Genetic characterization of a novel metallo-ß-lactamase gene *bla*$_{IMP-16}$: a highly divergent *bla*$_{IMP}$ with a unique genetic context. Report from the SENTRY Antimicrobial Surveillance Program.

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The JONES Group/JMI Laboratories
North Liberty, IA, USA; www.jmilabs.com
319.665.3370, fax 319.665.3371
ronald-jones@jmilabs.com

RE MENDES, J RIBEIRO, M CASTANHEIRA, M TOLEMAN, HS SADER, RN JONES, TR WALSH.
BCARE, Bristol, United Kingdom.
Hospital de Base do Distrito Federal, Brasilia, Brazil; The JONES Group/JMI Laboratories, North Liberty, IA.

AMENDED ABSTRACT

Background: As part of the SENTRY Antimicrobial Surveillance Program, *P. aeruginosa* strains resistant to carbapenems and ceftazidime have been screened for metallo-ß-lactamase (MßL) production and their resistant determinants analysed. *P. aeruginosa* 101-4704 was isolated from a 60-y-o patient who spent 151 days in the ICU and had numerous courses of antimicrobials for pneumonia (4 episodes) and a pleural empyema.
Methods: MßL production was confirmed by the Etest and further substantiated by biochemical assay techniques. Isolates demonstrating MßL activity were screened for bla$_{IMP}$, bla$_{VIM}$ and bla$_{SPM}$ using PCR. Gene banks were created from PSA 101-4704 and the novel MßL gene was cloned and analysed. Sequencing was accomplished using DuPont Automated systems and sequence analysis done by DNAstar.
Results: Sequence analysis revealed the MßL gene to be associated with a class 1 integron. The enzime, designated IMP-16, showed highest identity to bla$_{IMP-11}$ (91.0%) followed by blaIMP-8 (88.6%). Upstream of bla$_{IMP-16}$ lies a class 1 integron, but with an altered promoter site. Downstream of bla$_{IMP-16}$ lies an open reading frame, that has 52% identity to *aacA29b*, followed by *aacA4* and *aadA1* genes. IMP-16 poorly hydrolyses aztreonam and clavulanic acid and is inhibited by EDTA and dipicolinic acid.
Conclusions: bla$_{IMP-16}$ is a highly divergent IMP-like gene. Downstream of bla$_{IMP-16}$ lies a unique aminoglycoside resistance gene as well as standard genes associated with class 1 integrons. The altered promoter site of bla$_{IMP}$ is likely to be responsible for its poor expression and lack of high-level carbapenem resistance.

BACKGROUND

The mechanisms responsible for carbapenem resistance include decreased outer membrane permeability (porin deletion), up-regulation of multidrug efflux pumps, overproduction of AmpC, and production of classes A,B and/or class D ß-lactamase enzymes. The recent emergence of clinically relevant MßLs capable of hydrolyzing all classes of ß-lactams with the exception of aztreonam has caused great concern. Four different types of these zinc dependant enzymes are now described: IMP, VIM, SPM and GIM. IMP-type MßLs were initially reported in Japan in 1991 but since then they have been reported worldwide.

Most MßL enzymes thus far discovered are encoded by gene cassettes, which consist of a single gene and a downstream recombination site, known as 59-be (59- base element). These MßL gene cassettes are usually associated with integrons and the bla$_{IMP-1}$ gene cassette has been found on both class 1 and class 3 integrons. In the present study, we describe a novel bla$_{IMP}$ variant, bla$_{IMP-16}$ and its genetic context that was found recently in the *P. aeruginosa* strain 101-4704 from Brasilia, Brazil.

