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EMERGENCE OF MULTIDRUG-RESISTANT *PROTEUS MIRABILIS* IN A LONG-TERM CARE FACILITY IN CROATIA

SUMMARY

Purpose: 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 PCR followed by sequencing of *bla*_{ampC} genes. Quinolone resistance determinants (*qnr* genes) were characterized by PCR. Genotyping of the isolates was performed by rep-PCR and PFGE (pulsed-field gel electrophoresis).

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.

Key words: CMY-16, *Proteus mirabilis*, Amp C β -lactamases, conjugative plasmid, clonal dissemination

Introduction

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 [1]. Production of β -lactamases is one of the strategies adopted by bacteria to develop resistance to β -lactam class of antibiotics [1-2]. 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* [2]. 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 [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 is not 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 University Hospital Center Split [7-8]. Spread of multidrug-resistant *P. mirabilis* from

cefotaxime, ceftriaxone, ceftazidime, and aztreonam [10] and combined disk test using disks of ceftazidime, cefotaxime, ceftriaxone and cefepime with and without clavulanate (10 µg/l) according to CLSI were performed to detect ESBLs [9]. Deformation of the inhibition zone around 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 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-phenylboronic 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 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 [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

boiling method. PCR mix (50 μ l) contained 25 μ l of master mix (Roche), 20 μ l of ultrapure water, 1 μ l of each primer (10 pmol) and 3 μ l 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 in Microgene DNA sequencing service. CMY and TEM amplicons were sequenced. Sequences were analyzed using BLAST program (NCBI). Designation of *bla* genes based on identified mutations was done according to Bush, Jacoby and Medeiros scheme. The presence of *ISEcp1* and IS26 in the region upstream of *bla*_{CMY} genes was investigated by combining IS26 and *ISEcp1* forward primers with reverse primers for *bla*_{CMY} [19].

Detection of quinolone resistance determinants

Plasmid borne quinolone resistance genes-*qnrA*, *qnrB* and *qnrS* were determined by PCR as described previously [20].

Characterization of plasmids

Plasmids were extracted with 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 ng/L 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. Cut-off 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 UPGMA (unweighted pair-group method using arithmetical averages) 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 Godan long term care facility. Since Godan nursing home is located close to University Hospital Center Zagreb where the urine samples were processed we found in hospital internet system that 23 patients of 38 patients were previously hospitalized in University Hospital Center at the intensive care unit, pulmonary unit, abdominal surgery, haematology, ophthalmology, gastroenterology, cardiology and oncology. Three patients were only examined at 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*.

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 MIC₉₀ of 0.06 mg/L. In disk-diffusion test all isolates were resistant to sulfamethoxazole/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 inhibitor based test. An augmentation of the inhibition zones around cephalosporin disks of at least 5 mm was seen with PBA but not with clavulanic acid. All isolates tested phenotypically positive for AmpC but negative for ESBLs.

Conjugation

The isolates did not transfer ceftazidime resistance to *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 of the 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. *ISEcp1* was identified 110 bp upstream of *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*, *qnrB* 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) but 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 four singletons (6, 18, 32, 35) as shown in Fig. 2.

Rep-PCR showed better discriminatory effect because it identified subcluster among the large clone and this could explain small discrepancies between 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 disk test and E-test [25]. 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 isolates showed variable levels of susceptibility/resistance to piperacillin/tazobactam which would lead to 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 *E. coli* recipient isolate indicating that CMY genes were encoded on non-transferable plasmids. *P. mirabilis* lacks ampC gene and thus AmpC β -lactamases are always plasmid mediated in this species although some studies found incorporation of *bla*_{CMY} gene in the chromosome [26]. In our study the plasmid extract did not belong to any known PBRT but yielded amplicon 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 but three pairs, one triplet and four singleton isolates

were observed. This finding points out to clonal dissemination of related isolates within the nursing home probably due to the contaminated urinary catheters, but horizontal spread of *bla*_{CMY} gene also occurred most likely mediated by *ISEcp1* 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 the stay in the hospital, thus raising the possibility of multiple independent introduction 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]. The recent studies found plasmid-mediated AmpC β -lactamases of 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 additional β -lactamase in *Enterobacteriaceae* 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 *ISEcp1*, 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 *ISEcp1*, which can be found upstream of *bla*_{AmpC} genes, can facilitate their mobilization. Similar genetic context with *ISEcp1* 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 their study TEM-92 which is an ESBL and plasmid-mediated AmpC β -lactamase CMY-16 were found. Similarly as in 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 amoxicillin 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 were 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 wrong therapy in cronicly 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].

