Review

Evolution of β-Lactam Antibiotic Resistance in *Proteus* Species: From Extended-Spectrum and Plasmid-Mediated AmpC β-Lactamases to Carbapenemases

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Review

Evolution of β-Lactam Antibiotic Resistance in *Proteus* Species: From Extended-Spectrum and Plasmid-Mediated AmpC β-Lactamases to Carbapenemases

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Abstract: The management of infectious diseases has proven to be a daunting task for clinicians worldwide and the rapid development of antibiotic resistance among Gram-negative bacteria is making it even more challenging. The first-line therapy is empirical and it most often comprises β-lactam antibiotics. Among Gram-negative bacteria *Proteus mirabilis*, important community and hospital pathogen associated primarily with urinary tract and wound infection, holds a special place. This review's aim was to collate and examine recent studies investigating resistance phenotypes and mechanisms of *Proteus* species and the global significance of its resistance evolution. *P. mirabilis* as the dominant pathogen develops resistance to expanded-spectrum cephalosporins (ESC) by producing extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (p-AmpC). β-lactamase-mediated resistance to carbapenems in Enterobacterales including *Proteus* spp. is mostly due to expression of carbapenemases of class A (KPC), class B (metallo-β-lactamases or MBLs of IMP, VIM or NDM series) or class D or carbapenem-hydrolyzing oxacillinases (CHDL). Previously, a dominant ESBL type in *P. mirabilis* was TEM-52, yet lately it has been replaced by CTX-M variants, particularly CTX-M-3 and CTX-M-65. ESC resistance can also be mediated by p-AmpC with CMY-16 as the dominant variant. Carbapenem resistance in *Proteus* spp. is a challenge due to its intrinsic resistance to colistin and tigecyclin. The first carbapenemases reported, belonged to class B, most frequently VIM-1 and NDM-5. In Europe, predominantly France and Belgium, a clonal lineage positive for OXA-23 CHD spread rapidly undetected, due to its low-level resistance to carbapenems. Amazing capacity of *Proteus* spp. to accumulate a plethora of various resistance traits leading to multidrug or extensively- drug- resistant phenotype.

Keywords: *Proteus mirabilis*; extended-spectrum β-lactamases; plasmid-mediated AmpC β-lactamases; carbapenemases; fluoroquinolone resistance

2. Extended-Spectrum β-Lactamases

Ampicillin resistance is usually linked to production of TEM-1 or TEM-2 β-lactamases which do not hydrolyze ESC. Early studies found TEM-52 and PER-1 ESBLs to be dominant resistance determinants to expanded-spectrum cephalosporins in *P. mirabilis* [14,15]. TEM-52 was also found in

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P. mirabilis from southern Mediterranean regions of Croatia [16,17]. TEM-52 and PER-1 are both ceftazidimases which preferentially hydrolyze ceftazidime. In North Africa isolates positive for PER-1 were identified in *P. vulgaris* [18]. Switch to CTX-M variants was observed in early 2010s. *bla*$_{CTX-M-15}$ genes were preceded by an insertion element IS*Ecp* responsible for the mobilization of the gene and increased expression leading to high level resistance to all cephalosporins [19]. The isolates reported in Russia were found to harbor *bla*$_{CTX-M-65}$ gene with additional *bla*$_{VEB}$ encoding Vietnam-extended-spectrum β-lactamase, *aac*6-Ib genes encoding aminoglycoside resistance and *qnr*A1 for fluoroquinolone resistance [20]. Whole- genome sequencing (WGS) revealed that isolates belonged into two different clones. *bla*$_{CTX-M-3}$ and *bla*$_{CTX-M-65}$ gene in animal *P. mirabilis* isolates from Hong Kong were located in Tn7-like composite transposon [21]. Unlike previous studies, the ESBL encoding genes were chromosomally encoded. Genes responsible for sulphonamide resistance *sul*1 and *sul*2, and chloramphenicol *cat*B3 were located on the chromosome as well [21].

Rare type of ESBL found in *P. mirabilis* is VEB-1, previously reported in *Pseudomonas aeruginosa*, which coproduced NDM-5 MBL [22]. The patient with subphrenic abscess was previously treated with broad spectrum cephalosporins in Bangladesh [22].

Table 1. ESBL reported in *Proteus* species.

