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Antibiotic resistance in *Enterobacter cloacae* strains with derepressed/partly derepressed/inducible AmpC and extended-spectrum beta-lactamases in Zenica-Doboj Canton, Bosnia and Herzegovina

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ABSTRACT

Aim To investigate the prevalence of derepressed/partly derepressed/inducible and ESBL/AmpC-producing *Enterobacter cloacae* isolates and treatment options for infections associated with those isolates.

Methods Antibiotic susceptibility was determined by disc diffusion and broth microdilution according to CLSI guidelines. Double-disk synergy test (DDST) 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 Among 14 isolates with the ESBL positive *E. cloacae* producing isolates, four (28.6%), nine (64.3%) and one (7.1%) isolates were derepressed/partly derepressed and inducible AmpC producers. Eleven (out of 14) isolates were resistant to cefotaxime, ceftazidime, ceftriaxone, aminoglycosides and fluoroquinolones. All isolates were susceptible to imipenem and meropenem, 79% to cefepime. Five (out of 14; 35.7%) isolates (four derepressed and one inducible AmpC carrying *E. cloacae*) were negative in phenotypic test for ESBLs, but positive for broad spectrum TEM-1 β -lactamase. One (out of four derepressed) also produced CMY-2 β -lactamase. Four (out of nine) partly derepressed isolates were positive with the DDST, but did not yield PCR products with primers targeting TEM, SHV and CTX-M beta-lactamases. Four positive partly derepressed isolates carried a *bla*_{CTX-M-1} gene, two *bla*_{OXA-1} one *bla*_{CTX-M-15, OXA-1} and one *bla*_{CTX-M-28, OXA-1} (n=1).

Conclusion Microbiology laboratories must be able to detect and recognize AmpC-carrying isolates in a timely manner, especially those that are falsely susceptible *in vitro* to drugs that may be considered for therapy of infected patients.

Key words: chromosomal AmpC, ESBL, antibiotic resistance

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INTRODUCTION

Enterobacter cloacae is a nosocomial pathogen that can cause a range of infections such as bacteremia, lower respiratory tract infection, skin and soft tissue infections, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections (1). It has intrinsic chromosomal resistance to penicillins, first generation of cephalosporins, cephamycins and beta (β)-lactam/ β lactamase inhibitors due to the chromosomal AmpC β -lactamase (2). Overexpression of AmpC beta-lactamase causes resistance to third generation of cephalosporins. There are two main reasons for absence of guidelines for extended-spectrum beta-lactamase (ESBL) detection in Enterobacteriaceae with inducible chromosomal AmpC β -lactamases production, including *Enterobacter* spp. (3). First, AmpC expression may mask the synergy required for detection of ESBLs based on the synergy between third-generation cephalosporins and clavulanic acid (4), and second, the significance of ESBL detection in Enterobacteriaceae with inducible AmpC expression is considered to have limited therapeutic consequence (5,6). Cephalosporin resistance in *E. cloacae* is mainly due by overproduction (derepression) of the class-I beta-lactamase encoded by the chromosomal AmpC gene. Stable derepressed mutants are segregated from inducible strains at relatively high frequencies (7).

Classification of *E. cloacae* strains having derepressed, partly derepressed, or inducible AmpC production was determined by Sanders and Pai methods (8,9), in which cefoxitin-cefotaxime antagonist test should be performed (8,9). Prevalence of these mutants was largely investigated (10,11), because therapeutic failures with cephalosporin treatment have been associated with the selection and ultimate dominance of these variants (7,12). Cefepime was a drug of choice for the treatment of even infections caused by *E. cloacae* strains with AmpC overproduction (8), but reduced susceptibility to cefepime appeared very soon (13).

There has been no description of chromosomal AmpC- β -lactamases in *Enterobacter* spp. in Bosnia and Herzegovina (B&H) so far. During the 2009-2010 investigation of infections caused by ESBL-producing Gram-negative bacteria intermediate susceptibility to cefepime was noticed

in 43% (out of 30 ESBL-producing) *E. cloacae* isolates by disc-diffusion, as well as MIC of >16 $\mu\text{g/mL}$ for cefepime was found in 57% isolates (Ibrahimagić, unpublished data). It was prompted us to investigate mechanisms of reduced cefepime susceptibility, e.g. types of chromosomal AmpC beta-lactamases and molecular characteristics of ESBLs in these isolates.

