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Prevalence and antimicrobial resistance of beta-lactamase-producing Gram-negative isolates from outpatient clinical and environmental samples in the Zenica-Doboj Canton, Bosnia and Herzegovina

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ABSTRACT

Introduction: Infections due to extended-spectrum beta-lactamase (ESBL)-producing isolates in patients are hard to treat and cause high morbidity and mortality. ESBL-producing bacteria have been increasingly detected in environmental samples in different countries since 2002, and have gained considerable attention worldwide.

Methods: Antibiotic susceptibility of all isolates was determined using the disk diffusion method. The production of ESBLs was determined by the double-disk synergy test.

Results: Among the outpatient clinical samples, out of 2857 Gram-negative bacteria, 184 (6.5%) ESBL-producing bacteria were isolated. In this group, 143 (77.7%) were from urine samples, 26 (14.1%) from surgical wounds, 6 (3.3%) from umbilical swabs, and 9 (4.9%) from other patients sites (upper respiratory tract, cannula, eyes, genital swabs). *Escherichia coli* was isolated in 62 (33.7%), and *Klebsiella* spp. in 50 (27.8%) cases. Among the environmental samples, out of 381 Gram-negative bacteria, 52 (13.6%) were ESBL-producing isolates. In this group, 37 (71.2%) were sampled from water, 7 (13.5%) from food, and 8 (15.4%) from environmental surfaces. The most prevalent ESBL-producing bacteria isolated from the environmental samples were *E. coli* (isolated from 26 samples), *Klebsiella* spp. (10), non-fermenters (9), and other bacteria isolated from 7 samples. The clinical outpatient ESBL-producing isolates showed resistance to all cephalosporins, ranging from 25% (cefepime) to 100% (cefuroxime). The environmental ESBL-producing isolates showed resistance to cefuroxime, aztreonam, cefpodoxime, amoxicillin/clavulanate, and cefoxitin in the range of 65-100%.

Conclusions: Prevalence of antibiotic resistance of ESBL-producing strains is high and requires routine detection of ESBL-producing isolates in the laboratories, designing of appropriate antibiotic prescribing policies and control of the risk factors.

Keywords: Extended-spectrum beta-lactamase; water; food; antibiotic resistance

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INTRODUCTION

Over the past decades, antimicrobial resistance has been recognized as an important global health problem in many fields, such as human and veterinary medicine, livestock holdings, agriculture, and environment (1,2). In countries with low social and economic status, especially countries in South and Southeast Asia, antimicrobial resistance is more prevalent (3). The production of extended-spectrum beta-lactamases (ESBLs) or AmpC beta-lactamases is one of the most important mechanisms of resistance to extended-spectrum cephalosporins in the Gram-negative isolates (4). ESBLs are plasmid encoded enzymes and their genes are usually transported by plasmids. The enzymes are capable of inactivating a large number of beta-lactam antibiotics, including an extended spectrum and a very broad spectrum cephalosporins and monobactams. The overexpression of chromosomal or plasmid-mediated AmpC enzymes in patients can cause resistance to broad spectrum cephalosporins (5).

In the past decades, the ESBLs were mostly associated with hospitals and institutional care in humans, but they are now increasingly found in the community, in food producing animals, and in water (6-8). They have been detected in food for animals at farms, meat, water, and the environment in many countries such as Italy, China, Malaysia, Nigeria, Austria, and the Netherlands (5,9-14).

Investigations of prevalence and antibiotic resistance in Bosnia and Herzegovina (B&H) are scarce. ESBLs and AmpC beta-lactamases have been reported in isolates causing urinary tract infections and nosocomial infections in the Zenica-Doboj Canton and Tuzla Canton (6,15).

The aim of this study was to investigate the prevalence and antibiotic resistance of ESBL-producing isolates in the Zenica-Doboj Canton, B&H.

METHODS

Setting, bacterial isolates, and study design

Between December 2009 and May 2010, and between December 2013 and May 2014, a random sample of 184 clinically relevant and non-duplicate isolates was routinely collected in the Microbiology Laboratory at the Cantonal Hospital Zenica. The Cantonal Hospital Zenica is 849 bed tertiary level hospital admitting about 25 000 patients/year, with 240 000 hospital days, and covers a population of 331 229 in the Zenica-Doboj Canton.

