First report of SPM metallo-β-lactamasases producing *Acinetobacter baumannii* isolates in Morocco

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ABSTRACT

Background and Objectives: Carbapenem-resistant *Acinetobacter baumannii* has recently been identified by the World Health Organization as a critical pathogen. We propose to characterize the molecular characteristics of clinical isolates of *A. baumannii* resistant to carbapenems collected in a Moroccan hospital.

Materials and Methods: Seventy carbapenem-resistant *A. baumannii* isolates from various samples were received at the microbiology laboratory of the Hospital Center. Antibiotic susceptibility was tested by the diffusion disc method and molecular characterization of antimicrobial resistance was performed by PCR and sequencing.

Results: Carbapenemase genes were detected in our isolates: the OXA-51 gene and the ISbA1 sequence were detected in all isolates (100%), the OXA-23 and OXA-58 genes were detected in 82.85% and 10% of isolates respectively, MBL genes were dominated by VIM 39 isolates (55.7%), followed by GIM 26 isolates (37%), SIM 20 isolates (28.5%), IMP 8 isolates (11.4%), NDM 3 isolates (4%) and for the first time in Morocco SPM with 4 isolates (5.7%).

Conclusion: The emergence of resistance of *A. baumannii* to carbapenems is a serious problem in our hospital which requires the establishment of a prevention strategy and strict respect for hygiene to minimize their dissemination.

Keywords: *Acinetobacter baumannii*; Carbapenems; Resistance; Metallo-beta-lactamase

INTRODUCTION

*Acinetobacter baumannii* is an opportunistic pathogen that has become potentially fatal (1). It is the cause, in particular, of pneumonia, catheter infections, and septicemia in hospitalized patients in intensive care units and immunocompromised patients. These infections are particularly severe due to the ability of this bacterium to develop resistance to most of the antibiotics currently available on the market (2), which makes the management of these infections a real health problem and a source of concern for clinicians. In addition, the number of community infections caused by *A. baumannii*, such as pneumonia, bacteremia, endocarditis, and meningitis, has gradually increased in recent years (3, 4).

The overuse of antibiotics in the hospital setting has led to the acquisition of new genetic determinants...
for bacterial resistance. In addition to the intrinsic resistance in *A. baumannii* that confers resistance to a significant number of antibiotics, the acquired determinants have developed multi-resistant, and even pan-resistant, strains of *A. baumannii*. This limits the choice of antibiotics available for the treatment of carbapenem-resistant *A. baumannii* infections (5). However, *A. baumannii* has developed resistance to carbapenems, which has been reported worldwide and in Morocco (6).

Resistance to carbapenems in *A. baumannii* can be due to several mechanisms: intrinsic or chromosomal mechanism (loss or alteration of porin), the combination of mechanisms (extended-spectrum beta-lactamase ESBL with loss of permeability), or the production of carbapenem hydrolyzing enzymes. (7). The latter mechanism is the most frequent in *A. baumannii*.

The carbapenemase enzymes and metallo-β-lactamases are considered the most clinically threatening enzymes because of their efficient hydrolysis of carbapenems and high levels of resistance in the bacteria that produce them. Different types of MBLs have been described worldwide in *A. baumannii* isolates (IMP, VIM, SPM, GIM, SIM, AIM, and NDM) (8).

Detection of carbapenemases is very important to direct management and contribute to antibiotic stewardship to limit further evolution of new genetic resistance variants in *A. baumannii*. The objective of this study is to report the molecular profile of resistance to carbapenems in *A. baumannii* isolates in Morocco, thus we report the first detection of *A. baumannii* strains carrying SPM-1.

### MATERIALS AND METHODS

**Ethical statement.** This study was approved by the Joint Research Ethics Committee of Medical School and University Hospital Hassan II of Fez (Fez, Morocco).

**Bacterial strains.** Seventy-three isolates of *A. baumannii* from different specimens: distal protected lung specimen (PDP), blood, urine, pus, cerebrospinal fluid (CSF), collected at the microbiology laboratory of CHU Hassan II of Fez between September 2018 and June 2019. Duplicate samples were excluded. Identification is based on phenotypic methods, Api 20 gallery, and Phoenix 100Dickson galleries. Identification is confirmed by PCR of the OXA-51 gene-specific for *A. baumannii*. Antibiotic susceptibility including carbapenems of clinical isolates was verified by the standard disk diffusion technique on Müller-Hinton agar using imipenem disks (Oxoid, Basingstoke, UK), according to CLSI 2018 guidelines.

