First report of blaOXA-24 carbapenemase gene, armA methyltransferase and aac(6')-Ib-cr among multidrug-resistant clinical isolates of Proteus mirabilis in Algeria

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First report of *bla*$_{OXA-24}$ carbapenemase-encoding gene, *armA* Methyltransferase and *aac(6)-Ib-cr* producing multidrug-resistant clinical isolates of *Proteus mirabilis* in Algeria.

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Abstract

Background: 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 disease treatment. The aim of this study was to analyze antimicrobial resistance patterns and their encoding genes of *P. mirabilis* isolated in Constantine, Algeria.

Methods: A total of 106 *PMP* (*Proteus- Morganella- Providencia*) strains were isolated from a large variety of clinical specimens, at University Hospital of Constantine in Algeria, and identified by the API 20E system and Bruker MALDI Biotyper 2.0 (MALDI-TOF/MS) platforms for microbial identification. Diagnostic accuracy was determined by independent comparison of each method to phylogenetic analysis based on the 16S rRNA gene sequencing. Antimicrobial susceptibility was determined using disc diffusion and E-test methods. The presence of antibiotic resistance genes was screened for by PCR amplification and sequencing.

Results: a total of 72 *PMP* strains were multidrug- resistant. Among them, one isolate was resistant to imipenem with minimum inhibitory concentration $\geq$ 12 µg/ml. PCR and sequencing showed the presence of different antibiotic resistance encoding genes: *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, ant(2")-I*, and forming different profiles. Moreover, the *bla*$_{OXA-24}$ gene was detected in the imipenem-resistant strain.

Conclusion: in this study, we found for the first time in Algeria a multidrug resistant *P. mirabilis* isolates harboring *bla*$_{OXA-24}$, *armA* 16S rRNA methylase and *aac(6)-Ib-cr* gene.

Keywords: Multidrug-resistant; *bla*$_{OXA-24;}$ *arma* methyltransferase; *aac(6)-ib-cr, Proteus, Morganella* and *Providencia*.

1. Introduction

Members of the three genera *Proteus*, *Morganella* and *Providencia* (*PMP*) are components of the normal bacterial flora of the intestinal tracts 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. The 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 the species comprising these genera has occurred mainly that most infections are associated with prolonged hospitalization and in the case of *Proteus* and *Morganella spp*., colonization 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, drug resistance has been increasingly reported for these species, and the diffusion of resistance to extended-spectrum cephalosporins due to the production of extended-spectrum β-lactamases (ESBLs) has become of great concern (3) since ESBL production in *P. mirabilis* was first documented in 1987 (4). Carbapenems are now employed frequently 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 include oxacillin-hydrolyzing, or OXA-type enzymes has been reported (6). These class D carbapenemases have so far been associated with imipenem-resistant *A. baumannii* strains (7). However, the first and only detection of a clinical *P. mirabilis* strain producing class D carbapenemase was in France in 2002, producing an OXA-23 enzyme (6).

The purpose of the present study was (i) to determine the rate of antibiotic resistance of a large series of clinical isolates of the *PMP* group, from University Hospital of Constantine, Algeria, against molecules usually prescribed first intention and (ii) to detect for the first time the carbapenem-resistant *P. mirabilis* carrying the $bla_{OXA\text{-}24}$.

2. Materials and methods

2.1. Bacterial isolates

A total of 106 clinical isolates belonging to the *Proteus-Morganella-Providencia* group were isolated from outpatient and patients hospitalized between January and December 2011 in the University Hospital of Constantine, Algeria. A large variety of clinical specimens were issued from pus (n = 60), urine (n = 38), sonde (n = 5), catheter (n = 1), biological fluids (n = 3) and blood cultures (n = 1) with sex ratio=1. Strains were cultured on TSA (Trypticase Soja) agar plates at 37°C for 18 to 24 hours. Species identification was performed by standard biochemical tests using API20E system (BioMerieux, Marcy l'Etoile, France) and by use of the matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) method (Microflex; Bruker Daltonics) as previously described (8). Additionally, species identification was confirmed by sequencing of the 16S ribosomal RNA gene.

2.2. Antibiotic susceptibility testing

Antimicrobial susceptibility was tested with Mueller-Hinton agar by standard disk diffusion procedure as described by the Antibiogram Committee of the French society for microbiology (CA-SFM) (www.sfm.asso.fr). The following antibiotics were tested: Amoxicillin (25 µg), Amoxicillin/Clavulanic acid (20/10 µg), Cefotaxim (30 µg), Ceftazidim (30 µg), Ceftriaxon (30 µg), Aztreonam (30 µg), Imipenem (10 µg), Gentamicin (15 µg (10 UI), Kanamicin (30 UI), Tobramicin(10 µg), Amikacine (30 µg), Pefloxacin (5 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Triméthoprim/sulfamethoxazol (1,25/23,75 µg) and Colistin (50 µg).

