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Emergence of multidrug-resistant *Proteus mirabilis* in a long-term care facility in Croatia

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Summary

Background An increased frequency of *Proteus mirabilis* isolates resistant to expanded-spectrum cephalosporins was observed recently in a long-term care facility in Zagreb (Godan). The aim of this study was the molecular characterization of resistance mechanisms to new cephalosporins in *P. mirabilis* isolates from this nursing home.

Methods Thirty-eight isolates collected from 2013–2015 showing reduced susceptibility to ceftazidime were investigated. Antibiotic susceptibilities were determined by broth microdilution method. Inhibitor-based tests were performed to detect extended-spectrum (ESBLs) and AmpC β-lactamases. AmpC β-lactamases were characterized by polymerase chain reaction (PCR) followed by sequencing of *bla*ampC genes. Quinolone resistance determinants (*qnr* genes) were characterized by PCR. Genotyping of the isolates was performed by repetitive element sequence (rep)-PCR and pulsed-field gel electrophoresis (PFGE).

Results Presence of an AmpC β-lactamase was confirmed in all isolates by combined-disk test with phenylboronic acid. All isolates were resistant to amoxicillin alone and combined with clavulanate, cefotaxime, ceftriaxone, cefoxitin, and ciprofloxacin; but susceptible to cefepime, imipenem, and meropenem. PCR followed by sequencing using primers targeting *bla*ampc genes revealed CMY-16 β-lactamase in all but one strain. *Bla*cmy-16 was carried by a non-conjugative plasmid which did not belong to any known plasmid-based replicon typing (PBRT) group. Rep-PCR identified one large clone consisting of 15 isolates, three pairs or related isolates, one triplet, and four singletons. PFGE confirmed the clonality of the isolates.

Conclusions This is the first report of multidrug resistant *P. mirabilis* in a nursing home in Croatia. Cephalosporin resistance was due to plasmid-mediated AmpC β-lactamase CMY-16.

Keywords CMY-16 · *Proteus mirabilis* · AmpC β-lactamases · Conjugative plasmid · Clonal dissemination

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Introduction

The rapid emergence of antibiotic resistance among Gram-negative bacteria is a serious threat to the management of infectious diseases. β-lactam antibiotics are the most frequently used antimicrobials for empirical therapy [1]. Production of β-lactamases is one of the strategies adopted by bacteria to develop resistance to the β-lactam class of antibiotics [1, 2]. The development of highly stable expanded-spectrum cephalosporins at the beginning of the 1980s was quickly followed by the emergence of extended-spectrum β-lactamases (ESBL) in *Klebsiella pneumoniae* and other *Enterobacteriaceae* [2]. These enzymes are usually plasmid-mediated and most frequently derived from parental TEM-1, TEM-2, and SHV-1 β-lactamases by point mutations that alter the configuration of the active site to expand their spectrum of activity [3]. 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 [4]. *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 [5, 6]. Recently, an increased frequency of multidrug-resistant *P. mirabilis* isolates was observed in a long-term care facility in Zagreb (Godan). The role of *P. mirabilis* as an important multidrug-resistant pathogen in long-term care facilities has not been investigated yet. The previous reports on ESBLs in *P. mirabilis* in Croatia showed the clonal spread of TEM-52 β-lactamase-producing *P. mirabilis* isolates in the University Hospital Center Split [7, 8]. Spread of multidrug-resistant *P. mirabilis* from hospitals to nursing homes was observed recently. This prompted us to conduct the molecular characterization of antibiotic resistance in *P. mirabilis* isolates from a nursing home in Zagreb.

Materials and methods

Bacteria

Thirty-eight consecutive non-duplicate *P. mirabilis* isolates with reduced susceptibility to ceftazidime (zone diameter ≤22 mm) were isolated from urine samples during a period from April 29, 2013 until January 21, 2015 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 alone and combined with clavulanate; and piperacillin alone and combined with tazobactam, cefazoline, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, cefepime, cefoxitin, imipenem, meropenem, gentamicin, and ciprofloxacin was performed by a twofold microdilution technique according to Clinical Laboratory Standard Institution (CLSI) standard procedures [9].

Disk diffusion test was performed for all antibiotics which are routinely tested in our laboratory for diagnostic purposes (amoxycillin alone and combined with clavulanic acid; piperacillin alone and combined with tazobactam, cephalexin, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, cefepime, cefoxitin, gentamicin, netilmicin, amikacin, ciprofloxacin, norfloxacin, sulphametoxazole/trimethoprim, and nitrofurantoin) prior to microdilution.

Escherichia coli ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control isolates.