The aim of the study was to investigate the occurrence of derepressed, partly derepressed and inducible AmpC and extended-spectrum β -lactamases in *E. cloacae* isolates causing in- and outpatient infections in Zenica-Doboj Canton, Bosnia and Herzegovina, and their antibiotic susceptibility according to the breakpoint changes in CLSI-2009/CLSI-2014 documents.

MATERIALS AND METHODS

Setting, bacterial isolates and study design

During the period December 2009 to May 2010, a total of 9092 and 16037 samples from inpatients and outpatients, respectively, were collected in the Microbiology Laboratory of the Cantonal Hospital Zenica.

Among inpatients, Gram-negative bacteria were isolated from 1254 (13.8%) samples, of which ESBL and/or AmpC β -lactamase producing bacteria were detected in 126 (out of 1254, 10.0%) samples; *Enterobacter* spp. were isolated from 32 (out of 1254, 2.6%) samples, of which 14 (out of 32; 43.7%) were ESBL and/or AmpC β -lactamase producing isolates. Among outpatients, Gram-negative bacteria were isolated from 2857 (17.8%) samples, of which 184 (6.4%) were ESBL- and/or AmpC β -lactamase producing bacteria; *Enterobacter* spp. were isolated from 22 (out of 2857; 0.8%) samples, of which 16 (out of 22; 72.7%) were β -lactamase producing isolates.

Among 30 ESBL- and /or AmpC β -lactamase-producing *Enterobacter* spp. (14 in- and 16 outpatients), 14 (eight inpatient and six outpatient) were available for further analysis.

An 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 14 antimicrobials (Oxoid, Basingstoke, UK) initially was performed by

disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute) standard procedure: amoxicillin (AMX; 30 µg), amoxicillin+clavulanic acid (AMC; 20+10 µg), cefalexin (CN; 30 µg), cefazolin (CZ; 30 µg), cefuroxime (CXM; 30 µg), ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), ceftriaxone (CRO; 30 µg), cefoxitin (FOX; 30 µg), cefepime (FEP; 30 µg), imipenem (IMP; 10 µg), meropenem (MEM; 10 µg), gentamicin (GM; 10 µg), and ciprofloxacin (CIP; 5 µg) (14). *E. coli* ATCC 25922 were used as quality control strain.

Susceptibility testing to 12 antimicrobials was performed by a two-fold microdilution technique according to CLSI (Clinical and Laboratory Standards Institute) standard procedure (15), AMC, CZ, CXM, CAZ, CTX, CRO, FOX, FEP, IMP, MEM, GM, CIP. Multi-drug resistance (MDR) was defined as resistance to three or more antimicrobial classes (2nd, 3rd or 4th generation cephalosporins, aminoglycosides, fluoroquinolones and carbapenems (8).

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 centre to centre) 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 zone of ≥ 5 mm around cephalosporine disks toward amoxicillin/clavulanate disk was indicative of ESBL production (14).

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 iso-

late on a 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 (14).

Enterobacter 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 (16) 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 five 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-inhibition zone around a β -lactam disk with boronic acid was ≥ 5 mm larger than that around a disk containing the β -lactam substrate alone (16).

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 (17). 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 (17).

Classification of *E. cloacae* as having inducible, partially derepressed, or derepressed AmpC production was determined by Sanders et al. method using cefoxitin-cefotaxime antagonist test (8,9). The ability of cefoxitin to antagonize the activity of cefotaxime was determined in disk approximation tests. A cefoxitin disk producing no zone of inhibition was placed on a seeded plate at a distance from the cefotaxime equivalent to the radius of the zone produced by the cefotaxime when tested alone. After overnight incubation at 37 °C, the radii of the zone a) between the cefoxitin and cefotaxime, and b) on the far side of the cefotaxime were measured. If the radius of a) was smaller than that of b) by 4 mm or more, then antagonism was considered to have occurred (18).

