Characterization by phenotypic and genotypic methods of metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis

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Abstract. *Pseudomonas aeruginosa* continues to be a predominant cause of infections with high intrinsic resistance to antibiotics, resulting in treatment failure. *P. aeruginosa* is the leading cause of respiratory infections among cystic fibrosis (CF) patients. Resistance to carbapenem antibiotics among *P. aeruginosa* has been reported. Thus, this study was undertaken to characterize the metallo-β-lactamase (MBL) production of *P. aeruginosa* by phenotypic and genotypic methods. A total of 572 sputum samples were collected from cystic fibrosis patients along with the patient demographic details in a questionnaire. In total, 217 *P. aeruginosa* isolates were collected and an antibiogram revealed that 159 (73.3%) and 141 (64.9%) of these colonies exhibited resistance to imipenem and meropenem, respectively. Ceftazidime and tobramycin resistance were both identified in 112 (51.6%) isolates, and resistance to piperacillin-tazobactam, gatifloxacin and netilmicin was detected in 96 (44.2%) respective samples. A total of 62 (28.6%) respective samples were resistant to cefoperazone, cefepime and ceftriaxone. The least antibiotic resistance was shown to amikacin and ceftizoxime with 51 (23.5%) and 32 (14.7%) respective colonies resistant to the antibiotics. The minimum inhibitory concentration (MIC) for imipenem revealed a reduction in the MIC values. MBL screening by the zone enhancement method using ceftazidime plus EDTA discs demonstrated that 63 (56.25%) of the colonies were positive for MBL. A total of 53 (84.1%) samples expressed *blaVIM* and 48 (76.1%) expressed *blaIMP* genes, as detected by duplex polymerase chain reaction. In conclusion, carbapenem resistance is of great clinical concern in cystic fibrosis patients with *P. aeruginosa* infection. Therefore, mandatory regular screening and monitoring the resistance in *P. aeruginosa* among CF patients is required.

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Introduction

The fundamental aspect of interactions among microbes and the host organism is the ability of the pathogen to entrench itself and establish a persistent infection. Cystic fibrosis (CF) is an autosomal recessive genetic disease (6); the opportunistic pathogen *Pseudomonas aeruginosa* may cause chronic lung disease in CF patients, depending on the genetic adaptation of the pathogen, and is a prevalent pathogen in CF patients with pulmonary infection (1-5). Worldwide, 80% of CF patients were found to be infected with *P. aeruginosa* (7,8). *Pseudomonas* colonization in the lungs of CF patients results in tissue destruction and reduced respiratory function (9). Identification of CF isolates is difficult due to the phenotypic diversity, including the formation of mucoid colonies, loss of pigment and synthesis of rough lipopolysaccharides (10). However, identification using genotypic methods may evade this problem of identifying the variable phenotypes.

A high level of resistance has been exhibited by *P. aeruginosa* to numerous antimicrobials. Active efflux pump systems are of great importance in *P. aeruginosa* resistance. The MexA-mexB-oprM operon significantly contributes to the increased resistance of opportunistic pathogens (11). The rapid increase in antimicrobial resistance among *Pseudomonas* spp has resulted in extensive investigations aimed at understanding the factors that promote the emergence of antimicrobial resistance in *Pseudomonas*. Since *Pseudomonas* is inherently resistant to a number of antibiotics, infection with this bacterium is a serious problem in the treatment of CF patients. Carbapenem antibiotics, including imipenem and meropenem, are used in the treatment of infections caused by *P. aeruginosa* (12); however, carbapenem resistance among *P. aeruginosa* strains has been reported in recent years (13). The production of β-lactamase and metallo-β-lactamase (MBL), and reduced penetration of the drugs are challenging factors in the therapeutic management of these infections (13,14).

The MBLs are classified into three subgroups: B1, B2 and B3, according to their molecular structure. GIM, VIM,

SPM and IMP are genes in integrons, which integrate into chromosomes or plasmids (15,16). The genes involved in MBL production are either plasmid- or chromosome-mediated, and are transferred horizontally. This horizontal transfer of genes poses a health threat through spreading of resistance among other Gram-negative bacteria (17). Knowledge of these enzymes conferring resistance is required to prevent the spread of the infection among clinical samples. Numerous nosocomial outbreaks of *P. aeruginosa* producing metallo-lactamases have been reported, with an urgent requirement to implement infection control programs (18-21). Thus, the present study was undertaken to detect the presence of MBL-producing *P. aeruginosa* isolates from the sputa of CF patients by phenotypic and genotypic methods.

