ORIGINAL ARTICLE

Molecular epidemiology and antimicrobial susceptibility of AmpC- and/or extended-spectrum (ESBL) ß-lactamase-producing *Proteus* spp. clinical isolates in Zenica-Doboj Canton, Bosnia and Herzegovina

Selma Uzunović[1], Amir Ibrahimagić[1], Branka Bedenić[2,3]

[1]Department for Laboratory Diagnostics, Institute for Public Health and Food Safety, [2]School of Medicine, University of Zagreb, [3]Clinical Department of Clinical and Molecular Microbiology, University Hospital Center Zagreb; Zagreb, Croatia

ABSTRACT

Aim To investigate prevalence, antimicrobial susceptibility, molecular characteristics, and genetic relationship of AmpC- and/or extended spectrum beta lactamase (ESBL)- producing *Proteus* spp. clinical isolates in Zenica-Doboj Canton, Bosnia and Herzegovina.

Methods Antibiotic susceptibility was determined by disc diffusion and broth microdilution methods according to CLSI guidelines. Double-disk synergy test was performed in order to screen for ESBLs, and combined disk test with phenylboronic acid to detect AmpC β -lactamases. PCR was used to detect bla_{ESBL}/bla_{carb} genes. Genetic relatedness of the strains was determined by pulsed-field-gel-electrophoresis (PFGE).

Results Eleven ESBL-producing isolates were included in the study (six inpatients and five outpatients). Susceptibility rate to amoxicillin-clavulanic acid, imipenem and meropenem was 100%. Resistance rate to cefuroxime was 100%, gentamicine 90.9%, piperacillin/tazobactam 81.8%, cefotaxim, ceftriaxone and ceftazidime 72.7%, cefoxitine and ciprofloxacine 63.6% and to cefepime 45.5%. In five (out of 11) isolates multi-drug resistance (MDR) to cephalosporins, cefamicines, amynocligosides and fluoroquinolones was detected. Besides TEM-1 which was detected in all isolates, CTX-M+OXA-1 β-lactamases were detected in seven (out of 11; 63.6%) isolates (five $bla_{CTX-M-1}$ and two $bla_{CTX-M-15}$ genes), and CMY-2 β-lactamase in two isolates. PFGE showed no genetic relatedness.

Conclusion Because of high prevalence of MDR strains in epidemiologically unrelated patients with AmpC- and/or ESBL producing *Proteus* spp. infection, further surveillance is needed. Molecular characterization and strain typing, or at least phenotypic test for AmpC/ESBL production is important for appropriate therapy and the detection of sources and modes of spread, which is the main step in order to design targeted infection control strategies.

Key words: ESBL, CMY-2, antimicrobial resistance

Corresponding author:
Amir Ibrahimagić
Department of Laboratory Diagnostics,
Institute for Public Health and Food Safety
Zenica
Fra Ivana Jukića 2, 72000 Zenica,
Bosnia and Herzegovina
Phone: +387 32 443 580;
Fax: +387 32 443 530;
E-mail: ibrahimagic.amir@gmail.com

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INTRODUCTION

Proteus spp. cause various human infections and it was firstly documented as extended-spectrum beta-lactamase (ESBL)-producer in 1987 (1). *Proteus* spp. were susceptible to beta (β)-lactam antibiotics (2), but progressive increase of resistance to beta-lactam antibiotics mediated by the production of acquired beta-lactamases has occurred (3). Extended-spectrum β -lactamases showing activity to extended-spectrum cephalosporins have also started to spread, including most frequently TEM-type derivatives, but also other enzymes (4). Numerous outbreaks of infections with organisms producing ESBLs have been observed in many countries (5-7). Resistance to beta-lactam drugs is caused by chromosomal and plasmid encoded enzymes (5). Horizontal acquisition of AmpC β -lactamases represents an important driver of increasing resistance in Europe, and it is associated with the clonal expansion of resistant strains (8). The organism has achieved ability to cause infections leading to prolonged hospital stay, an increase of morbidity and mortality, and consequently an increase of health care associated costs (9).