ETHICAL STATEMENT: This was in vitro study which did not involve human or animal subject and the permission from the Ethical Committee was not necessary.

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REFERENCES

1. Grover CN, Sahni BAK, Bhattacharya CS. Therapeutic challenges of ESBLs and AmpC β -lactamase producers in a tertiary care center. *Medical Journal Armed Forces*. 2013;69:4-10.
2. Bush K. Is it important to identify extended-spectrum β -lactamase-producing isolates? *Eur J Clin Microbiol Infect Dis*. 1996;15:361-364.
3. Phillipon A, Arlet G, Lagrange H. Origin and impact of plasmid-mediated extended-spectrum β -lactamases. *Eur J Clin Microbiol Infect Dis*. 1994; 13(Suppl 1):17-29.
4. Tan TJ, Yong NG, Koh TK, Hsu LY. Evaluation of screening methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. *Antimicrob. Agents Chemother*. 2009; 53:146-149.
5. Datta P, Gupta V, Arora S, Garg S, Chander J. Epidemiology of extended-spectrum β -lactamase, AmpC, and carbapenemase production in *Proteus mirabilis*. *Jpn J Infect Dis*. 2014;67:44-46.
6. Pagani L, Migliavacca R, Pallechi L. Emerging extended-spectrum β -lactamases in

- Proteus mirabilis*. J Clin Microbiol. 2002;40:1549-1552.
7. Sardelić S, Bedenić B, Šijak D, Colinon C, Kalenić S. Emergence of *Proteus mirabilis* isolates producing TEM-52 β -lactamase in Croatia. Chemotherapy.2010;56: 208-13.
 8. Tonkic M, Mohar B, Sisko-Kraljević K, Mesko-Meglic, K, Goić-Barisić I, Novak A, Kovacić A, Punda-Polić V. High prevalence and molecular characterization of extended-spectrum β -lactamase-producing *Proteus mirabilis* isolates in southern Croatia. J Med Microbiol.2010; 59: 1185-90.
 9. Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing. 21st informational supplement.2011 Informational Supplement, M100-21, CLSI Wayne, PA, USA.
 10. Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum β -lactamases conferring transferable resistance to newer β -lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev Infect Dis.1998;10: 867-878.
 11. Coudron PE. Inhibitor-based methods for detection of plasmid-mediated AmpC β -lactamases in *Klebsiella* spp., *Escherichia coli* and *Proteus mirabilis*. J Clin Microbiol.2005;43:4163-4167.
 12. Elwell S, Falkow LP. The characterization of R plasmids and the detection of plasmid-specified genes. In: V. Lorian (ed.) Antibiotics in Laboratory Medicine. 2nd edn., Baltimore MD: Williams and Wilkins. 1986;683-721.
 13. Nüesch-Inderbinen MT, Haechler H, Kayser FH. Detection of genes coding for extended-spectrum SHV β -lactamases in clinical isolates by a molecular genetic method, and comparison with the E test. Eur J Clin Microbiol Infect Dis.1996;15:398-402.
 14. Arlet G, Bami D, Decre D, Flippo A, Gaillot O, Lagrange PH, Philippon A.

