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Multidrug-resistant *Acinetobacter baumannii* strains carrying the bla_{OxA-23} and the bla_{GES-11} genes in a neonatology center in Tunisia

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Multidrug-resistant and difficult-to-treat *Acinetobacter baumannii* may be responsible for nosocomial infections. The production of carbapenem-hydrolyzing class D β-lactamases (CHDLs) and extended-spectrum β-lactamase (ESBLs) of the GES type possessing a carbapenemase activity has been increasingly reported worldwide in *A. baumannii*. The aim of this study was to analyze the resistance mechanisms of two carbapenem resistant *A. baumannii* clinical isolates recovered in a neonatology center in the center-east of Tunisia.

Two carbapenem resistant *A. baumannii* isolates were recovered. The first isolate co-harbored the bla_{GES-11} ESBL gene and the bla_{OxA-23} CHDL gene. Analyses of the genetic location indicated that the bla_{GES-11} gene was plasmid located (Gr6). However, the bla_{OxA-23} gene was located on the chromosome. The second strain had only the bla_{OxA-23} CHDL gene, which was plasmid located.

This study showed the first description of the GES-type β-lactamase in *A. baumannii* in Tunisia.

1. Introduction

Acinetobacter baumannii is a glucose-non-fermentative Gram negative coccobacillus that has emerged over the last decade as a cause of healthcare associated infections with elevated morbidity and mortality. In addition, community-acquired *A. baumannii* infections are now reported [1]. Through the acquisition of antibiotic resistance genes by lateral gene transfer, *A. baumannii* has an extraordinary ability to become resistant to almost all antibiotics, leading to multidrug-resistant isolates [2,3]. The rapid and worldwide emergence of isolates resistant to carbapenems is now considered as a significant health problem because of limited therapeutic options.

Carbapenemases found in *Acinetobacter* spp. may belong to class A (KPC, GES), to metallo-beta-lactamases ((MBL): IMP, VIM, SIM, NDM) or to class D (carbapenem-hydrolyzing class D β-lactamases [CHDLs]), the latter being the most disseminated worldwide [1,2]. As elsewhere [4,5,6], occurrence of carbapenem resistance in *A. baumannii* in North Africa has been mainly attributed to the production of CHDLs, with OXA-23 and OXA-58-like enzymes being the most prevalent [3,7,8].

The aim of this study was to characterize the mechanisms responsible for carbapenem resistance in two clinical isolates of *A. baumannii* recovered from patients hospitalized in the maternity and neonatology center of Monastir, Tunisia.

2. Material and methods

2.1. Bacterial isolates

Two clinical isolates of multidrug-resistant *A. baumannii*, the Ab453 and the Ab537 were recovered in June 2011 from the maternity and neonatology center of Monastir, Tunisia. Ab453 was isolated from an intubation in a female infant suffering from congenital heart disease (single ventricle) and died after 65 days of hospitalization. The Ab537 isolate was recovered from a male infant suffering from a neonatal respiratory distress.

Isolates were identified by using the API 20 NE system (bioMérieux, Marcy l'Etoile, France) and 16S rRNA gene sequencing, as described previously [9].

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The rifampin-resistant *A. baumannii* BM4547 was used as host in conjugation assays. *A. baumannii* and *Klebsiella pneumoniae* carrying respectively the bla_{OxA-23} and bla_{GES-1} genes were used as reference strains [10,11]. *Escherichia coli* NCTC 50192 harboring four plasmids of 154, 66, 48 and 7 kb was used as a size marker.

2.2. Susceptibility testing and screening for MBL production

The antibiotic susceptibility of the *A. baumannii* isolates was determined by the disk diffusion method on Muller-Hinton agar plates with β-lactam and non-β-lactam antibiotic-containing disks (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). MICs were determined using Etest strips (AB bioMérieux, La Balme-les-Grottes, France) and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute [12]. MBL production was evaluated using Etest strips with imipenem and EDTA (AB Biodisk, Solna, Sweden) and confirmed by UV spectrophotometry [13].

2.3. PCR amplification and plasmid typing

Whole-cell DNA of *A. baumannii* was prepared by suspending one fresh colony into 100 µl of sterile distilled water and heating at 95 °C for 10 min. After centrifugation, the supernatant was stored at 4 °C before analysis. DNA in supernatant was used as a template under standard PCR procedure [9] with a series of different sets of primers. Detection of the known acquired CHDLs (bla_{OxA-23}, bla_{OxA-40}, and bla_{OxA-58}) [14], class A extended-spectrum β-lactamases (bla_{PER}, bla_{VEB}, and bla_{GES}) [14,15], MBL (bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{NDM}) [16,17] and the intrinsic $bla_{OxA-51-like}$ gene was performed as previously described [14].