MATERIALS & METHODS

Susceptibility testing. All isolates collected in the SENTRY Program are susceptibility tested by the reference broth microdilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS). Antimicrobial agents were obtained from the respective manufacturers and quality control was performed by concurrent testing of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212.
Phenotypic detection of ß-lactamases. Production of MßL was screened by the disk approximation test. Briefly, a 100mm Mueller-Hinton agar plate was inoculated using a 0.5 McFarland suspension from fresh cultures. Imipenem, meropenem, and ceftazidime disks were strategically aligned around disks contained either EDTA (750 µg) or thiolactic acid (0.3 µl). The test was read after 18-20 hs of incubation at 35ºC. The appearance of either an elongated or a phantom zone between the carbapenems and/or ceftazidime and either one of the disks containing a MßL inhibitor (EDTA or thiolactic acid) was considered a positive test. *Acinetobacter baumannii* 54/97 was used as a positive control. MßL Etest® strips (AB Biodisk, Solna, Sweden) were used to confirm the disk approximation test results. In addition, ceftazidime/ceftazidime-clavulanic acid and cefepime/cefepime-clavulanic acid ESBL Etest® strips were used to evaluate the production of extended-spectrum ß-lactamase (ESBL). The Etest® strips were used according to the manufacturer's instructions.
Antimicrobial resistance gene screening. Among other selected pathogens, *P. aeruginosa* strains resistant to imipenem (MIC, ≥ 16 µg/ml), meropenem (MIC, ≥ 16 µg/ml), and ceftazidime (MIC, ≥ 32 µg/ml) have been routinely examined for antimicrobial resistant genes through the amplification and sequencing of the variable region of class 1 integron. *P. aeruginosa* 101-4704 was grown overnight in nutrient agar with 10 µg/ml of imipenem at 37ºC. One loopful of bacterial growth was suspended in 300 µL of molecular biology grade water. One µL of this suspension was used as template in standard PCR conditions, with a low stringency annealing temperature (45ºC). Oligonucleotide primers targeting to conserved regions of bla$_{IMP}$, bla$_{VIM}$ and bla$_{SPM}$ genes were initially used to determine the genetic basis of the resistance. Additional primers designed for the 5' conserved segment (CS) and 3'CS regions of class 1 integrons were used to amplify the bla$_{IMP-16}$ containing integron resident in *P. aeruginosa* 101-4704.
DNA sequencing. Primers for the 5'CS and 3'CS of the class 1 integron, as well as primers for the gene cassette yielded PCR products that were sequenced on both strands using DuPont Automated systems. The sequences were found to overlap sequences, and were then assembled to produce a contiguous sequence of 4.332-bp.
Analytical IEF. The ß-lactamase extract from strain 101-4704 was obtained by cell lysis with BugBuster (Novagen, Nottingham, United Kingdom) and the experiment was performed with a NOVEX (Invitrogen, Paisley, United Kingdom) apparatus. The focused beta-lactamases were detected by overlaying the gel with nitrocefin solution (Microbiology Systems, Cockeysville, MD). Isoelectric points were estimated by linear regression obtained by comparison to reference proteins by using a pI 4.5 to 9.5 Standard IEF marker (Bio-Rad, Watford, United Kingdom).
Plasmid analyzes and transformation. Plasmid DNA of *P. aeruginosa* 101-4704 was extracted and electroporated as previously described. Transfer of ß-lactam resistance markers from strain 101-4704 into DH5α was performed using a Bio-Rad Gene Pulser apparatus (Bio-Rad, Richmond, CA) set at 2.5kV, 25µF and 400Ω. DH5α harboring the plasmid DNA was selected on nutrient agar plates containing ceftazidime (2ug/ml).
Computer sequence analysis. Nucleotides sequences and their deduced protein products, alignments and phylogenetic relationships were determined using the Lasergene software package (DNASTAR, Madison, WI).

CASE REPORT

DPD, a 60-year-old male, was first admitted at the Hospital de Base do Distrito Federal, Brasília, Brazil on May 17th, 2001 with a diagnosis of bronchogenic carcinoma. A pneumectomy of the right lung was performed on July 25th and on August 28th the patient developed a severe pneumonia requiring mechanical ventilation. An empyema was also diagnosed and drainage performed. The patient was empirically treated with meropenem, amikacin and vancomycin for 17 days. The patient's symptoms improved but he had three other episodes of pneumonia and remained in the ICU for 151 days. Several pathogens were recovered from respiratory specimens, including *P. aeruginosa* (Table 1) *Acinetobacter* spp. and *Stenotrophomonas maltophilia*, but all blood cultures were negative. During this period, the patient received meropenem 1g q8h (a total of 54 days), amikacin 500 mg q12h (a total of 42 days), trimethoprim/sulfamethoxazole 800 mg q6h (16 days), ciprofloxacin 400mg q12h (14 days), and vancomycin 1g q12h (21 days). The patient was transfered to the ward on January 28th, 2002 and went home on February 21st. On April 23rd, 2002 he was readmitted with cough and dyspnea. He received physiotherapy and the symptoms improved rapidly. Pulmonary secretion collected during a bronchoscopy yielded the *P. aeruginosa* strain 101-4704. No antibiotic was given to the patient and he was discharged on May 8th, 2002.

