EMERGENCE OF MULTIDRUG-RESISTANT *PROTEUS MIRABILIS* IN A NURSING HOME IN CROATIA

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BACKGROUND

The rapid emergence of antibiotic resistance among Gram-negative bacteria is serious threat to the management of infectious diseases. β-lactam antibiotics are the most frequently used antimicrobials for empirical therapy. Production of β-lactamases is one of the strategies adopted by bacteria to develop resistance to β-lactam class of antibiotics. The development of highly stable expanded-spectrum cephalosporins at the beginning of 1980s was quickly followed by the emergence of extended-spectrum β-lactamases (ESBL) in *Klebsiella pneumoniae* and other *Enterobacteriaceae*. These enzymes are usually plasmid-mediated and most frequently derived from parental TEM-1, TEM-2 and SHV-1 β-lactamases by point mutation that alter the configuration of active site to expand their spectrum of activity. AmpC enzymes hydrolyze first, second and third generation cephalosporins and cephamycins but spare cefepime and carbapenems. Unlike ESBLs they are not inhibited by clavulanic acid, sulbactam or tazobactam. *Proteus mirabilis* is an emerging cause of nosocomial infections, particularly of wounds and the urinary tract. The various types of P. *mirabilis* infections are difficult to treat because of acquisition of various resistance mechanisms such as ESBLs or AmpC β-lactamases. Recently, an increased frequency of multidrug-resistant *P. mirabilis* isolates was observed in nursing home in Zagreb. The role of *P. mirabilis* as an important multidrug-resistant pathogen in long-term care facilities is not investigated yet. In 2006, only 10% of *Proteus mirabilis* isolates were resistant to 3rd generation cephalosporins, while in 2007, the frequency of such strains increased to 24%

The previous report on ESBLs in *P. mirabilis* showed the high prevalence of TEM-52 β-lactamases in *P. mirabilis* strains from University Hospital Center Split. The aim of this study was the molecular characterization of cephalosporin resistance in *P. mirabilis* strains from a nursing home in Zagreb.

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MATERIAL AND METHODS

Bacteria

Twenty consecutive non-duplicate *P. mirabilis* strains with reduced susceptibility to ceftazidime (zone diameter of ⩽ 22mm) were isolated from urine samples during a period from October 1st 2013 until January 30 th 2014 from a nursing home Godan in Zagreb, Croatia. The isolates were identified by conventional biochemical tests using standard recommended techniques.

Susceptibility testing

The susceptibility testing to amoxicillin, cefuroxime, ceftazidime, gentamicin, tetracycline, chloramphenicol (Pliva, Zagreb), cefotaxime, ceftriaxone, cefepime, cefoxitin, aztreonam, ciprofloxacin (USP reference standard, Rockville, Maryland), piperacillin/tazobactam, sulfametoxazole, trimethoprim (Sigma), imipenem (MSD) and meropenem (AstraZeneca) was performed by a twofold microdilution technique according to CLSI standard procedures . *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strain. Detection of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases

A double-disk-synergy test (DDST) using the combination of amoxycillin/clavulanate with cefotaxime, ceftriaxone, ceftazidime, and aztreonam and combined disk test using disks of ceftazidime, cefotaxime, ceftriaxone and cefepime with and without clavulanate (10 µgl) were performed to detect ESBLs. Deformation of the inhibition zone aroung cephalosporin disks towards central disk with amoxicillin/clavulanate in DDST or augmentation of inhibition zone around cephalosporin disks for at least 5 mm in the presence of clavulanic acid compared to control disks without clavulanic acid in combined disk test indicated production of ESBL. *E. coli* ATCC 25922 was used as negative and *K. pneumoniae* ATCC 700603 as positive control.

Presumptive test for AmpC β-lactamases in considered positive if the inhibition zone for cefoxitin was ⩽ 18 mm. AmpC β-lactamases were phenotypically detected by combined disk test test using disks of ceftazidime, cefotaxime, ceftriaxone and cefepime with and without 3-amino-phenyboronic acid. AmpC production was indicated by an increase in zone size of 5 mm or more around cephalosporin disks containing 3-amino-phenyboronic acid compared to control disks containing only cephalosporins.

Conjugation

P. mirabilis strains were investigated for the transferability of their resistance determinants. Conjugation experiments were set up employing plasmid-free and rifampin-resistant *E. coli* A15 R- recipient strain. Transconjugants were selected on the combined plates containing ceftazidime (1 mg/L) and rifampicin (256 mg/L). The frequency of conjugation was expressed relatively to the number of donor cells.

