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Full Length Research Paper

Carbapenem-resistant and OXA-23 producing *Acinetobacter baumannii* isolate in Tunisian hospital

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We investigated resistance determinant of clinical isolate of carbapenem–resistant *Acinetobacter baumannii*, collected from patients hospitalized at the Military hospital in Tunisia. The isolate which was multidrug resistant produced OXA-23. The bla $_{OXA-23}$ gene was found to be adjacent to ISAbaI.

Key words: *Acinetobacter baumannii*, OXA-23, ISAbaI.

INTRODUCTION

Acinetobacter baumannii is a glucose-nonfermentative gram negative coccobacillus that is widely distributed in the hospital environment and is an important opportunistic pathogen.They can be associated with a wide range of clinical complications, such as pneumonia, septicaemia, urinary tract infections, wound infections and meningitis, especially in immunocompromised patients (Zarrilli et al., 2004; Beceiro et al., 2009). Treatment of *Acinetobacter* infections is often complicated by multidrug-resistant phenotypes, including resistance to broads pectrum β-lactams, aminoglycosides and fluoroquinolones (Wang et al., 2007). Carbapenems have been the mainstay of treatment for *Acinetobacter* infections for the past decade and still remain active in many centres, but reports of carbapenem resistance have now accumulated worldwide (Towner et al., 2008). Resistance against carbapenems is, in itself, considered sufficient to define an isolate of *A. baumannii* as highly resistant.

The resistance of *A. baumannii* to carbapenems can be mediated by one of the resistance mechanisms that are known to occur in bacteria, including enzymatic inactivation, active efflux of drugs and modification of target sites. The production of carbapenem-hydrolizing beta-lactamases is the most common mechanism responsible for carbapenem resistance in *A. baumannii* (Zarrilli et al., 2009). The commonest enzymatic mode of carbapenem resistance is the production of oxacillinases encoded by genes of the blaOXA-23, blaOXA-40 and blaOXA-58-like lineage. These may be plasmid or chromosomally located, are not inhibited by clavulanic acid and have been found in most regions of the world (Gordan and Wareham, 2010; Marti et al., 2008). *A. baumannii* also possesses an intrinsic carbapenem-hydrolysing oxacillinase, the expression of which may vary, that may play a role in carbapenem resistance: a naturally occurring AmpC β-lactamase, together with a naturally occurring oxacillinase, represented by the OXA-51/69 variants.

In addition to β-lactamases, carbapenem resistance in *A. baumannii* may also result from porin or penicillin-binding protein modifications. Several porins, including the 33- kDa CarO protein, that constitute a pore channel for influx of carbapenems, might be involved in carbapenem resistance (Poirel and Nordmann, 2006). OXA carbapenemases may not robustly hydrolyse imipenem, their presence in an organism that may have an insertion sequence element that acts as a promoter can result in imipenem resistance (Park et al., 2010). ISAbaI has been found upstream of bla_{ampC}, encoding the naturally occurring cephalosporinase, bla_{OXA-23} gene,

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encoding a carbapenem-hydrolyzing oxacillinase, *bla*OXA-58 and bla$_{OXA-51-like}$ (Corvec et al., 2007; Mugnier et al., 2010). The aim of this study was the identification of tle bla $_{OXA-23}$ in carbapenem- resistant *A. baumannii* and its association with the presence of the insertion sequence ISAbaI.

MATERIALS AND METHODS

Bacterial strain

One isolate of *A. baumannii* AB4968 is included in this study. This strain was isolated from a sampling Tracheal protection culture in intensive care unit of the military hospital of Tunis on December 2007. Bacterial strain was grown in Trypto–Caseine Soja broth (TCS) and incubated overnight at 37°C .The isolate was identified by the Vitek automated system (bioMérieux, Vitek 32) and API 20E system (bioMérieux, Marcy l'Etoile, France). Streptomycin resistance *Escherichia coli* HB101 (F-, D (gpt-proA) 62, leuB6, supE44, ara-14, galK2, lac Y1, D (mcrc-mrr), rps, L26, Xyl-rmtl 1, thi-1, IncFI, rec AB, strr) was used for the conjugation experiments. β-Lactamases with known pIs were used as standards: TEM-1 (pI 5.4), TEM-2 (pI 5.6), TEM-3 (pI 6.3) and SHV-1 (pI 7.6).

