Occurrence of extended-spectrum beta-lactamase- and AmpC-type cephalosporinase-producing *Escherichia coli* in mastitic cow's milk

HANNA RÓŻAŃSKA, MARIA KUBAJKA, MARCIN WEINER

Department of Microbiology, National Veterinary Research Institute, Partyzantów 57, 24-100 Puławy, Poland

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Różańska H., Kubajka M., Weiner M.

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Summary

The aim of the study was to evaluate the occurrence of *Escherichia coli* producing extended-spectrum beta-lactamases (ESBL and/or AmpC) in the milk of cows with *mastitis*. A total 2,500 milk samples from mastitic cows were tested in 2014-2018. The investigations included the culture of bacteria on MacConkey agar with cefotaxime, identification with the API Rapid 32 E test, synergy disc test D68C, assessment of antimicrobial resistance by the microdilution method and confirmation of the occurrence of genes encoding ESBL and AmpC. Out of 133 isolates identified as *E. coli*, 87 were recognized as ESBL producers and 46 as chromosomally encoded cephalosporinase AmpC producers. The *bla*_{TEM} was predominant in the ESBL producers. All 46 AmpC-positive strains had the *bla*_{CMY} gene. The results confirmed the occurrence of extended-spectrum beta-lactamase-producing *E. coli* in inflammatory secretions from mastitic bovine udders. This may impact the effectiveness of treatment of *mastitis* and create some risk for humans.

Keywords: cows, mastitis, *Escherichia coli*, extended-spectrum beta-lactamases

Escherichia coli (*E. coli*) is a natural and useful inhabitant of the digestive tract of humans and warm-blooded animals, responsible for digestion of some nutrient elements and synthesis of vitamins, such as group B, K, C and folic acid. Nevertheless, some strains of these bacteria are also responsible for various diseases of humans, such as serious food poisonings and infections of the urinary tract, bloodstream and the central nervous system. *E. coli* may be pathogenic for animals as well, including livestock (4, 12, 27, 34). These bacteria, among others, are one of the main causes of mastitis in lactating cows, which may take a subclinical, acute or peri-acute form and is called colimastitis (1, 8, 11, 14). The antimicrobial resistance of the *Enterobacteriaceae* family, including *E. coli*, seems to be one of major problems of medicine in the 21st century because of the dramatic reduction in therapeutic options for several infections caused by these bacteria. *E. coli* demonstrates some mechanisms of antimicrobial resistance to many substances. The ability to produce extended-spectrum beta-lactamases (ESBL) and AmpC-type cephalosporinases has a major significance because it is associated with resistance to

beta-lactams and usually to almost all other antibiotics (2, 10, 22, 24, 29). For this reason, the World Health Organization has included ESBL- and/or AmpC-producing strains of these bacteria in the list of "alert pathogens" (3, 10). Due to the fact that genes responsible for the production of these enzymes are often located on mobile genetic elements, such as plasmids, their dissemination in the environment is easy and rapid (13, 15, 20, 26, 32). Many authors have suggested a possible transfer of pathogenic, resistant bacteria from animals to humans via food chain or by contact with infected individuals (2, 3, 5, 8, 12, 25, 26). The genetic similarity between the isolates from animals and humans seems to confirm these opinions. From this point of view, the antimicrobial resistance may be perceived as zoonosis (19, 21, 35). However, some authors have also pointed out the differences between bacterial populations from animals and humans and the need for further, more detailed studies in this field (6, 18, 33). There is no sufficient data on the occurrence of ESBL- and/or AmpC-producing *E. coli* in the milk of cows suffering from mastitis in Poland. The aim of this study was thus to obtain up-to-date information.

Material and methods

A total of 2,500 milk samples from cows with clinical or subclinical mastitis were tested between 2014 and 2018. The samples were taken by veterinarians on farms, kept in sterile vials and transported to our laboratory under cooling conditions. The samples were frozen until analysis at $-18 \pm 1^\circ\text{C}$. To isolate ESBL- and/or AmpC-producing *E. coli*, 1 ml of the sample was incubated with 9 mL of buffered peptone water overnight at $37 \pm 1^\circ\text{C}$. Then, one loop (10 μL) was spread on the surface of MacConkey agar (Oxoid, UK) supplemented with cefotaxime (Sigma-Aldrich, USA) (2 mg/L). The plates were incubated overnight at $37 \pm 1^\circ\text{C}$. Bacteria from suspected colonies were identified with API Rapid 32 E Tests (bioMérieux, France) according to the manufacturer's instruction. Their capacity to produce ESBL and/or AmpC was initially established with the synergy disc test D68C (mast Diagnostica, Germany) and then confirmed by the minimal inhibitory concentration (MIC) technique applying Sensititre ESBL Plate Format (Trek Diagnostic Systems, USA). Additionally, for extension of the antimicrobials tested, CMV3AGNF plates (Trek) were used. The

configuration of the MIC plates is presented in Tab. 1. All tests were performed with a Sensititre auto reader device (Trek) according to the manufacturer's guide. The results, shown as R (resistant) or S (susceptible), were generated automatically on the basis of MIC values, according to clinical breakpoints proposed by the Clinical & Laboratory Standards Institute (CLSI, USA). For detection of the selected genes responsible for ESBL or/and AmpC production, the polymerase chain reaction was used. The details of DNA preparation and PCR conditions are described in our earlier paper (36). The characteristics of the primers used in PCR are shown in Tab. 2. The reference control was *E. coli* obtained from the European Union Reference Laboratory for Antimicrobial Resistance (EURL AR, Kings Lyngby, Denmark) under the proficiency testing programme EQAS (External Quality Assurance System), signed as EC – 4.5 (ESBL+), EC – 4.7 (AmpC+) and EC – 4.8 (ESBL+).

