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First report of bla_{OXA-24} carbapenemase gene, *armA* methyltransferase and *aac(6′)-Ib*-cr among multidrug-resistant clinical isolates of *Proteus mirabilis* in Algeria

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ABSTRACT

Objective: Carbapenemase-producing, or carbapenem-resistant, Enterobacteriaceae are an emerging threat to human and animal health because they are resistant to many of the last-line antimicrobials available for treatment of infection. The aim of this study was to analyse the antimicrobial resistance patterns and their encoding genes of *Proteus mirabilis* isolated in Constantine, Algeria.
Methods: A total of 108 *Proteus*, *Morganella* and *Providencia* (PMP) strains were isolated from a large variety of clinical specimens at University Hospital of Constantine in Algeria. Isolates were identified using the API 20E system and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). Diagnostic accuracy was determined by independent comparison of each method to phylogenetic analysis based on 16S rRNA gene sequencing. Antimicrobial susceptibility was determined by the standard disk diffusion and Etest methods. The presence of antimicrobial resistance genes was screened for by PCR amplification and sequencing.
Results: A total of 72 PMP strains were multidrug-resistant (MDR). Among them, one *P. mirabilis* isolate was resistant to imipenem with a minimum inhibitory concentration (MIC) of ≥12 μg/mL. PCR and sequencing showed the presence of various antimicrobial resistance genes, including $bla_{CTX-M-15}$, bla_{TEM-1}, bla_{TEM-2}, bla_{PER-1}, bla_{SHV-11}, *aadA1*, *aadA2*, *armA*, *aac(6′)-Ib*, *aac(6′)-Ib*-cr, *aac(3)-Ia* and *ant(2″)-I*, forming different resistance profiles. Moreover, the bla_{OXA-24} gene was detected in the imipenem-resistant *P. mirabilis* strain.
Conclusion: In this study, a MDR *P. mirabilis* isolate harbouring the bla_{OXA-24}, *armA* 16S rRNA methylase and *aac(6)-Ib*-cr genes was found for the first time in Algeria.

1. Introduction

Members of the genera *Proteus*, *Morganella* and *Providencia* (PMP) are components of the normal bacterial flora of the intestinal tract of humans and animals and are widespread in the environment [1]. Owing to their varied habitats, members of the PMP genera have many possible routes of human infection. Modes of transmission may include nosocomial sources such as hospital food and equipment, intravenous solutions and human contact through contaminated skin surfaces [2], causing primary and secondary infections [1]. Interest in species comprising these three genera has occurred mainly because most infections are associated with prolonged hospitalisation and, in the case of *Proteus* and *Morganella* spp., colonisation of indwelling catheters and associated urinary tract infections [2]. These organisms are intrinsically resistant to nitrofurantoin and tetracycline but are naturally susceptible to β-lactams, aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole [1,2]. However, antimicrobial resistance has been increasingly reported for these species, and the spread of resistance to extended-spectrum cephalosporins

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owing to the production of extended-spectrum β-lactamases (ESBLs) has become of great concern [3] since ESBL production in *Proteus mirabilis* was first documented in 1987 [4]. Carbapenems are now frequently employed in the treatment of serious nosocomial infections caused by Gram-negative bacteria, including ESBL-producing Enterobacteriaceae [5]. However, the emergence of clinical strains of various species producing class D carbapenemases, including oxacillin-hydrolysing or OXA-type enzymes, has been reported [6]. These class D carbapenemases have so far been associated with imipenem-resistant *Acinetobacter baumannii* strains [7]. However, the first and only detection of a clinical *P. mirabilis* strain producing class D carbapenemase was in France in 2002, which produced an OXA-23 enzyme [6].

The purpose of the present study was to determine the rate of antimicrobial resistance in a large series of clinical isolates of the PMP group isolated from University Hospital of Constantine (Constantine, Algeria) to agents usually prescribed as first intention. We also describe for the first time the detection of a carbapenem-resistant *P. mirabilis* strain carrying the bla_{OXA-24} gene.