Detection of the IS*Aba1* element upstream of bla_{OxA-51} and bla_{OxA-23} genes was also performed [9]. In addition Ab453 isolate and Ab537 isolate were also screened for 16S RNA methylase-encoding genes production (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *npmA*), as previously described [18].

The primers used in this study are listed in Table 1.

Plasmid typing was performed using the *A. baumannii* PCR-based replicon typing [AB-PBRT] method as described previously [19]. The PCR products fragments were purified with QIAquick column (Qiagen, Courtaboeuf, France) and sequenced with an Applied Biosystems sequencer (ABI 377). The nucleotide and deduced amino acid sequences were analyzed and compared to sequences available over the Internet on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Transfer of β-lactam resistance

Conjugation assays were performed with *A. baumannii* BM4547 as recipient strain. Briefly, one colony of each of the donor and recipient strains was cultured separately under weak agitation in 1 ml trypticase soy broth at 37 °C, and they were then used in the mating-out assays. Conjugation was done by incubating 800 µl of the recipient strain with 200 µl of the donor strain under low agitation at 37 °C for an additional 3 h step. The *A. baumannii* transconjugants were selected on trypticase soy agar plates containing ticarcillin (60 µg/ml) and rifampin (50 µg/ml).

2.5. Plasmid extraction and hybridization

Plasmid DNA was extracted by using Kieser method [20] and analyzed by electrophoresis on a 0.7% agarose gel. Southern transfer of the plasmid extract was carried out on a nylon membrane (Hybond N+; Amersham Pharmacia Biotech. Orsay, France), as described previously [21]. The membrane was hybridized with a probe specific for bla_{GES} (860 bp) gene and for bla_{OxA-23} (840 bp). Southern hybridization was carried out as described by the manufacturer using the ECL non-radioactive labeling and detection kit (Amersham Pharmacia Biotech).

Table 1
Primers used in this study.

Gene type	Primer	Sequences (5′ – 3′)	Ref.
OXA-40	OXA-40 A	GTACTAATCAAAGTTGTGAA	[14]
	OXA-40 B	TTCCCCTAACATGAATTTGT	
OXA 58	OXA-58A	CGATCAGAATGTTCAAGCGC	[14]
	OXA-58B	ACGATTCTCCCCTCTGCGC	
OXA-23	OXA-23A	GATGTGTCATAGTATTCGTCG	[14]
	OXA-23B	TCACAACAACTAAAAGCACTG	
OXA-51	OXA-51A	CTAATAATTGATCTACTCAAG	[14]
	OXA-51A	CCAGTGGATGGATGGATAGATTATC	
GES-1	GES-1A	ATGCGCTTCATTCACGCAC	[15]
	GES-1B	CTATTTGTCCGTGCTCAGG	
PER-1	PER-1	ATGAATGTCATTATAAAAGC	[15]
	PER-1	ATTTGGGCTTAGGGCAGAA	
VEB-1	VEB-1	CGACTTCCATTTCCCGATGC	[15]
	VEB-1	GGACTCTGCAACAAATACGC	
NDM	NDM-F	GGTTTGGCGATCTGGTTTTC	[17]
	NDM-R	CGGAATGGCTCATCACGATC	
IMP	IMP-A	GAAGGYGTTTATGTTCATAC	[17]
	IMP-B	GTAMGTTTCAAGAGTGATGC	
VIM	VIM2004A	GTTTGGTCGCATATCGCAAC	[16]
	VIM2004B	AATGCGCAGCACCAGGATAG	
SIM	SIM-F	TACAAGGGATTCGGCATCG	[17]
	SIM-R	TAATGGCCTGTTCCCATGTG	
ISAba1	ISAba1-B	CATGTAAACCAATGCTCACC	[14]
ArmA	ArmA-multiF	ATTTTAGATTTTGGTTGTGGC	[18]
	ArmAmultiR	ATCTCAGCTCTATCAATATCG	
RmtB	RmtB-multiF	ACTTTT ACAATCCCTCAATAC	[18]
	RmtB-multiR	AAGTATATAAGTTCTGTTCCG	
NpmA	NpmA-multi-F	GGGCTATCTAATGTGGTG	[18]
	NpmA-multi-R	TTTTTATTTCCGCTTCTTCGT	
RmtA	RmtA-multiF	AAACTATTCCGCATGGTTC	[18]
	RmtA-totR	TCATGTACACAAGCTCTTTCC	
RmtC	RmtC-multiF	CAGGGGTTCCAACAAGT	[18]
	RmtC-multiR	AGAGTATATAGCTTGAACATAAGTAGA	
RmtD	RmtD-multiF	GGAAAAGGACGTGGACA	[18]
	RmtD-multiR	TCCATCGATTCCACAGG	

2.6. PFGE

Ab453 and Ab537 were typed by using *ApaI* macrorestriction analyses and pulsed-field gel electrophoresis (PFGE) according to the manufacturer's recommendations (Bio-Rad, Marnes-la-Coquette, France). Electrophoresis was performed on an agarose gel using a CHEF-DR II apparatus (Bio-Rad) [14].