Phenotypic characterization of β-lactamases

A double-disk synergy test (DDST) using the combination of amoxycillin/clavulanate with cefotaxime, ceftriaxone, ceftazidime, and aztreonam [10], as well as a combined disk test using disks of ceftazidime, cefotaxime, ceftriaxone, and cefepime with and without clavulanate (10 μg/ml) according to CLSI were performed to detect ESBLs [9]. Deformation of the inhibition zone around cephalosporin disks towards the 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 a negative and *K. pneumoniae* ATCC 700603 as a positive control.

Presumptive test for AmpC β-lactamases is considered positive if the inhibition zone for cefoxitin was ≤18 mm [9]. AmpC β-lactamases were phenotypically detected by combined disk test using disks of ceftazidime, cefotaxime, and ceftriaxone with and without 3-amino-phenyboronic acid (PBA). AmpC production was indicated by an increase in zone size of 5 mm or more around cephalosporin disks containing PBA compared to control disks containing only cephalosporins [11].

Conjugation

P. mirabilis isolates were investigated for the transferability of their resistance determinants. Conjugation experiments were set up employing plasmid-free and sodium azide-resistant *E. coli* A15 R⁻ recipient strain [12]. Transconjugants were selected on the combined plates containing ceftazidime (1 mg/L) and sodium azide (100 mg/L). The frequency of conjugation was expressed relative 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 de-

scribed previously [13–17]. In order to amplify the whole coding sequence, additional primers were used for amplification of bla_{CMY} genes, as described previously [18]. Template DNA was extracted by the boiling method. PCR mix (50 µl) contained 25 µl of master mix (Roche, Medical Intertrade, Zagreb, Croatia), 20 µl of ultrapure water, 1 µl of each primer (10 pmol), and 3 µl of template DNA. Lysates from reference strains producing TEM-1, TEM-2, SHV-1, SHV-2, SHV-4, SHV-5, CTX-M-15, PER-1, CMY-4, MIR-1, DHA-1, FOX-1, and MOX-1 were used as positive controls for PCR. Nucleotide sequences were determined directly on PCR products on both strands by the Microgene (Macrogene, Seoul, South Korea, sDNA sequencing service) DNA sequencing service. CMY and TEM amplicons were sequenced. Sequences were analyzed using BLAST program (National Center for Biotechnology Information, NCBI). Designation of *bla* genes based on identified mutations was done according to the Bush, Jacoby, and Medeiros scheme. The presence of IS*Ecp1* and IS26 in the region upstream of bla_{CMY} genes was investigated by combining IS26 and IS*Ecp1* forward primers with reverse primers for bla_{CMY} [19].

Detection of quinolone resistance determinants
Plasmid borne quinolone resistance genes *qnr*A, *qnr*B and *qnr*S were determined by PCR as described previously [20].

Characterization of plasmids
Plasmids were extracted with the Macherey Nagel mini kit (Hilden, Germany) according to manufacturer's recommendations. Plasmids extractions were subjected to PCR-based replicon typing (PBRT) according to Carattoli et al. [21], and to PCR with primers specific for TEM and CMY β-lactamases to determine the location of *bla* genes.

Genotyping of isolates
Twenty-eight isolates were subjected to molecular typing by rep-PCR as described previously [22] DNA was isolated by Ultra-Clean microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), as recommended by the manufacturer. The DNA concentration was measured and set between 25 and 30 ng/L. Subsequently, the DNA was amplified using the Bacterial fingerprinting kit (Bacterial barcodes, bioMerieux, Athens, GA, USA), according to the manufacturer's instructions. PCR was performed using the following parameters: initial denaturation (94 °C) for 2 min; and then 35 cycles of 30 s of denaturation (94 °C), 30 s of annealing (60 °C), and 90 s of extension (70 °C); followed by 3 min of final extension (70 °C); and ending at 4 °C. The amplification products were separated with the Agilent B2100 bioanalyzer. Five microliters of DNA standard markers (used for normalization of sample runs) and 1 µl of the DNA product were used. All data were entered in the DiversiLab software system. Cutoff value of 97 % was used to define a clone.

Pulsed-field genotyping of SfiI-digested genomic DNA was performed on 30 isolates with a CHEF-DRIII system (Bio-Rad); the images were processed using the Gel-Compar software, and a dendrogram was computed after band intensity correlation using global alignment with 1.5 % optimization and tolerance and unweighted pair-group method using arithmetical averages (UPGMA) clustering. The strains were considered to be clonally related if they showed more than 80 % similarity of their PFGE patterns [23, 24].

