Carbapenem-resistant Acinetobacter baumannii producing the carbapenemase OXA-23 in Tunisia

Samia Hammami, Rafiaa Ghozzi, Mabrouka Saïdani , Saida Ben Redjeb

Laboratoire de Recherche « Résistance aux Antimicrobiens ». Faculté de Médecine de Tunis. Tunisie
Université Tunis El Manar

S. Hammami, R. Ghozzi, M. Saïdani, S. Ben Redjeb

Production d'une carbapénèmase OXA-23 chez Acinetobacter baumannii résistant aux carbapénèmes en Tunisie

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RÉSUMÉ

But: Détecter les mécanismes de résistance enzymatique chez A. baumannii résistant à l'imipénème isolé de différents services à l'hôpital Charles Nicolle de Tunis.

Méthodes: Cinquante souches d'A. baumannii résistantes à l'imipénème ont été recueillies de différents services durant l'année 2007 dans notre hôpital. Une étude de ces souches a été effectuée portant sur la détermination de la sensibilité aux antibiotiques, la production de metallo-ß-lactamase par test à l'EDTA, la production d'oxacillinase par PCR suivi d'un séquençage et le typage moléculaire par méthode d'électrophorèse en champ pulsé.

Résultats : Les souches sont isolées essentiellement dans le service de chirurgie (62%) et les unités de soins intensifs (22%). Elles présentent un haut niveau de résistance aux ß-lactamines testés : ticarcilline (CMI50 >2048µg/ml), ticarcilline-acide clavulanique (CMI50 >1024µg/ml), aztréonam (CMI50 = 512µg/ml), céftazidime (CMI50 = 512 µg/ml), imipénème (CMI50 = 512µg/ml), méropénème (CMI50 = 128µg/ml) et céfépime (CMI50 = 256µg/ml). Toutes les souches étaient non productrices de métallo-ß-lactamase. La coexistence des gènes blaOXA-51-like/ blaOXA-23-like a été détectée dans 82% des souches (n = 41). Aucunes des souches n'étaient productrices de blaOXA-24-like et blaOXA-58-like. Toutes les souches possédaient le gène blaOXA-51-like. Le séquençage a confirmé la présence des gènes blaOXA-23 et blaOXA-69. L'analyse des souches par électrophorèse en champ pulsé a permis de distinguer 8 profils différents dont un clone majoritaire A (n=41) suivi par les clones B (n=1), C (n=1), D(n=1), E (n=1), F(n=2), G (n=1) et H (n=2).

Conclusion : La production d'OXA-23 était le principal mécanisme de résistance aux carbapénèmes chez A. baumannii. Le renforcement des mesures d'hygiène sont exigés pour contrôler davantage la propagation des carbapénèmases en Tunisie.

Mots-clés

Acinetobacter baumannii ; résistance à l'imipénème ; épidémie ; OXA-23

SUMMARY

Background: Aim: To analyze the mechanisms of resistance to carbapenems among imipenem resistant A. baumannii recovered from different wards at Charles Nicolle Hospital.

Methods: From January to December 2007, 50 carbapenem-resistant A. baumannii isolates were recovered from hospitalized patients. MICs were performed by agar dilution method and interpreted according to CLSI guidelines. Metallo-ß-lactamase production was evaluated using imipenem-EDTA disk synergy test. PCR and DNA sequencing targeting blaOXA genes were performed and pulsed field gel electrophoresis was used for epidemiologic study.

Results: Most of the isolates were obtained from patients hospitalized in surgery (62%) and Intensive Care Units (22%). All strains showed high level of resistance to ticarcillin (MIC50 > 2048µg/ml), ticarcillin-clavulanic acid (MIC50 >1024µg/ml), aztreonam (MIC50 = 512µg/ml), ceftazidim (MIC50 = 512µg/ml), imipenem (MIC50 = 512µg/ml), meropenem (MIC50 =128µg/ml) and cefepime (MIC50 = 256µg/ml). Metallo-ß-lactamase production was negative for all isolates. The co-existence of blaOXA-51-like/ blaOXA-23-like was detected in 82% (n= 41). The genes blaOXA-24-like and blaOXA-58-like were not found in any isolate. All isolates harboured a blaOXA-51-like gene. Sequencing confirmed the presence of blaOXA-23 and blaOXA-69 genes. Eight distinct patterns were observed (A: 41 isolates, B: 1 isolate, C: 1 isolate, D: 1 isolate, E: 1 isolate, F: 2 isolates, G: 1 isolate, H: 2 isolates).

