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Original Article

Phenotypic and genotypic characterization of multidrug-resistant *Acinetobacter baumannii* isolated in Algerian hospitals

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Abstract

Introduction: the aim of this study was to investigate the drug-resistance and the molecular characterization of carbapenemases, ESBL, and aminoglycoside-modifying enzymes among *Acinetobacter baumannii* clinical isolates in Algerian hospitals.

Methodology: a total of 92 *A. baumannii* isolates were collected between 2012 and 2016. Antimicrobial susceptibility testings were performed for β-lactams, aminoglycosides, fluoroquinolones, trimethoprim-sulfamethoxazole, rifampicin and colistin. The phenotypic characterization of β-lactamases was investigated. For 30 randomly targeted strains, the carriage of the carbapenemases, ESBL and aminoglycoside-modifying enzymes -encoding genes was determined by PCR. Sequencing was carried out for carbapenemases and ESBL genes.

Results: most of the 92 isolates studied were recovered from hospitalized patients (93.5%) and were mainly from intensive care units (51.1%) and orthopedics (19.6%). The strains were collected primarily from low respiratory tract (33.7%), wounds (23.9%) and urine (16.3%). Multidrug-resistant *A. baumannii* strains were prevalent (96.7%). High rates of resistance were observed for almost all antibiotics tested (>70%) excluding rifampicin (7.6%) and colistin (5.4%). For the five colistin-resistant strains, MICs ranged between 4 and 128 µg/mL. Positive MBL (83.7%) and ESBL (23.9%) strains were identified. Regarding β-lactams, the bla_{NDM} and both bla_{SHV} and bla_{CTX-M1} genes were detected in five and two strains respectively. Sequencing of the genes revealed the presence of bla_{NDM-1}, $bla_{CTX-M-15}$, and bla_{SHV-33}. For aminoglycosides, *aac*(6')-Ib, *ant*(2'')-I and *aph*(3')-VI genes were detected in three, seven and six strains respectively.

Conclusions: here, we report the first co-occurrence of extended-spectrum β-lactamases SHV-33 and CTX-M-15, the carbapenemase NDM-1 and the emergence of colistin-resistant *A. baumannii* in Algerian hospitals.

Key words: Multidrug-resistant *Acinetobacter baumannii;* $bla_{CTX-M-15;}$ $bla_{SHV-33;}$ $bla_{NDM-1;}$ colistin-resistance; Algeria.

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Introduction

Acinetobacter baumannii is a Gram-negative opportunistic nosocomial pathogen causing clinical infections and outbreaks in healthcare settings especially in intensive care units (ICUs). This species account for almost 90% of all reported *Acinetobacter* infections, including respiratory tract infections, bacteremia, meningitis, wound infections and urinary tract infections [1-2].

A. baumannii is able to easily acquire resistance to different groups of antimicrobials and to survive in hospital environment, leading to its persistence and transmission in healthcare settings [1,3]. *A. baumannii* is a member of the ESKAPE group (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter species*) of six highly resistant pathogens that are a major cause of antibiotic-resistant infections [4,5].

Multidrug-resistant *A. baumannii* has become life threatening and is increasingly reported worldwide, including Europe, America, Asia and Africa [1,2]. Over the last decade, the emergence of *A. baumannii* resistant to carbapenems, a last-line group of β-lactams for treatment of patients infected with multidrug-resistant bacteria, was reported [6-9]. In this context, the World Health Organization recognize carbapenem-resistant *A. baumannii* (CRAB) as the first critical priority in its list of 12 resistant-bacteria that pose the greatest threat to human health [10].

The main resistance mechanism among *A. baumannii* isolates is the production of β-lactamases enzymes which include extended spectrum-β-

lactamases (ESBLs) as TEM-92, SHV-5, CTX-M-2 and CTX-M-15, metallo-β-lactamases (MBLs) as IMP-1, VIM-1 and NDM-1 and carbapenem-hydrolyzing class D β-lactamases (CHDLs), also called oxacillinases (OXAs) as OXA-23, OXA-24 and OXA-58 [11,12].