The institutional review board approval from the Ethics Committee of the Cantonal Hospital Zenica was obtained prior to the initiation of the study.

Between December 2013 and May 2014, a random sample of 52 Gram-negative isolates from environmental samples was collected in the Microbiology Laboratory of the Institute for Public Health and Food Safety in Zenica.

The environmental and clinical samples were analyzed according to the International Standardization Organization standards.

Antimicrobial susceptibility testing

The susceptibility testing to 13 antimicrobials was performed by a two-fold microdilution technique according to the Clinical and Laboratory Standards Institute (CLSI) procedure (16). *Escherichia coli* ATCC 25922 (ESBL negative) and *Klebsiella pneumoniae* 700603 (ESBL positive) were used as quality control strains.

Phenotype detection of ESBLs

ESBL production was determined by the double disk-synergy test (DDST). Overnight broth culture of the test strain was diluted in saline, adjusted to McFarland standard suspension 0.5 and inoculated onto Mueller-Hinton agar (MHA); disk containing amoxicillin/clavulanate (20/10 μg) was placed in the middle of the plate and surrounded (20 mm distance center to center) by disks containing cefotaxime (5 μg), ceftriaxone (30 μg), ceftazidime (10 μg), and cefepime (30 μg) (Becton-Dickinson, USA). Plates were incubated overnight at 37 °C. Any distortion or increase of the inhibition zones around cephalosporin disks toward amoxicillin/clavulanate disk was indicative of ESBL production (16). Production of ESBLs was confirmed by CLSI combined disk test. Disks containing 30 μg of cefotaxime and ceftazidime, and disks containing a combination of the two drugs plus 10 μL (10 μg) of clavulanic acid (Becton-Dickinson, USA) were placed independently, 20 mm apart, on a lawn

culture of 0.5 McFarland opacity of the test isolate on the Mueller-Hinton agar plate and incubated for 18-24 hours at 35°C. Isolates were considered ESBL positive if the inhibition zone measured around one of the combination disks after the overnight incubation was at least 5 mm larger than that of the corresponding cephalosporin disk (16). Isolates resistant to the extended-spectrum cephalosporins and β-lactam/β-lactamase inhibitor combination (amoxicillin/clavulanic acid) were screened for the production of AmpC β-lactamases by the combined disk test using 3-amino phenylboronic acid (PBA) (Sigma-Aldrich, Steinheim, Germany). The stock solution was prepared as previously recommended (17) by dissolving PBA (benzeneboronic acid; Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide at a concentration of 20 mg/mL. Twenty µL (containing 400 µg of boronic acid) of the solution was dispensed onto antibiotic disks. The disks were then dried and used within 60 min. The tests were performed by inoculating Mueller-Hinton agar by the standard diffusion method and placing disks containing four different β-lactams (CAZ, 10 µg; CRO, 30 µg; CTX, 5 µg; FEP, 30 µg) with or without boronic acid. The agar plates were incubated at 37°C overnight. The diameter of the growth-inhibitory zone around a β-lactam disk with boronic acid was compared to the zone around the corresponding β-lactam disk without boronic acid. The test was considered positive for the detection of AmpC production when the diameter of the growth-inhibitory zone around the β-lactam disk with boronic acid was ≥5 mm larger than the diameter around the disk without boronic acid (18).

Production of carbapenemases of the class A or class B was confirmed by the combined disk-test using meropenem disks with PBA and EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich, Steinheim, Germany), respectively (19). Three meropenem (MEM) disks were placed on Mueller-Hinton agar plate inoculated with the test strain. Ten µL of EDTA (300 mg) and PBA (300 mg) was added on the first and third disks, respectively. The difference of ≥5 mm in the zone size between the disks with and without EDTA was suggesting the production of carbapenemase class B, and the difference of ≥5 mm in the zone size between the disks with and without PBA was suggesting the production of carbapenemase class A (19).

RESULTS

From December 2009 until May 2010, 3532 samples (out of 16037, 22%) were bacteria-positive. Gram-negative bacteria were isolated from 2857 (80.9%) samples, out of which 184 (6.4%) phenotypically were ESBL/AmpC-producing bacteria. Among these, 33.7%, 27.8%, 14.1%, 10.9%, 8.7%, 2.7%, 1.6%, 0.5%, and 0.5% were *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Proteus* spp., *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Morganella morganii*, *Providencia rettgeri*, and *Acinetobacter* spp., respectively.