Conclusion: Production of OXA-23 was the important mechanism of resistance to carbapenem among A. baumannii. Strengthening of prevention measures are required to control further spread of carbapenemases in Tunisia.

Key-words

Acinetobacter baumannii; imipenem resistant; outbreak; OXA-23

A. baumannii is an opportunistic human pathogen that causes nosocomial infections and frequently exhibits multidrug resistance. Carbapenems are drugs of choice for treating nosocomial infections due to multidrug-resistant (MDR) *A. baumannii* [1]. However, their efficacy has been altered by the emergence of carbapenem-hydrolysing ß-lactamases (carbapenemases) [2-4] that are increasingly reported. They have been attributed to the Metallo-ß-lactamases (MBLs) and OXA type carbapenemases corresponding to Ambler class B and class D respectively. MBLs are powerful carbapenemases divided into nine types (IMP-like "default" size="100%">Metallo-</style><style face="normal" font="default" charset="161" size="100%">, </style><style face="norma, VIM-like </style><style face="normal" font="default" charset="161" size="100%">, </style><style face="normal" font="default" size="100%">, SIM-1 [6], SPM-1 ze="100%">Metallo-</style><style face="normal" font="default" charset="161" size="100%">, </style><style face="normal" font="def, GIM-1 [7], AIM-1 [8], KHM-1 [9], NDM-1 [10] and DIM-1 [11]), but only the first three of these groups have been identified in *A. baumannii*. Oxacillinases found in *A. baumannii* can be sub-classified into four distinct groups: OXA-23-like (OXA-23, OXA-27 and OXA-49); OXA-24-like (OXA-24, OXA-25, OXA-26, OXA-40 and OXA-72); OXA-58 and OXA-51-like enzymes. The last group constitutes a family of chromosomal enzymes typically present in *A. baumannii* [12, 13].

Resistance to carbapenems may also be explained by other mechanisms, such as porin loss or modification, as evidenced recently by the CarO protein, and rarely by modification of penicillin-binding proteins [4].

The aim of this study was to analyze the enzymatic mechanisms of resistance to carbapenems among carbapenem resistant *A. baumannii* pathogen isolates recovered from patients hospitalized in different wards of Charles Nicolle Hospital of Tunis.

MATERIAL AND METHODS

Bacterial isolates. from January to December 2007, 50 carbapenem-resistant *A. baumannii* isolates were recovered from different specimens.

Antimicrobial susceptibility testing and screening for MBL-producing strains. Routine antibiogram was performed by disk diffusion method on Mueller–Hinton agar. MIC values of ticarcillin, ticarcillin-clavulanic acid, ceftazidime, aztreonam, imipenem, meropenem and cefepime were determined by agar dilution technique. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains. Results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute [14]. MBL production was evaluated using imipenem-EDTA synergy test [15].

PCR amplification and sequencing. Genomic DNA of the isolates was extracted by boiling one to three colonies in 100μl of sterile ultrapure water for 10 mn followed by centrifugation for 1 mn at 14 000 rpm. To amplify the genes encoding oxacillinases, simplex PCR assays were run using primers listed in table 1. Amplification was performed in a final volume of 50 μl containing buffer 1X, 2 mM dNTP, 0.5 μM primers, 0.25 U**I** Taq polymerase (Promega) and 5 μl of DNA template. The thermo cycler (BioRad) was programmed at 94°C for 5 mn followed by 30 cycles of 25 s at 94°C, 40 s at 53°C, 50 s at 72°C, and a final cycle of 6 mn at 72°C. PCR products were separeted by agarose gel electrophoresis (1%).