3. Results

In 2011, 125 non-repetitive microbial isolates were recovered from neonates at the maternity and neonatology center of Monastir, Tunisia; among them 46 (36.8%) were multidrug resistant (resistance to more than three antibiotics) [22]. Eleven (24%) were identified as *Staphylococcus* spp. The other 35 (76%) were Gram-negative bacilli including 31 *Enterobacteriaceae*, twice *Pseudomonas* and twice *A. baumannii*: the Ab453 and the Ab537 which were used in this study (Table 2).

Ab453 and Ab537 were resistant to β-lactams (ticarcillin, ceftazidime, cefepime, aztreonam, and carbapenems), to aminoglycosides (amikacin, gentamicin, tobramicin and netilmicin), to fluoroquinolones, sulfonamides, and tetracyclines. Both of them showed susceptibility to colistin and rifampin.

Table 2
Case history of the two neonates with OXA-23 and/or GES-11 producing *Acinetobacter baumannii*.

Isolate ref	Date of hospitalization (mo/day/yr)	Age (day)	Sex	Date of isolation (mo/day/yr)	Underlying conditions	Site of isolation	Treatment	Outcome
Ab453	04/21/11	1	F	06/17/11	Congenital heart disease	Intubation	Ceftazidime, amikacin	Died (06/25/11)
Ab537	06/08/11	1	M	06/12/11	Infant respiratory distress syndrome	Catheter	Gentamicin, ceftazidime, ampicillin	Improved (06/25/11)

Results obtained with Etest strips containing imipenem and EDTA did not reveal any MBL production among the two isolates. However, hydrolysis assays performed by UV spectrophotometry revealed both of them as carbapenemase producers.

After PCR and sequencing of the amplicon, Ab453 and Ab537 were found to be harboring $bla_{\text{OxA-66}}$ (a variant of $bla_{\text{OxA-51}}$) and $bla_{\text{OxA-23}}$. In addition the Ab453 was carrying $bla_{\text{GES-11}}$ gene.

The insertion sequence IS*Aba1* was detected upstream of the $bla_{\text{OxA-23}}$ gene in Ab537 isolate.

PCR experiments for the detection of the bla_{IMP}-type, bla_{VIM}-type and $bla_{\text{SIM-1}}$ gene and for 16S RNA-methylase-encoding genes gave negative results.

Matting-out assays with *A. baumannii* BM4547 followed by selection on ticarcillin–rifampin containing plates gave different results for the two isolates (Table 3).

For Ab453, the transconjugant exhibited an ESBL phenotype but also showed reduced susceptibilities to carbapenems (Table 3) .In addition the transconjugant were resistant to aminoglycosides (amikacin, gentamicin, tobramycin and netilmicin), cotrimoxazole, and sulfonamides. PCR experiments performed for the transconjugant were positive for the bla_{GES} gene and negative for $bla_{\text{OxA-23}}$ gene. A DNA probe for the bla_{GES} gene, consisting of an 860 bp PCR fragment, was used for the hybridization experiment. A positive signal was observed with the Ab453 plasmid DNA with the bla_{GES} probe; however, no hybridization signal was observed with the $bla_{\text{OxA-23}}$ probe. The above results confirm the PCR results of the transconjugant isolate and suggest that $bla_{\text{OxA-23}}$ gene was located on the chromosome and $bla_{\text{GES-11}}$ gene was located on a plasmid of about 90 Kb (Fig. 1).

For Ab537, a transconjugant showing resistant to all tested antibiotics was found. After PCR the transconjugant was positive for $bla_{\text{OxA-23}}$, suggesting its plasmid location, which was confirmed by hybridization experiment.

AB-PBRT PCR performed for our isolates and their transconjugants revealed that plasmids p (Ab537) (OXA-23) and p (Ab453) (GES-11) harbored the aci6 replicase gene belonging to group GR6.

PFGE analysis showed that the two isolates clustered into 2 different PFGE types (data not shown).