According to the characteristics of chromosomal AmpC β -lactamase production, resistance types were defined as follows: derepressed AmpC producers were resistant to cefoxitin (zone diameter ≤ 14 mm; MIC ≥ 32 $\mu\text{g/mL}$), resistant or intermediately susceptible to cefotaxime (≤ 22 mm; MIC ≥ 16 $\mu\text{g/mL}$), have a negative cefoxitin-cefotaxime antagonist test and a negative ESBL production; partly derepressed AmpC producers were resistant to cefoxitin (≤ 14 mm; MIC ≥ 32 $\mu\text{g/mL}$), resistant or intermediately susceptible to cefotaxime (≤ 22 mm; MIC ≥ 16 $\mu\text{g/mL}$), have a negative cefoxitin-cefotaxime antagonist test and produced ESBL; inducible AmpC producers were susceptible to cefoxitin (≥ 18 mm; MIC ≤ 16), resistant to cefotaxime (≤ 22 mm; MIC of ≥ 32 $\mu\text{g/mL}$), have a positive cefoxitin-cefotaxime antagonist test and a negative ESBL production (8).

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

PCR was used to detect alleles encoding ESBL enzymes.

The presence of bla_{TEM} , bla_{SHV} , $bla_{\text{CTX-M}}$ genes was investigated by polymerase chain reaction (PCR) using primers and conditions as described previously (19). Designation of bla genes based on identified mutations was done according to Bush K and Jacoby GA (20). 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 $bla_{\text{CTX-M}}$ gene in CTX-M producing isolate (21, 22).

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 (17).

Pulsed-field gel electrophoresis (PFGE) of bacterial DNA

Isolation of genomic DNA, digestion with the *XbaI* restriction enzyme (Invitrogen) and PFGE of the resulting fragments was performed as described by Kaufman et al (23). 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 (24) and analysed by the GelComparII software (Applied Maths, St Martens, Belgium).

RESULTS

All AmpC- and/or ESBL producing *Enterobacter* spp. isolates

Infections caused by *Enterobacter* spp. were represented in our sample with 2.6% and 0.8% (32 and 22 isolates) prevalence among Gram-negative bacteria in inpatients and outpatients, respectively.

A total of 30 ESBL and/or AmpC beta-lactamase producing *Enterobacter* spp. (14 in- and 16 outpatients) were isolated: 18 (60.0%) were from urine samples (12 from outpatients), eight (26.7%) from wound infection (four from outpatients), and one in each cannula, upper respiratory tract, umbilicus and punctate. Samples were collected from six different municipalities of Zenica-Doboj Canton, predominantly from Zenica city (56.7%).

Ten (out of 14; 71.4%) inpatients were older than 60 years of age. The duration of hospitalization of patients was 6-40 days (median=15). Four patients were from Internal Medicine Department, four from Intensive Care Unit (ICU) and four from Neurology Department, and two from Paediatric Department.

Amoxicillin/clavulanic acid and cefazolin were mostly used for the treatment of infections associated with *Enterobacter* spp., in five cases each, respectively; seven inpatients received corticosteroid therapy. Seven patients had positive history of hospitalization in previous twelve months, and 12 inpatients had contacts with persons having positive history of recent hospitalization.

Eleven (out of 16; 68.7%) outpatients were older than 60 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 100.0%, 90.0%, 90.0%, 90.0%, 83.0% and 3.3% (50% were intermediate), respectively, were noticed in 30 AmpC- and/or ESBL producing strains. Resistance rates for amoxicillin, cefixime, trimethoprim/sulfamethoxazole, amoxicillin/clavulanic, nitrofurantoin, gentamicine, ciprofloxacin, and amikacin were 100.0%, 95.2%, 80.0%, 60.0%, 50.0%, 46.1%, 16.7% and 10.0%, respectively.

All isolates were susceptible to carbapenems (data have not shown).