- Molecular characterization by PCR restriction fragment polymorphism of TEM β -lactamases. *FEMS Microbiol Lett.* 1995;134:203-208.
15. Woodford N, Ward ME, Kaufmann ME, Turton J, Fagan EJ, James D, Johnson AP, Pike R, Warner M, Cheasty T, Pearson A, Harry S, Leach JB, Loughrey A, Lowes JA, Warren RE, Livermore DM. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum β -lactamases in the UK. *J Antimicrob Chemother.* 2004; 54: 735-743.
 16. Pagani L, Mantengoli E, Migliavacca R, Nucleo E, Pollini S, Spalla M, Daturi R, Romero E, Rossolini GM. Multifocal detection of multidrug-resistant *Pseudomonas aeruginosa* producing PER-1 extended-spectrum β -lactamase in Northern Italy. *J Clin. Microbiol.* 2004;42: 2523-2529.
 17. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC β -lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol.* 2002;40: 2153-2162.
 18. Hanson ND, Moland ES, Hossain A, Neville SA, Gosbell IB, Thomson KS. Unusual *Salmonella enterica* serotype typhimurium isolate producing CMY-7, SHV-9 and OXA-30 β -lactamases. *J Antimicrob Chemother.* 2002;49(6):1011-4.
 19. Saladin M, Cao VY, Lambert T. Diversity of CTX-M beta-lactamases and their promoter regions from *Enterobacteriaceae* isolated in three Parisian hospitals. *FEMS Microbiol Lett* 209.2002; 161-168.
 20. Robischek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis* 2006;6: 629-640.
 21. Carattoli A, Bertini L, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005;63: 219
 22. Overdevest S, Willemsen C, Elberts M, Verhulst P, Rijnsburger J, Savelkoulnd A, Kluytmans JW. Evaluation of the DiversiLab Typing Method in a Multicenter Study.

- Assessing Horizontal Spread of Highly Resistant Gram-Negative Rods. *J Clin Microbiol.*2011; 49:3551-3554.
23. Kaufman ME. Pulsed-Field Gel Electrophoresis. In: Woodford N and Johnsons A, eds. *Molecular bacteriology. Protocols and clinical applications.* 1st edn. New York: Humana Press Inc. Totowa; 1998; 33-51.
 24. Tenover F, R, Arbeit, RV Goering, P.A, Mickelsen, B.E. Murray, D.H. Persing, and B. Swaminthan.. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol*;33 2233-9.
 25. Black JA, Moland ES, Thomson KA. AmpC disk test for identification of plasmid-mediated-AmpC β -lactamases in *Enterobacteriaceae* lacking chromosomal AmpC β -lactamase. *J Clin Microbiol.*2005;43:3110-3113.
 26. D'Andrea MM, Literacka E, Zioga A, Giani T, Baraniak A, Fiett J, Sadowi E, Tassios T, Rossolini GM, Gniadkowski M, Miriagou V. Evolution and spread of multidrug-resistant *Proteus mirabilis* clone with chromosomal AmpC β -lactamase in Europe. *Antimicrob Agents Chemother.*2011;55:2735-2742.
 27. Giakoupi P, Tambić-Andrašević A, Vourli S, Škrilin J, Šestan-Crnek S, Tzouveleki LS, Vatoupoulos AC. Transferable DHA-1 cephalosporinase in *Escherichia coli*. *International Journal of Antimicrobial Agents* 2006;27: 77-80.
 28. Rubić Ž, Soprek S, Jelić M, Radić M, Novak A, Goić-Barišić I, Tonkić, M, Tambić-Andrašević, A. The first detection of plasmid-mediated AmpC β -lactamase in multidrug-resistant *Proteus mirabilis* isolates from University Hospital Split, Croatia . 24th European congress of clinical Microbiology and Infectious Diseases, Barcelona, Spain. 2014. Book of Abstracts; P 1027 www.ecmid.org.
 29. Bedenić B, Matanović K, Mekić S, Varda-Brkić D, Šeol-Martinac B. Coproduction

of CTX-M-15 and CMY-2 in animal *Escherichia coli* isolate from Croatia.24th European congress of clinical Microbiology and Infectious Diseases, Barcelona, Spain.2014 Book of Abstracts, P 1027, R060www.ecmid.org..