ESBL-producing Gram-negative isolates are resistant to extended-spectrum cephalosporins and monobactams, except cefamycins, carbapenems, and β-lactam/ β-lactamase inhibitors; AmpC beta-lactamases producing isolates are resistant to penicillins, cephalosporins, cephamycins, monobactams and β-lactam/β-lactamase inhibitor, but usually sensitive to carbapenems (10). *Proteus* spp. are intrinsically resistant to nitrofurantoin and tetracycline. Although *Proteus* spp. are intrinsically susceptible to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole, co-resistance to these drugs has been frequently reported among ESBL-producing *Proteus* spp. isolates, and the treatment is often limited to carbapenems (11). Reportedly, susceptibility of *Proteus* spp. to β-lactam/β-lactamase inhibitors, ciprofloxacin, and third generation cephalosporins varies widely, 74- 94%, 60- 90%, and 90- 99%, respectively, depending on patients' age, gender, hospital department, duration of hospitalization and infection type (12).

The ESBLs commonly reported among *Proteus* spp. are TEM and CTX-M, while other ESBLs (SHV, TEM β-lactamase inhibitors) are less frequent (13). TEM, CTX-M and other β-lactamases have been detected among isolates from Iran (1), France (2), Saudi Arabia (14), Italy (15), and Japan (16).

Investigations of molecular characteristics and epidemiology of β-lactamase producing *Proteus* spp. in Bosnia and Herzegovina (B&H) are scarce. TEM, CTX-M and AmpC genes have been recently reported in Gram-negative bacteria, including *Proteus* spp., causing urinary tract infections in Zenica-Doboj Canton (17).

The aim of the study was to investigate molecular epidemiology and genetic relatedness of AmpC β-lactamase- and ESBL-producing *Proteus* spp. inpatient and outpatient isolates, as well as their antimicrobial susceptibility.

MATERIALS AND METHODS

Setting, bacterial isolates and study design

During the period December 2009 - May 2010, the total of 9092 and 16037 samples from the inpatients and outpatients, respectively, were collected at the Microbiology Laboratory of the Cantonal Hospital Zenica. Among inpatients, Gram-negative bacteria were isolated from 1254 (72.7%) samples, of which ESBL and/or AmpC β-lactamase producing bacteria were detected in 126 (out of 1254, 10%) samples; *Proteus* spp. were isolated from 201 (out of 1254, 16.0%) samples, of which 27 (out of 201; 13.4%) were ESBL and/or AmpC β-lactamase producing isolates. Among outpatients, Gram-negative bacteria were isolated from 2857 (80.9%) samples, of which 184 (6.4%) were ESBL- and/or AmpC β-lactamase producing bacteria; *Proteus* spp. were isolated from 365 (out of 2857; 12.8%) samples, of which 26 (out of 365; 7.1%) were β-lactamase producing isolates.

Among 53 ESBL- and /or AmpC β-lactamase-producing *Proteus* spp. (27 in- and 26 outpatients), 11 (six inpatient and five outpatient) were available for further analysis.

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

Antimicrobial susceptibility testing

Susceptibility testing to 12 antimicrobials was performed by a twofold microdilution technique

according to CLSI (Clinical and Laboratory Standards Institute) standard procedure (18): amoxycillin+clavulanic acid (AMC), cefazolin (CZ), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), cefoxitin (FOX), cefepime (FEP), imipenem (IMP), meropenem (MEM), gentamicin (GM), and ciprofloxacin (CIP). The following MIC resistance breakpoints were used: ≥32 for amoxicillin/clavulanic, cefazolin, cefuroxime, ceftazidime, cefoxitime and cefepime; ≥64 for cefotaxime and ceftriaxone; ≥16 for imipenem and meropenem; ≥8 for gentamicine; and ≥4 for ciprofloxacin. *E. coli* ATCC 25922 (ESBL negative) and *K. pneumoniae* 700603 (ESBL positive) were used as quality control strains.

Detection of ESBLs, AmpC beta-lactamases and carbapenemases

ESBL production was determined by double-disk-synergy test (DDST). Overnight broth culture of test strain was diluted in saline, adjusted to McFarland standard suspension 0.5 and inoculated onto Mueller-Hinton agar (MH); 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 (18).