Resistance to aminoglycosides is prevalent worldwide. It is mainly mediated by aminoglycoside-modifying enzymes (AMEs) including acetyltransferases (AACs) as AAC(6')-I enzymes, phosphotransferases (APHs) as APH(3')-VI enzymes and nucleotidyltransferases (ANTs) as ANT(2'')-I [12]. Recently, 16S rRNA methylases have been reported worldwide among Gram negative bacilli, including *Acinetobacter* spp. [13].

The purpose of this study was to evaluate the antimicrobial resistance rates and molecular mechanisms of ESBLs, carbapenemases and AMEs to multidrug *A. baumannii* isolated in Algerian hospitals during a 5-year period starting from January 2012. Here, we report the first co-occurrence of extended-spectrum β-lactamases SHV-33 and CTX-M-15, the carbapenemase NDM-1 and the emergence of colistin-resistant *A. baumannii* in Algerian hospitals.

Methodology

Bacterial strains

Ninety two non-repetitive *A. baumannii* clinical isolates were collected from several hospitals from Algiers (n = 64), Blida (n = 6), Tipaza (n = 3), Setif (n = 2), Boumerdes (n = 1), Bejaia (n = 1), Oran, n = 13), Tiaret (n = 1) and Tamanrasset (n = 1) between January 2012 and December 2016.

All clinical isolates were identified by standard microbiological methods using Api 20NE identification system (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion method according to the CLSI guidelines [14]. The antibiotics tested, based on national drug nomenclature, were ticarcillin (75 µg), piperacillin (100 µg), ticarcillin-clavulanic acid (75/10 µg), ceftazidime (30 µg), imipenem (10 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), rifampicin (30 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg).

The minimum inhibitory concentrations (MICs) for netilmicin and colistin were determined using E-test method (bioMérieux, Marcy l'Etoile, France) and broth microdilution method respectively.

Interpretations were made according to CLSI guidelines. For rifampicin, EUCAST breakpoints were used [15]. Multidrug-resistance was defined according to Magiorakos *et al.* [16].

Quality control for the antimicrobial susceptibility analysis was performed with *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

Phenotypic detection of production of Extended-spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL)

Detection of ESBL and MBL production were performed by phenotypic confirmatory disc diffusion test (PCDDT) and combined disc test (CDT) as previousely described [17].

PCR assays

Thirty *A. baumannii* isolates were randomly targeted, using the "alea" function of the Excel 2013 software, among the 92 strains for the detection of the carbapenemase, ESBL and AME –encoding genes according to phenotypic test results.

Bacterial DNA was extracted by boiling. Conventional PCR simplex assay was performed in a 50 µL reaction mixture containing the final concentrations of 0.5 µM primer, 2.5 U/µL Taq polymerase, 2 mM $MgCl_2$, 0.2 mM dNTPs, 10 µL

Table 1. Primers sequence of the targeted genes.

Gene	Primer sequence (5'-3')		Size (bp)
	Forward	Reverse	
*bla*TEM	ATAAAATTCTTGAAGACGAAA	GACAGTTACCAATGCTTAATCA	1080
*bla*SHV-5	TTATCTCCCTGTTAGCCACC	GATTTGCTGATTTCGCTCGG	796
*bla*CTX-M-1	GGTTAAAAAATCACTGCGTC	TTGGTGACGATTTTAGCCGC	864
*bla*CTX-M-2	ATGATGACTCAGAGCATTCG	TGGGTTACGATTTTCGCCGC	867
*bla*NDM	AATGGAATTGCCCAATATTATGC	TCAGCGCAGCTTGTCGGC	621
*bla*VIM	GGTGTTTGGTCGCATATCGCAAC	TGTGCTKGAGCAAKTCYAGACCG	390
*bla*IMP	GCAGGAATAGAGTGGCTTAAY	GGTTTAAYAAAACAACCAACC	232
*bla*OXA-58-like	ATGAAATTATTAAAAATATTG	ATAAATAATGAAAAACACCCA	841
aac(6')-Ib	ATGACTGACCATGACCTTG	AACCATGTACACGGCTGG	476
aph(3')-VI	CGGAAACAGCGTTTTAGA	TTCCTTTTGTCAGGTC	716
ant(2'')-I	GACACAACGGAGGTCACATT	CGCATATCGCGACCTGAAAGC	524