Results

Patients

All patients were residents of the Godan long-term care facility. Since the Godan nursing home is located close to the University Hospital Center Zagreb where the urine samples were processed, we found in the hospital internet system that 23 of 38 patients were previously hospitalized in the University Hospital Center in the intensive care unit, pulmonary unit, abdominal surgery, hematology, ophthalmology, gastroenterology, cardiology, and oncology departments. Three patients were only examined in the emergency room in order to change the urinary catheter or to obtain the blood transfusion but did not stay in the hospital. They all had severe underlying diseases such as coronary artery disease; myocardial infarction; adenocarcinoma ventriculi; pancreatic cancer; chronic lymphocytic or myelocytic leukemia; prostatic adenocarcinoma; respiratory insufficiency; kidney failure; megaloblastic and hypochromic anemia; Morbus Alzheimer; pulmonary embolia; and diabetes mellitus. Two patients suffered from bronchopneumonia and were treated with azithromycine and ceftriaxone. All patients had urinary tract infection with $> 10^5$ CFU/ml of *P. mirabilis* and white blood cells in the urinary sediment. Ten patients had additional *E. coli* ESBL, three *K. pneumoniae* ESBL, and eight *E. faecalis*. The majority of patients received cefuroxime or ciprofloxacin for the treatment of urinary infections prior to isolation of *P. mirabilis*.

Isolates

Detection of ESBLs and susceptibility testing
The isolates were resistant to amoxicillin alone and combined with clavulanic acid, piperacillin, cefuroxime, cefoxitin, gentamicin, and ciprofloxacin, but susceptible to cefepime, imipenem, and meropenem with MICs of imipenem being slightly higher than those of meropenem according to microdilution test (Table 1). There were variable susceptibility/resistance patterns to ceftazidime, cefotaxime, ceftriaxone and to combination of piperacillin with tazobactam as shown in Table 1. Meropenem was the most potent antibiotic with Minimum Inhibitory