Among the ESBL/AmpC- producing isolates from the clinical outpatient samples, 143 (77.7%) were isolated from urines, 26 (14.1%) from surgical wounds, and 6 (3%) from other samples (skin and soft tissue infections [SSTIs], upper respiratory tract, ear swabs, genital tract, and eyes). Forty-five beta-lactamase-producing isolates were used for susceptibility testing.

From December 2013 until May 2014, 6279 environmental samples were collected, out of which 1141 were from water, 2405 from food, and 2733 from environmental surfaces. In this group, out of 381 Gram-negative bacteria, 52 (13.6%) were ESBL/AmpC-producing isolates. Among these, 37 (71.2%) were isolated from water, 7 (13.5%) from food, and 8 (15.4%) from environmental surfaces. The most prevalent ESBL/AmpC-producing bacteria isolated from environmental samples were *E. coli* (from 26 samples), *Klebsiella* spp. (10), non-fermenters (9), and other bacteria isolated from 7 samples.

Antibiotic susceptibility patterns of the clinical isolates

Table 1 demonstrates the antimicrobial resistance results of 45 phenotypically positive beta-lactamase producing isolates of which 19 were *E. coli* isolates, 17 were *Klebsiella pneumonia*, and 9 were *Klebsiella oxytoca*. The overall resistance of the isolates to the antibiotics demonstrated high resistance to amoxicillin (100%), cefazolin (100%), cefuroxime (100%), ceftazidime (≈85%), cefoxitin (≈80%), ceftriaxone

(≈70%), gentamicin (≈70%), cefotaxime (≈65%), ciprofloxacin (≈60%) and, cefepime (≈40%), while the resistance to imipenem and meropenem was low.

Antibiotic susceptibility patterns of the isolates from environmental samples

Table 2 demonstrates the antimicrobial resistance results of 52 phenotypically positive beta-lactamase-producing isolates, out of which 26 were *E. coli* isolates, 10 *Klebsiella* spp., 9 non-fermenters, and 7 were from other isolates (4 *Citrobacter* spp. and 3 *Enterobacter* spp.).

Cefoxitin, cefpodoxime, aztreonam, and cefuroxime resistance in *E. coli* were 100%, 80%, 76.9%, and 65.4%, respectively, while cefotaxime, cefepime, and ceftazidime resistance rates were lower (34.6%, 23.1%, and 19.2% of cases). Carbapenem resistance was found in one *E. coli* isolate.

Cephalosporins resistance rates were high in *Klebsiella* spp., following were the rates in *E. coli*. The bacteria showed 20 - 100% overall resistance rates, and all *Klebsiella* spp. isolates were susceptible to imipenem and meropenem (Table 2).

Non-fermenters and other isolates showed higher resistance rates to the 3rd and 4th generations of cephalosporins compared to *E. coli* and *Klebsiella* spp. (Table 2).

DISCUSSION

This study aimed to determine variability in the prevalence of ESBL-producing isolates causing infections in humans, and ESBL-producing isolates from environmental samples such as food, water, and environmental surfaces.

Detection of the ESBL production was performed with the combined disk method and showed that 6.5% of the clinical isolates were considered as potential producers of ESBLs, which was lower than in the reports from Serbia, the Republic of Macedonia, Turkey, and Egypt (20,21). However, our results are similar to those reported for Austria, Croatia, Slovenia, Japan, and Tunis (20,22,23).

The phenotypic cefoxitin resistance test showed a higher prevalence (70%) of the production of AmpC beta-lactamases which is contradictory to the report from Morocco (24), but similar to the report from the

TABLE 1. Antimicrobial resistance of beta-lactamase-producing isolates collected from clinical outpatient samples

Causative agent isolated	Setting	Number of isolates tested	Number (%) of antimicrobial resistance of various antibiotics*													
			AMX	AMC	CZ	CXM	CAZ	CTX	CRO	FOX	FEP	IMI	MEM	GEN	CIP	PIP	TZB
Escherichia coli	Outpatients	19	100	43.8	100	100	77.8	66.7	76.5	83.3	33.3	0	0	68.8	66.7	78.6	6.7
Klebsiella oxytoca	Outpatients	9	100	100	100	100	88.9	66.7	75	87.5	33.3	0	0	66.7	55.6	100	33.3
Klebsiella pneumoniae	Outpatients	17	100	90	80	100	88.2	64.7	58.8	58.8	52.9	0	0	88.2	52.9	58.8	5.9

