C2-1890

EMERGENCE OF AN IMP-LIKE METALLO-ENZYME IN *Acinetobacter baumannii* FROM A BRAZILIAN TEACHING HOSPITAL

Ana C. Gales[1*], Maria C. B. Tognim[1,2], Adriana O. Reis[1], Ronald N. Jones[3], Hélio S. Sader[1]

[1]Division of Infectious Diseases, Universidade Federal de São Paulo, São Paulo, Brazil; [2]Division of Microbiology, Universidade Estadual de Maringá, Paraná, Brazil; and [3]The Jones Group/JMI Laboratories, North Liberty, IA, USA.

ABSTRACT

Background: Although high carbapenem resistance (R) rates have been reported among *Acinetobacter* spp. isolated in Brazil, very little is known about their mechanisms. The knowledge of such mechanisms could contribute to a better understanding of the epidemiology and treatment. ***Objective:*** This study reports the first appearance of an IMP-like metallo--lactamase (MBL) in a clinical isolate of *A. baumannii* (strain A3227) from a Brazilian teaching hospital. ***Methods:*** MICs to diverse antimicrobials were determined using the NCCLS broth microdilution method. The hydrolysis of carbapenems was confirmed using a bioassay described by Gots. For detection of MBL production, phenotypic tests employed an Etest MBL strip and thiolatic acid/EDTA disks. Isoelectric bla_{imp-1} and bla_{imp-2} primers and the PCR product was purified and sequenced. Carbapenem R transferable rates were determined by mating experiments. ***Results:*** Strain A3227 was highly R to carbapenems (MIC, 32 g/ml), ceftazidime (MIC, > 256 g/ml) and cefepime (MIC, 128 g/ml). In contrast, this strain was susceptible to aztreonam (MIC, 4 g/ml), gentamicin (MIC, 1 g/ml) and ciprofloxacin (MIC, 0.12 g/ml). Two bands focusing at 5.4 and 8.6 were detected on IEF gel. The phenotypic test indicated that A3227 strain was a MBL producer. A 580-bp product was amplified utilizing bla_{imp-1} primers. The sequencing results showed 100.0% homology to bla_{imp-6}. However, the N-terminal region could not be amplified or sequenced. Transfer of carbapenem R by conjugation was not achieved. ***Conclusions:*** Our results show the presence of a bla_{imp} gene in the A3227 strain most resembling bla_{imp-6}.The emergence of a MBL in Latin America further compromises treatment of *Acinetobacter* infections.

INTRODUCTION

Multidrug-resistant (MDR) *Acinetobacter* spp. constitute a serious cause of nosocomial infection in Brazilian hospitals. Typically, carbapenems remain as the widest spectrum therapeutic option for treatment of such infections. However, resistance to these antimicrobial agents has increased, resulting in the use of potentially more toxic agents such as the polymyxins. Although the high carbapenem resistance rates have been reported among *Acinetobacter* spp. isolated in Brazil, very little is known about their mechanisms of resistance. The knowledge of such mechanisms could contribute to a better understanding of the emergence of resistance and the epidemiological scenario, which could guide the infection control services in implementing effective interventions to avoid the spread of carbapenem-resistant isolates between patients or organisms species.

This communication documents the first appearance of an IMP-like metallo- -lactamase found in a clinical isolate of *A. baumannii* from a Brazilian teaching hospital. The strain A3227 was isolated from a quantitative tracheal secretion sample in a male patient, who developed nosocomial pneumonia during his hospitalization at the Hospital São Paulo (São Paulo, Brazil). This hospital is a 600-bed teaching medical center, where carbapenem resistance among *Acinetobacter* spp. isolates has reached rates of approximately 10.0%.