30. Zujic-Atalic V, [Bedenic B](#), Kocsis E, Mazzariol A, Sardelic S, Barišic M, Plečko V, Bošnjak Z, Mijač M, Jajic I, Vranic-Ladavac M, Cornaglia G. Diversity of carbapenemases in clinical isolates of Enterobacteriaceae in Croatia-the results of the multicenter study. *Clinical Microbiology and Infection*.2004; 20:894-903.
31. Literacka E, Empel J, Baraniak A, Sadowy E, Hryniewicz W, Gniadkowski M. Four variants of the *Citrobacter freundii* AmpC type cephalosporinase including two novel enzymes, CMY-14 and CMY-15 in a *Proteus mirabilis* clone widespread in Poland. *Antimicrob Agents Chemother*.2004; 48:4136-4143.
32. Migliavacca R, Migliavacca A, Nucleo E, Ciaponi A, Spalla M, De Luca C, Pagani L. Molecular epidemiology of ESBL producing *Proteus mirabilis* isolates from a long-term care and rehabilitation facility in Italy.*New Microbiologica*.2007;30:362-366.
33. Tenover F, Emery SL, Spiegel CA. Identification of plasmid-mediated AmpC β -lactamases in *Escherichia coli*, *Klebsiella* spp and *Proteus mirabilis* can potentially improve reporting of cephalosporin susceptibility testing results. *J Clin Microbiol*.2009; 47: 294-299.
34. Luzzaro F. Spread of multidrug-resistant *Proteus mirabilis* isolates producing an AmpC-type β -lactamase:epidemiology and clinical management. *Int J Antimicrob Agents*.2009;33:328-333.