Type of ESBL	Substrate profile	reference
PER-1	Penicillins, ESC, cefepime, monobactams	[18]
VEB-1	Penicillins, ESC, cefepime, monobactams	[22]
TEM-52	Penicillins, ESC, cefepime, monobactams	[14–17]
CTX-M-3	Penicillins, ESC, cefepime, monobactams	[21]
CTX-M-65	Penicillins, ESC, cefepime, monobactams	[20]
CTX-M-15	Penicillins, ESC, cefepime, monobactams	[19]

3. AmpC β-Lactamases

Reports on p-AmpC β-lactamases are also very scarce in the medical bibliography. Most publications report β-lactamases belonging to CMY family [23]. Proteus species does not have chromosomal AmpC genes and thus all AmpC beta-lactamases are supposed to be plasmid mediated. However, some studies proved chromosomal integration of *bla*>$_{ampC}$ genes mediated by IS*Ecp1* insertion element [23].

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 [23]. CMY family is derived by chromosomal AmpC β-lactamases of *Citrobacter freundii*. 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 [23]. Simultaneous production of ESBLs and AmpC β-lactamases was also reported in *P. mirabilis* in recent studies [24]. CMY-16 was previously reported in *P. mirabilis* from a long-term care facility in Italy [25] and Croatia [26]. In the Italian 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 had similar resistance phenotypes, unlike those possessing ESBL [25]. CMY-16 producing organisms with similar properties as those present in the nursing homes were also identified in the hospital in southern Croatia [27]. Similarly, as the isolates from Zagreb, they demonstrated resistance to sulphonamides, fluoroquinolones and 80% to aminoglycosides. All isolates were susceptible to carbapenems, ceftazidime-avibactam and fosfomycin. *bla*$_{CMY}$ genes were associated with and IS*Ecp* insertion element 110 bp upstream of the *bla*$_{CMY-16}$ starting codon. [27]. The isolates were allocated into four clusters, as demonstrated by pulsed-field gel electrophoresis (PFGE).

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Table 2. Most frequent AmpC β-lactamases in *Proteus* species.

Type of AmpC	Substrate profile	reference
CMY-16	Penicillins, ESC, monobactams, cephamycins, β-lactam-inhibitor combinatons	[23,25–27]
CMY-2	Penicillins, ESC, monobactams, cephamycins, β-lactam-inhibitor combinatons	[28]
CIT	Penicillins, ESC, monobactams, cephamycins, β-lactam-inhibitor combinatons	[29]

4. Carbapenemases

P. mirabilis develops resistance to carbapenems due to production of carbapenemases, porin alteration or loss, hyperexpression of efflux pumps or alteration of PBP receptors. Carbapenemases in *Proteus* spp. emerged recently. The first report originated from Bulgaria in 2019 and described *P. mirabilis* isolates with chromosomally encoded VIM-1 carbapenemase, embedded in class 1 integron, containing IS26 insertion element. Increased resistance was related to the increased expression of the gene associated with increased gene copy number. The isolates showed variable resistance to carbapenems [30]. NDM was reported from Austria in combination with WEB-1 ESBL. The strain was imported from Bangladesh. IMP variants are the rarest [31]. Recently, OXA-48 with very unusual resistance phenotype was described in nine *P. mirabilis* in Germany [32]. The isolates demonstrated susceptibility to imipenem and ertapenem and in most cases to piperacillin-tazobactam due to weak hydrolytic activity, which complicates laboratory detection and enables the isolates to be missed in the routine diagnostic laboratories and create a hidden reservoir within hospitals which is a source for dissemination of *bla*$_{OXA-48}$ genes bypassing surveillance systems [32]. *bla*$_{OXA-48}$ genes were chromosomally encoded unlike those reported in other Enterobacterales. Unlike other Enterobacterales, diffusion of the isolate is consequence of the vertical transmission of related isolates. Three isolates were found to harbour *bla*$_{OXA-181}$ genes, related to *bla*$_{OXA-48}$, which were encoded on X3 plasmid. In France, 19 *P. mirabilis* isolates with slightly reduced susceptibility to carbapenems were analyzed and OXA-23 CHDL was found, which is usually associated with *Acinetobacter baumannii*. Emergence of such clone is worrisome as it could be misidentified as penicillinase producers due to its susceptibility to carbapenems [33]. This enables these isolates to escape laboratory surveillance and to disseminate in the hospitals and community. All 19 isolates were clonally related, but different from OXA-23 negative isolates. An outbreak of OXA-23 was also identified among 21 *A. baumannii* from Belgium and one isolate was found positive for OXA-58 [34] This indicates spread of CHDL which are typical for *A. baumannii* among Enterobacterales. OXA-23 encoding genes were located on chromosome while OXA-58 was plasmid-mediated [34]. The MICs of carbapenems are often in the susceptible range and thus the isolates are frequently not identified in the laboratory as carbapenemase producers, creating a potential reservoir for spread of CHDL encoding genes [34]. In addition to *bla*$_{OXA-23}$, the strains harboured genes conferring resistance to aminoglycosides (*aph*(3'' Ib,, *aph*(6)-Id), sulphonamides (*sul*1 and *sul*2), trimethoprim (*dfr*A) and chloramphenicol resistance (*cat*). Unlike *A. baumannii*, *bla*$_{OXA-23}$ genes in *P. mirabilis* were not preceded by IS*Aba1* element. This could explain very low carbapenem MICs. An accurate screening method for OXA-23 in *P. mirabilis* is based on the reduced inhibition zone size around amoxycillin-clavulanate disk (<11mm) and confirmation is usually done by immunochromatographic tests or PCR. In Europe OXA-58 was identified in *P. mirabilis* from Poland [35].