*AMX: Amoxicillin, AMC: Amoxicillin/clavulanate, CZ: Cefazolin, CXM: Cefuroxime, CAZ: Ceftazidime, CTX: Cefotaxime, CRO: Ceftriaxone, FOX: Cefoxitin, FEP: Cefepime, IPM: Imipenem, MEM: Meropenem, GEN: Gentamicin, CIP: Ciprofloxacin, PIP: Piperacilin, TZB: Tazobactam

TABLE 2. Antimicrobial resistance of beta-lactamase-producing isolates collected from environmental samples

ESBL-producing isolates	Total number of isolates	% Of antimicrobial resistance of various antibiotics*									
		AMC	ATM	CXM	CAZ	FOX	CTX	FEP	IMP	MEM	CPD
Escherichia coli	26	84.6	76.9	65.4	19.2	100	34.6	23.1	3.8	3.8	80
Klebsiella spp.	10	100	90	90	20	100	30	30	0	0	57.1
Non-fermenters	9	88.9	55.6	88.9	33.3	77.8	44.4	44.4	0	0	75
Others	7	100	100	71.4	57.1	100	42.9	42.9	0	28.6	66.7

*AMC: Amoxicillin/clavulanate, ATM: Aztreonam, CXM: Cefuroxime, CAZ: Ceftazidime, FOX: Cefoxitin, CTX: Cefotaxime, FEP: Cefepime, IPM: Imipenem, MEM: Meropenem, CPD: Cefpodoxime; ESBL: Extended-spectrum beta-lactamase

Netherlands (25). The number of potential producers of ESBLs and AmpC beta-lactamases in the isolates from the environmental samples was higher (13.6%) than in the isolates from the clinical outpatient samples, which is in agreement with the report from India (26). Patternel et al. reported a higher prevalence of ESBL-producing *E. coli* isolates from minced meat in 24% cases, in Graz, Austria, (13). Similarly, investigators from the Netherlands reported 55% of ESBL-producing *E. coli* isolates collected from retail chicken meat and surface water (14) and 100% from wastewater samples (27), which is contradictory to our report.

The ESBL-producing outpatient clinical isolates showed high rates of resistance to all cephalosporins, except to cefepime and high rates of resistance to aminoglycosides and fluoroquinolones, similar to the reports from India and Pakistan (28,29). In 2014, the World Health Organization reported that *E. coli*/*K. pneumoniae* was resistant to the 3rd generation of cephalosporins in 9.1/13.3% cases (Austria), 21.3/82.1% (Serbia), 8.8/30.2% (Slovenia), and 47.4/91% (the Republic of Macedonia) among others (20). In some instances, these results are similar to ours, however there are some differences as well. For the treatment of these infections in the community, first-line antibiotics were gentamicin or ciprofloxacin (cheap antibiotics without medical prescription), and this is similar to the report from Pakistan (29).

All isolates from our environmental samples showed high resistance rates to cefoxitin, cefuroxime, aztreonam, and amoxicillin/clavulanic acid, but low resistance to the 3rd and 4th generations of cephalosporins, which is also reported in Egypt (21).

Resistant bacteria can cause foodborne diseases, at first a silent carrier state but later may cause infections that are not recognized as being of foodborne origin. ESBL-producing isolates could be transmitted by the food production chain and environmental samples (30).

Boonyasiri et al. reported that basic sanitation and hygiene, including consumption of cooked food and clean water, as well as hand washing, are very important measures for containment and prevention of antibiotic resistance (30).

The main limitation of this study is the small number of ESBL-producing isolates that were collected/available for the analysis, because of the short time span (six months) and due to analysis of only one region-Canton, out of ten cantons in Bosnia and Herzegovina. It is not representative for whole B&H, because we have a different number of populations in each canton, different lifestyles with different social conditions, different climate, etc. However, reduced susceptibility of these isolates is a worldwide concern. Because of the high prevalence of the resistant ESBL-producing strains in the epidemiologically unrelated patients and environmental samples in this study, further local surveillance is needed. Molecular characterization, or at least phenotypic testing of the ESBL production of Gram-negative bacteria, is important for appropriate therapy and the detection of the sources and modes of the spread, which is further the main step in designing targeted infection control strategies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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