Characterization of β-lactamases

The presence of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{PER-1} and bla_{ampC} genes was investigated by polymerase chain reaction (PCR) using primers and conditions as described previously. Template. Amplicons were column-purified (Quiagen DNA purification kit) and sequenced directly using ABI PRISM 377 Genetic Analyser (Applied Biosystems). Sequences were analyzed using BioEdit v.7.0.9. (Ibis Biosciences) program. Designation of *bla* genes based on identified mutations was done according to Bush, Jacoby and Medeiros sheme.

Detection of quinolone resistance determinants

Plasmid borne quinolone resistance genes-qnrA, qnrB and qnrS were determined by PCR as described previously.

Characterization of plasmids

Plasmids were extracted with Macherey Nagel mini kit. Plasmids extractions were subjected to PCR-based replicon typing (PBRT) according to Carattoli et al.

Table 1. Minimum inhibitory concentrations of various antibiotics for multidrug-resistant *Proteus mirabilis* strains.

Strain number	Protocol number	date	ESBL	AMPC	BL	AMX	AMC	PIP	TZP	CXM	CZ	CAZ	CTX	CRO	FEP	FOX	IMI	MEM	CIP	GM	CIP
1.	77005	29.04. 2013	-	+	CMY-16, TEM-1	>128	>128	>128	4	>128	>128	16	>128	16	1	>128	0.12	0.06	8	16	>128
2.	77008	29.04.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	2	>128	>128	32	>128	16	1	>128	0.12	0.06	8	64	>128
3.	77889	30.04.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	2	>128	>128	32	>128	8	2	>128	0.5	0.06	16	32	>128
4.	77896	30.04.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	2	>128	>128	32	>128	32	0.5	>128	0.5	0.06	32	32	>128
5.	77018	29.04. 2013.	-	+	CMY-16, TEM-1	>128	>128	>128	4	>128	>128	64	>128	32	0.25	>128	0.5	0.06	4	32	>128
6.	77005	29.04.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	4	>128	>128	32	>128	64	0.5	>128	0.5	0.06	16	4	>128
7.	77896	30.04. 2013.	-	+	CMY-16, TEM-1	>128	>128	>128	2	>128	>128	32	>128	32	0.5	>128	0.5	0.06	32	32	>128
8.	87894	16.05.2014.	-	+	CMY-16, TEM-1	>128	>128	>128	2	>128	>128	16	>128	32	1	>128	0.5	0.06	8	16	>128
9.	50452	06.09. 2013.	-	+	CMY-16, TEM-1	>128	>128	>128	4	>128	>128	32	>128	32	1	>128	0.5	0.06	16	64	>128
10.	62950	27.09. 2013.	-	+	CMY-16, TEM-1	>128	>128	>128	32	>128	>128	16	>128	32	8	>128	1	0.06	>128	64	>128
11.	64004	30.09.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	64	>128	>128	16	>128	64	1	>128	1	0.06	>128	64	>128
12.	68478	07.10.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	8	>128	>128	>128	>128	64	0.5	>128	0.5	0.06	32	64	>128
13.	85073	04.11.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	8	>128	>128	>128	>128	32	0.5	>128	2	0.06	32	64	>128
14.	85058	04.11.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	4	>128	>128	>128	>128	32	1	>128	2	0.06	32	64	>128
15.	85109	04.11.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	16	>128	>128	>128	>128	64	2	>128	2	0.06	32	64	>128
16.	206382	9.12.2013.	-	+	CMY-16, TEM-1	>128	>128	>128	16	>128	>128	>128	>128	16	0.5	>128	2	0.06	32	64	>128
17.	401916	19.08.2013.	-	+	CMY-16, TEM-1	>128	32	>128	8	>128	>128	>128	>128	32	16	>128	2	0.06	32	64	>128
18.	026852	18.02. 2014	-	+	CMY-16, TEM-1	>128	>128	>128	4	>128	>128	32	16	16	0.5	>128	2	0.06	32	64	>128
19.	079495	19.05.2014.	-	+	CMY-16, TEM-1	>128	>128	>128	64	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
20.	77841	15.05.2014	-	+	CMY-16, TEM-1	>128	>128	>128	2	>128	>128	>128	>128	>128	0.5	>128	1	0.06	32	64	>128