Strains producing ESBL were detected by the test of synergy between a central disk of amoxicillin/clavulanic acid distant 30mm discs of cefotaxime, ceftriaxone, ceftazidime or aztreonam. The presence of ESBL was suspected in an appearance in "champagne cork".

The minimum inhibitory concentrations (MICs) for imipenem were determined using Etest method (AB Biodisk). Interpretations were made according to CA-SFM breakpoints.

2.3. PCR amplification of resistance-encoding genes

Detection of antimicrobial resistance genes was performed by Conventional PCR using the forward and reverse primers for the ESBL genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{VEB}, bla_{PER} and bla_{GES}) (9) , Fluoroquinolone resistance genes (*qnrA* and *qnrB*) (10), aminoglycoside-modifying enzymes (AMEs) (*armA*, *aac3*, *aac(6´)*, *ant(2´´)*, *aph(3´)*, *aad*, and *rmtA*) (11) and carbapenemases, for the strain resistant to imipenem, (bla_{VIM}, bla_{IPM}, bla_{KPC}, $bla_{NDM\text{-}1}$, bla_{OXA23}, bla_{OXA24}). Positive PCR products obtained were sequenced using the Big Dye® terminator chemistry on an automated ABI 3730 sequencer (Applied Biosystems, Foster City, California, United States). The sequences obtained were analyzed using BlastN and BlastP against the NCBI database (http:/www.ncbi.nlm.nih.gov/blast) for characterization.

2.4. Conjugation test

Conjugation experiments were carried out between *P. mirabilis* donor imipenem resistant and the azide-resistant recipient strain *E. coli J53* on TSA plates. Transconjugants were selected on TSA plates supplemented with 2 µg ceftazidime (CAZ) ml−1 or 2 µg cefotaxime (CTX) ml−1 and 100 µg sodium azide ml−1.

3. Results

3.1. Proteus, Morganella and Providencia isolates

During the study period, a total of 106 isolates were identified for 3 genera, *Proteus*, *Morganella* and *Providencia* both by the API20E identification system, by MALDI-TOF MS and by sequencing of the 16S rRNA gene. Four species were recovered, *Proteus mirabilis* is the species most frequently isolated and represents 89.62 % or 95 strains of all *PMP* isolated, followed by *Proteus vulgaris* and *Morganella morganii* with a frequency 4.72% (5 strains) for each one, and *Providencia stuartii* represent 0.94 % (1 strain). Among these strains, 84 isolates (79%) were isolated from hospitalized patient, and 22 isolates (21%) were isolated from outpatient

3.2. Antimicrobial susceptibility

Results of antibiotic susceptibility testing for the 106 isolates are summarized in table 1. A review of the antimicrobial resistance profile of isolates from the different clinical specimens showed that amoxicillin, amoxicillin/clavulanic acid, cefotaxim, tobramicin, ciprofloxacin, pefloxacin and trimethoprim/sulfamethoxazol were the most active antibiotics. Antibiotics with the lowest activities on all four species were aztreonam, gentamicin and amikacin. All strains were resistant to colistin, but just one strain of *P. mirabilis* was found to be resistant to imipenem (MIC $\geq$ 12 µg/ml, confirmed by E test).

3.3. Resistance gene determination

All 106 strains were checked for the presence of ESBL, resistance to fluoroquinolones and aminoglycoside-modifying enzymes encoding genes using the PCR methods described above. 72 strains were positive for different 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, ant(2″)-I*, and forming different profiles that it has been shown in table 2. Moreover, the sequencing amplification products confirmed the presence of the bla_{OXA-24} gene in the imipenem-resistant strain.

Conjugal studies between *E. coli J53* and the imipenem-resistant *P. mirabilis* isolate was successful with the transfer of *bla* $_{TEM-1}$, *aadA-2* and *armA* genes. However, it has failed to transfer carbapenemase resistance to *E. coli J53* by conjugation.

4. Discussion

The genera *Proteus, Morganella* and *Providencia* are considered as one of the most important human pathogens that often cause serious infections in hospitalized 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 antibiotic resistance. The occurrence of MDR and pan drug-resistant *PMP* is a growing concern. In this study, we investigated the molecular mechanism of antibiotic resistance in *PMP* clinical isolates recovered from University hospital of Constantine in Algeria. Our data revealed genetic diversity of genes that encode ESBL with the emergence of new genes.