Table 1 Minimum inhibitory concentrations and β-lactamase content of multidrug-resistant *Proteus mirabilis*
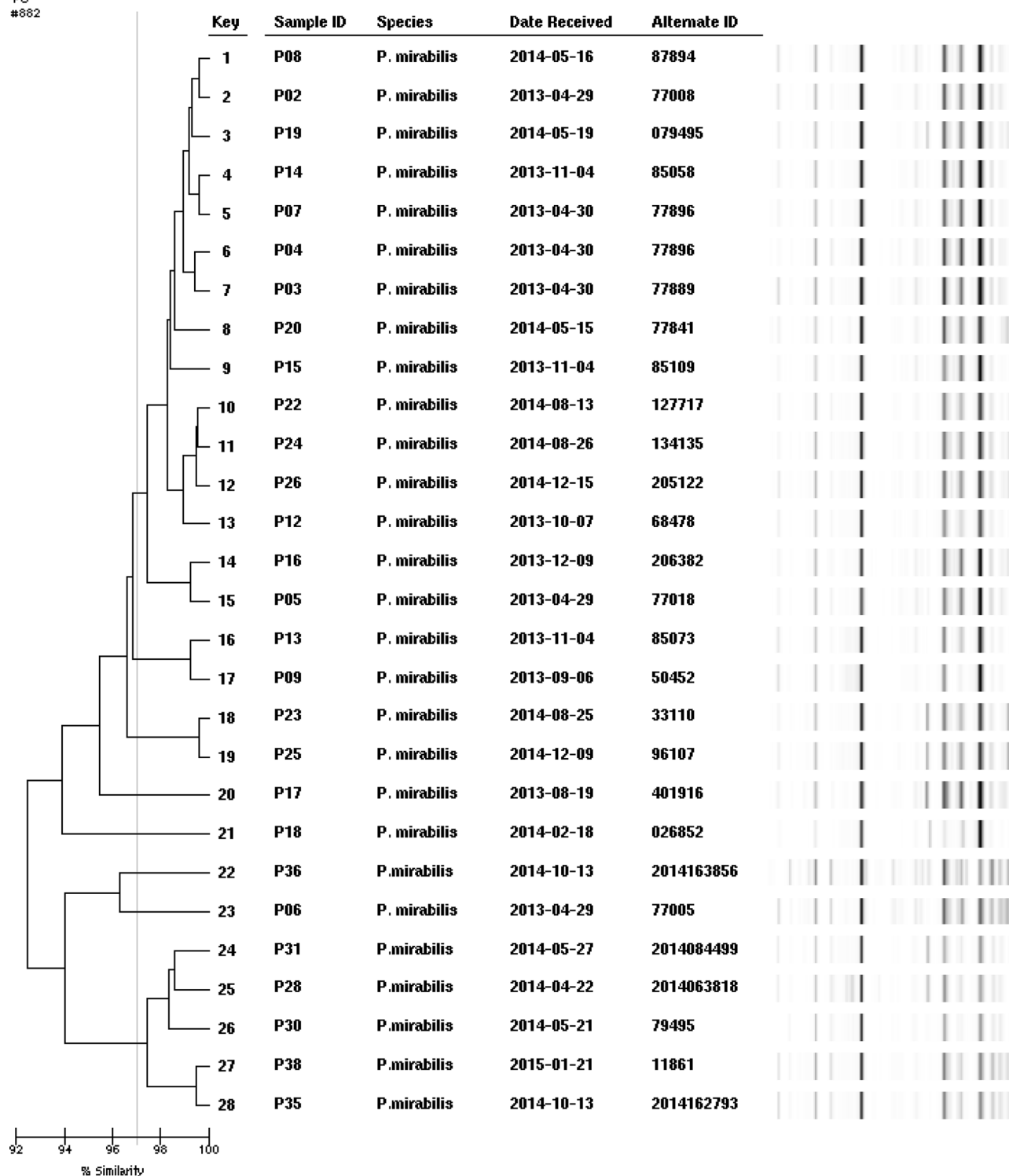
Strain no.	Protocol no.	Specimen	Date	ESBL	AmpC	BL	AMX	AMC	PIP	TZP	CXM	CZ	CAZ	CTX	CRO	FEP	FOX	IMI	MEM	CIP	GM	CIP
1	77005	Urine (catheter)	29.04.2013	–	+	CMY-16, TEM-1-1-1	>128	64	>128	8	>128	>128	16	32	16	1	>128	0.12	0.06	8	16	>128
2	77008	Urine	29.04.2013	–	+	CMY-16, TEM-1-1-1	>128	64	>128	8	>128	>128	32	64	16	1	>128	0.12	0.06	8	64	>128
3	77889	Urine	30.04.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	8	>128	>128	32	64	8	2	>128	0.5	0.06	16	32	>128
4	77896	Urine (catheter)	30.04.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	16	>128	>128	32	32	32	0.5	>128	0.5	0.06	32	32	>128
5	77018	Urine	29.04.2013	–	+	CMY,-16 TEM-1-1	>128	64	>128	8	>128	>128	64	32	32	0.25	>128	0.5	0.06	4	32	>128
6	77005	Urine (catheter)	29.04.2013	–	+	CMY,-16 TEM-1-1	>128	64	>128	4	>128	>128	32	64	64	0.5	>128	0.5	0.06	16	4	>128
7	77896	Urine (catheter)	30.04.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	2	>128	>128	32	64	32	0.5	>128	0.5	0.06	32	32	>128
8	87894	Urine (catheter)	16.05.2014	–	+	CMY-16 TEM-1-1	>128	64	>128	2	>128	>128	16	64	32	1	>128	0.5	0.06	8	16	>128
9	50452	Urine (catheter)	06.09.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	4	>128	>128	32	64	32	1	>128	0.5	0.06	16	64	>128
10	62950	Urine (catheter)	27.09.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	32	>128	>128	16	64	32	8	>128	1	0.06	>128	64	>128
11	64004	Urine (catheter)	30.09.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	64	>128	>128	16	>128	64	1	>128	1	0.06	>128	64	>128
12	68478	Urine	07.10.2013	–	+	CMY-112, TEM-1-1	>128	64	>128	32	>128	>128	>128	>128	64	0.5	>128	0.5	0.06	32	64	>128
13	85073	Urine (catheter)	04.11.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	8	>128	>128	>128	>128	32	0.5	>128	2	0.06	32	64	>128
14	85058	Urine (catheter)	04.11.2013	–	+	CMY-16, TEM-1-1	>128	32	>128	16	>128	>128	>128	>128	32	1	>128	2	0.06	32	64	>128
15	85109	Urine	04.11.2013	–	+	CMY-16, TEM-1-1	>128	32	>128	16	>128	>128	>128	>128	64	2	>128	2	0.06	32	64	>128
16	206382	Urine (catheter)	09.12.2013	–	+	CMY-16, TEM-1-1	>128	64	>128	32	>128	>128	>128	>128	16	0.5	>128	2	0.06	32	64	>128
17	401916	Urine (catheter)	19.08.2013	–	+	CMY-16 TEM-1	>128	64	>128	16	>128	>128	>128	>128	32	16	>128	2	0.06	32	64	>128
18	026852	Urine	18.02.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	4	>128	>128	32	16	16	0.5	>128	2	0.06	32	64	>128
19	079495	Urine (catheter)	19.05.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	64	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
20	77841	Urine	15.05.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	16	>128	>128	>128	>128	>128	0.5	>128	1	0.06	32	64	>128