Table 4 : Primers used for amplification of resistance genes by polymerase chain reaction

Primer	Sequence (5'–3')	Product size	Reference
OXA-51like-F	TAATGCTTTGATCGGCCTTG	353pb	[12]
OXA-51like-R	TGGATTGCACTTCATCTTGG		
OXA-23like-F	GATCGGATTGGAGAACCAGA	501pb	[12]
OXA-23like-R	ATTTCTGACCGCATTTCCAT		
OXA-24like-F	GGTTAGTTGGCCCCCTTAAA	246pb	[12]
OXA-24like-R	AGTTGAGCGAAAAGGGGATT		
OXA-58like-F	AAGTATTGGGGCTTGTGCTG	599pb	[12]
OXA-58like-R	CCCCTCTGCGCTCTACATAC		

PCR products from representative strains were purified using a Purification Kit (Qiagen). DNA sequencing was performed by the dideoxy chain terminator method with Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analysed using an ABI Prism 3100 genetic analyser (Applied Biosystems). Similarity searches and alignments for both the nucleotide sequences were performed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Pulsed-field gel electrophoresis (PFGE). Isolates were typed by PFGE following digestion of intact genomic DNA with *Apa*I (BioFaster). DNA fragments were separated on 1% (w/v) agarose gels in 0.5% TBE buffer using a CHEF DRIII apparatus (Bio-Rad, Hercules, CA) with 6 V/cm, pulsed from 5 s to 20 s, for 22 h at 14°C. Gels were stained with ethidium bromide and photographed under ultraviolet light. The *Apa*I restriction profiles were compared by visual inspection according to the criteria of Van Belkum et al [16].

RESULTS

Carbapenem resistant *A. baumannii* strains were isolated from blood cultures (32%), pulmonary specimens (26%), pus (18%), materials (14%) and urines (10%), mainly recover from patients hospitalized in surgery (62%) and Intensive Care Units (22%). The fifty carbapenem resistant *A. baumannii* isolates, screened by antimicrobial susceptibility testing using disk diffusion method were multiresistant to a wide range of antibiotics tested: netilmicin (62%), amikacin (94%), tobramycin (96%) and gentamicin (96%). However they remained susceptible to colistin (100%).

Table 2 : Characteristics of A. baumannii isolates

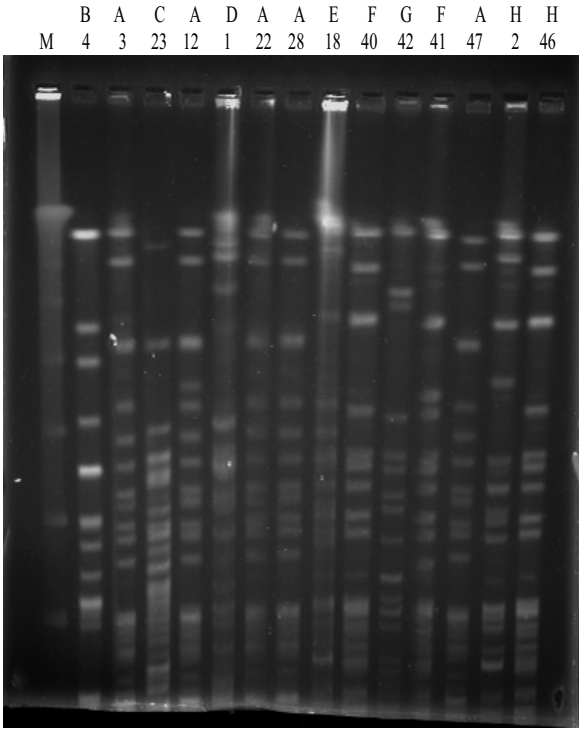
Strains	Date (day / month)	Gender	Specimen[a]	Ward[b]	Susceptibility[c]	PCR OXA-23 like	MIC[d] (μg/ml) Tic	Tcc	Caz	Azt	Imp	Mem	Fep	PFGE
1	12/02	M	PS	Medecine	Cs,An	-	>2048	>1024	2048	>512	>512	>512	256	D
2	01/03	M	PS	Pneumology	Cs, Gn,Tm,Net,An	-	>2048	>1024	>2048	512	>512	256	64	H
3	03/04	M	PS	ICU	Cs, Net	+	>2048	>1024	512	>512	>512	64	256	A
4	07/04	M	PS	Surgery	Cs	-	>2048	>1024	256	>512	512	>512	128	B
5	11/04	F	Urine	Medecine	Cs	+	>2048	>1024	256	128	512	64	256	A
6	16/04	M	PS	Surgery	Cs,Net	+	>2048	>1024	1024	512	512	128	>256	A
7	18/04	M	Blood	ICU	Cs,Net	+	>2048	>1024	256	256	512	128	512	A
8	21/04	M	Blood	Surgery	Cs	+	>2048	>1024	512	>512	512	512	256	A
9	23/04	M	Blood	Surgery	Cs,Net	+	>2048	>1024	256	256	512	512	512	A
10	24/04	M	Blood	Surgery	Cs,Tm,Net	+	>2048	>1024	512	>512	256	128	>256	A
11	23/04	M	Materiel	Surgery	Cs,Net	+	>2048	>1024	512	512	>512	64	256	A
12	23/04	M	PS	Surgery	Cs,Net	+	>2048	>1024	512	512	512	64	256	A
13	30/04	M	Blood	Surgery	Cs,Net	+	>2048	>1024	512	512	>512	512	512	A
14	02/05	F	PS	Surgery	Cs	-	>2048	>1024	256	128	512	256	128	A
15	03/05	M	Materiel	Surgery	Cs,Net	+	>2048	>1024	512	>512	512	>512	256	A
16	15/05	M	Pus	Surgery	Cs	+	>2048	>1024	512	128	512	256	64	A
17	22/05	F	Materiel	ICU	Cs	+	>2048	>1024	512	256	512	512	256	A
18	25/05	M	PS	ICU	Cs	+	>2048	>1024	1024	>512	>512	64	256	E
19	01/06	M	Blood	ICU	Cs	+	>2048	>1024	32	512	512	128	512	A
20	01/06	M	Blood	Surgery	Cs	+	>2048	>1024	512	512	512	64	256	A
21	02/06	M	Materiel	Surgery	Cs	+	>2048	>1024	1024	512	512	256	64	A
22	06/06	M	Pus	Surgery	Cs	-	>2048	>1024	512	128	512	64	512	A
23	15/06	F	Pus	ORLC	Cs, Net	-	>2048	>1024	512	256	128	32	64	C
24	19/06	F	PS	ICU	Cs	+	>2048	>1024	512	128	128	64	32	A
25	20/06	M	Blood	ICU	Cs, Net	+	>2048	>1024	512	>512	512	>512	>256	A
26	23/06	M	Materiel	Surgery	Cs	+	>2048	>1024	1024	512	512	256	64	A
27	06/07	F	Materiel	Surgery	Cs	+	>2048	>1024	512	256	512	512	64	A
28	10/07	M	Urine	Surgery	Cs, An	+	>2048	>1024	512	256	128	512	64	A
29	13/07	M	Urine	Orthopedy	Cs, Net	+	>2048	>1024	512	256	128	32	512	A