5×buffer, 3 µL DNA template and nuclease-free water. Amplification conditions consisted of denaturation at 94 °C for 7 minutes and 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes. PCR products were detected in 2% agarose gel. All PCR primers targeting resistance genes used in this study are listed in Table 1.

DNA sequencing of carbapenemases and ESBL genes detected was performed using Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed using an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, CA, USA).

Results

The strains were collected from various clinical specimens, including lower respiratory tract (33.7%), wounds (23.9%), urine (16.3%), blood (9.8%), cerebrospinal fluid (4.3%), upper respiratory tract (3.3%), catheter and drain (2.2%) respectively, pericardial liquid, rectal swab, tracheostomy cannula and urinary catheter (1.1%) respectively. These samples were from intensive care units (ICUs) (51.1%), orthopedics (19.6%), medicine (13.0%), surgery (9.8%) and 6 outpatients (6.5%).

Antimicrobial susceptibility testing

The 92 isolates investigated showed high resistance rates to β-lactams: ticarcillin (95.6%), piperacillin (98.9%), ticarcillin-clavulanic acid (90.2%), ceftazidime (96.7%) and imipenem (83.7%), to aminoglycosides: gentamicin (86.9%), amikacin (79.3%), tobramycin (83.7%) and netilmicin (61.9%),

Figure 1. Antimicrobial resistance of MDR *A. baumannii* isolates

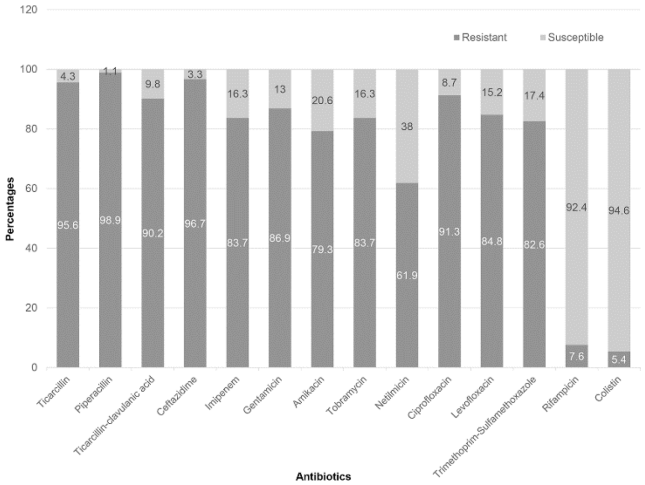

Table 2. Distribution of aminoglycosides resistance profiles among MDR *A. baumannii* isolates.

Aminoglycosides resistance profiles	Number (%)
Susceptible*	3 (3.3)
Gm	1 (1.1)
Ak	6 (6.5)
Tm	1 (1.1)
Nt	1 (1.1)
Gm-Ak	3 (3.3)
Gm-Tm	13 (14.1)
Gm-Tm-Nt	1 (1.1)
Gm-Ak-Nt	1 (1.1)
Gm-Ak-Tm	8 (8.7)
Gm-Ak-Tm-Nt	54 (58.7)

Gm: gentamicin; Ak: amikacin; Tm: tobramycin; Nt: netilmicin. *Strains were susceptible to the four aminoglycosides tested.

to fluoroquinolones: ciprofloxacin (91.3%) and levofloxacine (84.8%) and to trimethoprim-sulfamethoxazole (82.6%). However, resistance rates to rifampicin (7.6%) and colistin (5.4%) were low (Figure 1).