2. Materials and methods

2.1. Bacterial isolates

A total of 108 clinical isolates belonging to the PMP group were isolated from outpatient and patients hospitalised (patient sex ratio = 1) between January–December 2011 in the University Hospital of Constantine. A large variety of clinical specimens were obtained, including pus ($n = 60$), urine ($n = 38$), probe ($n = 5$), catheter ($n = 1$), biological fluids ($n = 3$) and blood cultures ($n = 1$). Strains were cultured on trypticase soy agar (TSA) plates at 37 °C for 18–24 h. Species identification was performed by standard biochemical tests using an API20E system (bioMérieux, Marcy-l'Étoile, France) and by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) using a Bruker microflex™ system (Bruker Daltonik GmbH, Bremen, Germany) with Bruker MALDI Biotyper 2.0 software as previously described [8]. In addition, species identification was confirmed by 16S rRNA gene sequencing.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on Mueller–Hinton agar by the standard disk diffusion procedure as described by the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (www.sfm-microbiologie.org/) to the following antibiotics: amoxicillin (25 μg); amoxicillin/clavulanic acid (AMC) (20/10 μg); cefotaxime (30 μg); ceftazidime (30 μg); ceftriaxone (30 μg); aztreonam (30 μg); imipenem (10 μg); gentamicin [15 μg (10 UI)]; kanamycin (30 UI); tobramycin (10 μg); amikacin (30 μg); pefloxacin (5 μg); ciprofloxacin (5 μg); ofloxacin (5 μg); trimethoprim/sulfamethoxazole (SXT) (1.25/23.75 μg); and colistin (50 μg).

ESBL-producing strains were detected by synergy test between a central disk of AMC at a distant of 30 mm from disks of cefotaxime, ceftriaxone, ceftazidime or aztreonam. The presence of an ESBL was suspected by the appearance of a 'champagne cork'.

Minimum inhibitory concentrations (MICs) for imipenem were determined by Etest (AB bioMérieux, Marcy-l'Étoile, France). Interpretations were made according to CA-SFM breakpoints.

2.3. PCR amplification of antimicrobial resistance genes

Detection of antimicrobial resistance genes was performed by conventional PCR using forward and reverse primers for ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB}, bla_{PER} and bla_{GES}) [9], fluoroquinolone resistance genes (*qnrA* and *qnrB*) [10] and genes encoding aminoglycoside-modifying enzymes (AMEs) [*armA*, *aac(3)*, *aac(6′)*, *ant(2″)*, *aph(3′)*, *aad* and *rmtA*] [11] as well as carbapenemase genes for the strain resistant to imipenem (bla_{VIM}, bla_{IMP}, bla_{KPC}, bla_{NDM-1}, bla_{OXA-23} and bla_{OXA-24}). Positive PCR products obtained were sequenced using BigDye® Terminator chemistry on an ABI 3730 Automated Sequencer (Applied Biosystems, Foster City, CA). The sequences obtained were analysed using BlastN and BlastP against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Conjugation experiment

Conjugation experiments were carried out on TSA plates between the imipenem-resistant *P. mirabilis* donor strain and the azide-resistant recipient strain *Escherichia coli* J53. Transconjugants were selected on TSA plates supplemented with 2 μg/mL ceftazidime or 2 μg/mL cefotaxime and 100 μg/mL sodium azide.

3. Results

3.1. Proteus, Morganella *and* Providencia *isolates*

During the study period (January–December 2011), a total of 108 isolates were identified belonging to three PMP genera by the API20E identification system, MALDI-TOF/MS and 16S rRNA gene sequencing. Four species were recovered. *Proteus mirabilis* was the most frequently isolated species, representing 89.8% (97/108) of all PMP isolated, followed by *Proteus vulgaris* and *Morganella morganii* [4.6% (5/108) each] and *Providencia stuartii* [0.9% (1/108)]. Among these strains, 86 isolates (79.6%) were isolated from hospitalised patients and 22 isolates (20.4%) were isolated from outpatients.

3.2. Antimicrobial susceptibility testing

Results of antimicrobial susceptibility testing for the 108 isolates are summarised in Table 1. A review of the antimicrobial resistance profile of isolates from the different clinical specimens showed that amoxicillin, AMC, cefotaxime, tobramycin, ciprofloxacin, pefloxacin and SXT were the least active antibiotics.

Table 1
Phenotypic antimicrobial resistance of 108 *Proteus*, *Morganella* and *Providencia* (PMP) isolates.

Species	Percent resistant															
	AMX	AMC	CTX	CAZ	CRO	ATM	IPM	GEN	KAN	TOB	AMK	PEF	CIP	OFX	SXT	COL
Proteus mirabilis ($n = 97$)	65.98	25.77	10.31	5.15	9.28	1.03	1.03	11.34	16.49	53.61	10.31	36.08	30.93	36.08	56.70	100
Proteus vulgaris ($n = 5$)	100	0	40	0	20	0	0	0	0	20	0	0	0	0	20	100
Morganella morganii ($n = 5$)	80	80	20	20	0	0	0	0	20	0	0	40	40	80	80	100
Providencia stuartii ($n = 1$)	0	0	0	0	0	0	0	0	0	0	0	100	100	0	100	100

AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; ATM, aztreonam; IPM, imipenem; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; AMK: amikacin; PEF, pefloxacin; CIP, ciprofloxacin; OFX, ofloxacin; SXT, trimethoprim/sulfamethoxazole; COL, colistin.

