

## First Isolation of Metallo- $\beta$ -Lactamase-Producing Multiresistant *Klebsiella pneumoniae* from a Patient in Brazil

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**A multiresistant *Klebsiella pneumoniae* isolate was taken from the blood of a 75-year-old patient with nosocomial pneumonia who developed septic shock and failed therapy with imipenem. The isolate presented an MIC of imipenem of 128  $\mu$ g/ml, and the production of a metallo- $\beta$ -lactamase was confirmed by phenotypic and genotypic techniques. We here report, for the first time, the detection of a metalloenzyme (IMP-1)-producing *K. pneumoniae* clinical strain in Latin America. The gene responsible for this phenotype was found to be *bla*<sub>IMP-1</sub>, carried in a class 1 integron.**

### CASE REPORT

In April 2003, a 75-year-old man with a previous history of diabetes and hypertension was admitted with a hemorrhagic cerebral vascular accident to the Hospital Universitário da Universidade de São Paulo, a secondary university hospital. He was put under mechanical ventilation and was transferred to a tertiary care hospital, the Hospital das Clínicas da Universidade de São Paulo, for specialized medical care. Four days after his admission, he developed pneumonia and was treated with cefepime (2 g/day). The antibiotic doses were adjusted based on the patient's creatinine clearance, which was estimated to be of 30 ml/min. Two days later, he was readmitted to the Hospital Universitário, where therapy was changed to ceftazidime at 2 g/day and clindamycin at 2.4 g/day. In our hospital ceftazidime and clindamycin are the antibiotics indicated for aspirative pneumonia. Cefepime was not available.

Taking into account that there was no clinical improvement and that a bronchoalveolar-lavage culture yielded *Acinetobacter baumannii* resistant to cephalosporins and ciprofloxacin, the treatment was changed to meropenem (2 g/day) (due to an adjustment for renal failure), which was taken for 14 days. Persistence of fever and a positive urine culture for *Candida tropicalis* led to the administration of fluconazole (400 mg/day). The patient then presented skin infection secondary to phlebitis and was treated with vancomycin at 500 mg/day for 7 days, once *Staphylococcus epidermidis* was isolated in two blood samples.

After a week without antibiotics fever and leucocytosis returned and treatment with vancomycin and imipenem (IMP) was empirically initiated, with respective doses of 500 mg/day and 250 mg times a day. *Stenotrophomonas maltophilia*, *Kleb-*

*siella pneumoniae*, and *Staphylococcus aureus* isolates were recovered from a tracheal aspirate culture and were managed as colonizers. The *S. maltophilia* strain was resistant to multiple antibiotics, whereas the *K. pneumoniae* strain was susceptible to carbapenems and cephalosporins. The *S. aureus* strain was also multiresistant.

Because the patient presented sepsis without a focus and had received broad-spectrum antibiotics a few days before, the multiresistant colonizers could be associated to sepsis and were treated empirically.

The patient showed clinical improvement, and the treatment was completed after 14 days. On the same day, fever returned, and 3 days later the patient presented a positive blood culture for a multidrug-resistant *K. pneumoniae* strain (strain KPBr1).

Because the only new sign of infection was fever, which was under investigation, the patient was not treated. Unfortunately, the patient deceased within a few hours after a positive blood culture result due to septic shock after being hospitalized for 53 days was reported.

Initially, the identification and the antimicrobial susceptibility profile of strain KPBr1 were evaluated using a Vitek system (BioMérieux, Hazelwood, Mo.). The MICs were subsequently determined using both an agar dilution method and an Etest according to NCCLS recommendations (11) and the manufacturer's instructions (AB Biodisk, Solna, Sweden). Hydrolysis of IMP was evaluated with bioassays (7) using either *S. aureus* ATCC 25923 or *Micrococcus luteus* ATCC 9341; these bioassays involved satellite growth of these strains around the *K. pneumoniae* strain growing on Mueller-Hinton agar plates containing 10<sup>8</sup> CFU of ATCC strains/ml and IMP at a concentration of 0.06 or 0.12  $\mu$ g/ml. The isolate was screened for metallo- $\beta$ -lactamase (MBL) production by a double disk-synergy test using ceftazidime and IMP as substrates and EDTA and thiol compounds (2-mercaptopropionic acid and 2-mercaptoacetic acid) as  $\beta$ -lactamase inhibitors (1). The MIC of IMP with and without EDTA was then measured by dilution in agar (11). Isoelectric focusing was performed using crude  $\beta$ -lactamase extracts in polyacrylamide gels containing ampholines