Eighty nine (96.7%) *A.baumanni* strains showed multidrug-resistance. Combined resistance to carbapenems, aminoglycosides and fluoroquinolones was observed in 74 (80.4%) cases and accounted for 37/74 (50.0%) of the invasive isolates.

Regarding aminoglycosides, eleven resistance profiles including resistance to one to four aminoglycosides were defined among MDR *A. baumannii* isolates. The resistance to all aminoglycosides was prevalent (n = 54; 58.7%) strains, followed by the resistance to gentamicin and tobramycin (n = 13; 14.1%) strains (Table 2). MICs to netilmicin were ranged between 0.016 µg/mL to 256 µg/mL with MIC_{50} and MIC_{90} both at 256 µg/mL.

MICs to colistin were ranged between 0.064 µg/mL and 128 µg/mL with MIC_{50} at 0.5 µg/mL and MIC_{90} at 1 µg/mL. For the five colistin-resistant strains, the MICs were 4 µg/mL for four strains and 128 µg/mL for one strain.

Phenotypic detection of β-lactamases (ESBL and MBL)

Among the 92 strains screened for ESBL and MBL production, 22 (23.9%) were PCDDT positive and 77 (83.7%) were CDT positive, indicating the probable production of ESBL or MBL.

Molecular detection of resistance genes

Carbapenemase and extended-spectrum β-lactamase-encoding genes

Out of the eight resistance genes investigated, five genes (*bla*$_{TEM}$, *bla*$_{CTXM-2}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{OXA-58-like}$) were not detected in any of the tested strains.

Among the 21/30 CDT positive isolates, the *bla*$_{NDM}$ gene was detcted in five strains. The strains were recovered from different hospitals in Algiers, Boumerdes, Blida and Tipaza.

Two strains were positive for both *bla*$_{SHV}$ and *bla*$_{CTX-M-1}$ genes among 8/30 PCDDT positive isolates and were collected from an Algiers hospital.

Sequencing of the genes revealed the presence of *bla*$_{NDM-1}$, *bla*$_{CTX-M15}$, and *bla*$_{SHV-33}$. Detailed data are shown in Table 3.

Aminoglycoside-modifying enzymes-encoding genes

The screening of 26/30 aminoglycosides resistant isolates for resistance genes revealed the presence of the *aac(6')-Ib* (n = 6), *ant(2'')-I* (n = 7), and *aph(3')-VI* (n = 6) genes.

Combined aminoglycoside-modifying enzyme (AME) resistance genes was observed with *ant(2'')-I-aph(3')-VI* (n = 2) and association of ESBL, MBL and AME resistance genes was also identified, namely *bla*$_{SHV}$-*bla*$_{CTX-M-1}$-*aac(6')-Ib* (n = 2), *bla*$_{NDM}$-*ant(2'')-I-aph(3')-VI* (n = 1), *bla*$_{NDM}$-*ant(2'')-I* (n = 3) and *bla*$_{NDM}$-*aph(3')-VI* (n = 1). Detailed data are shown in Table 3.

Discussion

Multidrug resistant *A. baumannii* represent a major threat in nosocomial infections, mostly in ICUs.

In this study, the most frequently strains were obtained from lower respiratory tract (33.7%), followed by wounds (23.9%) and were mostly from intensive care units (51.1%) and orthopedics (19.6%).

Antimicrobial susceptibility testing

In our study, most of the isolates showed high antibiotic resistance rates to β-lactams, aminoglycosides, fluoroquinolones and trimethoprim-sulfamethoxazole and low resistance rates to rifampicin and colistin. Our findings are moderately higher than the Algerian Antimicrobial Resistance Network (AARN) data recorded in *Acinetobacter* spp. during the same period (ceftazidime 85.6%, imipenem 73.4%, amikacin 65.7%, ciprofloxacin 80.1% and trimethoprim-sulfamethoxazole 72.2%) [18], and studies carried out in Westen Algeria in 2013 and Algiers in 2015 [19,20].