Antimicrobial susceptibility testing

The antibiotics susceptibilities were determined on Muelluer-Hinton agar by the standard disk diffusion procedure as described by the antibiogram committee of the French society for microbiology (www.sfm.asso.fr) (Cavallo et al., 2007). The following antibiotics were tested, ampicillin,amoxilin/acide clavulanique, cefotaxime, cefepime, ceftazidime, ciproflaxacine, aztrenam, imipinem, piperacilin and acid nalidixique (Biorad, Marnes-la-Coquette, France). Minimum inhibitory concentration (MICs) of selected antimicrobial agents were determined by broth microdilution. Custom-designed microdilution trays containing dilutions of selected antimicrobial agents in cation-adjusted Muller–Hinton broth (Diagnostic Pasteur) were inoculated and analyzed using guidelines of the National Committee for Clinical Laboratory Standards (CASFM).

Analytical isoelectric focusing (IEF)

IEF was performed as follows, bacterium growing exponentially at 37°C in tryticase soy broth was harvested at 10000 rpm for 10 min (Beckman centrifuge, FO650 Rotor) and cell-free lysate was prepared by sonication (sonicator UP 400S cycle: 25 and amplitude 50 Hz). After centrifugation, supernatants of sonicates were subjected to isoelectric focusing for 3 h by using a 111Mini IEF Cell, (Bio-Rad), and a gradient made up of polyampholytes with a pH range of 3 to 10 (Bio-Rad). Extracts from TEM-1-, TEM-2-, TEM-3- and SHV-1-producing strains were used as standards for pIs of 5.4, 5.6, 6.3, and 7.6, respectively. β-Lactamases activities were revealed by the iodine method, with benzylpenicillin (0.5 mM) in phosphate buffer (25 mM; pH 7) (Labia and Barthelemy, 1979).

β-lactamase assay

β-Lactamase activities were determined by spectrophotometric method. Briefly, the decrease in absorbance of the antibiotics at an appropriate concentration and wavelength was measured in a temperature controlled spectrophotometer (Varian R CARY 50 Bio UV- visible) at 37°C. Specific activity is calculated on depending of Ross et al. equation in 1975.

Effect of inhibitor

Ethylenediamine tetraacetic acid (EDTA) is used at a fixed concentration of 1 mM. Residuals βlactamases activities are determined by the spectrophotometric method using cephalothin.

β-lactam resistance transfer assay

Conjugation was carried out on Luria Bertani broth, with *E. coli* HB101Str^R as the recipient. Culture mixtures were incubated for 16 h at 37°C at a 1:2 ratio (donor to recipient). Transconjugants were selected on Luria Bertani agar containing ampicillin (100 µg/ml) and streptomycin (256 µg/ml). The resulting transconjugants were purified and identified with API 20 E strips.

Polymerase chain reaction (PCR) assay and sequencing

Sequences of primers used for the detection of genes encoding *bla*$_{OXA-23}$ like, *bla*$_{OXA-24}$ like, *bla*$_{OXA-51}$ like and *bla*$_{OXA-58}$ like genes are given in Table 1. The PCR conditions were as follows: Initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1min, 53°C for 1min and 72°C for 1 min, followed by an elongation step at 72°C for 10 min. The presence of the ISAba1 insertion sequence, upstream of the resistance genes and in the correct orientation to promote resistance-gene expression, was sought using a forward primer homologous to sequences located within ISAba1 and reverse primers homologous to the respective resistance genes. Primers are listed in Table 1. PCR products were purified using a PCR purification kit (Promega) and subsequently sequenced with the primers used for PCR. The aligned sequences were then analysed with the Bioedit sequence program and similarity searches for the nucleotide sequences were performed with the BLAST program (http://www.ncbi.nlm.nih.gov).