MATERIAL AND METHODS

Susceptibility testing: **The minimum inhibitory concentrations (MICs) were determined using broth microdilution method according to the National Committee for Clinical Laboratory Standards.**

Carbapenem-hydrolysis: **The hydrolysis of carbapenems was evaluated using a bioassay described by Gots. This test utilizes *Micrococcus luteus* ATCC 9341 (yellow pigmented) as an indicator of antimicrobial hydrolysis by the challenge strains. Briefly, using the Stiers inoculator, the clinical strain A3227, the positive and negative control strains were plated on Mueller-Hinton agar plates containing approximately 10^9 cfu/ml of *M. luteus* ATCC 9341 and meropenem at concentrations of 0.06 or 0.12 g/mL (Figure 1).**

MATERIALS AND METHODS

Metallo- -lactamase phenotypic detection: **An investigational Etest strip (MBL strip; AB BIODISK, Solna, Sweden) was used as a screening test for the detection of metallo-enzyme producing isolates, as well as a disk-approximation test using 2-mercaptopropionic acid (2-MPA) or EDTA. A reduction of imipenem MIC by three $\log_2$ dilutions in the presence of EDTA was considered suggestive of a metallo- -lactamase production by the Etest. According to the disk-approximation test, an isolate was categorized as metallo- -lactamase producer if an enhanced zone of inhibition was observed between the disks containing 2- MPA or EDTA and imipenem or ceftazidime.**

Isoelectric focusing (IEF): **It was performed utilizing crude -lactamase extracts prepared by freeze-thaw lysis of bacterial cultures grown to exponential phase in tryptic soy broth. Isoelectric focusing tests utilized a Multiphore II electrophoresis system in ampholine-polyacrylamide gels, pI 3.5-9.5 (Pharmacia Biotech, Sweden).**

Bla_{IMP} detection by PCR and DNA sequencing: **PCR was performed on total DNA after boiling the bacterial cells using bla_{imp-1} (*imp*1, sense: 5'-CTACCGCAGCAGAGTCTTTGC-3', antisense: 5'-GAACAACCAGTTTTGCCTTACC-3') and bla_{imp-2} (*imp2*, sense: 5'-TGCCGCGGGAGCGCGTTTG-3', antisense: 5'-GCCCTTTAACAGCCTGTTCCC-3') primers. The amplification PCR conditions were as follows: 35 cycles of denaturation at 94C for 1.0 minute, annealing at 61°C for 1.0 minute, and amplification at 72C for 1 minute. PCR products were purified using the Concert Rapid PCR Purification System (Gybco, USA). DNA sequence analysis was performed using Big Dye terminator cycle sequencing chemistry for ABI BioPrism 377/310 (Applied Biosystems, California, USA).**

Conjugation: **To examine whether the carbapenem resistance was transferable, mating experiments were carried out using *Escherichia coli* K12 (streptomycin-resistant, MIC 1024 g/ml) and *P. aeruginosa* ATCC 27853 mutant (streptomycin-resistant, MIC 1024 g/ml) as recipients. Overnight cultures were mixed together and plated onto selective media containing streptomycin 1000 g/ml, ampicillin 60 g/ml, and imipenem 24 g/ml.**

Quality Control Strains: **The IMP-1-producing *Pseudomonas aeruginosa* (PSA319) and *A. baumannii* (ACB 17-4) were used as positive control strains, while *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 29212) were used as negative control strains.**

RESULTS

Susceptibility testing: **The strain A 3227 was highly resistant to imipenem and meropenem (MIC, 32 g/ml), extended-spectrum cephalosporins (ceftazidime MIC, > 256 mg/ml; cefepime MIC, 128 g/ml). In contrast, this isolate was susceptible to aztreonam (MIC, 4 g/ml), gentamicin (MIC, 1 g/ml) and ciprofloxacin (MIC, 0.12 g/ml). This isolate exhibited intermediate resistance to ampicillin/sulbactam (MIC, 16 g/ml) and amikacin (MIC, 32 g/ml).**

Metallo- -lactamase phenotypic detection: **The phenotypic evidence that the strain A3227 produces an metallo- -lactamase can be observed in the Figure 1, 2 and 3. The growth of the *M. luteus* ATCC 9341 was routinely inhibited at 0.12 g of meropenem. Thus, the growth of *M. luteus* ATCC 9341 around strain A3227 colonies on the 0.12 g/ml meropenem agar dilution plates indicated carbapenem inactivation (Figure 1). Using the Etest MBL strip, the A3227 strain was considered to be a metallo- -lactamase producer since there was a 64-fold reduction of the imipenem MIC in the presence of EDTA (Figure 2). A enhanced zone of inhibition was also observed between the 2-MPA/EDTA and both ceftazidime and imipenem disks (Figure 3), confirming that A3227 was a possible metallo- -lactamase producer.**