In accordance with results reported around the world [11], almost all *A. baumannii* strains in our study were MDR and carbapenem-resistant *A. baumannii* (CRAB) were prevalent (83.7%). The widespread of theses strains has described worldwide [11]. The high rate of CRAB isolates is a concern, as carbapenems represent the treatment of choice for infections caused by *A. baumannii*, leading to limited therapeutic options.

In this study, five strains were resistant to colistin. In recent years, the emergence of colistin resistance in *A.baumanni* clinical isolates has been increasingly reported in several countries worldwide, becoming a public health concern as this antibiotic is used for the treatment of infections due to CRAB [21,22].

In the North African countries, a few cases of colistin resistance among *A. baumannii* has been described in Tunisia and Egypt [23,24]. In Algeria, Bakour *et al.* in 2014, described one colistin-resistant *A. baumannii* clinical strain that harbored a single mutation in the *pmrB* gene in an Algiers hospital [25].

Table 3. Detection of ESBL, MBL, aminoglycosides resistance and their related genes among MDR *A. baumannii* isolates.

Code of isolates	Year of isolation	Phenotypic tests			Resistance genes
		ESBL test	MBL test	Aminoglycosides resistance	
Aba.1	2012	+	+	Ak	*bla*$_{NDM}$, *aph(3')-VI*
Aba.2	2012	-	+	Gm-Ak-Tm	*bla*$_{NDM}$, *ant(2'')-I, aph(3')-VI*
Aba.3	2012	+	-	Ak	*aph(3')-VI*
Aba.7	2014	+	-	Gm-Ak-Tm-Nt	*bla*$_{SHV}$, *bla*$_{CTX-M-1}$, *aac(6')-Ib*
Aba.8	2014	+	-	Gm-Ak-Tm-Nt	*bla*$_{SHV}$, *bla*$_{CTX-M-1}$, *aac(6')-Ib*
Aba.11	2015	-	+	Gm-Tm	*bla*$_{NDM}$, *ant(2'')-I*
Aba.12	2015	+	+	Gm-Tm	*bla*$_{NDM}$, *ant(2'')-I*
Aba.15	2015	-	+	Gm-Ak-Tm-Nt	*ant(2'')-I, aph(3')-VI*
Aba.19	2015	-	+	Gm-Ak-Tm-Nt	*aph(3')-VI*
Aba.20	2016	-	-	Gm-Tm-Nt	*ant(2'')-I*
Aba.21	2016	-	+	Gm-Ak-Tm-Nt	*bla*$_{NDM}$, *ant(2'')-I*
Aba.22	2016	-	+	Gm-Ak-Tm-Nt	*aph(3')-VI*
Aba.23	2016	+	-	Gm-Tm	*ant(2'')-I*
Aba.25	2016	-	+	Gm-Ak-Tm-Nt	*aac(6')-Ib*

ESBL: extended-spectrum β-lactamase; MBL: metallo-β-lactamase; ICU: intensive care unit; CSF: cerebrospinal fluid; Gm: gentamicin; Ak: amikacin; Tm: tobramycin; Nt: netilmicin.

To delay the emergence of this resistance, combination therapies using tigecycline or rifampicin with colistin were found to be effective [26,27]. In addition, new drugs developed such as eravacycline and cefiderocol are promising but need to be evaluated in further studies [28].

Seven (7.6%) isolates were resistant to rifampicin. This rate is higher than that (2.8%) reported by Bakour *et al.*in 2013 in Setif and Tizi-Ouzou [29]. So, this increased resistance rate should prompt attention and be continuously monitored.

Molecular detection of resistance genes

Carbapenemases and extended-spectrum β-lactamases-encoding genes

Five strains harbored the bla_{NDM} gene, identified as bla_{NDM-1}. The MBL NDM-1, detected originally in *Enterobacteriaceae*, was later described in *Acinetobacter* spp. Currently, this enzyme has spread in several countries around the world [30-32].

In Algeria, the first descriptions of NDM-1 in Algerian patient were reported by Boulanger and Bogaerts in 2011 [33,34]. After, this MBL was increasingly reported by other authors in Western: Oran [18], Eastern: Annaba [35,36] and Central Algeria: Tizi-Ouzou (Azzam *et al.*34th RICAI 2014, France), Algiers, Bejaia and Setif [19,37-39].