Antibiotics with the highest activities against all four species were aztreonam, gentamicin and amikacin. All strains were resistant to colistin, and just one strain of *P. mirabilis* was found to be resistant to imipenem (MIC ≥ 12 μg/mL confirmed by Etest).

3.3. Determination of antimicrobial resistance genes

All 108 strains were searched by PCR for the presence of ESBL genes, fluoroquinolone resistance genes and AME-encoding genes. A total of 72 strains were positive for different genes, including $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{TEM\text{-}2}$, $bla_{PER\text{-}1}$, $bla_{SHV\text{-}11}$, *aadA1*, *aadA2*, *armA*, *aac(6′)-Ib*, *aac(6′)-Ib*-cr, *aac(3)-Ia* and *ant(2″)-I*, forming different resistance profiles (Table 2). Moreover, sequencing of the amplification products confirmed the presence of the $bla_{OXA\text{-}24}$ gene in the imipenem-resistant *P. mirabilis* isolate.

Conjugation studies between *E. coli* J53 and the imipenem-resistant *P. mirabilis* isolate was successful, with the transfer of $bla_{TEM\text{-}1}$, *aadA2* and *armA* genes. However, transfer of carbapenem resistance to *E. coli* J53 by conjugation failed.

4. Discussion

The PMP genera are considered as one of the most important human pathogens that frequently cause serious infections in hospitalised patients and immunocompromised persons. Different methods of isolation and identification have been developed for most PMP species. However, the treatment of infected patients is often problematic due to the development of antimicrobial resistance. The occurrence of multidrug-resistant (MDR) and pandrug-resistant PMP isolates is a growing concern. In this study, the molecular mechanisms of antimicrobial resistance in PMP clinical isolates recovered from University Hospital of Constantine in Algeria were investigated. The data revealed genetic diversity of genes encoding ESBLs, with the emergence of new genes.

Epidemiological data regarding ESBLs available for Algeria report the presence of different genes such as $bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}110}$, $bla_{SHV\text{-}1}$, $bla_{SHV\text{-}12}$, $bla_{SHV\text{-}28}$, $bla_{PER\text{-}1}$ and $bla_{VEB\text{-}1}$ in various species of Gram-negative bacteria [12]. In the current series of PMP isolates, the main molecular support explaining resistance to extended-spectrum cephalosporins was the presence of $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, $bla_{TEM\text{-}2}$, $bla_{PER\text{-}1}$ and $bla_{SHV\text{-}11}$ ESBL-encoding genes, along with the coexistence of the $bla_{OXA\text{-}24}$ carbapenemase-encoding gene for one strain of imipenem-resistant *P. mirabilis*.

The presence of $bla_{SHV\text{-}11}$ was observed in only one strain of *P. vulgaris*. This gene differs from the $bla_{SHV\text{-}1}$ gene by a Leu35 → Gln substitution, and it differs only at position 1 of codons 238 and 240 [13]. This gene was previously described in *Klebsiella pneumoniae* [14] but never in a strain belonging to the PMP group.

Currently, carbapenems are the most potent antimicrobial agents used in the treatment of serious infections caused by MDR Gram-negative bacteria. especially in the current context of massive diffusion of CTX-M-type ESBLs, but it is necessary to preserve this class of antibiotics [15]. This is especially true as there is no current perspective to placing new antibiotics on the market. These antibiotics have good activity against the PMP group. Unfortunately, in recent years the emergence and spread of strains resistant to carbapenems has been observed [16]. This resistance is mainly due to the production of carbapenemases, essentially of class D (oxacillinases) and sometimes class B (metallo β-lactamases).

According to the literature, genes encoding carbapenemases have been widely detected in many bacterial groups in different countries. However, reports on *Proteus* spp. producing carbapenemases are rare. Bonnet et al. first reported the chromosomally-encoded class D β-lactamase OXA-23 in *P. mirabilis* in 2002 in France, which was exclusively found in *Acinetobacter* spp. [6]. Different studies have reported *P. mirabilis* isolates producing a VIM-1 molecular class B metallo-β-lactamase resulting in carbapenem resistance [5,17]. In 2008, Tibbetts et al. first reported a single isolate of *P. mirabilis* harbouring bla_{KPC} in the USA [18]. Hu et al. reported for the first time the emergence of *P. mirabilis* harbouring $bla_{KPC\text{-}2}$ and *qnrD* in the same strain in a Chinese hospital [19], and Cicek et al. reported the first identification of a $bla_{OXA\text{-}320}$–*aadA1* gene cassette, a novel variant of class D β-lactamase, in *P. mirabilis* from Turkey [20]. Recently, Girlich et al. described for the first time the $bla_{NDM\text{-}1}$ gene in a *P. mirabilis* clinical

Table 2
Genotypic profiles of antimicrobial resistance of *Proteus*, *Morganella* and *Providencia* (PMP) isolates (n = 108).