**Detection of carbapenemases: Modified Hodge test (MHT).** MHT was performed as described by Lee et al. (9). One to ten dilutions of 0.5 McFarland suspension of carbapenem-susceptible *E. coli* was aseptically blotted onto a sterile Müller-Hinton agar plate. A disk of imipenem (10 μg) was aseptically placed in the center of the plate. In a straight line from the inside to the outside of the plate, each isolate tested was streaked. carbapenemase-producing *Klebsiella pneumoniae* was used as a positive control. Plates were then incubated for 18-24 h at 37°C and then examined for a clover leaf-like indentation in the inhibition zone of the carbapenem disc at the intersection of the test organism and susceptible *E. coli*.

**Double disc synergy test (DDST).** The DDST test was performed according to Lee et al. (11) for the detection of MBL. Briefly, an overnight culture of a clinical isolate was diluted to 10^5 CFU / ml and spread on an MH agar plate using a cotton swab. Two IPM discs (Oxoid, UK) were placed on the agar surface 4.5 cm (center to center) apart, then 10 mL of 0.5 M EDTA was added to one of the two discs. The plate was incubated overnight. The presence of a synergistic inhibitory zone was considered positive for MBL.

**Molecular detection of carbapenemase genes: Total DNA extraction.** DNA extraction was performed by heat shock by preparing a suspension of strains to be studied in 500 μl of distilled water. After boiling for 10 minutes this suspension, then put it on ice for 2 min, then centrifugation for 10 minutes at a speed of 14000 rpm. We recovered 300 μl of supernatant in a new Eppendorf tube of 1.5 ml and stored it at 20°C.

**Gene amplification by PCR.** Gene amplification was performed using specific primers (Table 1). The amplification procedure starts with the preparation of mix in a final volume of 50 μl containing 2 μl of DNA, 5 μl of 10 x PCR buffer, 2.5 mmol / μl of MgCl₂, 100 μm of deoxynucleotide triphosphates (dNTPs), 0.4 μL of each primer, DreamTaq DNA polymerase.
PCR conditions were programmed as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing (59°C for 1 min for NDM and VIM, and 53°C for 1 min for OXA-23, OXA-24, OXA-48, OXA-51, OXA-58, SPM, SIM, GIM, and IMP), and extension at 72°C for 1 min, ending with a final extension at 72°C for 6 min. The amplicons products were separated in 1.5% agarose gel electrophoresis.

**Sequence analysis.** PCR products positive for VIM, NDM, and SPM were purified by the BigDye kit, and sequencing was performed. Nucleotide sequence analysis and BLAST are performed in NCBI (www.ncbi.nlm.nih.gov/BLAST).

**RESULTS**

Of the 73 A. baumannii isolated in the laboratory, 70 were resistant to imipenem, corresponding to a carbapenem resistance rate of 97%. The majority of isolates were isolated in intensive care units (81.4%). Pulmonary specimens were the dominant ones by 44.3% (Table 2).

Regarding the modified Hodge test, 71% of isolates showed a positive phenotype, while the DDST test for MBL was positive in 76% (Table 3).

As for the molecular detection of the genes encoding the different carbapenemases and the ISba1 insertion sequence, the OXA-51 gene, and the ISba1 sequence were detected in all isolates (100%), the OXA-23 and OXA-58 gene were detected in 82.85% and 10% of the isolates respectively, MBL genes were predominated by VIM 39 isolates (55.7%), followed by GIM 26 isolates (37%), SIM 20 isolates (28.5%), IMP 8 isolates (11.4%), NDM 3 isolates (4%) and SPM with 4 isolates (5.7%). Noting that this is the first report of the gene coding for SPM in Morocco (Table 4 and Fig. 1).