Epidemiological data regarding ESBLs available for Algeria report the presence of different genes such as *bla* $_{CTX-M-3}$, *bla* $_{CTX-M-14}$, *bla* $_{CTX-M-15}$, *bla* $_{TEM-110}$, *bla* $_{SHV-1}$, *bla* $_{SHV-12}$, *bla* $_{SHV-28}$, *bla* $_{PER1}$ and *bla* $_{VEB-1}$ in various species of Gram-negative bacteria (12). In our series of *PMP* isolates, the main molecular support explaining the resistance to ESBL was the presence of *bla* $_{CTX-M-15}$, *bla* $_{TEM-1}$, *bla* $_{TEM-2,}$ *bla* $_{PER-1}$ and *bla*$_{SHV-11}$ ESBL encoding genes along with the coexistence of *bla*$_{OXA-24}$ carbapenemase encoding gene for one strain *P. mirabilis* imipenem-resistant.

The presence of *bla* $_{SHV-11}$ gene has been observed in only one strain of *P. vulgaris*. This gene is different from Gln - Leu substitution at amino acid 35 to the *bla* $_{SHV-1}$ gene, and it's differing only at position 1 of codon 238 and 240 (13). This gene was previously described in *K. pneumonia* (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 multidrug-resistant gram-negative bacteria. especially in the current context of massive diffusion of $bla_{CTX\text{-}M}$ type ESBLs, but are antibiotics which it is necessary to preserve (15) . This especially that there is no current perspective to placing on the market of new antibiotics. These antibiotics have good activity against the *PMP* group. Unfortunately, there has been in recent years the emergence and spread of resistant strains to carbapenems (16). This resistance is due, mainly, to the production of carbapenemases essentially of class D (oxacillinases) sometimes class B (metallo β-lactamases).

According to the literature, genes encoding carbapenemases have widely been detected in many bacterial groups in different countries. However, reports on *Proteus spp.* producing carbapenemases are rare. Bonnet et al. first reported chromosome-encoded class D beta-lactamase *OXA-23* in *P. mirabilis* in 2002 in France, which was exclusively found in *Acinetobacter* spp (6). Different studies reported the *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* harboring bla_{KPC} in USA (18). Hu et al. reported for the first time emergence of *P. mirabilis* producing $bla_{KPC\text{-}2}$ and *qnrD* in the same strain in a Chinese hospital (19), and Cicek et al. reported the first identification of $bla_{OXA\text{-}320}$ *-aadA1* gene cassette, a novel variant of Class D β-lactamase, in *P. mirabilis* from Turkey (20). Recently, Girlich et *al.* describe for the first time *the* $bla_{NDM\text{-}1}$ gene in a *Proteus mirabilis* clinical isolate (21), in addition, the production of $bla_{OXA\text{-}58}$ in *P. mirabilis* has been reported from France (22) and Germany (23). Another recent study has demonstrated the presence of $bla_{OXA\text{-}58}$ in Multidrug-Resistant *Proteus mirabilis* Strain from Gaza, Palestine (24).

In our study, the main molecular support of resistance to carbapenems is the presence of bla_{OXA-24} gene encoding a class of carbapenemase D, gene typically present in *A. baumannii*. This gene was identified in isolates in 1997, which were part of an outbreak in Spain, and since, it has never been detected in strains other than *A. baumannii (25).* In Algeria, this gene is still rare, 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) and 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 to 2012 (27). However, the existence of *P. mirabilis* isolate resistant to carbapenems with a class D carbapenemase bla_{OXA24} type 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 *Proteus mirabilis* positive for the bla_{OXA-24} gene. It is a disturbing trend, given the relatively recent discovery of this family of carbapenemase. While extended-spectrum β-lactamase and carbapenemase activities have previously been documented in *Proteus* species (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.

Conjugating experiments reveal the association of genes bla_{TEM-1}, *aadA-2* and *armA* on a same genetic structure, since they were found in the transconjugant after transfer of a single plasmid. But the carbapenemase resistance of *P. mirabilis* encoded by bla_{OXA-24} is mainly referred to the chromosomal gene rather than plasmid-mediated factors. According to the literature, the bla_{OXA-23} and bla_{OXA-58} genes are mostly found on plasmids, whereas the bla_{OXA-24} genes have been identified as chromosomally encoded (28). It is tempting to speculate that genes encoding bla_{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 by both Gram-positive (*Staphylococcus spp.*, *Enterococcus spp.*) and Gram-negative, including *Proteus spp*. strains. In the past decade, these antibiotics are no longer used because of the spread of AMEs worldwide (34). In this research we observed a high rates of AME among multidrug resistant isolates (30,55%). Acquisition of new resistance mechanisms by strains already resistant to particular antimicrobials creates serious concern, due to the propagation of multidrug resistant. In our survey, the most prevalent gene encoding AME was aac(6)-Ib, present in 59.09% 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-negatives, as well as Gram-positives (29), but resistance of *Proteus* to aminoglycosides still remains low over other bacteria (30) . In Algeria similar results are reported in previous studies on aminoglycoside resistance mechanisms among different clinical strains Gram-negative (12;27;31), except that none of these reports has studied resistance to aminoglycosides in *PMP* strains. From these results it is suggested that during the period from 2007 to 2013, genes coding for AME 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 carries neither the gene *qnrA* nor *qnrB* gene. The results of resistance to ciprofloxacin, observed in our strains, suggest that the main mechanism of fluoroquinolone resistance is probably due to the mutations in genes encoding topoisomerases or gyrases which express high levels of resistance (32). However, one strain of *P. mirabilis* is found carrier gene *aac(6')- Ib-cr*.