Table 1 Minimum inhibitory concentrations and β-lactamase content of multidrug-resistant *Proteus mirabilis* (Continued)

Strain no.	Protocol no.	Specimen	Date	ESBL	AmpC	BL	AMX	AMC	PIP	TZP	CXM	CZ	CAZ	CTX	CRO	FEP	FOX	IMI	MEM	CIP	GM	CIP
21	108998	Urine (catheter)	08.07.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	64	>128	>128	64	64	>128	0.5	>128	1	0.06	64	64	>128
22	127717	Urine	13.08.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	16	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	32
23	33110	Urine	25.08.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	16	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
24	134135	Urine	26.08.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	8	>128	>128	64	64	>128	0.5	>128	1	0.06	64	64	>128
25	96701	Urine	19.12.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	4	>128	>128	>128	32	>128	0.5	>128	1	0.06	64	64	>128
26	205122	Urine (catheter)	15.12.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	16	>128	>128	>128	64	>128	0.5	>128	1	0.06	64	64	>128
27	2014057567	Urine (catheter)	09.04.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
28	2014063818	Urine (catheter)	22.04.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
29	2014064696	Urine	23.04.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
30	79495	Urine	21.05.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	4	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
31	2014084499	Urine	27.05.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
32	2014084497	Urine (catheter)	27.05.2024	–	+	CMY-16, TEM-1-1	>128	>128	>128	64	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
33	201462795	Urine (catheter)	13.10.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
34	2014162797	Urine	13.10.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
35	2014162793	Urine (catheter)	13.10.2014	–	+	CMY-16, TEM-1-1	>128	64	>128	64	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
36	2014163856	Urine	14.10.2014	–	+	CMY-16, TEM-1-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
37	2014205122	Urine	15.12.2014	–	+	CMY-16, TEM-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128
38	11861	Urine (catheter)	21.01.2015	–	+	CMY-16, TEM-1	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64	>128

Clavulanic acid was added to AMX in the fixed concentration of 4 mg/L
AMX amoxycillin, *AMC* amoxycillin/clavulanic acid, *CAZ* ceftazidime, *CTX* cefotaxime, *CRO* ceftriaxone, *FEP* cefepime, *FOX* cefoxitin, *TZP* piperacillin/tazobactam, *IPM* imipenem, *MEM* meropenem, *GM* gentamicin, *CIP* ciprofloxacin, *ESBL* phenotypic test for detection of extended-spectrum β-lactamases, *AmpC* phenotypic test for AmpC β-lactamases, *BL* β-lactamase content