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 (10μg) 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 overnight incubation was at least 5 mm larger than that of the corresponding cephalosporin disk (18).

Proteus spp. isolates resistant to extended-spectrum cephalosporins and β-lactam/ β-lactamase inhibitor combination (amoxicillin/clavulanic acid) were screened for production of AmpC β -lactamases by combined disk test using 3-amino phenylboronic acid (PBA) (Sigma-Aldrich, Steinheim, Germany). The stock solution was prepared as previously recommended (19) by dissolving PBA (benzeneboronic acid; Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide at a concentration of 20 mg/mL. 20 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 onto the agar. 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 with that 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 a β-lactam disk with boronic acid was ≥5 mm larger than that around a disk containing the β-lactam substrate alone (20).

Production of carbapenemases of group A or group B was confirmed by combined disk-test using meropenem disks with PBA and EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich, Steinheim, Germany), respectively (21). Three meropenem (MEM) disks were placed on Mueller-Hinton agar plate inoculated with test strain. 10 μL of EDTA (300 mg) and PBA (300 mg) was added on the first and third disks, respectively. The difference in zone size of ≥5 mm between disks with and without EDTA was suggesting production of carbapenemase group B, and the difference in zone size of ≥5 mm between disks with and without PBA was suggesting production of carbapenemase group A (21).

PCR detection of bla_{CTX-M}, bla_{SHV}, bla_{TEM}, and bla_{KPC} genes

PCR was used to detect alleles encoding ESBL enzymes.

The presence of bla_{TEM}, $bla_{SHV,}$ bla_{CTX-M} genes was investigated by polymerase chain reaction (PCR) using primers and conditions as described previously (22). Designation of *bla* genes based on identified mutations was done according to Bush K and Jacoby GA (23).

Primers IS26F (5'-GCG-GTA-AAT-CGT-GGA-GTG-AT-3) and IS26R (5'-ATT-CGG-CAA-GTT-TTT-GCT-GT-3') were used to amplify 400 bp fragment spanning the link between IS26 insertion sequence and bla_{CTX-M} gene in CTX-M producing isolate (24, 25).

Genes encoding carbapenemases of class A (KPC), class B (MBLs belonging to VIM, IMP and NDM family) and OXA-48 was detected by PCR as described previously (21).

Pulsed-field gel electrophoresis (PFGE) of bacterial DNA

Isolation of genomic DNA, digestion with the *Xba*I restriction enzyme (Invitrogen) and PFGE of the resulting fragments was performed as described by Kaufman et al (26). The electrophoresis was carried out with a CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, CA). The PFGE patterns were compared following the criteria of Tenover et al. (27) and analyzed by the GelComparII software (Applied Maths, St Martens, Belgium).

RESULTS

All *Proteus* spp. isolates

Infections caused by *Proteus* spp. were represented in our sample with 16% and 12.8% prevalence among Gram-negative bacteria in the inpatients and outpatients, respectively.

A total of 53 ESBL and/or AmpC beta-lactamase producing *Proteus* spp. (27 in- and 26 outpatients) were isolated: 22 (41.5%) were from urine samples (15 from outpatients), 18 (34.0%) from wound infections (nine from outpatients), three (5.7%) from stoma, two in each catheter, skin, and upper respiratory tract (URT, outpatient), and one in each cannula, ear, genital tract (outpatient vaginal swab) and umbilicus. Samples were collected from six different municipalities of Zenica-Doboj Canton, predominantly from Zenica city (56.6%).

Fourteen (out of 27; 51.8%) inpatients were older than 60 years of age. The duration of hospitalization of patients was 3-53 days (median=15). Six patients were from the Surgery Department, four from Internal Medicine and four from Neurology Department, three from Intensive Care Unit (ICU) and from the Department for Ear, Nose and Throat each, two from Pediatric and Physical Medicine and Rehabilitation Departments–each and one from Oncology, Infection and Urology Departments each.