Results and discussion

In total, 133 strains growing on MacConkey agar with cefotaxime were identified as *E. coli*. Based on synergy disc test D68C results, 87 isolates were suspected of producing ESBL and 46 of producing AmpC. In all ESBL-positive strains, the presence of at least one of the genes encoding extended-spectrum beta-lactamases was detected (Tab. 3). *Bla_{TEM}* was predominant, as it occurred in 83 strains. One of the strains had *bla_{TEM}* as well as *bla_{CTX-M}*. Genes belonging to the CTX-M group were detected in 4 strains. *Bla_{CMY}* group genes were detected in all 46 isolates suspected of being AmpC producers. Two isolates had *bla_{TEM}* and *bla_{CMY}* genes simultaneously. *Bla_{SHV}* genes were not detected in any of the strains. The results of the antimicrobial resistance analyses are presented in Tab. 4. For the strains classified as ESBL-positive, the level of resistance was 100% for ceftazidim, cefpodoxin, cefotaxime, ceftriaxone, ciprofloxacin, tetracycline, sulfisoxazole and trimethoprim/sulfamethoxazole, 98.8% for ceftiofur and streptomycin, 97.7% for cefepime, 92.0% for ampicillin and cefotaxime with clavulanic acid, 87.4% for ceftiofur, 74.7% for gentamycin, 35.6% for a combination of amoxicillin with clavulanic acid and 19.5% for chloramphenicol. All these strains were susceptible to imipenem and meropenem. All AmpC-positive strains were resistant to all of the penicillins and cephalosporines, the combination of amoxicillin with clavulanic acid, tetracycline, sulfisoxazole and trimethoprim/sulfamethoxazole, and were partially inhibited by ceftazidime/clavulanic acid (29 resistant isolates out of 46), and cefotaxime/clavulanic acid (3 resistant strains). Forty-three strains showed resistance to chloramphenicol (93.5%), 30 (65.2%) to

Tab. 1. Configuration of MIC plates used in the study

ESBL	CMV3AGNF
Ceftazidime (TAZ)	Cefoxitin (FOX)
Cefazoline (FAZ)*	Azithromycin (AZI)
Cefepime (FEP)	Chloramphenicol (CHL)
Cefoxitin (FOX)	Tetracycline (TET)
Cephalotin (CEP)	Ceftriaxone (AXO)
Cefpodoxime (POD)	Amoxicillin/clavulanic acid 2:1 ratio (AUG2)
Cefotaxime (FOT)	Ciprofloxacin (CIP)
Ceftriaxone (AXO)	Gentamicin (GEN)
Imipenem (IMI)	Nalidixic acid (NAL)*
Meropenem (MERO)	Ceftiofur (XNL)
Gentamicin (GEN)	Sulfisoxazol (FIS)
Ampicillin (AMP)	Trimethoprim/sulfamethoxazole (SXT)
Ciprofloxacin (CIP)	Ampicillin (AMP)
Piperacillin/tazobactam constant 4 (P/T4)	Streptomycin (STR)
Ceftazidime/clavulanic acid (T/C)	
Cefotaxime/clavulanic acid (F/C)	

Explanation: * – not applicable for *Enterobacteriaceae*

Tab. 2. Characteristics of primers used in PCR

Genes	Sequence (5'→3')	Annealing temp. (°C)	Amplicon size (bp)
<i>bla_{CTX}</i>	ATGTGCAGYACAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	60	593
<i>bla_{TEM}</i>	TGAGTATTCAACATTTCCGTGT TTACCAATGCTTAATCAGTGA	53	861
<i>bla_{SHV}</i>	CAAAACGCCGGGTTATTC TTAGCGTTGCCAGTGCT	53	937
<i>bla_{CMY-2-group}</i>	GCACTTAGCCACCTATACGGCAG GCTTTTCAAGAATGCGCCAGG	60	758
<i>bla_{CMY}</i>	GACAGCCTCTTTCTCCACA TGGAACGAAGGTACGTA	50	550

Tab. 3. Occurrence of genes encoding ESBL and/or AmpC in the isolates

Genes	Number of isolates
<i>bla</i> _{CTX}	4
<i>bla</i> _{TEM}	83
<i>bla</i> _{CTX} + <i>bla</i> _{TEM}	1
<i>bla</i> _{SHV}	0
<i>bla</i> _{CMY}	46
<i>bla</i> _{TEM} + <i>bla</i> _{CMY}	2