Diversilab v3.6
PC
#882

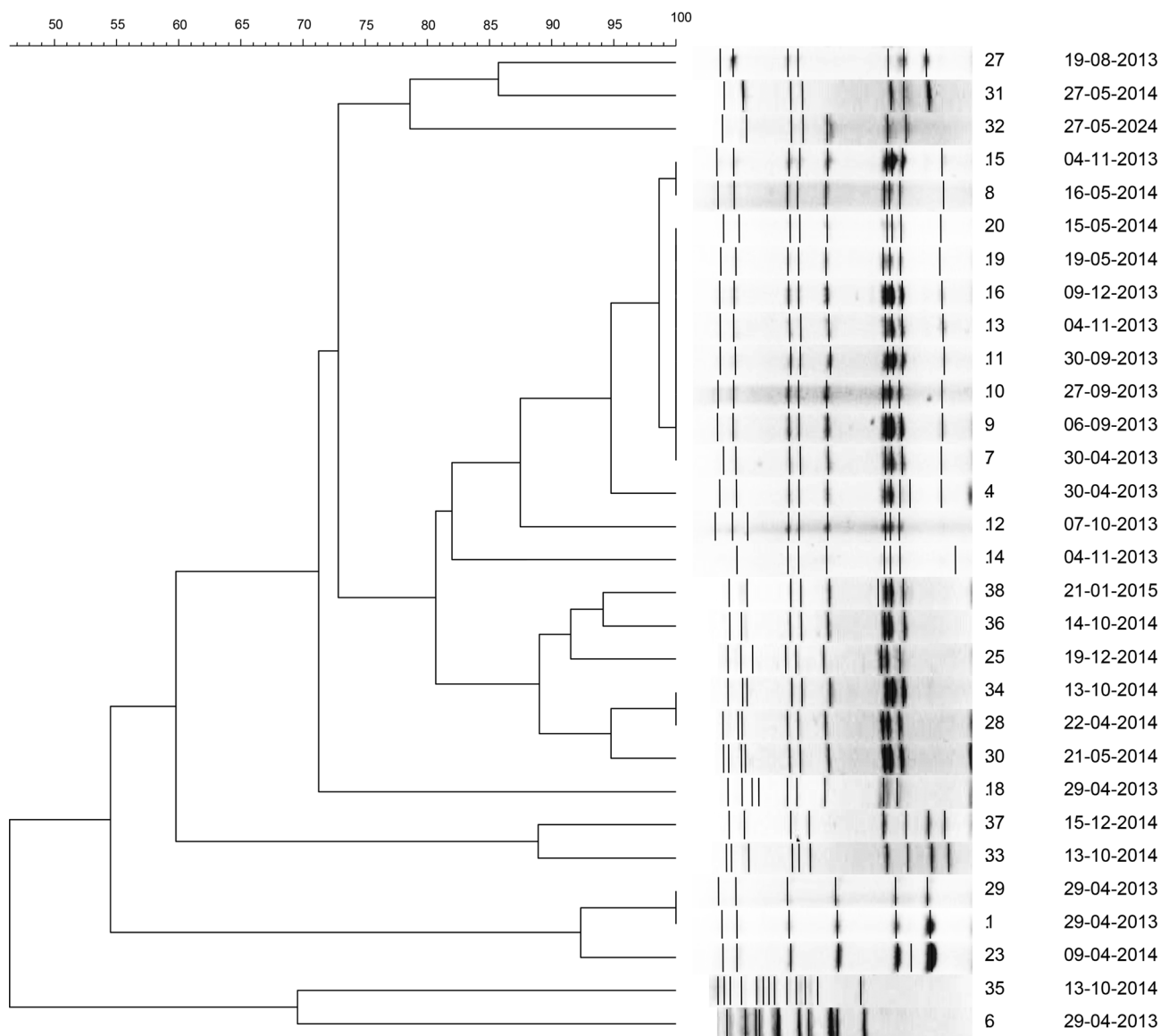| Key	Sample ID	Species	Date Received	Alternate ID
1	P08	P. mirabilis	2014-05-16	87894
2	P02	P. mirabilis	2013-04-29	77008
3	P19	P. mirabilis	2014-05-19	079495
4	P14	P. mirabilis	2013-11-04	85058
5	P07	P. mirabilis	2013-04-30	77896
6	P04	P. mirabilis	2013-04-30	77896
7	P03	P. mirabilis	2013-04-30	77889
8	P20	P. mirabilis	2014-05-15	77841
9	P15	P. mirabilis	2013-11-04	85109
10	P22	P. mirabilis	2014-08-13	127717
11	P24	P. mirabilis	2014-08-26	134135
12	P26	P. mirabilis	2014-12-15	205122
13	P12	P. mirabilis	2013-10-07	68478
14	P16	P. mirabilis	2013-12-09	206382
15	P05	P. mirabilis	2013-04-29	77018
16	P13	P. mirabilis	2013-11-04	85073
17	P09	P. mirabilis	2013-09-06	50452
18	P23	P. mirabilis	2014-08-25	33110
19	P25	P. mirabilis	2014-12-09	96107
20	P17	P. mirabilis	2013-08-19	401916
21	P18	P. mirabilis	2014-02-18	026852
22	P36	P.mirabilis	2014-10-13	2014163856
23	P06	P. mirabilis	2013-04-29	77005
24	P31	P.mirabilis	2014-05-27	2014084499
25	P28	P.mirabilis	2014-04-22	2014063818
26	P30	P.mirabilis	2014-05-21	79495
27	P38	P.mirabilis	2015-01-21	11861
28	P35	P.mirabilis	2014-10-13	2014162793

92   94   96   98   100
% Similarity

Fig. 1 Rep-PCR of *P. mirabilis* isolates. Cutoff value of 97 % was applied to define a clone. Date of isolation is shown

Concentration (MIC)$_{90}$ of 0.06 mg/L. In disk diffusion tests, all isolates were resistant to sulfametoxazole/trimethoprim (cotrimoxazole) and norfloxacin. The phenotype of resistance, including resistance or reduced susceptibility to expanded-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone), cefoxitin, and amoxicillin/clavulante, but preserved susceptibility to cefepime was consistent with production of plasmid-mediated AmpC β-lactamase which was confirmed by an inhibitor-based test. An augmentation of the inhibition zones around cephalosporin disks of at least 5 mm was seen with PBA but not with

Dice (Opt:3.00%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE01   **PFGE01**