The β-lactam susceptibility pattern indicated resistance to penicillins (including temocillin), their β-lactamase inhibitor combinations, and carbapenems (with ertapenem, imipenem, and meropenem MICs of 8, 32, and 16 g/ml, respectively) and susceptibility to oxyimino compounds (1, 2). The strain was resistant to fluoroquinolones and chloramphenicol and susceptible to amikacin, gentamicin, tobramycin, co-trimoxazole, and fosfomycin [35].

Class A carbapenaemases are rare in *Proteus* spp. The first report on KPC-2 harboring *P. mirabilis* causing bloodstream infections originated from China [36]. *Bla*$_{KPC-2}$ gene was located on incN plasmid

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[36]. Antimicrobial susceptibility testing revealed the strain was resistant to imipenem, meropenem, amoxicillin-clavulanic acid, ampicillin, ampicillin-sulbactam, cefotaxime, piperacillin, cefazolin, ciprofloxacin, levofloxacin, moxifloxacin, gentamicin and sulfamethoxazole-trimethoprim but susceptible to ceftazidime, amikacin, aztreonam and piperacillin-tazobactam. In keeping with its multidrug-resistant profile, *P. mirabilis* XH983 had a number of ARGs, conferring resistance to aminoglycosides [aph(3')-Ia, *aph*(3'')-Ib, *aph*(6)- Id, *aac*(3)-IId, *aad*A5, *aad*A1], β-lactams (*bla*$_{KPC-2}$, *bla*$_{TEM-1B}$), phenicol (cat, catA1), sulphonamide/trimethoprim (*drf*A1, *drf*A17, *sul*1, *sul*2), and tetracycline (tet(J). Except in human samples carbapenemase producing *P. mirabilis* isolates were found in broilers in China [37] reinforcing one health theory.

Coproduction of double carbapenemases (KPC-2 and NDM-1) was reported in Brazil [38]. The isolates harbored a plethora of different virulence gene in addition to blaKPC-2, *bla*$_{NDM-1}$ and *bla*$_{OXA-10}$.

Table 3. Most frequent carbapenemases in *Proteus* species.

Type of carbapenemase	Substrate profile	reference
VIM-1	Penicillins, ESC, carbapenems	[30]
NDM-5	Penicillins, ESC, carbapenems	[22]
OXA-48	Penicillins, monobactams, carbapenems	[32]
OXA-162	Penicillins, monobactams, carbapenems	[32]
OXA-181	Penicillins, monobactams, carbapenems	[32]
OXA-23	Penicillins, monobactams, carbapenems	[33,34]
OXA-58	Penicillins, monobactams, carbapenems	[34]

5. Fluoroquinolone Resistance

High level fluoroquinolone resistance in *P. mirabilis* is usually mediated by mutations in *gyr*A and *par*C genes. Low level resistance is in most cases associated with plasmid-mediated qnr genes (*qnr*A, qnrB, *qn*rC, *qnr*D, *qnr*S). However, recently qnrA6 was found to be chromosomally encoded in *P. mirabilis* [39].

6. Laboratory Detection of Extended-Spectrum β-Lactamases, Plasmid Mediated AmpC β-Lactamases and Carbapenemases in *Proteus* spp.

Laboratory detection of ESBLs in *Proteus* species is done by double-disk synergy test according to Jarlier [40], and combined disk test with clavulanic acid (CLSI, 2018).