Characterisation of AmpC/ESBL in 14 *Enterobacter cloacae* isolates

Fourteen (eight inpatient and six outpatient) out of 30 AmpC- and/or ESBL-producing *E. cloacae* isolates were available for molecular analysis. Three isolates originated from Intensive Care Unit (ICU) (two wound infections and one aspirate), two from Internal Medicine Department (upper respiratory tract and urine), two from Paediatric (smear of umbilicus and urine), and one from Neurology Department (urine).

Table 1. Antibiotic susceptibility of 14 AmpC- and/or ESBL-producing *Enterobacter cloacae* strains

Protocol	Gen-Isolate	Age	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)			
17192/10	M	Wound	70	ICU	>32 (R/R)	>32 (R/R)	128 (R/R)	2 (S/S)	32 (S/R)	256 (R/R)	>256 (R/R)	16 (S/R)	<0.06 (S/S)	<0.06 (S/S)	16 (R/R)	4 (R/R)	dAmpC, TEM-1	NA
17200/10	M	Aspirate	66	ICU	>32 (R/R)	>32 (R/R)	<0.12 (S/S)	4 (S/S)	<0.12 (S/S)	<0.12 (S/S)	128 (R/R)	<0.12 (S/S)	0.25 (S/S)	<0.06 (S/S)	<0.12 (S/S)	<0.06 (S/S)	dAmpC, TEM-1	NA
8549/10	M	Resp.	68	Internal	8 (S/S)	>32 (R/R)	>256 (R/R)	>256 (R/R)	>256 (R/R)	>256 (R/R)	>128 (R/R)	128 (R/R)	64 (R/R)	0.12 (S/S)	4 (S/S)	8 (R/R)	pdAmpC, TEM-1, CTX-M-1, OXA-1	S
22040/10	M	Umbilicus	<01	Paediatrics	16 (S/S)	>32 (R/R)	>256 (R/R)	>256 (R/R)	>256 (R/R)	>256 (R/R)	>128 (R/R)	64 (R/R)	<0.06 (S/S)	<0.06 (S/S)	>256 (R/R)	256 (R/R)	CTX-M-15, OXA-1, SHV-1	NA
30322/10	F	Urine	61	Internal	16 (S/S)	>32 (R/R)	>256 (R/R)	256 (R/R)	128 (R/R)	256 (R/R)	>128 (R/R)	1 (S/S)	<0.06 (S/S)	<0.06 (S/S)	4 (S/S)	64 (R/R)	dAmpC, TEM-1, CMY-2	S
8851/10	F	Wound	73	ICU	>32 (R/R)	>32 (R/R)	>256 (R/R)	8 (S/S)	32 (S/R)	32 (S/R)	>128 (R/R)	8 (S/S)	<0.06 (S/S)	0.5 (S/S)	>256 (R/R)	2 (S/S)	pdAmpC, SHV-1	A
34356/10	M	Urine	<01	Paediatrics	128 (R/R)	>32 (R/R)	>256 (R/R)	256 (R/R)	>256 (R/R)	>256 (R/R)	8 (S/S)	128 (R/R)	<0.06 (S/S)	<0.06 (S/S)	32 (R/R)	32 (R/R)	pdAmpC, CTX-M-28, OXA-1	B
13590/10	F	Urine	82	Neurology	16 (S/S)	>32 (R/R)	>256 (R/R)	256 (R/R)	256 (R/R)	>256 (R/R)	>128 (R/R)	16 (S/R)	<0.06 (S/S)	1 (S/S)	256 (R/R)	128 (R/R)	dAmpC, TEM-1	B
84874	F	Wound	65	Outpatient	16 (S/S)	>32 (R/R)	>256 (R/R)	256 (R/R)	128 (R/R)	256 (R/R)	>256 (R/R)	16 (S/R)	<0.06 (S/S)	8 (S/R)	32 (R/R)	16 (R/R)	pdAmpC, SHV-1	B
13819	F	Wound	85	Outpatient	>32 (R/R)	>32 (R/R)	32 (R/R)	128 (R/R)	32 (S/R)	4 (S/S)	128 (R/R)	16 (S/R)	<0.06 (S/S)	<0.06 (S/S)	16 (R/R)	1 (S/S)	pdAmpC	S
10336	M	Urine	65	Outpatient	>32 (R/R)	>32 (R/R)	>256 (R/R)	64 (R/R)	64 (R/R)	>256 (R/R)	>256 (R/R)	16 (S/R)	0.25 (S/S)	<0.06 (S/S)	>256 (R/R)	4 (R/R)	pdAmpC, CTX-M-1, OXA-1	B
18730	M	Urine	70	Outpatient	>32 (R/R)	>32 (R/R)	>256 (R/R)	128 (R/R)	8 (S/R)	32 (S/R)	>256 (R/R)	<0.12 (S/S)	<0.06 (S/S)	<0.06 (S/S)	16 (R/R)	128 (R/R)	pdAmpC	A
30812	F	Urine	55	Outpatient	>32 (R/R)	>32 (R/R)	>256 (R/R)	64 (R/R)	16 (S/R)	64 (R/R)	>256 (R/R)	0.5 (S/S)	0.12 (S/S)	<0.06 (S/S)	16 (R/R)	2 (S/S)	pdAmpC	NA
14423	M	Wound	60	Outpatient	16 (S/S)	>32 (R/R)	>256 (R/R)	16 (S/R)	4 (S/R)	0.25 (S/S)	8 (S/S)	1 (S/S)	0.25 (S/S)	<0.06 (S/S)	128 (R/R)	64 (R/R)	iAmpC, TEM-1	B