4. Discussion

Carbapenem resistance in *A. baumannii* is most often mediated by acquired CHDLs (OXA-23-like, OXA-40-like, and OXA-58-like) and less frequently by MBLs. OXA-type carbapenemases have predominated in *A. baumannii*, especially worldwide outbreaks of OXA-23 [1]. Recently the GES-1-like carbapenemases have also been reported for *A. baumannii* [9,14,23,24,25].

In Tunisia, resistance to carbapenem in *A. baumannii* isolates has been found to be associated with the production of CHDLs (OXA-97 and OXA-23) [3,7,8]. However, recently Bonnin et al. reported *A. baumannii* carrying NDM-1 MBL. The strain was recovered in France from a Tunisian patient [26].

In our study, two carbapenem-resistant *A. baumannii* isolates recovered from two neonates hospitalized in the maternity and neonatology center of Monastir, Tunisia in June 2011 were resistant to almost all antibiotics but colistin and rifampin.

Ab453 and Ab537 isolates produced the intrinsic $bla_{\text{OxA-51-like}}$ gene ($bla_{\text{OxA-66}}$) supporting the identification of the strains as *A. baumannii* [25]. Noteworthy, IS*Aba1* was detected upstream to $bla_{\text{OxA-23}}$ gene in Ab537 isolate.

The involvement of OXA-51-like enzymes in carbapenem-resistance in our stains could be ruled out since IS*Aba1* was not detected upstream to $bla_{\text{OxA-66}}$ [26].

For Ab453, association of the $bla_{\text{OxA-23}}$ with IS*Aba1* could be responsible for high levels of carbapenem resistance since IS*Aba1* element may act as a strong promoter and could be responsible for higher $bla_{\text{OxA-23}}$ expression resulting in a high level of carbapenem resistance as previously reported [26].

Occurrence of GES-11 among clinical isolates of *A. baumannii* has been described for the first time in France, the clinical isolate was harboring both $bla_{\text{OxA-58}}$ and $bla_{\text{GES-11}}$ genes [20]. Since then, several GES-1 mutants have been detected in *A. baumannii*, such as GES-12, GES-14, and GES-22 [9,14,22,23,24].

Table 3
β-Lactams MICs for *A. baumannii* clinical isolates Ab453, the *A. baumannii* BM4547 (pGES-11) transconjugant, *A. baumannii* clinical isolates Ab537, the *A. baumannii* BM4547 (pOXA-23) transconjugant and the *A. baumannii* BM4547reference strain.

MIC (μg/ml)					
	A. baumannii Ab453 (GES-11, OXA-23)	*A. baumannii* BM4547 (pAb453) (GES-11)	*A. baumannii* BM4547	*A. baumannii* AB537 (OXA-23)	*A. baumannii* BM4547 (pAb537) (OXA-23)
β-lactams					
Ticarcillin	>256	>256	8	>256	>256
Ticarcillin +CLA[a]	>256	256	8	>256	>256
Piperacillin	>256	>256	4	>256	>256
Piperacillin + TZB[a]	>256	256	4	>256	>256
Cefoxitin	>256	>256	256	>256	>256
Cefotaxime	>64	>64	>32	64	64
Ceftazidime	>64	>64	4	>64	8
Cefepime	>64	32	16	>64	>32
Aztreonam	>64	64	32	>64	>64
Meropenem	32	2	0.5	>32	>24
Imipenem	32	1	0.25	>32	>24

[a] CLA, Clavulanic acid (4 μg/ml); TZB, Tazobactam (4 μg/ml).