Amoxicillin/clavulanic acid, cefazolin and metronidazole were mostly used for the treatment of infections associated with *Proteus* spp., in eight, seven and six cases, respectively; twelve inpatients received corticosteroid therapy. Nine patients had positive history of hospitalization in previous twelve months, and 21 inpatients had contact with persons with positive history of recent hospitalization.

Fifteen (out of 26; 57.7%) outpatients were 19-59 years of age. Other data for outpatients were missing.

Overall resistance rates to cephalexin, cefuroxime, ceftazidime, ceftriaxone, cefotaxime, and cefepime by disk-diffusion method of 80.4%, 71.7%, 67.9%, 67.9%, 56.4% and 45.3, respectively, were noticed in 53 AmpC- and/or ESBL producing strains. Resistance rates for cefixime, trimethoprim/sulfamethoxazole, gentamicine, ciprofloxacine, nitrofurantoin, amoxicillin/clavulanic and amikacin were 80.0%, 75.5%, 68.7%, 60.8%, 52.6%, 41.5% and 26.4%, respectively (data have not shown).

AmpC- and/or ESBL producing *Proteus* spp.

Eleven (six inpatients and five outpatients) AmpC- and/or ESBL producing *Proteus* spp. (seven *Proteus mirabilis* and four *Proteus vulgaris*) isolates were available for further analysis: four from urine, two wound swabs and one swab of each stoma, cannula, ear, skin and soft tissue infection (SSTI) and genital tract (vaginal swab).

Antibiotic susceptibility

Susceptibility rate for 11 AmpC- and/or ESBL-producing *Proteus* spp. (seven *Proteus mirabilis* and four *Proteus vulgaris*) isolates was 100% for amoxicillin-clavulanic acid, imipenem and meropenem.

Resistance rate to cefuroxime was100%, to gentamicine 90.9%, cefotaxim, ceftriaxone and ceftazidime 72.7% each, cefoxitine and ciprofloxacine 63.6% each, and for cefepime 45.5%.

In five (out of 11) isolates multi-drug resistance (MDR) to cephalosporins, cefamicines, amynocligosides and fluoroquinolones was detected.

Seven cefoxitin-resistant isolates (five in- and two -outpatients) additionally showed resistance to amoxicillin (100%), gentamicine (100%), the first, second, third cephalosporin generation (85.7%), ciprofloxacin (71.4%), and cefepime (42.9%), but they remained sensitive (100%) to amoxicillin/clavulanic acid.

All outpatient isolates were 100% resistant to aminoglycosides.

Detection and characterisation of ß-lactamases

Eleven *Proteus* spp. isolates were positive on β-lactamases by phenotypic test. All isolates produced nature TEM-1 β-lactamase. No isolates possessed SHV β-lactamase.

All six inpatient isolates, besides TEM-1 β-lactamase, produced CTX-M β-lactamase (four isolates harboured $bla_{\text{CTX-M-1}}$ and two $bla_{\text{CTX-M-15}}$ genes) and OXA-1 β-lactamase; one isolate additionally co-produced CMY-2 AmpC β-lactamase (Table 1).

One outpatient isolate, besides TEM-1 β-lactamase, was positive for CTX-M-and OXA-1 β-lactamase, and one isolate harbored, except TEM-1, AmpC β-lactamase too ($bla_{\text{CMY-2}}$ gene) (Table 1).

PFGE typing

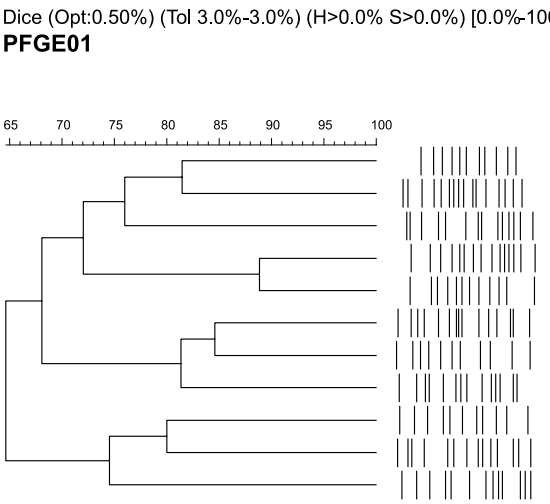
Eleven isolates were tested for genetic relatedness by PFGE typing. Four clones (A-D) and two singletons were identified among 11 *Proteus* spp. using a similarity threshold of 80%. Both clones A and B consisted of two outpatient isolates. Clone C comprised three, and clone D consisted of two inpatient isolates (Figure 1, Table 1).