*AMC, amoxicillin+clavulanic acid; CZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; FEP, cefepime; IMI, imipenem; MEM, meropenem; GM, gentamicin; CIP, ciprofloxacin; F, female, M, male; ICU, Intensive Care Unit; dAmpC, derepressed AmpC; pdAmpC, partly derepressed AmpC; iAmpC, inducible AmpC beta-lactamase; NA, not applicable; S, singleton

Of 14 ESBL-producing *Enterobacter cloacae* isolates, derepressed, partly derepressed and inducible AmpC β -lactamase was detected in four (28.6%), nine (64.3%) and one (7.1%) isolate, respectively. Four (out of nine) strains with partly derepressed AmpC beta-lactamase were also positive for bla_{CTX-M} gene (two isolates were encoding $bla_{CTX-M-1}$, one isolate $bla_{CTX-M-15}$ and one $bla_{CTX-M-28}$) and additionally co-produced TEM-1, SHV-1 or OXA-1. One strain (out of four, 25%) with the derepressed AmpC beta-lactamase was positive for bla_{CMY-2} and bla_{TEM-1} . Six isolates (three dAmpC, two pdAmpC and one iAmpC) produced only natural beta-lactamases (four isolates harboured bla_{TEM-1} and two bla_{SHV-1}). Among 14 AmpC chromosomally beta-lactamase producing isolates available for the analysis, four (out of 14; 28.6%) isolates did not possess any of natural beta-lactamases (TEM or SHV) (Table 1).

Antibiotic susceptibility – a comparison of CLSI-2009 and CLSI-2014

According to CLSI-2009/CLSI-2014 (14, 15, 31), a high prevalence of resistance to all cephalosporin antibiotics was noticed among 14 AmpC and/or ESBL-producing *Enterobacter cloacae* isolates ranging from 71.4-100%/78.6-100%, even to cefepime, of 21.4%/57.7%. According to both CLSI, gentamicin and ciprofloxacin also showed the same low activity, 78.6% and 71.4%, respectively. Resistance to imipenem and meropenem was noticed in 7.1%/7.1% and 0/7.1% (one isolate for each).

Multi drug resistance was detected in 78.6% (11 out of 14)/92.9% (13 out of 14), according to CLSI-2009/CLSI-2014, and all MDR isolates were resistant to cephalosporins, cefamicines, aminocyclitolides, fluoroquinolones and/or carbapenems.

MIC₉₀ for cefepime was 128 μ g/mL.

Two (out of four) strains with derepressed AmpC beta-lactamase were resistant to cefuroxime, ceftazidime, ceftriaxone, cefotaxime, and ciprofloxacin; one isolate was resistant to cefotaxime, but none had an MIC >64 mg/L (Table 1).