Materials and methods

Bacterial strains. A total of 572 CF patients were included in the study from Henan Hospital of Traditional Chinese Medicine (Zhengzhou, China). Sputum samples from patients with CF were collected and processed according to standard methods (Clinical and Laboratory Standards Institute®; CLSI Guidelines, 2012). All demographic details, including age, gender and history of antibiotic usage were collected. *P. aeruginosa* was isolated and characterized using biochemical methods. The present study was approved by the ethical committee of Henan Hospital of Traditional Chinese Medicine. Written informed consent was obrained from the patient's families.

Antimicrobial susceptibility analysis. The following antibiotics (Oxoid Ltd., Basingstoke, UK) were used for the antimicrobial susceptibility analysis by Kirby Bauer's disc diffusion method according to CLSI guidelines (23): Amikacin (30 mg), gentamicin (10 mg), netilmicin (30 mg), tobramycin (10 mg), cefoperazone (75 mg), cefepime (30 mg), ceftazidime (30 mg), ceftriaxone (30 mg), ceftizoxime (30 mg), ciprofloxacin (5 mg), gatifloxacin (5 mg), imipenem (10 mg), meropenem (10 mg) and piperacillin-tazobactam (100/10 mg). *P. aeruginosa* ATCC 27853, obtained from the Microbology Laboratory at Henan Hospital of Traditional Chinese Medicine, served as a control.

Minimum inhibitory concentration (MIC). The MIC was determined on imipenem-resistant isolates using the agar dilution method, with serial dilution of the imipenem powder at a concentration range of 0.06-512 μg/ml. A volume of 1 ml of the appropriate dilution of imipenem was added to 19 ml Muller Hinton agar, cooled to 55˚C and subsequent to mixing thoroughly, and the mixture was poured onto Petri dishes. The culture grown overnight was collected and the turbidity was matched to McFarland's standard 0.5 (23). A sample of 2 μl culture was delivered onto a Petri dish, which was divided into quadrants, and the plate was incubated for 18-24 h at 37˚C. Following incubation, the highest dilutions exhibiting no visible growth were considered as the MIC of the particular strain (CLSI 2012 guidelines) (23).

Detection of MBLs. Phenotypic detection of MBLs was conducted by the zone enhancement method using ceftazidime discs (Oxoid Ltd) with EDTA (22). Muller Hinton agar plates were seeded with the test organism matched to 0.5 McFarland's standard, according to the CLSI 2012 guidelines (23). A 0.5 M EDTA solution was prepared with 186.1 g disodium EDTA dissolved in 1.0 ml distilled water at pH 8.0 using NaOH. Subsequent to sterilization by autoclaving, EDTA solution was added to 750-μg ceftazidime discs. The discs impregnated with EDTA were dried in the incubator and stored in airtight vials at –20˚C. The ceftazidime (30 μg) discs and ceftazidime-EDTA discs (750 μg) were placed upon the agar surface and incubated for 16-18 h at 35˚C. The zone of enhancement surrounding the ceftazidime EDTA disc was considered to be positive for MBL production.

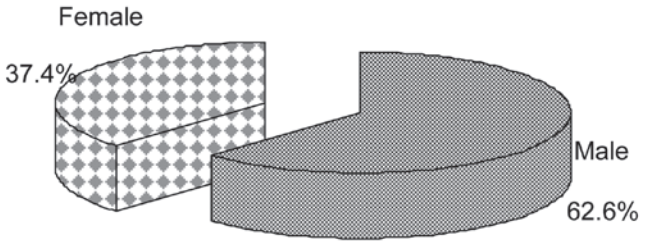

Figure 1. Gender distribution among the cystic fibrosis patients.