The augmentation of the inhibition zones of cephalosporin disks of at least 5 mm by clavulanic acid confirmed ESBL production. Screening for p-AmpC beta-lactamases in *Proteus* spp. is based on reduced susceptibility to cefoxitin.

Confirmation of p-AmpC is carried out by double-disk synergy test with a disk supplemented with 500 μg cloxacillin placed between the disks containing ceftazidime and cefotaxime on a lawn of P. mirabilis isolates with reduced susceptibility to cefoxitin in order to detect p-Amp-C [9]. The distortion of the inhibition zones around ESC disks toward the central disk with cloxacillin was considered a positive result [9]. The other method for confirmation of P-AmpCs is an AmpC disk test according to Black [41]. A blank paper disk is impregnated with 20 μL Tris-EDTA to permeabilize bacterial cells. Three to five colonies of the test organism are applied to the surface of the disk. The disk is placed on the surface of Mueller–Hinton (MH) agar previously inoculated with cefoxitin susceptible *E. coli* ATCC 25922.–The distortion of the inhibition zone around the cefoxitin disk indicated the enzymatic inactivation of cefoxitin [41].

Isolates demonstrating reduced susceptibility to carbapenems are subjected to screening for carbapenemase production by modified Hodge test, CIM (carbapenem-inactivation method) [42], eCIM or CarbaNP test. Isolates suspicious for MBL positivity are tested by imipenem-EDTA inhibitor- based test.

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Overnight culture of the carbapenem-resistant test strain is suspended in saline and an ertapenem disk (10 μg) is placed in the suspension which was incubated for 2 hours at 37 °C. As an indicator strain, *E. coli* ATCC 25922 is inoculated on Mueller–Hinton (MH) agar plates. The disk is removed after 2 h and placed in the middle of the plate. Carbapenem hydrolysis is confirmed if there is no inhibition zone, the zone is smaller than 14 mm or if there are colonies within the inhibition zone [42].

For MHT, an overnight culture of carbapenem-susceptible indicator strain *E. coli* ATCC 25922 is inoculated on the surface of MacConkey agar plates to avoid swarming. After drying, an ertapenem disk (10 μg) is placed in the middle of the plate. Overnight Proteus cultures are streaked as a single line from the periphery of the disc to the edge of the plate. The plates are incubated overnight at 37 °C. Carbapenemase is suspected if the clover-leaf indentation of the indicator organism was observed toward the ertapenem disc [43].

For imipenem-EDTA inhibitor based test overnight Proteus culture is spread on the MH agar plate. Imipenem and meropenem disks with and without EDTA are placed on the plate. Cultures are incubated overnight at 37 °C. The augmentation of the inhibition zone around the carbapenem disk for at least 7 mm in the presence of EDTA is considered a positive result [44].

7. Therapeutic Options

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 [45] 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 [46,47]. 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 [46].

P. mirabilis has intrinsic resistance to colistin, nitrofurantoin and tigecycline which limits therapeutic options.

8. Conclusions

This review demonstrated amazing capacity of *Proteus* species to acquire various resistance determinant in addition to intrinsic resistance and to develop multidrug or extensively-drug resistance phenotype with a few or no therapeutic options left. Accurate and fast laboratory identification of resistance determinants is mandatory to avoid spread of resistance isolates and hospital outbreaks. Confirmation of genes encoding ESBLs, AmpC β- lactamases and carbapenemases is of high epidemiological relevance order to choose the appropriate therapy for bacterial infections due to multidrug-resistant *Proteus* species. The same allelic variants of ESBL and p-AmpC genes were found in both human and animal isolates from different geographic areas and continents, reinforcing one health approach. The dominant animal species harbouring ESBL and AmpC positive *P. mirabilis* are broilers which are food producing animals and can serve as a source of human intestinal colonization. *P. mirabilis* is an important host organism for CHDL previously identified in *A. baumamannii* such as OXA-23 and OXA-58, unlike other Enterobacterales, but with much weaker expression, and developing clinically relevant resistance only in the presence of other resistance determinants like porin loss or hyperexpression of efflux systems.

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Abbreviations: ESBL-extended-spectrum β-lactamases, p-AmpC-plasmid-mediated AmpC β-lactamases, ESC: expanded-spectrum cephalosporins;CHDL-carbapenem-hydrolyzing class D oxacillinases: MIC-minimum inhibitory concentration; WGS-whole genome sequencing, PFGE-pulsed-field gel electrophoresis.

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