DISCUSSION

The prevalence of ESBLs in *Proteus* spp. has an increasing trend in worldwide, including the United States, Asia and Europe (28). The results of this study have shown 9.4% ESBL and/or AmpC producers among 566 *Proteus* spp. isolated from clinical specimens, which indicates a low prevalence. This result is similar with reports from Tehran (12%), Japan (7-13%) and Nigeria (8%) (1,16,29,30). Higher prevalence of ESBL-producing *Proteus* spp. was noticed in India (60%) (31), Croatia (21%) (32) and Uganda (14%) (33).

Risk factors for infection caused by ESBL and/or AmpC- beta lactamase producing isolates are long hospitalization, female sex, older age and previous treatment with antibiotics (1). Elderly patients and previous treatment with antibiotics in this study were factors associated with the presence of ESBL-producing isolates, and it is similar to a report from Taiwan (12). Wang et al. reported that both prior antibiotic use and/or broad-spectrum antibiotic exposure could result in acquisition of drug-resistance genes (12). Based on our results, 82% ESBL-producers were obtained from males, and it is similar to reports from France (2) and Italy (34).

ESBL and/or AmpC- beta lactamase producing *Proteus* spp. isolates in this research have shown



PFGE01

Gender	Isolate origin	Age	Department	PFGE clone
M	Urine	02	Outpatient	03.02.2010.
M	Wound	28	Outpatient	29.01.2010.
M	Cannula	66	Neurology	26.12.2009.
M	Wound	28	Outpatient	05.03.2010.
F	Vagina	58	Outpatient	05.02.2010.
M	Stoma	54	Neurology	30.03.2010.
M	SSTI	53	Oncology	07.01.2010.
M	Ear	49	ENT	09.03.2010.
M	Urine	40	Physiatry	29.01.2010.
F	Urine	58	Neurology	22.01.2010.
M	Urine	01	Outpatient	26.01.2010.

Figure 1. Dendogram shows genetic relatedness of 11 *Proteus* spp. isolates by PFGE typing using the 80% similarity. Clones A and B consisted of two outpatient isolates. Clone C consisted of three inpatient, and clone D of two inpatient isolates SSTI, skin and soft tissue infection; ENT, Ear, nose and Throat;

Table 1. Antibiotic susceptibility of 11 AmpC- and/or ESBL-producing *Proteus* spp. strains