RESULTS

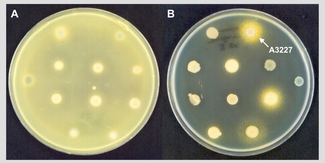

Figure 1: (a) The growth of *M. luteus* ATCC 9341 is shown on a 0.06 g/ml meropenem agar plate. (b) The growth of *M. luteus* ATCC 9341, on a 0.12 g/ml meropenem plate, can be observed only around the strain A3227 colonies and the positive control strains (positive Gots ' test).

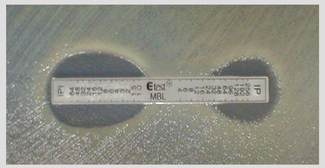

Figure 2: A reduction of 64-fold in the imipenem MIC in the presence of EDTA indicated that A3227 was a possible metallo- -lactamase producer according to the Etest MBL strip.

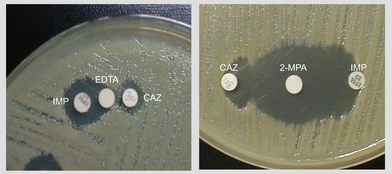

Figure 3: An enhanced zone of inhibition can be observed between the EDTA or 2-MPA and the ceftazidime or imipenem disks in the plates a and b, respectively. It confirms that the strain A3227 was a possible metallo- -lactamase producer by the disk approximation test.

Isoelectric focusing (IEF): **Two bands focusing at pI 5.4 and 8.6 were detected on the IEF gel. Among *Acinetobacter* spp. isolates, enzymes with isoelectric point (pI) 5.4 have been described as derivatives of TEM- or *PER*-genes and have not been capable of hydrolyzing carbapenems. Thus, the pI 8.6 enzyme could be responsible for the carbapenem hydrolysis.**

Bla_{IMP} detection by PCR: **The PCR utilizing bla_{imp-2} primers failed to produce any products; however, a 580-bp product was amplified utilizing bla_{imp-1} primers. A total of 508-bp were sequenced, showing 100.0% of homology to IMP-6. However, the N-terminal region could not be amplified or sequenced yet.**

Conjugation: **Transfer of carbapenem resistance to *E. coli* K12 or *P. aeruginosa* ATCC 27853 mutant was not detected despite several attempts.**

DISCUSSION

There have been increasing number of reports, especially from the Asia-Pacific region, of gram-negative organisms that carry the transferable carbapenem resistance gene bla_{imp} . The majority of these isolates produce IMP-1 type metallo- -lactamases. Variants of bla_{imp} , such as IMP-2 through -9, have been described in a variety of species in diverse parts of the world (IMP-2, *A. baumannii*, Italy; IMP-3, *Shigella flexneri*, Japan; IMP-4, *Acinetobacter* spp., China; IMP-5, *A. baumannii*, Portugal; IMP-6, *Serratia marcescens*, Japan; IMP-7, *P. aeruginosa*, Canada; IMP-8, *Klebsiella pneumoniae*, Taiwan; and IMP-9, *P. aeruginosa*, China).

Recently, a new metallo- -lactamase (SPM-1) was detected from a *P. aeruginosa* strain isolated from the same Brazilian institution. The N-terminal sequence from SPM-1 was completely different from that described for IMP-derivative enzymes.

To determine the sequence of the N-terminal of the IMP-like metallo- -lactamase described in this report (*A. baumannii* A3227), cloning experiments are underway and new transference attempts using transformation techniques have been carried out. Only after completing these procedures, will we be able to conclude if this index strain carries a new variant of the bla_{imp} gene and if the gene was inserted into the bacterial chromosome or carried by a plasmid. Finally, expanded surveillance studies are necessary to evaluate the prevalence of these emerging IMP- or SPM-producing isolates in the Brazilian hospitals and in this geographic region as a component of already existing carbapenem-resistant strains of non-fermentative gram-negative bacilli.