Species	Resistance profile	Gene(s)	No. of strains	Rate of isolates (%)
Proteus mirabilis	1	$bla_{CTX\text{-}M\text{-}15}$	6	5.56
	2	$bla_{CTX\text{-}M\text{-}15}$ + $bla_{TEM\text{-}1}$	3	2.78
	3	$bla_{CTX\text{-}M\text{-}15}$ + $bla_{TEM\text{-}2}$	1	0.93
	4	$bla_{TEM\text{-}1}$	29	26.85
	5	$bla_{TEM\text{-}1}$ + *aadA2*	3	2.78
	6	$bla_{TEM\text{-}1}$ + $bla_{OXA\text{-}24}$ + *aadA2* + *armA*	1	0.93
	7	$bla_{TEM\text{-}1}$ + *aadA1* + *aac(3)-Ia*	1	0.93
	8	$bla_{TEM\text{-}1}$ + *aac(6′)-Ib*	1	0.93
	9	$bla_{TEM\text{-}2}$	9	8.33
	10	$bla_{TEM\text{-}2}$ + *aac(6′)-Ib*-cr	1	0.93
	11	*aac(6′)-Ib*	6	5.56
	12	*aac(6′)-Ib* + *ant(2″)-I*	1	0.93
	13	*aac(3)-Ia*	1	0.93
Proteus vulgaris	1	$bla_{TEM\text{-}1}$	1	0.93
	2	$bla_{TEM\text{-}2}$ + $bla_{PER\text{-}1}$ + $bla_{SHV\text{-}11}$ + *aadA1* + *aac(6′)-Ib* + *ant(2″)-I*	1	0.93
	3	$bla_{TEM\text{-}2}$ + $bla_{PER\text{-}1}$ + *aadA1* + *aac(6′)-Ib* + *ant(2″)-I*	1	0.93
	4	$bla_{PER\text{-}1}$ + *aac(6′)-Ib*	1	0.93
	5	*armA*, *aadA2*	1	0.93
Morganella morganii	1	$bla_{TEM\text{-}1}$	1	0.93
	2	*aac(6′)-Ib*	1	0.93
	3	*aac(3)-Ia* + *aadA2* + *ant(2″)-I*	1	0.93
Providencia stuartii	1	*aadA1*	1	0.93
Total			72	66.67

isolate [21]; in addition, production of OXA-58 in *P. mirabilis* has been reported from France [22] and Germany [23]. Another recent study has demonstrated the presence of bla_{OXA-58} in a MDR *P. mirabilis* strain from Gaza, Palestine [24].

In the current study, the main molecular support for resistance to carbapenems was the presence of bla_{OXA-24} encoding a class of carbapenemase D, a gene typically present in *A. baumannii*. This gene was identified in isolates in 1997 that were part of an outbreak in Spain, and since then it has never been detected in strains other than *A. baumannii* [25]. This gene is still rare in Algeria, with only a very few reports. It was previously reported in 6 strains of *A. baumannii* isolated in 2011 in different hospitals in Tlemcen, Setif, Sidi Bel Abbes, Oran and Tizi Ouzou [26] as well as in 17 other strains of *A. baumannii* isolated in three different hospitals in the west of Algeria (Tlemcen, Oran and Sidi Bel Abbes) from 2008–2012 [27]. However, the existence of a *P. mirabilis* isolate resistant to carbapenems with a class D OXA-24-type carbapenemase has never been described before. In this report we describe what we believe to be the first reported case of infection caused by a strain of carbapenem-resistant *P. mirabilis* harbouring the bla_{OXA-24} gene. It is a disturbing trend given the relatively recent discovery of this family of carbapenemase. Whilst ESBL and carbapenemase activities have previously been documented in *Proteus* spp. [5,6,17–20], the addition of bla_{OXA-24} to the spectrum of resistance factors carried by an organism that traditionally has been placed in the low-level endogenous resistance category is equally troubling.