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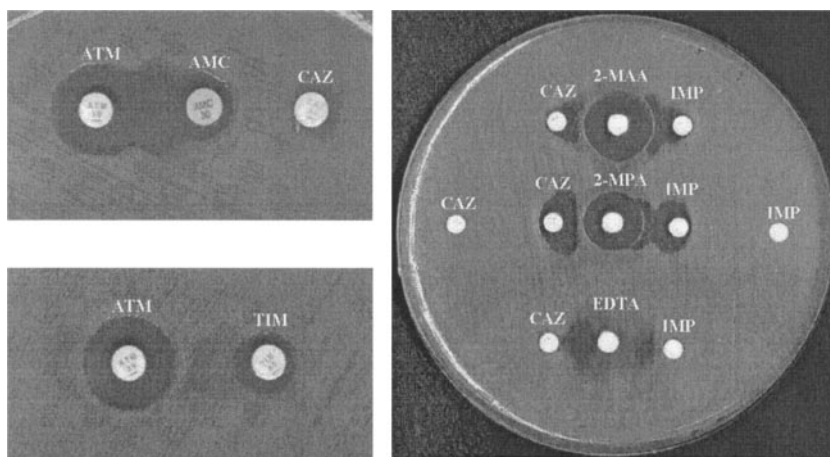


FIG. 1. Appearance of a zone of growth inhibition in CTX-M- and IMP-producing *K. pneumoniae* strain KpBr-1. An assay using AZT (ATM), amoxicillin-clavulanic acid (AMC), and ceftazidime (CAZ) (top left panel) and an assay using AZT (ATM) and ticarcillin-clavulanic acid (TIM) for ESBL screening (bottom left panel) for ESBL screening are shown. (Right panel) Results of an assay for MBL screening using CAZ, 2-mercaptoacetic acid (2-MAA), 2-mercaptopropionic acid (2-MPA), EDTA, and IMP. Marked growth inhibition of KpBr-1 was observed for both  $\beta$ -lactamases.

with a pH range of 3.5 to 9.5 as previously described (5), and DNA amplification by PCR was carried out using primers specific to the *bla*<sub>IMP-1</sub> gene and *bla*<sub>CTX-M</sub> genes (3, 13). Both strands of the amplification products of the *bla*<sub>IMP</sub> gene were sequenced by the standard Sanger dideoxynucleotide method (17).

The resistant organism was confirmed to be *K. pneumoniae* by standard biochemical tests. Imipenemase activity was inhibited by thiol compounds or EDTA but not by clavulanic acid or tazobactam (Fig. 1). The KpBr1 strain presented an MIC of IMP of 128  $\mu$ g/ml in the absence of EDTA and an MIC of IMP of 1  $\mu$ g/ml in the presence of EDTA (Table 1), thereby suggesting the production of an MBL. The pI of this enzyme was

estimated to be >9.5 (13). DNA amplification by PCR yielded a fragment of approximately 600 bp, and nucleotide sequencing showed that the *bla*<sub>IMP</sub> gene of strain KpBr1 was identical to the *bla*<sub>IMP-1</sub> gene first described for *Serratia marcescens* (13) (GenBank accession no. S71932).

Isoelectric focusing analysis showed that this strain (KpBr1) produced another  $\beta$ -lactamase with a pI of 7.9, which corresponds to a cefotaximase (most likely CTX-M-2), since this strain also possessed an extended-spectrum  $\beta$ -lactamase (ESBL) phenotype (Table 1) and genotype (DNA amplifications by PCR using primers specific to the *bla*<sub>CTX-M</sub> gene yielded a fragment of approximately 550 bp).

The KpBr1 isolate was tested for the presence of class 1 integrons by PCR using primers for the 5' and 3' conserved sequences of class 1 integrons. Moreover, the presence of *bla*<sub>IMP-1</sub> and *bla*<sub>CTX-M</sub> genes in these integrons was tested by PCR by using (i) a forward primer for the conserved sequence of class 1 integrons with a reverse primer for either *bla*<sub>IMP-1</sub> or *bla*<sub>CTX-M</sub> genes and (ii) a forward primer for either *bla*<sub>IMP-1</sub> or *bla*<sub>CTX-M</sub> genes with a reverse primer for the conserved sequence of class 1 integrons (GenBank accession no. AF133699).