Protocol No	Agent	Gender	Isolate origin	Age (years)	Hospital department at present stay	Minimal inhibitory concentration (MIC, mg/L) of antibiotics* according to CLSI 2009/2014 (2009/2014 breakpoint)												β-lactamase	PFGE clone
						AMC (≥32/≥32)	CZ (≥32/≥32)	CXM (≥32/≥32)	CAZ (≥32/≥16)	CTX (≥64/≥4)	CRO (≥64/≥4)	FOX (≥32/≥32)	FEP (≥32/≥16)	IMI (≥16/≥4)	MEM (≥16/≥4)	GM (≥8/≥16)	CIP (≥4/≥4)		
6858	*P. mirabilis*	F	Urine	58	Neurology	8 (S/S)	>32 (R/R)	>256 (R/R)	64 (R/R)	128 (R/R)	64 (R/R)	>256 (R/R)	16 (I/R)	2 (S/I)	<0.06 (S/S)	64 (R/R)	128 (R/R)	TEM-1, CTX-M-15, OXA-1, CMY-2	D
7627	*P. mirabilis*	M	Cannula	66	Neurology	16 (S/S)	>32 (R/R)	>256 (R/R)	32 (R/R)	64 (R/R)	64 (R/R)	256 (R/R)	16 (I/R)	2 (S/I)	<0.06 (S/S)	64 (R/R)	256 (R/R)	TEM-1, CTX-M-15, OXA-1	S
26351	*P. mirabilis*	M	Stoma	54	Neurology	8 (S/S)	>32 (R/R)	>256 (R/R)	32 (R/R)	64 (R/R)	128 (R/R)	>256 (R/R)	32 (R/R)	2 (S/I)	<0.06 (S/S)	64 (R/R)	<0.12 (S/S)	TEM-1, CTX-M-1, OXA-1	C
9314	*P. vulgaris*	M	Urine	40	Physiatry	16 (S/S)	>32 (R/R)	>256 (R/R)	<0.12 (S/S)	128 (R/R)	>256 (R/R)	16 (I/I)	64 (R/R)	<0.06 (S/S)	<0.06 (S/S)	0,5 (S/S)	64 (R/R)	TEM-1, CTX-M-1, OXA-1	D
13011	*P. mirabilis*	M	SSTI	53	Oncology	8 (S/S)	>32 (R/R)	>256 (R/R)	64 (R/R)	128 (R/R)	32 (I/R)	>256 (R/R)	16 (I/R)	2 (S/I)	<0.06 (S/S)	64 (R/R)	128 (R/R)	TEM-1, CTX-M-1, OXA-1	C
23421	*P. mirabilis*	M	Ear	49	ENT	16 (S/S)	>32 (R/R)	>256 (R/R)	64 (R/R)	256 (R/R)	256 (R/R)	256 (R/R)	16 (I/R)	<0.06 (S/S)	<0.06 (S/S)	32 (R/R)	32 (R/R)	TEM-1, CTX-M-1, OXA-1	C
8608	*P. vulgaris*	F	Vaginal swab	58	Outpatient	4 (S/S)	>32 (R/R)	>256 (R/R)	64 (R/R)	8 (S/R)	8 (S/R)	>256 (R/R)	256 (R/R)	<0.06 (S/S)	0.25 (S/S)	8 (R/I)	<0.25 (S/S)	TEM-1	B
10913	*P. vulgaris*	M	Urine	02	Outpatient	4 (S/S)	>32 (R/R)	>256 (R/R)	256 (R/R)	128 (R/R)	64 (R/R)	4 (S/S)	16 (I/R)	0,25 (S/S)	2 (S/I)	64 (R/R)	32 (R/R)	TEM-1, CMY-2	A
6627	*P. mirabilis*	M	Urine	01	Outpatient	1 (S/S)	8 (S/S)	>256 (R/R)	<0.12 (S/S)	<0.12 (S/S)	<0,12 (S/S)	1 (S/S)	<0.12 (S/S)	0.12 (S/S)	<0.06 (S/S)	8 (R/I)	0.25 (S/S)	TEM-1	S
22367	*P. mirabilis*	M	Wound	28	Outpatient	16 (S/S)	>32 (R/R)	>256 (R/R)	32 (R/R)	>256 (R/R)	>256 (R/R)	128 (R/R)	128 (R/R)	<0.06 (S/S)	<0.06 (S/S)	>256 (R/R)	>128 (R/R)	TEM-1	A
52158	*P. vulgaris*	M	Wound	28	Outpatient	16 (S/S)	>32 (R/R)	>256 (R/R)	4 (S/S)	32 (I/R)	64 (R/R)	4 (S/S)	16 (I/R)	2 (S/I)	8 (S/R)	32 (R/R)	0.5 (S/S)	TEM-1, CTX-M-1, OXA-1	B

*AMC, amoxicillin+clavulanic acid; CZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; FEP, cefepime; IMI, imipenem; MEM, meropenem; GEN, gentamicin; CIP, ciprofloxacin; F, female, M, male; ENT, Ear, Nose and Throat Department

highest susceptibility rate to cefepime (64%), which is far higher than in the Tijjani et al. report (38%) (30), but susceptibility to cefoxitin and ciprofloxacin (36% and 40%, respectively) was similar in both studies. Besides, ten (out of 11) ESBL-producing isolates were resistant to gentamicin, which is similar to the reports from Tehran (1) and France (2).

