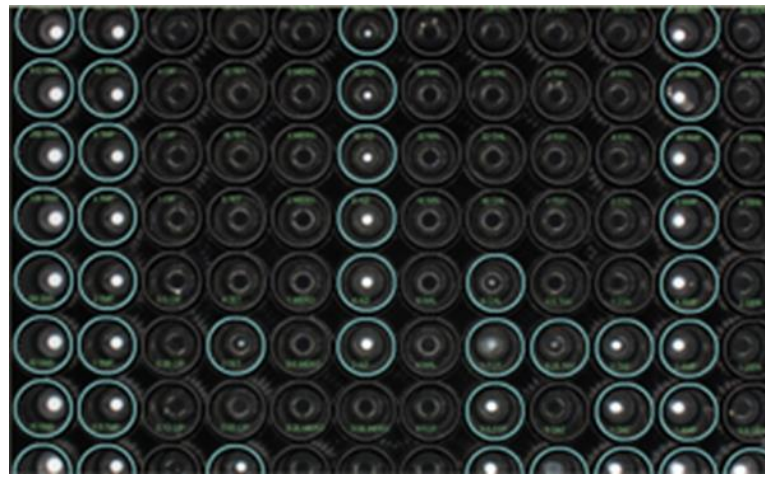


Fig. 4. *Sensititre E. coli* EUVSEC plate (TrekDiagnostic System, Thermofisher)

Beta-lactamase *E. coli* spectrum isolates identified as resistant to cefotaxime and/or ceftazidime and/or meropenem, were subsequently tested with a second antimicrobial panel *Sensititre E. coli* EUVSEC2 plate (TrekDiagnostic System, Thermofisher) necessary for phenotypic verification of presumably carbapenemase-producing *E. coli* strains. Minimum inhibitory concentrations (MIC) (mg/l) were determined for the following antibiotics: cefoxitin (FOX), ertapenem (ETP), imipenem (IMI), meropenem (MERO), ceftazidime (TAZ), cefepime (FEP), cefotaxime/clavulanic acid (F/C), ceftazidime / clavulanic acid (T/C), Cefotaxime (TRM), temocillin (TRM).

Results and discussions

Swine is one of the major food-producing animals in several European countries including Romania. More important, swine has been implicated as a source of antimicrobial-resistant bacteria indicating the importance of identification of antimicrobial resistance in food-producing animals. Although, data on the occurrence of ESBL-producing *E. coli* strains from healthy swine are very limited in Romania. In this study, we screened for ESBL-producing *Escherichia coli* in swine luminal contents of gut sections (cecal) from Botoșani County. The results highlighted that 45 out of 87 isolates were positive for ESBL-producing *E. coli* (Fig. 5).

Using broth microdilution method for certain antibiotics such as cefotaxime and ceftazidime, testing was performed using two different panel plates. Although most ESBL-producing *E. coli strains* have resistance to both compounds, some ESBL enzymes primarily confer resistance to only one of the compounds. Confirmatory synergy testing was also provided so that the ESBL phenotype could be identified. Cefoxitin was also included to identify the AmpC phenotype. Antibiotics, meropenem, imipenem and ertapenem were included to identify suspected carbapenemase-producing *E. coli* strains. The effectiveness of temocillin (6-α-methoxy-ticarcillin) is not affected by most ESBL and AmpC enzymes and this antibiotic may be especially useful in human medicine to treat urinary tract infections caused by gram-negative organisms producing ESBL (Oteo, 2008 ; Livermore and Tulkens, 2009). Temocillin susceptibility allows for additional phenotypic characterization of carbapenemase-producing *E. coli* strains.

The aim of the study, by using both panel testing, was to deduce the ESBL-producing *E. coli* strains that are responsible for conferring the phenotypic profile of resistance to third-generation cephalosporins or meropenem, providing additional important epidemiological information.

If we analyze to the total number of confirmed isolates, the highest prevalence was reported in 2016 representing 57,14% (20 out of 35 samples), followed by 2017 with a percentage of 56,52% (13 out of 23 samples). The lowest prevalence of ESBL-producing *Escherichia coli* was registered in 2018 representing 41,37% (12 out of 29 samples) observing a slightly decreasing of the number of ESBL-producing *E. coli* samples.