RESULTS

Resistance phenotype for *A. baumannii* strain AB 4968

Antibiogram data determined by the disk diffusion method and interpreted according to the Antibiogram Committee of the French Society for Microbiology (www.sfm.asso.fr) revealed that *A. baumannii* AB 4968 was resistant to penicillins, extended-spectrum cephalosporins and imipenem.

The enhancement of the inhibition zone between the disks containing amoxicillin-clavulanate and cefotaxime, ceftriaxone and cefpirome indicated the presence of ESBL production was not detected.

The resistance to β-lactams of *A. baumannii* AB 4968 strain is confirmed by the MIC. The MIC of the penicillins (ampicillin and ticarcillin) and the extended spectrum cephalosporins (cefoxitin, cefotaxim, cephalotin and ceftriaxone) which exceed 512 µg/ml, whereas the MIC for ceftazidim was 512 µg/ml and 128 µg/ml for

Table 1. Primers used for the detection of carbapenemase gene.

Primer	Sequence	Target	Reference
OXA-23-likeF	5'-GAT CGG ATT GGA GAA CCA GA-3'	BlaOXA-23-like	Turton et al. (2006)
OXA-23-likeR	5'-ATT TCT GAC CGC ATT TCC AT-3'		
OXA-24-likeF	5'-GGT TAG TTG GCC CCC TTA AA-3'	BlaOXA-24-like	
OXA-24-likeR	5'-AGT TGA GCG AAA AGG GGA TT-3'		
OXA-51-likeF	5'-TAA TGC TTT GAT CGG CCT TG-3'	BlaOXA-51-like	
OXA-51-likeR	5'-TGG ATT GCA CTT CAT CTT GG-3'		
OXA-58-likeF	5'-AAG TAT TGG GGC TTG TGC TG -3'	BlaOXA-58-like	
OXA-58-likeR	5'-CCC CTC TGC GCT CTA CAT AC-3'		
ISAbaI-F	5'-CAC GAA TGC AGA AGT TG-3'	ISAbaI	Ségal et al. (2005)
ISAbaI-R	5'-CGA CGA ATA CTA TGA CAC-3'		

Table 2. Antibiotics susceptibility of the *A. baumannii* AB 4968 strain.

Antibiotics	MIC (µg/ml)
Ampicilline	>512
Ticarcilline	>512
Imipénème	>32
Céfoxitine	>512
Ceftriaxone	>512
Ceftazidime	>512
Aztréonam	128
Cefpirome	>512
Streptomycine	16
Chloramphénicol	16
Tétracycline	>2
Acide nalidixique	>512
Ciprofloxacine	128

aztreonam. The isolate was highly resistant to imipenem (MIC> 32 µg/ml). It was also resistant to quinolones but remained susceptible to tetracycline (CMI<2 µg/ml) (Table 2).

Isoelectric focusing

The supernatants of *A. baumannii* AB 4968 was collected and subjected to IEF in broad range (pH 3 - 10) polyacrylamide gel 7% at 4°C. The β-lactamase activity was detected according to the iodometric method in the presence of the benzylpenicillin as substrate revealed that this strain produces two β-lactamases with pI of 6.64 and another with pI>8,6 (Figure 1). The resistance determinant of representative strain *A. baumannii* AB 4968 was failed to a recipient strain, *E. coli* HB101 and this finding indicated that the genetic determinant was chromosomal location.

Table 3. Specific activities β-lactamase of *A. baumannii* strain AB4968 (µmol of substrate hydrolyzed/min/mg of protein).