FIGURE LEGEND

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

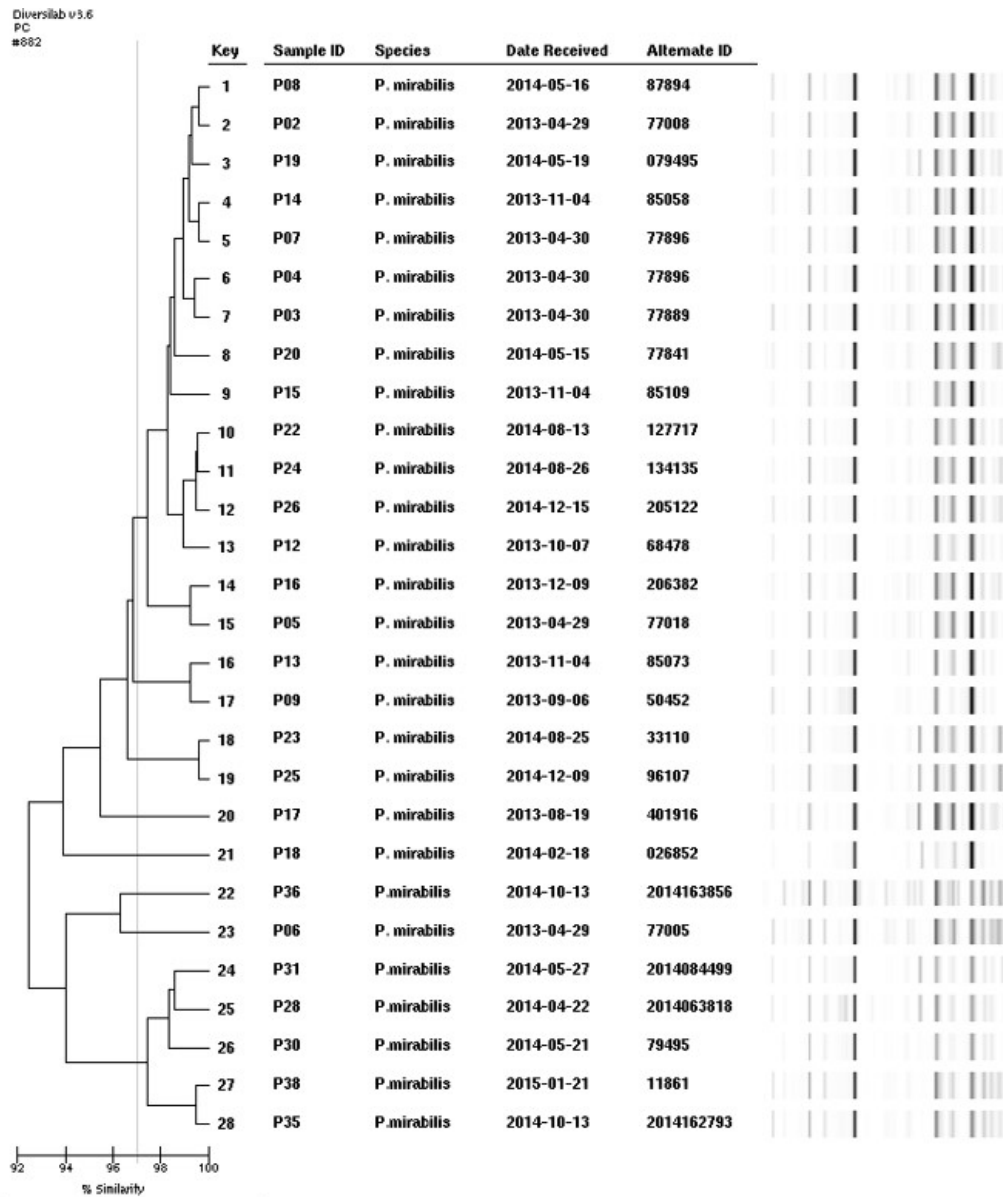


Fig. 2. PFGE dendrogram of *P. mirabilis* isolates. Cut of value of 80% was applied to define a clone. Date of isolation is shown.