Genotypic characterization of the MBL gene. DNA was extracted from the *P. aeruginosa isolates* by the boiling method (24). Cultures of *P. aeruginosa* were grown overnight in Trypticase soy broth (Difco Laboratories, Inc., Detroit, MI, USA). A sample of 1.5 ml overnight culture was transferred to an Eppendorf tube and centrifuged at 17,310 x g in an Eppendorf cooling centrifuge for 5 min. Following centrifugation, the supernatant was decanted and the pellet was suspended in 500 μl MilliQ water (Millipore Corp., Billerica, MA, USA). The suspension was boiled at 95˚C for 10 min and cell debris was removed by centrifugation at 17,310 x g for 5 min. The supernatant served as a template for amplification. Duplex polymerase chain reaction (PCR) was performed to detect the presence of *blaIMP* and *blaVIM* β-lactamase in a Thermal Cycler 9600 instrument (Applied Biosystems, Norwalk, CT, USA). The reaction was prepared in a final volume of 50 μl, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM deoxyribonucleotide triphosphate, 0.75 mM deoxyuridine triphosphate (Roche Diagnostics, Quebec, Canada), 0.125 U uracil-DNA glycosylase and 2 U *Taq* polymerase (Roche Diagnostics). Concentrations of 0.16 mM of each primer were used in PCR .The primer sequences were as follows: IMP-A, forward 5'-GAAGGYGTTTATGTTCATAC-3' and IMP-B, reverse, 5'-GTAMGTTTCAAGAGTGATGC-3' with a product size of 587 bp (33) and VIM2004A, forward 5'-GTTTGGTCGCATATCGCAAC-3' and VIM2004B, reverse 5'-AATGCGCAGCACCAG GATAG-3' with a 382 bp amplicon size (Roche Diagnostics) A 2-μl sample served as a template. The following thermocycling conditions were used for amplification: Initial denaturation step at 94˚C for 5 min, for 30 cycles followed by denaturation at 94˚C for 1 min, annealing at 54˚C for 1 min and primer extension at 72˚C for 1.5 min. Subsequent to amplification, the amplicons were visualized on 1.5% agarose gel in TAE buffer [containing 0.04 M Tris-acetate and 0.002 M EDTA (pH 8.5)] to detect the presence of bands and the gels were scanned under ultraviolet illumination, visualized and digitized with a Bio-Rad Gel Doc imaging system (Bio-Rad,

Table I. Number of *Pseudomonas aeruginosa* imipenem-resistant isolates at different concentrations of MIC analyzed using the agar dilution method for imipenem.

MIC (μg)	512	256	128	64.0	3.02	16.0	8.00	4.00	2.00	1.00	0.500	0.250	0.125	0.0600
Imipenem-resistant isolates (n=159)	22	16	6	2	8	2	1	5	2	3	1	1	1	2

MIC, minimum inhibitory concentration.

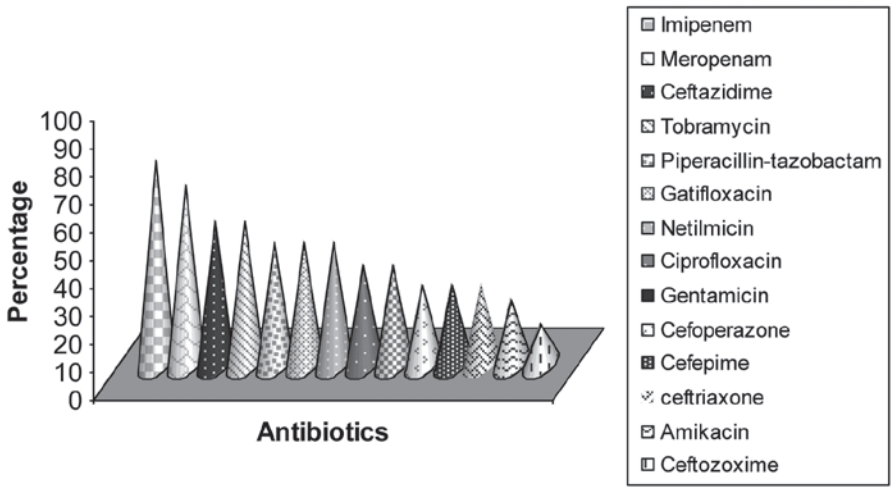

Figure 2. Proportion of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients resistant to various antibiotics. The present study reported Imipenen resistance as 73.3%. Certain strains were multidrug resistant.

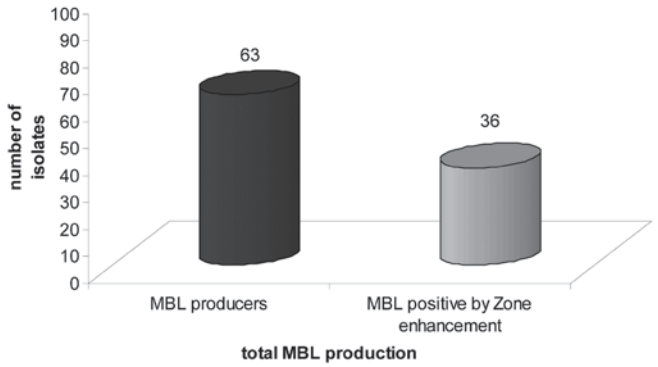

Figure 3. MBL positivity of *Pseudomonas aeruginosa* samples isolated from cystic fibrosis patients. MBL, metallo-β-lactamase.