Antibiotiques	Specific activity (U/mg)
Benzylpénicilline	0.812
Imipénème	0.32
Céfoxitine	No detected
Céfotaxime	No detected
Ceftriaxone	No detected
Ceftazidime	No detected
Oxacillin	1.62

β-Lactamase activities detection and inhibitors effect

A. baumannii AB 4968 hydrolyzed oxacilline faster than benzylpenicillin and imipenem. The specific activity ranged from 1, 62 to 0, 32 U/mg of protein. The hydrolysis of extended-spectrum cephalosporins by *A. baumannii* AB 4968 was not detectable (Table 3). *A. baumannii* AB 4968 activity was not inhibited by EDTA (Bush et al., 1995); consequently, these enzymes were not defined as metallo-enzymes but they belonged to class with a serine active β-lactamases.

PCR amplification of β-Latamase genes and DNA sequencing analysis

PCR experiments using primers specific for oxacillinases with carbapenemase activity genes were performed and the isolate was found positive for bla_{OXA-23} and bla_{OXA-51}. None of the isolates was found to harbor bla_{OXA-24} or $bla_{OXA-58-like}$ genes. The presence of the insertion sequence ISAba1, upstream of a carbapenemase gene, reportedly can affect the gene expression. To determine if this association applied among our isolate, the isolate was screened by PCR for linkage of ISAba1 to blaOXA.ISAba1 was present upstream of the $bla_{OXA-23-like}$

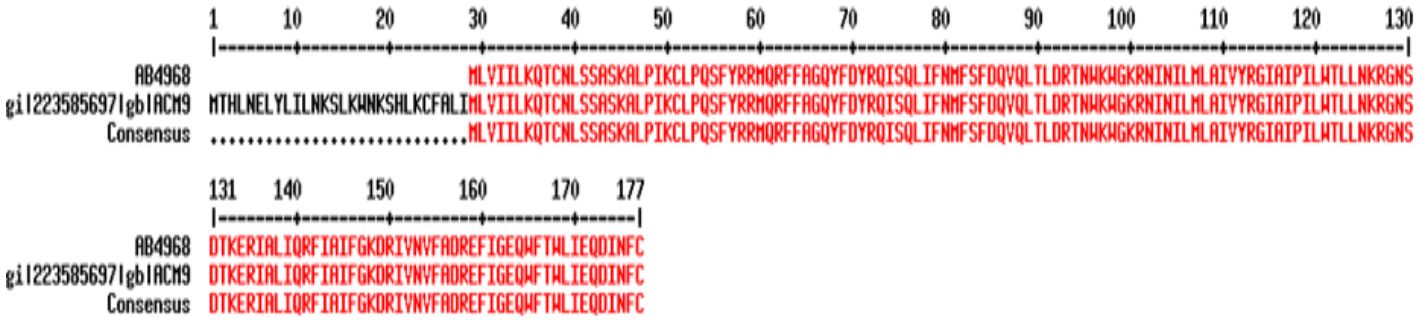

Figure 2. Nucleotides sequence alignment of the OXA23/ISAbaI gene from *A. baumannii* AB 4968 with the published OXA23/ISAbaI variant.

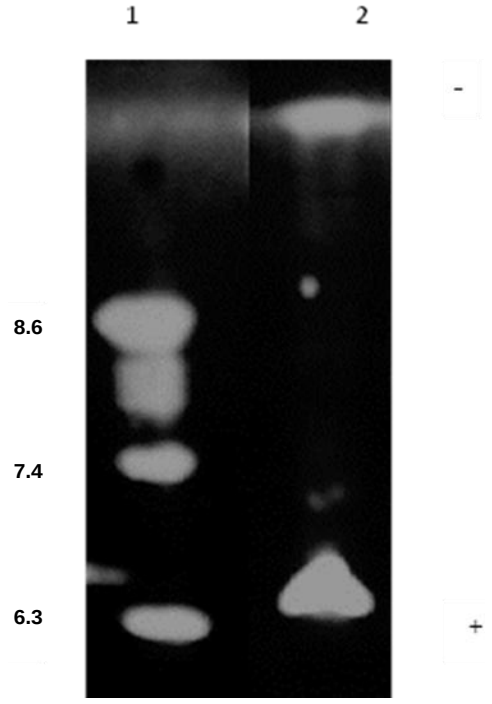

Figure 1. Isoelectric focusing of *Abaumannii*. Lane 1, Markers; lane 2, *Abaumannii* AB 4968.

gene; Sequencing of the amplified fragments revealed perfect identity with the bla$_{OXA-23/ISabaI}$ variant, the blast of the amino acid sequences of this β-lactamase were performed with the website: http://www.ncbi.nlm.nih.gov/ (Figure 2).