Conjugation experiments revealed the association of bla_{TEM-1}, *aadA2* and *armA* genes on the same genetic structure as they were found in the transconjugant following transfer of a single plasmid. However, the carbapenemase resistance of the *P. mirabilis* strain encoded by bla_{OXA-24} is mainly referred to the chromosomal gene rather than plasmid-mediated factors. According to the literature, bla_{OXA-23} and bla_{OXA-58} are mostly found on plasmids, whereas the bla_{OXA-24} has been identified as being chromosomally encoded [28]. It is tempting to speculate that genes encoding OXA-24 enzymes could belong to a subspecies of *P. mirabilis* that had acquired this type of gene in the distant past. The reservoir (natural producer) of these genes is unknown, as is the location of the genetic exchange.

Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of human infections caused both by Gram-positive strains (*Staphylococcus* spp., *Enterococcus* spp.) and Gram-negative strains including *Proteus* spp. In the past decade, these antibiotics are no longer used owing to the spread of AMEs worldwide [29]. In this study, high rates of AMEs were observed among MDR isolates (22/72; 30.6%). Acquisition of new resistance mechanisms by strains already resistant to particular antimicrobials creates a serious concern owing to the propagation of multidrug resistance. In the current study, the most prevalent gene encoding an AME was *aac(6′)-Ib* present in 59.1% (13/22) of AME-positive isolates. These results are consistent with the literatures data in which *aac(6′)*-Ib is considered as the most common variant of AME among Gram-negative as well as Gram-positive isolates [30]. However, resistance of *Proteus* spp. to aminoglycosides still remains low compared with other bacteria [29]. In Algeria, similar results have been reported in previous studies on aminoglycoside resistance mechanisms among different Gram-negative clinical strains [12,27,31], although none of these reports studied resistance to aminoglycosides in PMP strains. From these results it is suggested that during the period 2007–2013, genes coding for AMEs have become endemic in Algeria and have spread among different species of Gram-negative bacteria.

Despite the existence of strains resistant to fluoroquinolones, no strain carried the *qnrA* or *qnrB* genes. The resistance to ciprofloxacin observed in strains in the current study suggests that the main mechanism of fluoroquinolone resistance is probably due to mutation(s) in genes encoding topoisomerases or gyrases, which confer high levels of resistance [32]. However, one strain of *P. mirabilis* was found to carry the *aac(6′)-Ib*-cr gene. This variant is an acetyltransferase that is part of the AMEs [33].

Fluoroquinolone resistance genes have been recently identified in Algeria. The first study published in 2008 reported the presence of *qnr* genes in *Enterobacter cloacae* strains [34]. Thereafter, several studies have identified different variants of *qnr* determinants often in combination with the *aac(6′)*-Ib-cr gene among Enterobacteriaceae strains [12,27,31]. This determinant was discovered for the first time in 2006 in a *qnrA*-positive *E. coli* strain in China [33]. In Algeria, the *aac(6′)*-Ib-cr gene was detected for the first time in 2009 in an *E. cloacae* strain [31]. Since then, two other studies have reported the presence of this gene both in hospitals and in the community [27,35]. However, this gene has not been reported in Algeria in strains belonging to the PMP group, although it was recently reported in North Africa in clinical strains of *P. mirabilis* and *M. morganii* isolated in Tunisia [36]. The current study is the first description in Algeria of the *aac(6′)*-Ib-cr determinant in clinical strains of *P. mirabilis*, suggesting that there has been spread of this gene between bacterial groups and clonal spread within PMP strains in North Africa. This plasmid-mediated quinolone resistance mechanism confers low-level resistance to fluoroquinolones but its presence could further encourage the development to a higher level of resistance by mutation selection in the target of these molecules [31].

In conclusion, acquisition of resistance to carbapenems in *P. mirabilis* may be of significant concern for physicians because this organism is usually resistant to colistin and is poorly susceptible to tigecycline, which represents an important option for treating infections caused by MDR Gram-negative bacilli.

This study is the first report describing imipenem-resistant *P. mirabilis* isolated from patients in Algeria. We report for the first time the emergence of bla_{OXA-24} as well as the co-occurrence of *armA* 16S rRNA methylase with bla_{OXA-24} in Eastern Algeria. We also report the first identification of MDR PMP isolates harbouring bla_{SHV-11} and *aac(6)-Ib*-cr genes in Algeria.

The emergence of a combination of resistance genes in PMP group isolates could pose a public-health problem, thus substantially restricting therapeutic alternatives. Based on this finding, it would be prudent to systematically review all clinically relevant Enterobacteriaceae isolates for resistance to carbapenem, even in circumstances where the use of this class of drug for the treatment of infection would be less likely, i.e. uncomplicated urinary tract infection.

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Competing interests

None declared.

Ethical approval

Not required.

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