27	19-08-2013
31	27-05-2014
32	27-05-2024
15	04-11-2013
8	16-05-2014
20	15-05-2014
19	19-05-2014
16	09-12-2013
13	04-11-2013
11	30-09-2013
10	27-09-2013
9	06-09-2013
7	30-04-2013
4	30-04-2013
12	07-10-2013
14	04-11-2013
38	21-01-2015
36	14-10-2014
25	19-12-2014
34	13-10-2014
28	22-04-2014
30	21-05-2014
18	29-04-2013
37	15-12-2014
33	13-10-2014
29	29-04-2013
1	29-04-2013
23	09-04-2014
35	13-10-2014
6	29-04-2013

Fig. 2 PFGE dendrogram of *P. mirabilis* isolates. Cutoff value of 80 % was applied to define a clone. Date of isolation is shown

clavulanic acid. All isolates tested phenotypically positive for AmpC but negative for ESBLs.

Conjugation
The isolates did not transfer ceftazidime resistance to an *E. coli* recipient strain.

Characterization of β-lactamases
All 38 *P. mirabilis* strains yielded an amplicon of 1432 bp with primers specific for CMY-β-lactamase genes. Sequencing of amplicons revealed the bla_{cmy16} β-lactamase allele in all strains except strain 12, which was found to produce CMY-112. The isolates were positive for bla_{TEM-1}, but negative for bla_{SHV}, bla_{CTX-M},

and bla_{PER-1} genes. IS*Ecp1* was identified 110 bp upstream of the bla_{CMY-16} starting codon.

Characterization of plasmids
Plasmid encoding CMY-16 did not belong to any known PBRT. The plasmid extractions were positive for bla_{TEM} and bla_{CMY} genes.

Detection of quinolone resistance determinants
Plasmid borne quinolone resistance genes-*qnrA*, *qnr*B, and *qnrS* were not found.

Genotyping of the isolates

Rep-PCR of 28 isolates identified one large clone consisting of 15 isolates (8, 2, 19, 14, 7, 4, 3, 20, 15, 22, 24, 26, 12, 16, 5); however, a certain degree of diversification was observed within the clone, with seven subclusters containing two or three identical isolates as shown in Fig. 1. The first one with strains 2, 8, and 19; the second one with strains 7 and 14; the third with strains 3 and 4; the fourth one with strains 22, 24 and 26; and the fifth one with strains 5 and 16.

Three pairs of related isolates (13 and 9, 23 and 25, and 38 and 35) and one triplet (28, 30, 31) were identified (Fig. 1). Four isolates were singletons: 6, 17, 18 and 36.

PFGE identified one large clone with 19 isolates out of 30 (isolates 15, 8, 20, 19, 16, 13, 11, 10, 9, 7, 4, 12, 14, 38, 36, 25, 34, 28, 30), one small cluster with three strains (1, 23, 29), two pairs (37, 33 and 27 and 31), and and four singletons (6, 18, 32, 35), as shown in Fig. 2.

Rep-PCR showed a better discriminatory effect because it identified a subcluster among the large clone and this could explain small discrepancies between the two genotyping methods.

Discussion

Previous studies found TEM-52 and PER-1 ESBLs to be dominant resistance determinants to expanded-spectrum cephalosporins in *P. mirabilis* [6]. This study demonstrated predominance of plasmid-mediated AmpC β-lactamase CMY-16 among tested isolates. AmpC β-lactamases detection is not routinely carried out in many microbiology laboratories. This could be attributed to lack of awareness, or lack of resources and facilities to conduct β-lactamase identification. Currently available tests for detection of plasmid-mediated AmpC β-lactamases are inconvenient, subjective, and lack sensitivity and specificity [4, 11]. AmpC β-lactamases are inhibited by PBA and cloxacillin. There are several inhibitor-based tests for identification of AmpC β-lactamases, including the disk test and the E-test [25]. The production of CMY β-lactamase was associated with resistance or reduced susceptibility to third-generation cephalosporins and the combination of amoxicillin with clavulanic acid. The isolates showed variable levels of susceptibility/resistance to piperacillin/tazobactam, which would lead to the conclusion that this combination 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, with meropenem having slightly lower MICs. Ceftazidime resistance was not transferred by conjugation to an *E. coli* recipient isolate, indicating that CMY genes were encoded on non-transferable plasmids. *P. mirabilis* lacks the ampC gene and, thus,