DISCUSSION

A. baumannii included in this study was isolated in July 2007, from a 70 –year-old man with respiratory distress hospitalized in a Tunisian hospital. The patient was treated with Tienam and Colimycin. The MIC of various antibiotics was determined, which showed a high level of resistance for this isolate. The strain was highly resistant to imipenem. *A. baumannii* is frequently associated with nosocomial infections. Its acquired resistance too many antibiotics may complicate significantly the choice for antibiotic treatment. Carbapenems have been the drug of choice for treatment of infections caused by *A. baumannii*. However, in recent years, the number of isolates showing resistance to carbapenems has increased worldwide (Amudhan, 2011). Carbapenem resistance associated with class D β-lactamases is an increasing problem in *A. baumannii* (Brown et al., 2005). *A. baumannii* isolate hydrolyzed oxacillin faster than benzylpenicilline hence the name of oxacillinases (Poirel et al., 2010).

The hydrolysis of extended-spectrum cephalosporins by *A. baumannii* was not detectable. However we detected the hydrolysis of imipenem .The activity was not inhibited by EDTA which confirms the presence of active-serine-site enzymes. Class D β-lactamases, also known as oxacillinases or OXA type β-lactamases (OXAs), are active-serine-site enzymes (Poirel et al., 2010).

Isoelectric point determination with benzylpenicclin as substrate, revealed the presence of a band of pI 6.64, which is identical to the reported value for OXA-23 (Donald et al., 2000). *A. baumannii* was positive for both bla$_{OXA-23-like}$ and bla$_{OXA-51-like}$ genes. The genes encoding the blaOXA-51-like β-lactamases are chromosomally located in all of the *A. baumannii* isolates studied to date (Poirel and Nordmann, 2006). Conjugaison experiments were failed suggesting the chromosomal location of the bla $_{OXA-23}$ gene. In addition, the insertion sequence ISAba1 was found immediately upstream of bla$_{OXA-23}$. ISAba1 was not present upstream of the bla$_{OXA-51-like}$ gene in the *A. baumannii* isolate.

The first identified OXA-type enzyme with carbapenem-hydrolyzing activity was from an *A. baumannii* strain isolated in 1985 from Scotland and was originally named ARI-1, but was renamed as *bla*$_{OXA-23}$. Analysis of the bla OXA-23 genetic environment showed that this gene was associated with insertion sequence ISAba1 located upstream of this gene (Mansour et al., 2008). Insertion sequences (ISs) may enhance β-lactamase gene expression by providing promoters. Insertion sequences (IS) are the smallest and the most abundant transposable

elements (<2.5 kb) capable of independent transposition in microbial genomes and has been identified in association with several antibiotic resistance genes in *A. baumannii* (Mugnier et al., 2009).

ISAba1 has frequently been found upstream of the ADC AmpC β-lactamase and OXA carbapenemase genes in *A. baumannii* (Lee et al., 2011). ISAbal, which is adjacent to blaOXA, plays a major role in the development of resistance to carbapenem (Lin et al., 2011). In the isolate that were PCR positive for both blaOXA-23-like and blaOXA-51-like genes, ISAba1was only associated with blaOXA-23-like genes. This implies that the expression of these two genes might be associated with different promoters (Turton et al., 2006). Additional mechanisms of resistance that have not been investigated in this study, such as loss of membrane porins and multidrug efflux pump, may also play important roles in the development of carbapenem resistance.

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