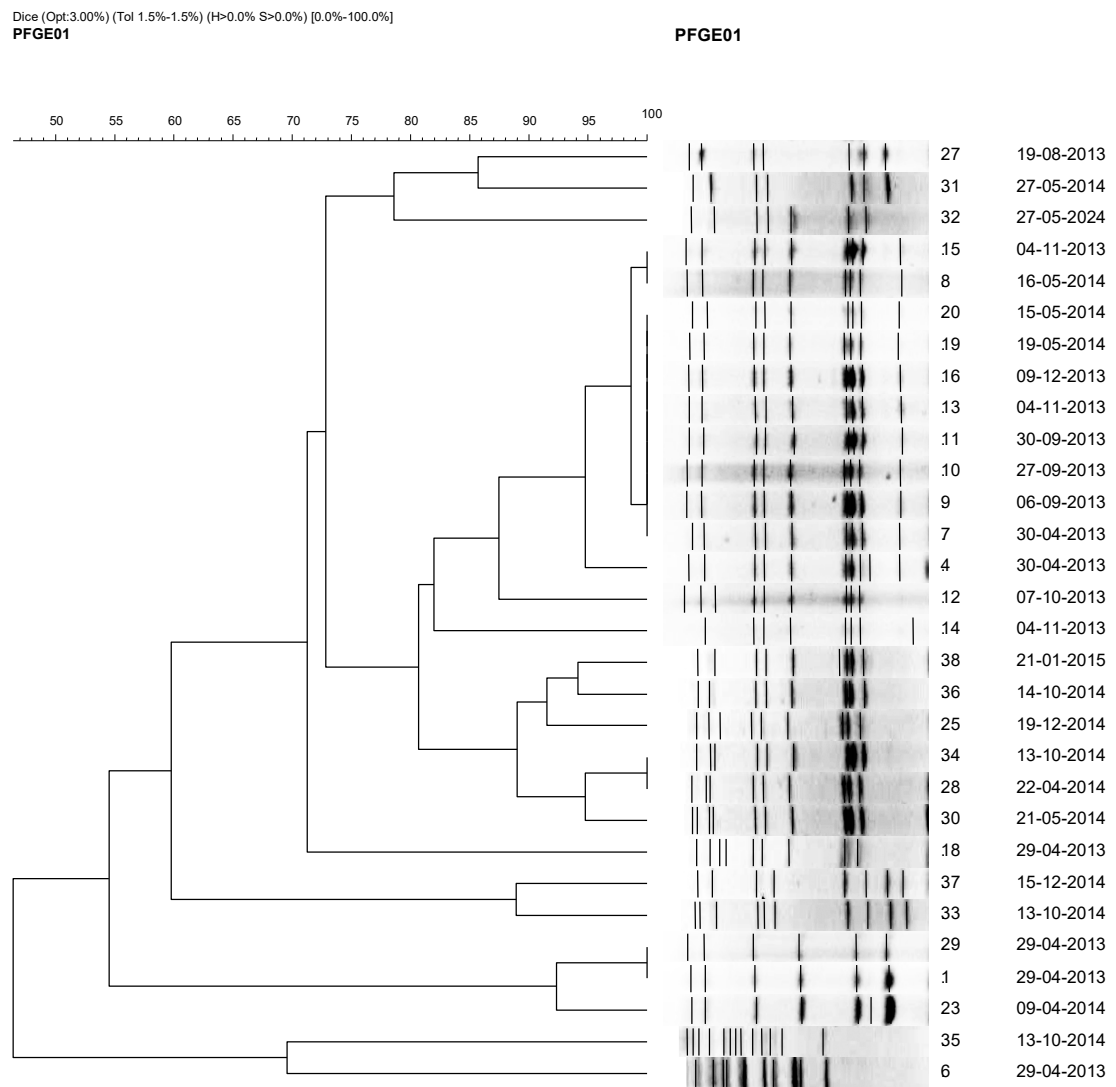


Table 1.Minimum inhibitory concentrations and β -lactamase content of multidrug-resistant *Proteus mirabilis*.