RESULTS

- Only polymyxin B (MIC$_{90}$, 2 µg/ml and 96.9% susceptibility) showed good activity against strains of *P. aeruginosa* isolated in the Hospital de Base do Distrito Federal in 2001 and 2002 (Table 1). The second most active compound was amikacin (82.8% susceptibility), followed by piperacillin/tazobactam (73.4%) and ciprofloxacin (68.8%).
- Although several other *P. aeruginosa* isolates with similar susceptibility patterns were recovered from the patient prior to the index strain, these isolates were not available for epidemiologic characterization (Table 2).
- Initial PCR screening of this strain with primers designed to detect conserved regions of bla$_{IMP}$, bla$_{SPM}$ and bla$_{VIM}$ MßL genes yielded a PCR product from the bla$_{IMP-2}$ primer set. Further sequence analysis of this PCR product revealed a new MßL gene, which was designated bla$_{IMP-16}$.
- The new bla$_{IMP-16}$ gene is located in a class 1 integron. Two promoters probably drive expression of the gene cassettes: P1 (-35[TGGACA]; -10[TAAGCT]), containing two hexamers spaced by 17-bp; and P2 (-35[TTGTTA]; -10[TACAGT]) located 82-bp downstream of P1.
- This integron harbored four antibiotic resistance gene cassettes (Figure 1). The MßL bla$_{IMP-16}$ gene was located at the firstposition downstream of the 5'-CS, which encoded a putative protein of 246 amino acids (GC content of 38.5%).
- IMP-16 displays greatest identity to IMP-11 (91.0%) and IMP-8 (88.6%) with 22 and 28 amino acids differences, respectively (Figure 2).
- The IMP-16 MßL active site was conserved as previously described for all other IMP-type enzymes (Figure 3).
- The ß-lactam resistance markers could not be transferred from *P. aeruginosa* 101-4704 to *E. coli* DH5α. Thus, the bla$_{IMP-16}$ gene is more likely to be located in the chromosome.
- The analytical IEF experiment showed that *P. aeruginosa* 101-4704 produced three ß-lactamases with pI value of 6.4, 8.1 and 6.9, the last being in accordance with the theoretical pI value of the bla$_{IMP-16}$ (data not shown).
- A novel aminoglycoside acetyltransferase gene cassette (*aacA*) was found downstream of the bla$_{IMP-16}$. This 401-bp gene is preceded by a recognizable ribosome-binding site and potentially encodes a protein of 15.1-kDa. This protein contains 133 amino acids and shows greatest identity (52.7%) with the previously described AAC(6')-29b (Figure 4).
- This new *aacA* gene cassette is flanked upstream by a core site (GTTAGGT) and downstream by an inverse core site (ACCTAAC); however, it does not appear to possess an associated 59-be, but instead is followed 5-bp downstream by an additional aminoglycoside acetylthransferase gene, namely, the *aacA4*. The *aacA4* has a core site (ATTAGGC) with a 1-bp mismatch (Figure 1). The fourth gene cassette encodes the aminoglycoside adenyltransferase enzyme (*aadA1*).

Table 1. Antimicrobial susceptibility of *P. aeruginosa* isolates (n = 64) from the Hospital de Base do Distrito Federal, Brasilia, Brazil (SENTRY Program, 2001-2002).

Antimicrobial agent[a]	MIC$_{50}$ (µg/ml)	MIC$_{90}$ (µg/ml)	% susceptible	% resistant
Polymyxin B	≥1	2	96.9	3.1
Amikacin	4	>32	82.8	15.6
Piperacillin/tazobactam	8	>64	73.4	26.6
Ciprofloxacin	0.25	>4	68.8	26.6
Meropenem	0.5	>8	62.5	28.1
Ceftazidime	4	>16	62.5	31.3
Imipenem	1	>8	60.9	31.3
Aztreonam	8	>16	56.2	31.3

a. Sorted by % susceptibility.

Table 2. Antimicrobial susceptibility of *P. aeruginosa* strains isolated from the patient during hospitalization.

Date of isolation	Specimen	Susceptibility category[a] IMI	AZT	CAZ	P/T	GNT	AMK	CIP
9/30/01	Tracheal secretion[b]	S	S	R	R	R	S	S
9/30/01	Urine	I	S	R	R	R	S	S
10/22/01	Tracheal secretion[b]	R	S	R	R	R	S	S
10/31/01	Catheter tip	R	S	R	R	R	S	S
12/03/01	Urine	I	S	R	R	R	S	S
12/04/01	Catheter tip	R	R	R	R	R	S	S
05/07/02[c]	Tracheal secretion[b]	R	R	R	R	R	R	R

a. The isolates were tested by the Vitek® System at the Hospital de Base do Distrito Federal. Abbreviations: IMI: imipenem; AZT: aztreonam; CAZ: ceftazidime; P/T: piperacillin/tazobactam; GNT: gentamicin; AMK: amikacin; CIP: ciprofloxacin.
b. Specimen obtained by tracheal aspiration.
c. Strain 101-4704.