Abbreviations: AMX, amoxycillin; AMC, amoxycillin/clavulanic acid; CXM, cefuroxime; CZ cefazolin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; FOX, cefoxitin; AZT, aztreonam; PIP, piperacillin; TZP, piperacillin/tazobactam; IPM, imipenem; MEM, meropenem; GM, gentamicin; CIP, ciprofloxacin,
ESBL: phenotypic test for ESBLs, ampC-phenotypic test for AmpC beta-lactamases, BL: beta-lactamase content

RESULTS

Phenotypic detection of beta-lactamases and susceptibility testing

All strains tested phenotypically positive for AmpC but negative for ESBLs. An augmentation of the inhibition zones around cephalosporin disks of at least 5 mm was seen with PBA but not with clavulanic acid.

The strains were resistant to amoxicillin alone and combined with clavulanic acid, piperacillin, cefotaxime, ceftriaxone, cefoxitin, gentamicin, tetracycline, chloramphenicol, sulfametoxazole, trimethoprim and ciprofloxacin, but susceptible to cefepime, imipenem, and meropenem with MICs of imipenem being slightly higher than those of meropenem (Table 1). There was variable level of susceptibility/resistance to ceftazidime, ceftriaxone and to combination of piperacillin with tazobactam In disk-diffusion test all strains except one were resistant to sulfametoxazole /trimethoprim (cotrimoxazole).

Conjugation

The strains did not transfer resistance to E. coli recipient strain.

Characterization of β-lactamases

All twenty *P. mirabilis* strains yielded an amplicon of 853 bp with primers specific for CMY- β-lactamase genes. Sequencing of amplicons revealed the $blabla_{CMY-16}$ β-lactamase allele in all strains. The strains coharboured TEM-1 β-lactamase.

Detection of quinolone resistance determinants

QnrA, B and S genes were not found.

Characterization of plasmids

Plasmid encoding CMY-16 were did not belong to any known PBRT.

CONCLUSIONS

The study demonstrated predominance of plasmid-mediated AmpC β-lactamase CMY-16 among tested strains. AmpC β-lactamases detection is not routinely carried ot in many microbiology laboratories. This could be attributed to lack of awareness or lack of resources and facilities to conduct β-lactamase identification. AmpC β-lactamases are inhibited by phenylboronic acid and cloxacillin. There are severeal inhibitor-based tests for identification of AmpC β-lactamases including disk test and E-test .

The production of CMY β-lactamase was associated with resistance or reduced susceptibility to 3rd generation cephalosporins and combination of amoxicillin with clavulanic acid. The strains showed variable levels of susceptibility/resistance to piperacillin/tazobactam which would lead to conclusion that this comination is less affected by production of AmpC β-lactamase compared to amoxicillin/clavulanate. This could be attributed to better intrinsic activity of piperacillin against *P. mirabilis* compared to amoxicillin. The susceptibility to cefepime and carbapenems was maintained.

The recent studies found plasmid-mediated AmpC β-lactamases of CMY family in University Hospital Split and among *E. coli* isolates from companion animals in Croatia. Moreover, CMY-4 was identified as additional β-lactamase in Enterobacteriacea producing VIM or NDM metallo-β-lactamases in University Hospital Center Zagreb.

In the present study, we found an alarming number of AmpC-producing *P. mirabilis* in a nursing home in Zagreb. CMY β-lactamases originate from chromosomal AmpC β-lactamases of *Citrobacter freundii*.

The acquired bla_{CMY} genes have escaped from the chromosome of *C. freundii* folowing mobilization mediated by ISEcp1, IS26 or ISCR1. CMY-1, CMY-12 and CMY-16 were found to be the most prevalent variants of plasmid-mediated AmpC beta-lactamases in Europe. Simultaneous production of ESBLs and AmpC β-lactamases was also reported in *P. mirabilis* in recent studies.

From the therapeutic point of view, it is important to distinguish between ESBLs and AmpC β-lactamases because infections caused by AmpC positive strains can be effectively treated with cefepime and cefpirome. On the other hand uncomplicated urinary tract infections due to ESBL positive organisms can be treated with β-lactam/inhibitor combinations which are not recommended for AmpC producing organismsalthough our strains demonstrated in vitro susceptibility to piperacillin/tazobactam.

Considering the gravity of the implication of wrong therapy in cronically ill and debilitated patients in long-term care facilities, looking for AmpC β-lactamases must be mandatory in all microbiogical laboratories and clinicans should be educated on the importance of ESBLs and AmpC β-lactamases and therapeutic challenges that they pose.