AmpC β-lactamases are always plasmid mediated in this species, although some studies found incorporation of the bla_{CMY} gene in the chromosome [26]. In our study, the plasmid extract did not belong to any known PBRT but yielded amplicons with primers specific for TEM and CMY β-lactamases. However, it is not possible to exclude the possibility of chromosomal contamination of plasmid extract and chromosomal location of the bla_{ampC} gene. Fifteen of the isolates were found to be clonally related, although three pairs, one triplet, and four singleton isolates were observed. This finding points to clonal dissemination of related isolates within the nursing home, probably due to the contaminated urinary catheters, but horizontal spread of the bla_{CMY} gene also occurred, most likely mediated by the IS*Ecp1* insertion sequence upstream of the gene. All the patients had severe underlying diseases and were previously hospitalized in one of the large hospital centers in Zagreb (University Hospital Center Zagreb, Sisters of Mercy University Hospital, and University Hospital Merkur) and there is a possibility that they were colonized with multiresistant *P. mirabilis* during their stay in the hospital, thus raising the possibility of multiple independent introduction routes of AmpC-positive *P. mirabilis* into the long-term care facility. The first plasmid-mediated AmpC β-lactamase reported in Croatia was DHA-1, identified in *E. coli* in 2003 [27]. Recent studies found plasmid-mediated AmpC β-lactamases of the CMY family among hospital *P. mirabilis* isolates from Split [28] and among *E. coli* isolates from companion animals in Croatia [29]. Moreover, CMY-4 was identified as an additional β-lactamase in *Enterobacteriacea* producing VIM or NDM metallo-β-lactamases [30]. 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* [26]. The acquired bla_{CMY} genes have escaped from the chromosome of *C. freundii* following mobilization mediated by IS*Ecp1*, IS26, or ISCR1. CMY-1, CMY-12, and CMY-16 were found to be the most prevalent variants of plasmid-mediated AmpC β-lactamases in Europe [26]. In addition, mobile insertion sequences such as IS26 and/or IS*Ecp1*, which can be found upstream of bla_{AmpC} genes, can facilitate their mobilization. Similar genetic context with IS*Ecp1* preceding bla_{CMY-16} was previously reported [31]. Simultaneous production of ESBLs and AmpC β-lactamases was also reported in *P. mirabilis* in recent studies [5]. CMY-16 was previously reported in *P. mirabilis* from a long-term care facility in Italy [32]. In the latter study, TEM-92, which is an ESBL, and plasmid-mediated AmpC β-lactamase CMY-16 were found. Similar to our study, CMY-16-producing organisms were clonally related, unlike those possessing ESBL [32]. The production of additional TEM-1 β-lactamase could increase the level of resistance to amoxycillin combined with clavulanate.

From the therapeutic point of view, it is important to distinguish between ESBLs and AmpC β-lactamases because infections caused by AmpC positive isolates 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 organisms [33], although our isolates demonstrated in-vitro susceptibility to piperacillin/tazobactam. Some authorities recommend all expanded-spectrum cephalosporins to be reported as resistant if the isolate produces plasmid-mediated AmpC β-lactamase, regardless of the in-vitro susceptibility results, to avoid therapeutic failures [33]. CLSI has yet to establish a testing and reporting algorithm specifically for organisms containing AmpC β-lactamases. Identification of AmpC β-lactamases in *E. coli, P. mirabilis*, and *Klebsiella* spp. can increase the accuracy of antimicrobial testing reports for expanded-spectrum cephalosporins if the results are used to modify the interpretations of cephalosporin results [33]. Recent studies demonstrated a high rate of clinical failure among patients who were infected in the bloodstream with AmpC-producing organisms and who received cephalosporin treatment [33, 34]. There are no data on efficacy of cephalosporin therapy for urinary tract infections associated with AmpC-producing organisms. The spread of AmpC-producing *P. mirabilis* in Europe pose a serious laboratory and therapeutic challenge [34]. Recently, *P. mirabilis* has demonstrated great capacity to accumulate resistance genes such as those encoding ESBLs, plasmid-mediated AmpC β-lactamases, carbapenemases, and fluoroquinolone resistance genes.

Considering the gravity of the implication of inappropriate therapy in chronically ill and debilitated patients in long-term care facilities, looking for AmpC β-lactamases must be mandatory in all microbiological laboratories, and clinicians should be educated on the importance of ESBLs and AmpC β-lactamases and therapeutic challenges that they pose [33, 34].

Compliance with ethical guidelines

Conflict of interest B. Bedenić, N. Firis, V. Elveđi-Gašparović, M. Krilanović, K. Matanović, I. Štimac, J. Luxner, J. Vraneš, T. Meštrović, G. Zarfel and A. Grisold state that there are no conflicts of interest.

Ethical standards This was in vitro study which did not involve human or animal subject and the permission from the Ethical Comittee was not necessary.

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