These studies support our results and confirm that NDM-producing *A. baumannii* isolates are nowadays endemic in Algerian hospitals.

$bla_{OXA-58-like}$ was not detected in any strain tested. In recent years, several studies from Algeria described carbapenem resistance among *A. baumannii* isolates, mainly due to CHDLs OXA-23 and OXA-24 in several regions of Algeria: Tizi-Ouzou (Azzam *et al.* 34th RICAI 2014, France), Setif, Tlemcen, Sidi Bel Abbes, Algiers and Bejaia [18,19,39]. OXA-58 was reported only in two studies from Annaba [40] and Tizi-Ouzou (Azzam *et al.*34th RICAI 2014, France).

For 16 CDT positive strains, none of bla_{VIM}, bla_{IMP}, bla_{NDM} and bla_{OXA-58} genes were detected. Several studies reported false MBL-positive results using this test, particularly in CHDLs strains likely due to chelators as EDTA that may inhibit the activity of oxacillinases [41,42].

In our research, two isolates harbored both $bla_{CTX-M15}$, and bla_{SHV-33} genes. Several previous studies reported ESBLs-producing *A. baumannii* strains [11,12]. In Egypt and Pakistan, TEM, CTX-M or SHV genes were previously reported [43,44]. In Algeria, $bla_{TEM-128}$ was previously described [29].

To the best of our knowledge, this is the first description of SHV-33 and co-occurrence of extended spectrum β-lactamase SHV-33 and CTX-M-15-producing *A. baumannii*.

For the six PCDDT positive strains, bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$ and $bla_{CTX-M-2}$ genes were negative. As previously described by Beceiro et al., the intrinsic susceptibility of some strains of *A. baumannii* to β-lactamase inhibitor clavulanic acid could yield false ESBL-positive results in *A. baumannii* [45].

Aminoglycoside-modifying enzymes-encoding genes

Among 26/30 aminoglycosides resistant isolates, the three types of AMEs genes were identified with *aac(6')-Ib* (n = 6), *ant(2'')-I* (n = 7), and *aph(3')-VI* (n = 6) genes, alone or associated. As previously described in several studies [12], these findings are in accordance with three aminoglycosides resistance profiles identified: (amikacin), (gentamicin-tobramycin) and (gentamicin-amikacin-tobramycin).

The most frequent resistance profile (gentamicin-amikacin-tobramycin-netilmicin) is probably due to the 16S rRNA methylase ArmA [12].

In Algeria, several previous studies reported the presence of aminoglycoside encoding genes *aac(6')-Ib*, *aph(3')-VI*, *ant(2'')-I*, *aac(3)-Ia* and *aadA* [18,19,25,38].

Combined AME resistance genes was observed with *ant(2'')-I-aph(3')-VI* (n = 2) and association of ESBL, MBL and AME resistance genes was also identified (n = 7).The association of aminoglycosides resistance, MBL and ESBL genes identified, highlight the multiple mechanisms involved leading to multidrug resistant *A. baumannii* strains.

Conclusions

Our results highlight that (i) the high prevalence of multidrug resistant *A. baumannii* in Algerian hospitals is alarming, (ii) NDM-1 isolates are actually endemic in Algerian hospitals, (iii) co-occurrence of ESBLs SHV-33 and CTX-M-15 is described for the first time in Algeria, (iv) AMEs, often combined, are mediated by *aac(6')-Ib*, *aph(3')-VI* and *ant(2'')-I*and (v) colistin-resistant strains have emerged in Algerian hospitals.

These findings report a critical situation in Algerian hospitals that requires rational use of antibiotics, strict compliance with hygiene rules, rapid and accurate screening of inpatients and implementation of early precautions for MDR carriers to decrease nosocomial transmission in hospitals. Further investigations are needed to better understand the molecular

epidemiology of this nosocomial opportunistic pathogen.

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