A patient with the infection caused by ESBL and/or AmpC- beta lactamase producing *Proteus* spp. showed a high-prevalence of MDR to various classes of antibiotics, which is similar to a report from Ireland (8). Therefore, infections caused by such multidrug-resistant strains are more likely to result in therapeutic failure (1,17).

Clinical and Laboratory Standards Institute (CLSI) revised the criteria for interpretation of breakpoints for some beta-lactam antibiotics in Enterobacteriaceae to achieve easier identification of ESBLs and AmpC production (18, 35). Our results showed that the revised 2014 CLSI breakpoints (35) have significant (increasing) impact to resistance in *Proteus* spp. comparing to 2009 CLSI (18) for both cefotaxime and ceftriaxone, 90.9% /72.7%, for cefepime 90.9 /45.5, and even meropenem, 9.1/ 0, respectively. Similar results were obtained in the report from Taiwan (12). Resistance for gentamicine was lower considering CLSI 2014 revised version, 72.7%/90.9%, respectively, because breakpoint in 2009 was ≥8, but in revised CLSI 2014 it was ≥16. Unfortunately, only 11 AmpC/ESBL- producing *Proteus* spp. isolates were available for the analysis, and accordingly, there was no possibility to compare the differences in resistance between *Proteus* spp. isolates with and without ESBL/AmpC production in light of recent 2014 CLSI breakpoint revision toward CLSI 2009 breakpoint. Additionally, analysis of very low number of AmpC/ESBL producing strains obtained in this study could resulte in resistance rates bias, but it has not affected obvious overall increase of resistance rates considering 2014 CLSI revision breakpoints comparing to the 2009 one.

All AmpC/ESBL-producing *Proteus* spp. isolates from this study were susceptible to imipenem and meropenem according to CLSI 2009 document; similarly to the reports from Tanzania (36). But, resistance to meropenem showed up when considering CLSI 2014 (in one strain, which has shown MIC of 8μg/mL). It has usually described large difference in carbapenem susceptibility using imipenem different CLSI breakpoints (12). According to CDC document Facility Guidance for Control of Carbapenem-resistant Enterobacteriaceae (37) for bacteria that have intrinsic imipenem non-susceptibility, including *Morganella morganii*, *Proteus* spp., and *Providencia* spp., reporting of resistance to carbapenems other than imipenem is recommended, which is confirmed in our study.

Many ESBL genes are situated on large plasmids which carry multiple resistance genes, and this can explain an appearance of resistance to multiple antibiotics (36). TEM-1 (natural) β-lactamase was noticed in all *Proteus* spp. isolates in this study, and it is similar to Tehran and Nigeria studies with 100% TEM-1 prevalence (1,38). On the other hand, TEM-52 was found in all *Proteus* ESBL-producing isolates in the report from Croatia (32). In this study, CTX-M-1 and CTXM-15 were detected in 64% cases, similarly to the report from Tehran (1), but contradictory with the report from Japan, where CTX-M-2 gene was noticed in 67% cases (16).

The CMY-2 AmpC beta-lactamase was found in two *Proteus* spp. isolates in this study (one in- and one outpatient, both from urine), and both isolates were resistant to all cephalosporins tested, aminoglycosides and fluroquinolones, but susceptible to carbapenems and amoxicillin/clavulanic acid, which is in concordance with the report from Taiwan (12). AmpC β-lactamase producing isolates are usually resistant to several groups of antimicrobials, such as penicillins, oxyimino-, 7-α-methoxycephalosporins and monobactams (39). This could result in treatment failure as a consequence of inadequate empirical therapy in patients with unrecognized AmpC-carrying *Proteus* infections (31). It has been recently shown that ceftazidime-avibactam (non β-lactam/β-lactamase inhibitor) demonstrated potent activity against (molecularly confirmed) ESBL-, plasmid-mediated AmpC-, and ESBL/AmpC producing isolates of *P. mirabilis* (MIC_{90} of 0.06 μg/mL), as well as for other Gram-negative bacteria (40,41).