MICs value determination showed high level of resistance to ticarcillin (MIC_{50} > 2048μg/ml), ticarcillin-clavulanic acid (MIC_{50} >1024μg/ml), aztreonam (MIC_{50} = 512μg/ml), ceftazidime (MIC_{50} = 512μg/ml), cefepime (MIC_{50} = 256μg/ml), imipenem (MIC_{50} = 512μg/ml) and meropenem (MIC_{50} =128μg/ml) (table 2).

Using imipenem-EDTA disk synergy test, the presence of MBL was not detected in all isolates. The ***bla***$_{OXA-51-like}$ gene was detected in 100% (50/50) of the isolates. Sequencing confirmed the presence of the *bla*$_{OXA-69}$ gene. The *bla*$_{OXA-23-like}$ gene was found in 41 isolates (82%) (table 2), with full sequence homology (100%) to *bla*$_{OXA-23}$ gene. Other carbapenem-hydrolysing class D enzymes, OXA-24-like and OXA-58-like, were not detected in any of the 50 isolates.

PFGE used to determine the genomic diversity of carbapenem-resistant *A. Baumannii* isolates showed eight distinct profiles defining clones A to H : type A (41 strains), type F (2 strains), type H (2 strains), types B, C, D, E, G (one strain for each type) (figure1). The predominant clone A (82 %) was mainly observed in intensive care units (21 strains) and surgery (16 strains) (table 2) suggesting a wide spread of this clone in the two wards. The OXA-23-producing isolates belonged to patterns A (n=39), E (n=1) and G (n=1) (table 2).