This variant is an acetyltransferase which is part of aminoglycoside modifying enzymes (AME) (33).

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

In conclusion, The 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 multi-drug-resistant 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 $bla_{OXA\text{-}24}$, and the cooccurrence of 16S rRNA methylase *armA* with $bla_{OXA\text{-}24}$ in Eastern Algeria. We also report the first identification of multidrug-resistant *PMP* isolates harboring $\mathit{bla}_{\mathit{SHV-11}}$ *and aac(6)-Ib-cr* genes in Algeria.

The emergence of a combination of resistance genes in *PMP* group could pose a public health problem, thus substantially restricting the 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 the infection would be less likely , i.e., uncomplicated urinary tract infection.

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Ethical Statement

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Table1. Phenotypic antimicrobial resistance of 106 *PMP* isolates

	AMX	AMC	CTX	CAZ	CRO	ATM	IMP	GN	K	TOB	AK	PEF	CIP	OFX	STX	CT
P.mirabilis	65,62	25	10,42	4,16	9,38	1,04	1,04	10,42	15,63	54,16	9,38	36,46	31,25	36,46	56,25	100
P.vulgaris	100	16,66	50	16,67	33,33	16,67	0	0	16,66	33,33	0	16,67	16,67	16,67	33,33	100
M.morganii	80	80	20	20	0	0	0	0	20	0	0	40	40	80	80	100
P.stuartii	0	0	0	0	0	0	0	0	0	0	0	100	100	0	100	100

AMX: amoxicillin; AMC : amoxicillin/clavulanic acid; CTX : cefotaxim; CAZ : ceftazidim; CRO : ceftriaxon; ATM : Aztreonam; IMP : imipenem; GN : gentamicine; K : kanamicin; TOB : tobramicin; AK : amikacin; PEF : pefloxacin; CIP : ciprofloxacin; OFX : ofloxacin; SXT : triméthoprim/sulfaméthoxazol; CT : colistine.

Table 2. Genotypic profiles of antimicrobial resistance of *PMP* isolates

Species	Groups	Genes	Nb of Strains	Rate of isolates (%)
	1	*bla*$_{CTX\text{-}M\text{-}15}$	6	5.45
	2	*bla*$_{CTX\text{-}M\text{-}15}$ + *bla*$_{TEM\text{-}1}$	3	2.73
	3	*bla*$_{CTX\text{-}M\text{-}15}$ + *bla*$_{TEM\text{-}2}$	1	0.91
	4	*bla*$_{TEM\text{-}1}$	29	26.36
	5	*bla*$_{TEM\text{-}1}$ +*aadA2*	3	2.73
	6	*bla*$_{TEM\text{-}1}$ + *bla*$_{OXA\text{-}24}$ + *aadA2* + *armA*	1	0.91
P. mirabilis	7	*bla*$_{TEM\text{-}1}$ + *aadA1* + *aac(3)-Ia*	1	0.91
	8	*bla*$_{TEM\text{-}1}$ + *aac(6′)-Ib*	1	0.91
	9	*bla*$_{TEM\text{-}2}$	9	8.18
	10	*bla*$_{TEM\text{-}2}$ + *aac(6′)-Ib-cr*	1	0.91
	11	*aac(6′)-Ib*	6	5.45
	12	*aac(6′)-Ib* + *ant(2″)-I*	1	0.91
	13	*aac(3)-Ia*	1	0.91
	1	*bla*$_{TEM\text{-}1}$	1	0.91
	2	*bla*$_{TEM\text{-}2}$ +*bla*$_{PER\text{-}1}$ + *bla*$_{SHV\text{-}11}$+ *aadA1*+ *aac(6′)-Ib* + *ant(2″)-I*	1	0.91
P. vulgaris	3	*bla*$_{TEM\text{-}2}$ +*bla*$_{PER\text{-}1}$ + *aadA1*+ *aac(6′)-Ib* + *ant(2″)-I*	1	0.91
	4	*bla*$_{PER\text{-}1}$ + *aac(6′)-Ib*	1	0.91
	5	*armA, aadA2*	1	0.91
	1	*bla*$_{TEM\text{-}1}$	1	0.91
M. morganii	2	*aac(6′)-Ib*	1	0.91
	3	*aac(3)-Ia* + *aadA2* + *ant(2″)-I*	1	0.91
P. stuartii	1	*aadA1*	1	0.91
Total			72	67.92