RESULTS

Figure 1. Schematic representation of the class 1 integron containing the bla$_{IMP-16}$ gene cassette from *P. aeruginosa* 101-4704. The horizontal arrows indicate the gene cassette and their respective translation orientation. The conserved core and inverse core sites are boxed, and the cassette boundaries are represented by vertical arrows. The possible stop codons are indicated by asterisks. The vertical yellow arrow indicates the *aacA* cassette boundaries with a core site presenting a 1-bp mismatch and the site of fusion between the *aacA* and *aacA4* genes.

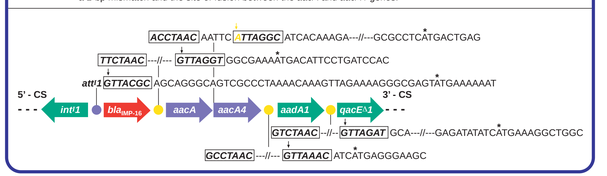


Figure 2. Phylogenetic tree displaying the relationship between members of the IMP family of MßLs. The tree is based on a CLUSTALW alignment of IMP MßLs generated using the PAM 250 matrix.

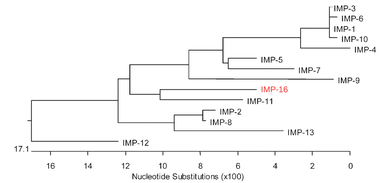


Figure 3. Comparison of the amino acid sequences of the IMP-16 and the previously described IMP-type MßLs. The active site implicated in the binding of Zn^{2+} ions are indicated by blue boxes.

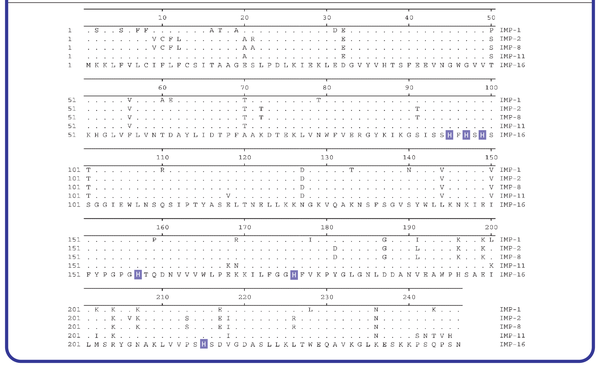
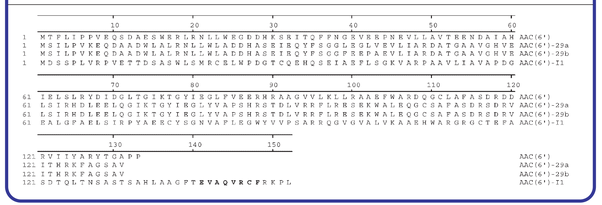


Figure 4. Comparison of the deduced amino acid sequence from the new aacA gene cassette with those closely related aminoglycoside acetyltransferase AAC(6')-29b, AAC(6')-29a, and AAC(6')-I1, showing 52.7%, 51.1% and 34.6% identity, respectively. The carboxy terminal end of the protein, which are conserved in most of the AAC(6')-type enzymes and absent in AAC(6')-29b, AAC(6')-29a and AAC(6') are represented in bold letters.



CONCLUSIONS

- After a very long stay in the ICU (151 days) to treat several episodes of nosocomially acquired pneumonia, the patient remained colonized with a multi-drug resistant (and MßL producing) strain of *P. aeruginosa*.
- bla$_{IMP-16}$ is the first IMP-type gene to be well characterized in Latin America. This new IMP variant is most similar to bla$_{IMP-11}$ (91.0%) and bla$_{IMP-8}$ (88.6%) with 22 and 28 amino acids differences, respectively.
- The bla$_{IMP-16}$ integron has a unique structure containing two aminoglycoside genes fused into a single gene cassette, one of which has not been described previously.
- Three MßL genes have already been described by the SENTRY Program in *Pseudomonas aeruginosa* clinical isolates from Brazil (bla$_{IMP-1}$, bla$_{IMP-16}$, and bla$_{SPM}$). The emergence and dissemination of MßLs among P. aeruginosa in Brazil may be responsible for the high rates of ß-lactam resistance among this pathogen in Brazilian hospitals.

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