As observed by electrophoresis, the KpBr1 strain contains plasmids. Despite repeated attempts, however, experimental transformation of plasmid DNA into *Escherichia coli* DH5 $\alpha$  was unsuccessful even by electroporation. Conjugation experiments between the clinical isolate KpBr1 and *E. coli* K12 did not yield any transconjugants. Although the *bla*<sub>IMP-1</sub> gene was found to be on a mobile element, a class 1 integron, in strain KpBr1, it seems that this gene is carried chromosomally.

Production of the IMP enzyme caused reduced susceptibility to almost all  $\beta$ -lactam antibiotics, including penicillins, cephalosporins, and carbapenems. The reduced susceptibility to aztreonam (AZT) observed was most likely due to CTX-M production. A ghost zone between the amoxicillin-clavulanic acid disk and the AZT disk was observed with a Kirby-Bauer disk diffusion test (Fig. 1). The ESBL phenotype was thus verified

TABLE 1. Antimicrobial susceptibility profile of the *K. pneumoniae* strain KpBr-1 carrying the *bla*<sub>IMP-1</sub> and *bla*<sub>CTX-M</sub> genes

Antimicrobial agent	MIC ( $\mu$ g/ml)		
	Vitek system	Etest	Agar dilution method
Piperacillin	>64		
Piperacillin-tazobactam	>64		
AZT	>16, ESBL <sup>c</sup>	12	
Cefoxitin	>16	>256	>512
Ceftriaxone	>32, ESBL	>256	512
Cefotaxime	>32, ESBL		512
Cefotaxime-clavulanic acid			512 <sup>a</sup>
Cefotaxime-EDTA			256 <sup>b</sup>
Ceftazidime	>16	>256	256
Ceftazidime-clavulanic acid		>32-4.0	256 <sup>a</sup>
Ceftazidime-EDTA			2 <sup>b</sup>
Cefpodoxime	>1		
Cefepime	>16	>256	
Ciprofloxacin	>2	>32	
IMP	>8	>32	128
IMP-clavulanic acid			128 <sup>a</sup>
IMP-EDTA			1 <sup>b</sup>

<sup>a</sup> Clavulanic acid was tested at a fixed concentration of 4  $\mu$ g/ml.

<sup>b</sup> EDTA was tested at a fixed concentration of 320  $\mu$ g/ml.

<sup>c</sup> ESBL, carrying an ESBL phenotype.

by clavulanic acid inhibition, in the presence of AZT, which is not a substrate for MBL enzymes. The resistance to AZT in KpBr1 was encoded by an additional CTX-M-type gene, as determined by PCR.

The production of these two enzymes by the strain contributed to treatment failure and death of the patient.

**Discussion.** As opportunistic pathogens, *Klebsiella* spp. primarily thrive in immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction (14). Nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, the medically most important species of the genus. The principal reservoirs for transmission of *Klebsiella* are the gastrointestinal tract and the hands of hospital personnel (14). Because of their ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks. The main problem concerning these infections is that ESBL-producing strains among clinical *Klebsiella* isolates appear to be common in Latin American countries. For example, in a worldwide study including isolates from Europe, the Americas, and the Western Pacific region (21) the SENTRY Antimicrobial Surveillance Program noted the highest prevalence (45.4%) of ESBL production by *K. pneumoniae* in South America. In Brazil, CTX-M is the most prevalent class of ESBL found and is widely disseminated (2, 3, 21). The intensive use of broad-spectrum cephalosporins such as cefotaxime could account for the emergence and spread of the CTX-M enzymes whose genes are carried in plasmids among nosocomial pathogens in Brazilian hospitals (3).

Carbapenems, such as IMP and meropenem, are used ever more frequently for the treatment of multiresistant gram-negative nosocomial pathogens, especially strains that produce ESBLs, once they are stable with respect to these enzymes (16). Because of the high prevalence of ESBL production in nosocomial strains, selective pressure inflicted by the frequent use of carbapenems has led to high levels of resistance to these drugs among strains of gram-negative bacilli (9, 12).