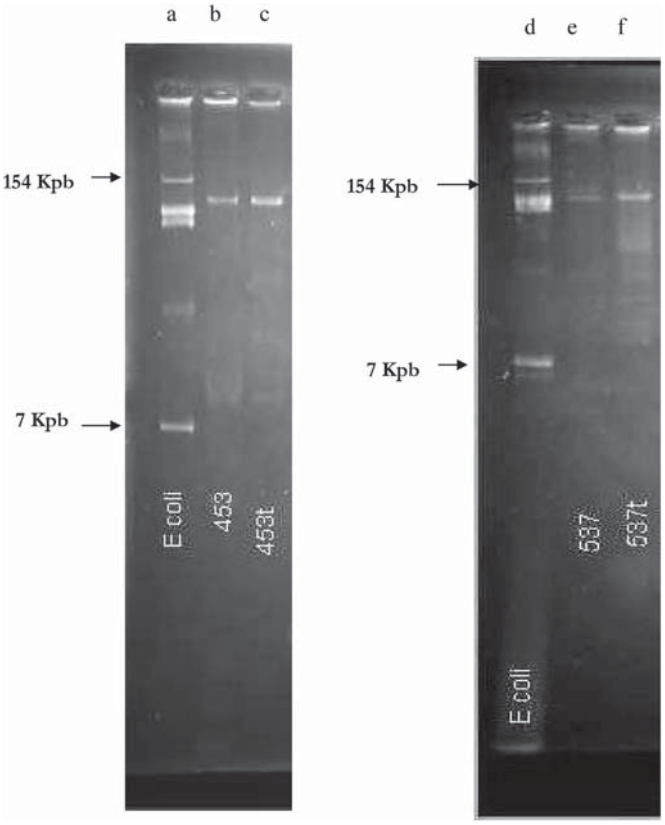

Fig. 1. Plasmid analysis of *Acinetobacter baumannii* isolates. a; d: Plasmids of reference size (*Escherichia coli* NCTC 50192). b: Plasmid analysis of Ab453 isolate. c: Plasmid analysis of the transconjugant BM4547 harboring bla_{GES-11} gene. e: Plasmid analysis of Ab537 isolate. f: Plasmid analysis of the transconjugant BM4547 harboring bla_{OxA-23} gene.

DNA sequencing of the bla_{GES} gene identified in Ab453 gave 100% identity with GES-11. GES-11 β-lactamase possesses increased activity against aztreonam [14,23]. However the bla_{GES-11}-carrying transconjugant showed reduced susceptibilities to carbapenems, suggesting the presence of weak hydrolysis of carbapenems by this enzyme potentiated by the efflux overproduction in *A. baumannii* BM4547 [14,23].So, GES-11 may contribute to play a synergistic role toward resistance to carbapenems in *A. baumannii*, together with OXA-23 [14].Several studies reported the association between the bla_{GES} and $bla_{OxA\text{-}like}$ gene, especially bla_{GES-11} and bla_{OxA-23} genes [14,23,24]. To our knowledge, however, carbapenem resistant *A. baumannii* isolate producing the carbapenem-hydrolyzing oxacillinase OXA-23 in association with the GES-11 class A β-lactamase in Tunisia have not been reported previously.

Plasmid analysis and AB-PBRT PCR showed that both plasmids (pAb453 and pAB537) were of ca. 90 Kb in size and belonged to the plasmid group Gr6 which has been shown to be highly prevalent in *A. baumannii* strains and was previously found to be associated with the bla_{OxA-23}, bla_{OxA-40},,bla_{OxA-58} CHDL genes and the $bla_{GES-11/GES-14}$ genes [2,14,24].

Unfortunately, in the current study, MLST PCR which could be interesting to compare clonality of our *A. baumannii* GES-11 producing with other GES-type recovered elsewhere was not performed.

Clinical data presented in Table 2 explain a hypothesis concerning the origin of isolates. The Ab537 was isolated from a neonate hospitalized the 8 of June 2011 because of a neonatal respiratory distress after 7 h of birth. He recovered after two days antibiotics therapy and left the hospital on the 25th of June. The origin of this strain is internal and concerns the neonatology center.

The Ab453 strain was isolated from a neonate hospitalized in April 2011 because of heart disease. In June, he was transferred to another hospital (University hospital Sahloul, Sousse) for an urgent surgery. He was re-admitted in the neonatology center and received imipenem and amikacin. Five days after surgery he died. The bla_{OxA-23} was previously described in the university hospital Sahloul in Tunisia [3] and it was chromosome located. However, numerous other studies showed the plasmid location of the bla_{OxA-23} gene [6,24].So, a possible transfer of this strain from the Sahloul university hospital to the neonatology center is strongly probable.

In conclusion, this study re-emphasizes the worldwide dissemination of OXA-carbapenemase genes in multidrug-resistant clinical isolates of *A. baumannii* and supports our understanding of carbapenem resistance in *A. baumannii* strains from Tunisia, showing that acquisition of bla_{OxA-23} gene seems to be the most prevalent mechanism of carbapenem resistance among *A. baumannii* in Tunisia [3,4].

Emergence of GES-type ESBLs in *A. baumannii* [9,14,24,25] highlights the propensity of *A. baumannii* to acquire new resistance mechanisms and it further underlines the absolute necessity to control inpatients transferred from hospitals. However the occurrence of GES enzymes in this species in Tunisia is probably underestimated, since synergism between extended-spectrum cephalosporins and clavulanic acid may be masked by the presence of the intrinsic AmpC cephalosporinase and OXA-51-like oxacillinase that are frequently associated with other â-lactamases.

Author disclosure statement

None to declare.

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