All nine strains with partly derepressed AmpC beta-lactamase were resistant to cefuroxime and gentamicin; all isolates were resistant to ceftazidime, the MIC did not exceed 64 mg/L. Six (66.7%) isolates were resistant to ciprofloxacin.

One strain with inducible AmpC beta-lactamase was susceptible to third and fourth cephalosporin generation, but resistant to gentamicin and ciprofloxacin.

PFGE typing

Ten isolates were tested for genetic relatedness by PFGE typing. Two clones and three singletons were identified among *E. cloacae* (A-B), using a similarity threshold of 80%. Clone A consisted of one inpatient and one outpatient isolate. Clone B comprised two inpatient and three outpatient isolates (Figure 1). *E. cloacae* isolates of clone A were resistant to cefazolin, cefoxitin, and gentamicin, but susceptible to cefotaxime and ceftriaxone. In clone B, all isolates were resistant to cefotaxime, ceftriaxone, ceftazidime, aminoglycosides and fluoroquinolones.

DISCUSSION

In this study an occurrence of derepressed, partly derepressed and inducible AmpC beta-lactamases was presented as well as characterisation of ESBLs in *E. cloacae* isolates obtained from in- and outpatients, and their antibiotic susceptibility according to the breakpoint changes in CLSI-2009/CLSI-2014 documents.

The partly derepressed AmpC β -lactamases were the most prevalent type in AmpC carrying *E. cloacae* isolates in this study which is in the contrast to the report from Vienna (Austria) with a high prevalence of inducible AmpC β -lactamases in *E. cloacae* (44%) (10), but it is similar to reports from France, Greece, Italy, Latvia, Luxembourg, Slovenia, Israel and Spain (25). Partly derepressed AmpC β -lactamase producing isolates in this study co-harboured CTX-M-1, CTX-M-15 and CTX-M-28.

In bacterial genera, such as *Enterobacter* spp., with the presence of inducible AmpC chromosomal enzymes, a detection of ESBLs is difficult (26). AmpC beta-lactamases in *E. cloacae* are mostly produced in the presence of clavulanate resulting in difficulties to detect ESBLs, because they mask a synergy between amoxicillin-clavulanate and cephalosporin disks (27).

Isolates co-producing both ESBL and AmpC β -lactamases have become more frequent worldwide (28). Prevalence of isolates co-producing both beta-lactamases in this study is low

(five, out of 14, 36%) and it is similar to a report from Asian-Pacific region (23%) (28). According to Jeong et al. if ESBL double disc synergy test is negative or inconclusive, and expanded-spectrum cephalosporins or aztreonam susceptibility is reduced ($\text{MIC} \geq 4 \mu\text{g/ml}$), the isolate should be retested by a method that is unaffected by AmpC beta-lactamases, e. g. with addition of cloxacilin in the culture medium or AmpC inhibitor (boronic acid) (29). In this study there were no ambiguous results.

Strains with AmpC genes are often resistant to multiple agents making therapeutic selection of effective antibiotic difficult (30). Most cephalosporins and penicillins should be avoided because of development of resistance during the therapy due to the potential for AmpC induction or selection of mutants (30). Unsatisfactory clinical outcome, with ceftazidime, cefotaxime and other cephalosporins is well documented (30).

Available inpatients data from this study showed that ceftazolin was mostly used drug in the treatment of infections caused by *E. cloacae* isolates carrying AmpC β -lactamases, but no data available for clinical outcome of the infections. Clinical failure even in cefotaxime treatment of *E. cloacae* infections was reported by Crowey et al. showing that although cefotaxime had good *in vitro* susceptibility ($\text{MIC} 2 \text{ mg/L}$) on MIC testing at the standard inoculum of 10^5 cfu/mL , the MIC rose dramatically when the inoculum was increased to 10^7 cfu/mL (64 mg/L) (26). Resistance rate for cefotaxime in the present study was double rose applying CLSI-2014 guideline (31) comparing to CLSI-2009 guideline (50%/93%) leading in therapeutic failure.