Tab. 4. Antimicrobial resistance of ESBL- and AmpC-producing *E. coli* isolated from mastitic milk

Antimicrobials	Number/% of resistant strains	
	ESBL producers (n = 87)	AmpC producers (n = 46)
Amoxicillin/clavulanic acid	31/35.6	46/100
Ampicillin	80/92.0	46/100
Ceftazidim/clavulanic acid	0	29/63.0
Cefepime	85/97.7	46/100
Cefoxitin	76/87.4	46/100
Cefpodoxim	87/100	46/100
Cefotaxime	87/100	46/100
Cefotaxime/clavulanic acid	80/91.9	3/6.5
Ceftiofur	86/98.8	46/100
Ceftriaxone	87/100	46/100
Piperacillin/tazobactam	38/43.7	0
Chloramphenicol	17/19.5	43/93.5
Imipenem	0	0
Meropenem	0	0
Ciprofloxacin	87/100	46/100
Gentamicin	65/74.7	22/47.8
Streptomycin	86/98.8	30/65.2
Tetracycline	87/100	46/100
Sulfisoxazole	87/100	46/100
Trimethoprim/sulfamethoxazole	86/98.8	46/100

streptomycin, 29 (63.4%) to ceftazidim with clavulanic acid, and 3 (6.52%) to cefotaxime in combination with clavulanic acid.

E. coli is one of the main etiological factors causing mammary gland infections in milking cows. It is estimated that these bacteria may be responsible for the majority of mastitis cases. The abattoir environment and farm animals are reservoirs of *E. coli*. The frequent occurrence of mastitis, especially in its subclinical and clinical forms, leads to the increasing use of antibiotics and selection of resistant bacteria, including *E. coli*. Over recent decades, a global dissemination of ESBL- and AmpC-producing *Enterobacteriaceae*, including *E. coli*, has occurred, and these bacteria have increasingly been isolated from animals, including livestock. From the clinical point of view, the ability of *Enterobacteriaceae* family members to produce

AmpC-type enzymes seems to be more significant than their ability to produce ESBL because the growth of these bacteria is poorly inhibited by combinations of antimicrobials with clavulanic acid, sulbactam and tazobactam. In our studies, ESBL- and/or AmpC-producing *E. coli* were isolated from 133 mastitic milk samples (5.32%). Among them, 87 strains produced ESBL, and 46 produced AmpC-type enzymes. Two strains were capable of producing both types of extended-spectrum beta-lactamases. There are no data from Poland for comparison. The available data from other countries are diversified. According to data from France (4), ESBL-producing *E. coli* and *Klebsiella pneumoniae* occurred in 6 out of 1427 isolates from mastitis. In Greece, Filliouis et al. (7) isolated 18 ESBL-producing strains of *E. coli* from 400 samples of mastitic milk. In studies carried out in Switzerland by Geser et al. (9), one strain of *E. coli* out of 67 isolates from mastitic milk was able to produce ESBL. Locatelli et al. (17) recovered ESBL-producing *E. coli* from 5 (0.9%) out of 550 samples of mastitic milk tested in Italy. Data on ESBL-producing *E. coli* strains from mastitis are also available from Turkey (23) and the United Kingdom (31). In our studies, two strains of *E. coli* demonstrated a potential ability to produce ESBL as well as AmpC enzymes (the presence of *bla*_{TEM} and *bla*_{CMY} genes simultaneously). The ability to produce both types of enzymes was also observed by Schmid et al. (26) and Tekiner and Özpinar (30). It is worth noting that in our experiments the *bla*_{TEM} genes were detected most frequently, whereas other authors argue rather that the predominant group are CTX-M (4, 7). The *bla*_{TEM} genes were dominant in Iran (16) and in isolates from raw milk in Czech Republic (28). Many scientists have reported the occurrence of ESBL- and/or AmpC-producing *E. coli* strains in raw milk samples from cows without clinical signs of mastitis (8, 28, 30). This may confirm the potential risk of transferring these bacteria to humans via food chain due to the growing popularity of consumption of raw milk or products from unpasteurised milk. The results of our investigations presented in tab. 4 indicate that many isolates able to produce extended-spectrum beta-lactamases demonstrated resistance not only to beta-lactams, but also to many other substances. All ESBL- and AmpC-positive strains were resistant to ciprofloxacin, tetracycline and sulfisoxazole. Moreover, all strains producing AmpC-type cephalosporinases and almost all ESBL producers were resistant to trimethoprim with sulfamethoxazole. Detailed investigations by many authors also indicate the simultaneous occurrence of different genetic mechanisms responsible for resistance to many antimicrobials in strains expressing ESBL or AmpC (4, 8, 9, 26, 31, 32). This multi resistance is particularly noteworthy, as it impacts the effectiveness of therapy against infections caused by such microorganisms.

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Corresponding author: Marcin Weiner, DVM, PhD, ScD, Department of Microbiology, National Veterinary Research Institute, Al. Partyzantów 57, 24-100 Puławy, Poland; e-mail: mpweiner@piwet.pulawy.pl