Different PFGE profiles among inpatient and outpatient AmpC- and/or ESBL-positive *Proteus* spp. strains indicate that in our settings the spre-

ading of these bacteria was not due to a single clone, but rather to the horizontal transfer of plasmids containing different genes between different species of Enterobacteriaceae. In the hospital environment, under selection pressure, plasmids could be transferred between the patients and hospital personnel by hands (24).

The main limitation of this study is the small number of AmpC- and/or ESBL-producing *Proteus* spp. isolates collected/available for the analysis because of short time span (six months) and their low prevalence in infections. Still, reduced susceptibility of *Proteus* isolates is a matter of an increasing clinical concern worldwide. Because of high prevalence of MDR strains in epidemiologically unrelated patients with AmpC- and/or ESBL producing *Proteus* spp. infection in this study, further local surveillance is needed. Molecular characterization and strain typing, or at least phenotypic test for AmpC/ESBL production of *Proteus* spp, as well as other Gram-negative bacteria, is important for appropriate therapy and the detection of the sources and modes of spread, which is the main step in order to design targeted infection control strategies.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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Molekularna epidemiologija i antimikrobna osjetljivost kliničkih izolata *Proteus* spp. koji produciraju AmpC- i/ili beta-laktamaza proširenog spektra djelovanja u Zeničko-Dobojskom kantonu, Bosna i Hercegovina

Selma Uzunović[1], Amir Ibrahimagić[1], Branka Bedenić[2,3]

[1]Služba za Laboratorijsku dijagnostiku, Institut za zdravlje i sigurnost hrane Zenica, [2]Medicinski fakultet, Sveučilište u Zagrebu, [3]Klinički zavod za kliničku i molekularnu mikrobiologiju, Klinički bolnički centar Zagreb, Zagreb, Hrvatska

SAŽETAK

Cilj Istražiti prevalenciju, molekularne karakteristike i klonsku pripadnost kliničkih izolata *Proteus* spp. koji produciraju AmpC- i(li) beta (β)-laktamaze proširenog spektra (ESBL), te njihovu antimikrobnu osjetljivost u Zeničko-dobojskom kantonu u Bosni i Hercegovini.

Metode Za određivanje antimikrobne osjetljivosti korištena je disk-difuzijska i mikrodilucijska metoda prema CLSI-standardima, a za detekciju ESBL-a dvostruki disk-sinergistički test i kombinirani disk-test s fenil-boroničnom kiselinom za detekciju AmpC β-laktamaza. Za potvrdu lučenja ESBL-a i AmpC-a korišten je PCR. Klonska pripadnost izolata ispitivana je uz pomoć elektroforeze u pulsirajućem polju (PFGE).

Rezultati U analizu je uključeno 11 (6 bolničkih i 5 vanbolničkih) AmpC- i(li) ESBL-producirajućih *Proteus* spp. izolata. Svi izolati su pokazali 100% osjetljivost na amoksicilin/klavulansku kiselinu, imipenem i meropenem. Rezistencija na cefuroksim zabilježena je u 100%, na gentamicin 90,9%, piperacilin/tazobaktam 81,8%, cefotaksim, ceftriakson i ceftazidim 72,7%, cefoksitin i ciprofloksacin 63,6% i na cefepim u 45,5% slučajeva. TEM-1 β-laktamaza detektirana je kod svih izolata, a kod 7 (od 11; 63,6%) izolata dodatno iCTX-M (pet CTX-M-1 i dvije CTX-M-15) i OXA-1 β-laktamaze. Dva izolata su producirala CMY-2 β-laktamazu, dok SHV beta-laktamaze nisu zabilježene. Nije zabilježena klonska pripadnost.

Zaključak Molekularna karakterizacija i tipizacija, te fenotipski testovi za detekciju AmpC i ESBL-producirajućih *Proteus* spp. izolata, kao i drugih gram-negativnih bakterija, važni su koraci u dizajniranju i primjeni terapije, u detekciji izvora i širenju izolata, kao i za pripremu strategije u sprečavanju širenja ovih infekcija.

Ključne riječi: ESBL, CMY-2, antimikrobna otpornost