Cefepime is a poor inducer of AmpC beta-lactamase and it is stable to hydrolysis by these β -lactamases, thus the majority of AmpC-producing isolates are cefepime susceptible (30).

In this study, 21.4% (three isolates, all ESBL producers) were resistant to cefepime using $\text{MIC} \geq 32 \text{ mg/L}$ breakpoint, and it is in accordance with reports from Austria (13 %) (10) and the Netherlands (14 %) (32), but in the report from China 100% resistance to cefepime is noticed (33). It is very worrying, because in this study additional five isolates (out of 14) have shown intermediate susceptibility to cefepime with the MIC of $16 \mu\text{g/mL}$, which is even higher than from Pittsburgh report (three out of 15, MIC of 16 mg/L) (13).

MIC_{90} of $128 \mu\text{g/mL}$ for cefepime in the present study was higher than in the report from Pittsburgh where the MIC_{90} of $64 \mu\text{g/mL}$ was noticed (13) suggesting cefepime could not be used in the first line therapy.

According to CLSI 2014 and EUCAST 2014 guidelines (31, 34), prevalence of resistance to cefepime in the present study was 57% ($\text{MIC} \geq 16$) and 64% ($\text{MIC} \geq 4$), respectively, which is more than double comparing to CLSI 2009 (21%; $\text{MIC} \geq 32$).

Almost all isolates in this study were susceptible to meropenem and imipenem, similarly to the report from the Netherlands (32). Resistance rates for aminoglycosides and fluoroquinolones of 79% and 71%, respectively, are similar to the report from China (33) and Spain (35). Usually, carbapenem and cefepime therapy has been successful (33).

The definition of MDR (acquired non-susceptibility to at least one agent in three or more antimicrobial categories) is most frequently used for Gram-negative bacteria (36). In the case of *Enterobacter* spp. which has intrinsic resistance to the first and second cephalosporin generation, cefamycine, and penicillin+beta-lactam inhibitors, these agents/category should not be considered for detection of MDR (36). In this study, according to CLSI-2009/2014 guidelines (15, 31, 36) and EUCAST-2014 (34), 79%, 93% and 86% were detected as MDR isolates. Having in mind that *Enterobacter* spp. are either very resistant to many agents or could develop resistance during the course of antimicrobial therapy, the choice of appropriate antimicrobial agents is complicated (37). Colistin, polymyxin B or tigecycline are being used more frequently to treat serious infections caused by MDR *E. cloacae*, as monotherapy or in combination with other antibiotics (37).

Two PFGE clones were detected among both inpatient and outpatient AmpC carrying beta-lactamase producing *E. cloacae* isolates, indicating that beta-lactamase production was not due to the spreading of a single clone, but rather due to the horizontal transfer of plasmids containing different genes between different species (21). Different antibiotic resistance phenotypes of *E. cloacae* isolates in two clusters, as well as in isolates in the same cluster containing different genes (bla_{TEM} , bla_{SHV} , $bla_{\text{CTX-M}}$) has been found in this study. It is similar to the report from Spain (35).

Investigation of risk factors and identification of infections caused by *E. cloacae* with the chromosomal AmpC beta-lactamases are important for the management and control of health care associated infections (38-40).

The main limitation of this study is a small number of AmpC- and/or ESBL-producing *Enterobacter* spp. isolates collected/available for the analysis because of the short time span (six months) and their low prevalence in infections.

In conclusion, the prevalence of ESBLs in *E. cloacae* isolates causing infections in this study is low. Partly derepressed mutants are most frequent ones. Reduced susceptibility among *E. cloacae* isolates is a matter of an increasing concern worldwide. Molecular characterization of these strains is important for the detection of the sources of infections and mode of their spre-

ading, which is the main step in order to design targeted infection control strategies. It is important to include phenotypic detection of AmpC beta-lactamases in routine laboratory practice. Further clinical studies are needed to evaluate large numbers of patients treated with cefepime or carbapenems to assess the efficacy of these drugs in the treatment of AmpC beta-lactamase producing *E. cloacae* infections.

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TRANSPARENCY DECLARATION

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