**DISCUSSION**

The emergence of carbapenemase-producing A. baumannii is a serious health problem worldwide, especially in intensive care units, which is clear after analysis of our results since 82.85% of the sam-

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Table 1. Lists of primers used in this study

| Gene | Primers | Sequence 5′ 3′ | Base pair size bp | References |
|------|---------|---------------|------------------|------------|
| Oxa 23 | Oxa-F | 5′-GATCGGATTGAGACCTCAGA-3′ | 501 | |
| | Oxa-R | 5′-ATCTTGCAGCGATATTTCCAT-3′ | | |
| Oxa 51 | Oxa-F | 5′-TATGCTTTGACCGGTTG-3′ | 353 | |
| | Oxa-R | 5′-TGGATTCGACTCTGATCCTTG-3′ | | |
| Oxa24 | Oxa-F | 5′-GGTGGTTGGCCCACTAATG-3′ | 246 | |
| | Oxa-R | 5′-AGTTGAGCAAAGACGGATT-3′ | | |
| Oxa 58 | Oxa-F | 5′-AAGTATGCTGCTTCTGACCTTG-3′ | 599 | |
| | Oxa-R | 5′-CCCCTCTGCGCTTATACATAC-3′ | | |
| VIM | VIM-F | 5′-GATGGTGTGTCGTCGACATA-3′ | 390 | |
| | VIM-R | 5′-CGAATGCGACAGACCAG-3′ | | |
| NDM | NDM-F | 5′-CGTTTGGCCGATCTGGTCTTCT-3′ | 621 | (10, 11) |
| | NDM-R | 5′-CGAATGCGCTACGACGATC-3′ | | |
| IMP | IMP-F | 5′-GGAATAGATGCTTAATCTC-3′ | 188 | |
| | IMP-R | 5′-CCAAAYACTASGTTACT-3′ | | |
| GIM | GIM-F | 5′-TCGACAACCTTGGTCTGATC-3′ | 477 | |
| | GIM-R | 5′-AACCTCAACCTGCTGATC-3′ | | |
| SIM | SIM-F | 5′-TACAGGAGTTGTCGATCTA-3′ | 570 | |
| | SIM-R | 5′-TATGACCTGCTGAC-3′ | | |
| SPM | SPM-F | 5′-AACATCTGCTGACGAAACG-3′ | 271 | |
| | SPM-R | 5′-ACATTATCCGCTGACGCAAGG-3′ | | |
| ISba1 | ISba1-F | 5′-CGAAATTGAGGAAGTTG-3′ | 520 | |
| | ISba1-R | 5′-CGACGGAATCTATGACACG-3′ | | |
ACINETOBACTER BAUMANNII IN MOROCCO

Table 2. Distribution of isolates by service and sampling site

| Workforce Percentage | Percentage |
|----------------------|------------|
| Service              | ICUS 61    |
|                      | Other 9    |
| Sampling site        | CATHETER 13|
|                      | CSF 2      |
|                      | PDP 32     |
|                      | PUS 7      |
|                      | BLOOD 13   |
|                      | URINE 3    |
|                      | Total 70   |

ICU: intensive care unit; PDP: protected distal sampling, CSF: cerebrospinal fluid

Table 3. Positivity rate of phenotypic tests for the detection of carbapenems

| Test  | Positive | Negative | Positivity % |
|-------|----------|----------|--------------|
| MHT   | 50       | 20       | 71%          |
| DDST  | 53       | 17       | 76%          |

MHT: modified test Hodg; DDST: double-disc synergy test

Table 4. Percentage of carbapenemases genes found

| OXA51  | 100 | 0 | 70 |
| OXA23  | 83  | 21| 58 |
| OXA24  | 1.4 | 69| 1  |
| OXA58  | 10  | 63| 7  |
| ISbA1  | 100 | 0 | 70 |
| IMP    | 11.4| 62| 8  |
| VIM    | 55.7| 31| 39 |
| NDM    | 4   | 67| 3  |
| SPM    | 5.7 | 66| 4  |
| SIM    | 28.5| 50| 20 |
| GIM    | 37  | 44| 26 |

ples came from intensive care units in our institution, which confirms that A. baumannii is a frequent bacterium in intensive care units (12). This bacterial species possesses an extremely large and diverse group of enzymes which gives it resistance to several antibiotics including carbapenems (12, 13). The resistance of A. baumannii to imipenem in our hospital has an alarming rate, 97%, and it is similar to that (100%) reported recently in Morocco by Uwingabile et al. (14). This high rate may be related to the empirical prescription of imipenem and third-generation cephalosporins. Indeed, several studies have shown a relationship between the pressure exerted by third-generation cephalosporins and the selection of A. baumannii strains resistant to imipenem (15).