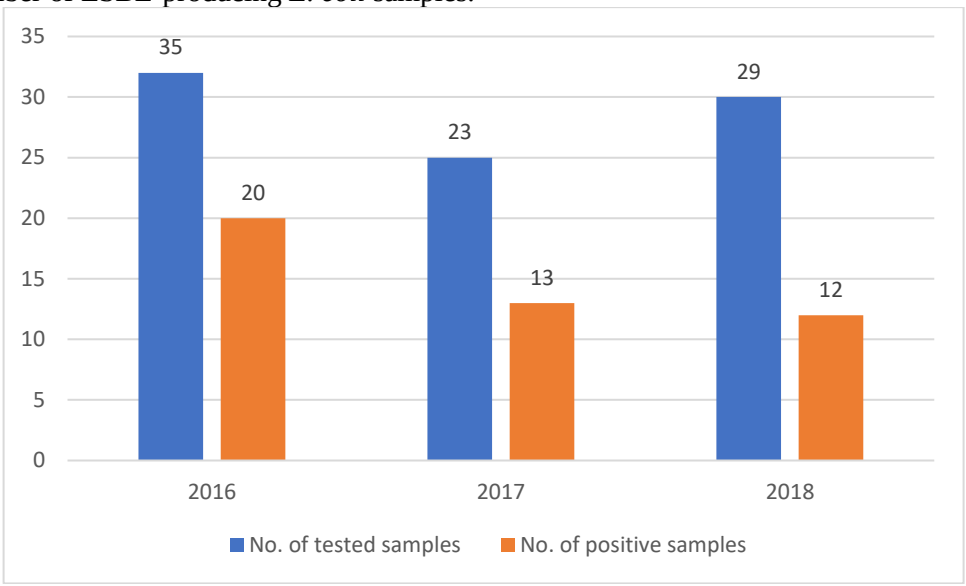


Fig. 5 Distribution of the positive ESBL-producing *Escherichia coli* identified in swine from Botoşani County

In Romania, a similar study published by Miliță et al. revealed that in 2015 the prevalence of ESBL/AmpC producing *E. coli* isolates in swine cecal samples was 65,78% (223 out of 339 samples). Based on MIC obtained for 21 antimicrobials, the selected 223 strains were resistant to cefotaxime (100%), ampicillin (100%), cefepime (91.93%), ceftazidime (90.13%), sulfamethoxazole (73.54%), tetracycline (71.30%), trimethoprim (62.33%), ciprofloxacin (53.81%), chloramphenicol (41.70%), nalidixic acid (39.91%), gentamicin (20.63%), cefoxitin (20.63%), azithromycin (14,35%) and colistin (3.59%) (Miliță et al, 2017).

ESBL-producing Enterobacteriaceae are reported in livestock, companion animals and wildlife. In the European program for monitoring antimicrobial resistance, cefotaxime-resistance was shown among *E. coli* isolates randomly selected from broilers at the slaughterhouse (6.6%), from pigs (1.3%), and from cattle (1.2%) (EFSA/ECDC, 2015).

In 2017, the overall prevalence of presumptive ESBL producing *E. coli* in fattening pigs in the EU was 30.62%, that is slightly higher than reported in 2015 (30.2%). Within Eastern European countries, the data collected in 2017 revealed that Hungary had the highest prevalence rate (56.2%), while in 2015 Bulgaria was the country with the highest one (49.8%). The lowest rates in 2017 were registered in the Czech Republic (Bergšpica et al., 2020).

The transmission and spread of infectious diseases through population mobility, including multidrug-resistant organisms such as *Escherichia coli* that produce extended-spectrum- β lactamases is an emerging phenomenon. In Europe from 2010 to 2013, a 9.5% to 12.6% significant increase in resistance to third-generation cephalosporins in *E. coli*, which is an indicator for ESBL-production, was observed in invasive human isolates (Karanika S. et al, 2016). Antimicrobial resistance in the EU/EEA published in the Annual epidemiological report for 2019 that the most

commonly reported bacterial species was *Escherichia coli* (44.2%). Moreover, more than half of the *E. coli* isolates reported in 2019 were resistant to at least one antimicrobial group under surveillance, and combined resistance to several antimicrobial groups was frequent (EARS-Net, 2020).

Humans and swine may be exposed to antibiotic resistant bacteria by direct physical contact or indirect contact via the environment, that is fomites and the natural environment. Exposure to ESBL-producing *E. coli* could lead to infection, carriage or disease in both humans and pigs. Studies looking at transmission of β-lactamase-producing *Enterobacteriaceae* between humans and livestock farms are limited to pig farms, but results suggest that working with positive pigs is associated with an increased risk of carriage (Dohmen *at al*., 2015). Moreover, at slaughterhouses, a risk of cross-contamination of meat exists, especially during evisceration, where carcasses can be contaminated by AMR bacteria from the fecal content of the same or different pigs (Wu et al, 2009). Food processing environments are considered to be important intermediate reservoirs and vectors of multi resistance bacteria, and also food handlers pose a risk of transmission of ESBL producing bacteria (Oniciuc et al, 2019).

Conclusions

Antimicrobial resistance poses a growing threat to public health. Overall, the prevalence of ESBL producing *E. coli* isolated from pigs is increasing in Europe over the years. The information published the literature highlights the urgent need to reconsider the responsible use of antimicrobials at farm level. Moreover, the antimicrobial resistance surveillance should provide up-to-date and relevant information to monitor the appropriateness of therapy guidelines, public health interventions, infection control policies, and antimicrobial development.

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