ACKNOWLEDGMENTS

We are grateful to Douglas J. Biedenbach, Paul Rhomberg, Alan Mutnick for critically reviewing this manuscript; and to Elisabeth Travalieri and Mariana Castanheira for their technical assistance during the sequencing tests.

REFERENCES

1. Gales AC, Sader HS, Sinto S, Santos OP, Mendes CF. In vitro activity of ampicillin-sulbactam against multiresistant *Acinetobacter baumannii* strains. J Chemother 1986;8:416-419.
2. Levin AS, Barone AA, Penco J, Santos MV, Marinho IS, Arruda EA, et al. Intravenous colistin as therapy for nosocomial infections caused by multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Clin Infect Dis 1999;28:1008-1011.
3. Gales AC, Jones RN, Forward KR, Linares J, Sader HS, Verhoef J. Emerging importance of multidrug-resistant *Acinetobacter* species and *Stenotrophomonas maltophilia* as pathogens in seriously ill patients: geographic patterns, epidemiological features, and trends in the SENTRY Antimicrobial Surveillance Program (1997-1999). Clin Infect Dis 2001;32(Suppl 2):S104-113.
4. Costa, SF, Woodcock J, Gill M, Wise R, Barone AA, Caiaffa H, et al. Outer-membrane proteins pattern and detection of beta-lactamases in clinical isolates of imipenem-resistant *Acinetobacter baumannii* from Brazil. Int J Antimicrob Agents 2000;13:175-182.
5. National Committee for Clinical Laboratory Standards Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically, fifth edition. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, 2000, Wayne, PA.
6. Gots JS, The detection of penicilinase-producing properties of microorganisms. Science 1945;102:309.
7. Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H, et al. Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds. J Clin Microbiol 2000;38:40-43.
8. Walsh TR, Bolmström A, Qwärnström A, Gales AC. Etest MBL: A new approach for detection of metallo–lactamase producing isolates by routine clinical microbiology laboratories. J Clin Microbiol (*in press*).
9. Osano E, Arakawa Y, Wacharotayankun R, Ohta M, Horii T, Ito H, et al. Molecular characterization of an enterobacterial metallo -lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob Agents Chemother 1994;38:71-78.
10. Riccio ML, Franceschini N, Boschi L, Caravelli B, Cornaglia G, Fontana R, et al. Characterization of the metallo-beta-lactamase determinant of *Acinetobacter baumannii* AC-54/97 reveals the existence of bla(IMP) allelic variants carried by gene cassettes of different phylogeny. Antimicrob Agents Chemother 2000; 44:1229-1235.
11. Bou G, Martínez-Beltrán J. Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC -lactamase in *Acinetobacter baumannii*. J Clin Microbiol 2000;44:428-432.
12. Da Silva GJ, Leitao R, Peixe L. Emergence of carbapenem-hydrolyzing enzymes in *Acinetobacter baumannii* clinical isolates. J Clin Microbiol 1999;37:2109-2110.
13. Chu YW, Afzal-Shah M, Houang ET, Palepou MI, Lyon DJ, Woodford N, et al. IMP-4, a novel metallo-beta-lactamase from nosocomial *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. Antimicrob Agents Chemother 2001;45:710-714.
14. Gibb AP, Tribuddharat C, Moore RA, Louie TJ, Krulicki W, Livermore DM, et al. Nosocomial outbreak of carbapenem-resistant *Pseudomonas aeruginosa* with a new bla_{exp} allele, bla_{IMP-7}. Antimicrob Agents Chemother 2002;46:255-258.
15. Yano H, Kuga A, Okamoto R, Kitasato H, Kobayashi T, Inoue M. Plasmid-encoded metallo-beta-lactamase (IMP-6) conferring resistance to carbapenems, especially meropenem. Antimicrob Agents Chemother 2001;45:1343-1348.
16. Walsh TR, Simm A., Gales AC, Biedenbach DJ, Jones RN. First Appearance of a mobile metallo--Lactamase in Latin America. Antimicrob Agents Chemother (*in press*).