Different mechanisms can contribute to carbapenem resistance, however, MBL and oxacillinase production remain the most common and widespread mechanisms among A. baumannii isolates (16). In this study, the modified Hodg test (MHT) was able to diagnose carbapenemase production with a sensitivity of 71%, which was confirmed by molecular tests. Furthermore, the sensitivity of this test in the study is in agreement with previously published reports in which MHT was able to detect carbapenemase production in 83.3%, 71% and 73% of the carbapenem-resistant isolates screened (16-18) respectively.

Class D carbapenemes (oxacillinases) are by far the most prevalent carbapenemases in A. baumannii (17, 18). Our results are consistent with many studies that confirm the emergence of oxacillinases in A. baumannii. In addition, the coexistence of OXA-51, OXA-23, and ISbA-1 is emerging in our institution which explains the high resistance of our isolates to all antibiotics including carbapenems, except colistin which remains the only effective antibiotic to treat A. baumannii infections. Indeed, the molecular characterization of carbapenem-resistant isolates shows that all isolates have OXA-51, this intrinsic gene has a weak carbapenemase activity that requires additional genetic elements, such as ISbA-1 genes thus favoring an overexpression of the gene and the production of carbapenemase (19, 20). We have noted the coexistence of oxa-51 and oxa-23 genes in 83% of clinical isolates, which is consistent with other studies (21).

MBLs are particularly problematic because their genes are harbored on mobile elements, allowing easy dissemination among clinical isolates (22). The DDST synergy test showed a positivity rate of 76%. This prevalence of MBL is high compared to other Moroccan studies (23) which reported a prevalence rate of 75%. The search for genes encoding MBLs showed high diversity in these enzymes in our institution. We found that the gene coding for VIM is predominant by 55.7%, this rate is higher compared to other studies (24, 25) which reported respectively rates of 5.7% and 7%. However, it is still slightly in agreement with that reported by PeshattiWar et al. (26) indicating a percentage of 62.5%. The first MBL.
identified in A. baumannii strains was the IPM-2 reported in 2000 in Italy (27). Since then, IMP, VIM, and SIM have been sporadically reported in some parts of the world (28), including Mediterranean countries, especially Greece and Italy (27-32).

Unfortunately, there are few studies in this field in Morocco to know the prevalence of MBL genes in our country. In our study, we were able to detect VIM in 39 isolates, a frequency of 56% of isolates, a very significant rate compared to Moroccan studies. SIM and GIM were detected in 26 (39%) and 20 (37%) isolates respectively. These three enzymes predominate the metallo-beta-lactamases in our isolates, but the rate is comparable with other studies that report a dominance of VIM (33).

We found that VIM coexists predominantly with SIM and GIM in the genome of our isolates. The IMP gene was found in 8 isolates or 11%, this rate is low compared to other studies that reported 95% (34). Concerning NDM producers, A. baumannii strains harboring these enzymes are increasingly observed in the world (35) especially in Mediterranean countries. They have been detected in North Africa: Algeria (36, 37) and Libya (isolated from a patient transferred from Libya to Denmark) (38); and in Morocco (6, 39). In our study, we detected 3 isolates (4%) carrying NDM-I. This rate of NDM in this study, is low compared to other studies in Africa including Ghana, Egypt, and Kenya with 8%, 19%, and 6% respectively.

The SPM gene was detected in 4 isolates (5.7%) this represents the first appearance, to our knowledge, of this gene in A. baumannii isolates in Morocco. The first report of SPM-I (São Paulo Metallo-β-lactamase) was reported in Brazil in a P. aeruginosa isolate, and after this enzyme spread throughout Brazil (40).

CONCLUSION

To conclude, MBL production represents an important mechanism of carbapenem resistance among A. baumannii isolates in our hospital. The genetic diversity observed among isolates producing each type of MBL indicates both horizontal and clonal dissemination. However, it remains to complete our work by a study of clonality to have an idea on the dynamics of multi-resistant A. baumannii strains.

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