Figure 1 : Representative PFGE patterns of carbapenem-resistant A. baumannii PFGE patterns are indicated by the letters above the strains numbers Lane M: Lambda ladder (Bio-Rad)



DISCUSSION

Carbapenems are widely used for the treatment of nosocomial infections caused by *A. baumannii*. However, in recent years, the number of isolates resistant to these antibiotics has increased [17]. The frequency of carbapenem resistant *A. baumannii* isolates at Charles Nicolle hospital has increased from 2% in 2000 to 33% in 2007. A multicentric study in Tunisia in 2008 showed that 34.5% of *A. baumannii* isolates were imipenem resistant (unpublished data) which poses an increased threat to hospitalized patients. These isolates showed high MICs of carbapenems and were resistant to the most of antibiotics tested but remained susceptible to colistin (100%) which is being used to treat infections caused by MDR *A. baumannii*. The production of carbapenemase was the main mechanism involved. High prevalence of MBL genes was reported in different geographic regions [18]. However, in our study, the MBL production detected by imipenem-EDTA disk synergy test was negative for all isolates that was also reported in another Tunisian hospital [19]. OXA-types carbapenemases were the only enzymes detected among our *A. baumannii* isolates and *bla*$_{OXA-23}$ was the common gene, which accounted for 82% of the class D carbapenemase-encoding genes detected. The OXA-23 subgroup was first reported in *A. baumannii* in Scotland in 1995, originally named ARI-1 [20, 21] and then has been increasingly reported worldwide with reports from Africa [22], Europe [23], Asia [15], China [24] and South America [25]. Recently, Zhou *et al* [18] reported that 94.2% of imipenem-resistant *A. baumannii* isolates from China harboured *bla*$_{OXA-23}$. Carbapenem resistant *A. baumannii* isolates producing OXA-23 have been reported in many countries, underlining the large distribution of that mechanism of resistance to carbapenems. In Africa, single reports of OXA-23–producing *A. baumannii* isolates were from Algeria and from Libya [22].

In the Asia-Pacific region and among the carbapenem non-susceptible isolates, class D carbapenemase- encoding genes were detected in 70% of the isolates. The ***bla***$_{OXA-23}$ was the most common gene, which accounted for 95% of the class D carbapenemase-encoding genes detected, followed by a lower occurrence of *bla*$_{OXA-58}$ (11.9%) and *bla*$_{OXA-24/40}$ (5.6%) [7].

Analysis of the molecular epidemiology of carbapenem-resistant *A. baumanni* in the various parts of the world indicated a considerable degree of geographic specificity in the spread of various carbapenem-hydrolyzing oxacillinases [4]. Qi et al [26] showed association of imipenem resistance with the presence of *bla*$_{OXA-23}$ and *bla*$_{OXA-40}$ genes. The *bla*$_{OXA-40}$ gene has been identified in the outbreak strains recovered in several regions of Europe [27, 28] from 1999 to 2004 and lately in the United States [29].

Other carbapenem-hydrolysing class D enzymes, OXA-24-like and OXA-58-like, were not detected in any of the 50 isolates. However, OXA-24 has been reported in Spain [30] and OXA-58 worldwide ormal' font='default' charset='161' size='100%'>, </style><style face='normal' font='default' size='100%'>-lactamase in a hospit.

Concerning the 9 remaining strains, resistance to carbapenem may be explained by non enzymatic mechanisms (e.g., increased efflux of ,-lactam antibiotics, reduced affinity of

penicillin-binding proteins for carbapenems and decreased permeability of the outer membrane). Recent reports have demonstrated that *A. baumannii* possesses outer-membrane proteins (OMPs) that play a role in carbapenem resistance. In 2002, Limansky et al [33] showed that imipenem resistance was associated with the loss of a 29-kDa OMP in clinical isolates of *A. baumannii*, designated CarO [34].

The fifty *A. baumannii* isolates exhibited 8 distinct PFGE profiles defining clones A to H. The predominant clone A had disseminated among 2 wards (intensive care units and surgery), suggesting a cross-contamination. Transfer of infected or colonised patients between surgery and intensive care units and sharing of common healthcare staff may explain the clonal dissemination of MDR *A. baumannii*.

The diversity of PFGE patterns among isolates with $bla_{\text{OXA-23-like}}$ genes suggests horizontal gene spread. Outbreaks of OXA-23-producing *A. baumannii* isolates have been reported repeatedly in Europe, South America and Asia [18, 25, 35].

CONCLUSION

Carbapenem resistant *A. baumannii* strains are spreading in our hospital, $bla_{\text{OXA-23}}$ genotype is an important concern. However, other mechanism may be associated. These results emphasize the importance of continuous surveillance to evaluate the prevalence of carbapenemase genes in MDR *A. baumannii*. Strengthening of prevention measures are required to control further spread of carbapenemases in Tunisia.

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