Resistance to carbapenems is still rare in *Enterobacteriaceae* strains, where it is generally due to an AmpC enzyme hyperproduction, coupled with alteration in outer membrane permeability (9, 12). On the other hand, it has been observed more frequently among nonfermentative gram-negative bacilli. In *Acinetobacter* strains, it is mostly due to class D serine  $\beta$ -lactamases in Europe and MBLs in Asia (12). Currently there have been a growing number of reports indicating an increase in the prevalence of carbapenemases (4, 8, 13, 19). Basically, two molecular classes of carbapenem-hydrolyzing enzymes, classes A (Bush group 2f) and B (Bush group 3), have been described. Class B enzymes or MBLs are clinically relevant, since they are able to degrade virtually all  $\beta$ -lactams except monobactams. In contrast to ESBLs, MBLs are not inhibited by  $\beta$ -lactamase inhibitors such as clavulanic acid and tazobactam; however, they are inhibited by EDTA and/or thiol compounds (1, 15). Three different types of mobile MBLs have been described in the literature: IMP, VIM, and SPM. IMP and VIM enzymes have been found in various gram-negative clinical isolates, mostly in the Far East and the Mediterranean region (12, 13, 19).

In Latin America, recent studies have characterized the appearance of metalloenzymes such as IMP and SPM (a novel

MBL) in Brazilian clinical isolates of *A. baumannii* and *Pseudomonas aeruginosa*, respectively (4, 5, 20). Moreover, VIM-type MBLs from *Pseudomonas* sp. isolates from Chile and Venezuela have been reported by the SENTRY Antimicrobial Surveillance Program (R. E. Mendes, M. Castanheira, P. Garcia, M. Guzman, M. A. Toleman, T. R. Walsh, and R. N. Jones, Letter, Antimicrob. Agents Chemother. **48**:1433–1434, 2004).

The detection of MBLs in *K. pneumoniae* has occurred sporadically. Among these MBLs, IMP-1 and IMP-8 have been described in studies of *K. pneumoniae*, the former in Japan (18) and Singapore (T. H. Koh, G. S. Babini, N. Woodford, L. H. Sng, L. M. C. Hall, and D. M. Livermore, Letter, Lancet **353**:2162, 1999) and the latter in Taiwan (23). VIM-1 and VIM-4 have recently been described in Greece and Italy (6, 10). Gram-negative bacilli producing both IMP-like and CTX-M enzymes have been recently reported (22). From this study, we report the presence of both enzymes in the KpBr1 strain, coded by *bla*<sub>IMP-1</sub> and *bla*<sub>CTX-M</sub>; the former is found to be in a class 1 integron.

To our knowledge, this is the first report of a clinical isolate of *K. pneumoniae* producing an MBL in Latin America. The occurrence of carbapenem-resistant *K. pneumoniae* in Brazil is alarming, since it jeopardizes treatment with carbapenems. This class of antimicrobial agents is the main therapeutic option for treating infections caused by both ESBL-producing strains and nonfermentative gram-negative bacilli, which are highly prevalent in most Brazilian hospitals. Microorganisms are notorious for their ability to spread among patients and to act as harborers of resistance plasmids.

CTX-M-encoding genes have primarily been found on plasmids; however, secondary chromosomal insertions of *bla*<sub>CTX-M</sub> genes have also been observed in clinical *E. coli* strain HK56 in Japan (2). These data suggest mobility of the *bla*<sub>CTX-M</sub> and *bla*<sub>IMP-1</sub> genes, which allows for their transference between plasmids and from plasmids to chromosomes (2). Since integrons are carried on plasmids and transposons, a strong antibiotic selective pressure can potentially result in the mobilization and dissemination of antibiotic resistance genes.

Certainly, the mobility of gene cassettes (*bla*<sub>CTX</sub> or *bla*<sub>IMP</sub>) combined with the flexibility of class 1 integrons suggests that resistance to cephalosporins and carbapenems will continue to increase and would confer an enormous potential for the dissemination of these genes into other nosocomial pathogens. Mobile  $\beta$ -lactamases will pose a serious threat to broad-spectrum beta-lactam therapies within Brazilian hospitals.

Nosocomial Infection Control Committees in Brazilian hospitals should thus be on the lookout for potential outbreaks involving this kind of strain.

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