Strain no	Protocol number	specimen	date	ESBL	AmpC	BL	AMX	AMC	PIP	TZP	CXM	CZ	CAZ	CTX	CRO	FEP	FOX	IMI	MEM	CIP	GM
1	77005	Urine (catheter)	29.04. 2013	-	+	CMY-16, TEM-1- >128	>128	64	>128	8	>128	>128	16	32	16	1	>128	0.12	0.06	8	16
2	77008	urine	29.04.2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	8	>128	>128	32	64	16	1	>128	0.12	0.06	8	64
3	77889	Urine	30.04.2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	8	>128	>128	32	64	8	2	>128	0.5	0.06	16	32
4	77896	Urine (catheter)	30.04.2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	16	>128	>128	32	32	32	0.5	>128	0.5	0.06	32	32
5	77018	urine	29.04. 2013.	-	+	CMY-16 TEM-1- >128	>128	64	>128	8	>128	>128	64	32	32	0.25	>128	0.5	0.06	4	32
6	77005	Urine (catheter)	29.04.2013.	-	+	CMY-16 TEM-1- >128	>128	64	>128	4	>128	>128	32	64	64	0.5	>128	0.5	0.06	16	4
7	77896	Urine (catheter)	30.04. 2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	2	>128	>128	32	64	32	0.5	>128	0.5	0.06	32	32
8	87894	Urine (catheter)	16.05.2014.	-	+	CMY-16 TEM-1- >128	>128	64	>128	2	>128	>128	16	64	32	1	>128	0.5	0.06	8	16
9	50452	Urine (catheter)	06.09. 2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	4	>128	>128	32	64	32	1	>128	0.5	0.06	16	64
10	62950	Urine (catheter)	27.09. 2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	32	>128	>128	16	64	32	8	>128	1	0.06	>128	64
11	64004	Urine (catheter)	30.09.2013.	-	+	CMY-16 TEM-1- >128	>128	64	>128	64	>128	>128	16	>128	64	1	>128	1	0.06	>128	64
12	68478	urine	07.10.2013.	-	+	CMY-112,, TEM- >128	>128	64	>128	32	>128	>128	>128	>128	64	0.5	>128	0.5	0.06	32	64
13	85073	Urine (catheter)	04.11.2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	8	>128	>128	>128	>128	32	0.5	>128	2	0.06	32	64
14	85058	Urine (catheter)	04.11.2013.	-	+	CMY-16, TEM-1- >128	>128	32	>128	16	>128	>128	>128	>128	32	1	>128	2	0.06	32	64
15	85109	Urine	04.11.2013.	-	+	CMY-16, TEM-1- >128	>128	32	>128	16	>128	>128	>128	>128	64	2	>128	2	0.06	32	64
16	206382	Urine (catheter)	9.12.2013.	-	+	CMY-16, TEM-1- >128	>128	64	>128	32	>128	>128	>128	>128	16	0.5	>128	2	0.06	32	64
17	401916	Urine (catheter)	19.08.2013.	-	+	CMY-16 TEM-1 >128	>128	64	>128	16	>128	>128	>128	>128	32	16	>128	2	0.06	32	64
18	026852	urine	18.02. 2014	-	+	CMY-16, TEM-1- >128	>128	64	>128	4	>128	>128	32	16	16	0.5	>128	2	0.06	32	64
19	079495	Urine (catheter)	19.05.2014.	-	+	CMY-16, TEM-1- >128	>128	64	>128	64	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
20	77841	urine	15.05.2014	-	+	CMY-16, TEM-1- >128	>128	64	>128	16	>128	>128	>128	>128	>128	0.5	>128	1	0.06	32	64
21	108998	Urine (catheter)	8.07. 2014	-	+	CMY-16, TEM-1- >128	>128	64	>128	64	>128	>128	64	64	>128	0.5	>128	1	0.06	64	64
22	127717	Urine	13.08.2014.	-	+	CMY-16, TEM-1- >128	>128	64	>128	16	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
23	33110	Urine	25.08.2014.	-	+	CMY-16, TEM-1- >128	>128	64	>128	16	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
24	134135	Urine	26.08. 2014	-	+	CMY-16, TEM-1- >128	>128	64	>128	8	>128	>128	64	64	>128	0.5	>128	1	0.06	64	64
25	96701	urine	19.12. 2014.	-	+	CMY-16, TEM-1- >128	>128	>128	>128	4	>128	>128	>128	32	>128	0.5	>128	1	0.06	64	64
26	205122	Urine (catheter)	15.12.2014	-	+	CMY-16, TEM-1- >128	>128	>128	>128	16	>128	>128	>128	64	>128	0.5	>128	1	0.06	64	64
27	2014057567	Urine (catheter)	09.04. 2014	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
28	2014063818	Urine (catheter)	22.04. 2014.	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
29	2014064696	urine	23. 04. 2014.	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
30	79495	Urine	21.05. 2014	-	+	CMY-16, TEM-1- >128	>128	>128	>128	4	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
31	2014084499	urine	27.05. 2014.	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
32	2014084497	Urine (catheter)	27.05. 2024	-	+	CMY-16, TEM-1- >128	>128	>128	>128	64	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
33	201462795	Urine (catheter)	13.10. 2014	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
34	2014162797	urine	13.10. 2014.	-	+	CMY-16, TEM-1- >128	>128	64	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
35	2014162793	Urine (catheter)	13. 10. 2014.	-	+	CMY-16, TEM-1- >128	>128	64	>128	64	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
36	2014163856	Urine	14.10. 2014.	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
37	2014205122	urine	15.12. 2014.	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64
38	11861	Urine (catheter)	21.01. 2015	-	+	CMY-16, TEM-1- >128	>128	>128	>128	32	>128	>128	>128	>128	>128	0.5	>128	1	0.06	64	64

Abbreviations: AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CXM, cefuroxime; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; FOX, ceftazidime; PIP, piperacillin; TZP, piperacillin/tazobactam; IPM, imipenem; MEM, meropenem; GM, gentamicin; CIP, ciprofloxacin, ESBL: phenotypic test for detection of ESBLs, AmpC-phenotypic test for AmpC β -lactamases, BL: β -lactamase content

^bclavulanic acid was added to AMX in the fixed concentration of 4 mg/L