Sydney, Australia). SPSS 11 software (SPSS, Inc., Chicage, IL, USA) was used for statistical analysis of data.

Results

Patient characteristics. Of the 572 CF patients, 358 (62.6%) were male and 214 (37.4%) were female (Fig. 1). *P. aeruginosa* infection among the CF patients was more prevalent in the 0-5 year-old group compared with the >15 year-old group. Among the 572 patients, 298 (52.1%) were hospitalized and were aged <12 years and found to be culture negative for *P. aeruginosa*. Out of 572 patients recruited, 217 (37.9%) were infected with *P. aeruginosa*. No significant correlation was observed between *Pseudomonas* infection and CF.

Resistance pattern. Of the 217 *P. aeruginosa* isolates, 159 (73.3%) were resistant to imipenem and 141 (64.9%) to meropenem. Ceftazidime and tobramycin resistance was detected in 112 (51.6%) respective samples, and 96 (44.2%) isolates were resistant to piperacillin-tazobactam, gatifloxacin and netilmicin, respectively. A total of 78 (35.9%) colonies were resistant to ciprofloxacin and gentamicin, and 62 (28.6%) were resistant to cefoperazone, cefepime and ceftriaxone. The least resistance was observed for amikacin, with 51 (23.5%) resistant isolates, followed by ceftizoxime with 32 (14.7%) resistant samples (Fig. 2).

MIC. The MIC for the antibiotics was determined by the agar dilution method according to the CLSI 2012 guidelines (23). Among the 159 imipenem-resistant isolates examined, 72 exhibited a four-fold reduction in MIC values (Table I).

Detection of MBLs. Of the 112 isolates resistant to ceftazidime, 63 (56.25%) were found to be positive for MBL production. A total of 36 (57.1%) showed enhancement of the zone surrounding the ceftazidime-EDTA discs (Fig. 3). One notable feature among the 63 MBL-producing *P. aeruginosa* samples was that all strains were found to be resistant to meropenem and ceftazidime. Out of the 63 MBL-producing isolates, 38 (60.3%) isolates were from male patients and

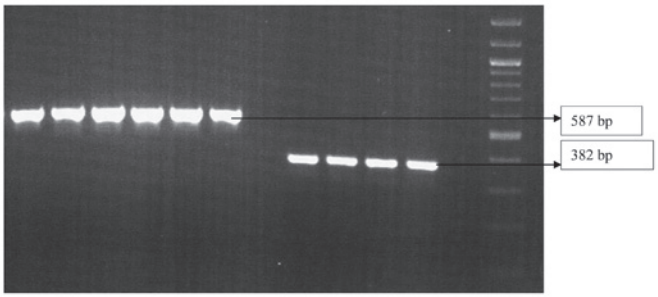

Figure 4. Gel image of DNA extracted from *Pseudomonas aeruginosa* isolates from cystic fibrosis patients, amplified using polymerase chain reaction. Lanes 1-6 reveal the presence of a *blaIMP* gene of amplicon size 587 bp. Lane 7 is a negative control, with no amplicon. Lanes 8-11 reveal the presence of a *blaVIM* gene of 382 bp length. Lane 12, 100 bp ladder.

25 (39.7%) were from female patients; no statistical significance (P<0.05) was identified between gender and MBL production. However, statistical significance (P<0.05) was detected in the association between ceftazidime resistance and MBL production in the isolates.

Genotypic detection of MBL genes. Primers were designed to detect the presence of the β-lactamase genes *blaVIM* and *blaIMP*. Of the 217 *P. aeruginosa* strains screened, only 63 isolates were found to produce MBL. These 63 strains were then analyzed by PCR. Out of 63 isolates, 53 (84.1%) exhibited the presence of *blaVIM* genes and 48 (76.1%) exhibited the presence of *blaIMP* genes (Fig. 4).

Discussion

Knowledge of the susceptibility of *P. aeruginosa* to antimicrobial agents is urgently required, since understanding of the pattern of antibiotic resistance may aid in treatment of this infection, particularly in CF patients. The prevalence of resistant strains among CF patients may be elucidated by testing these antibiotics on isolates collected from patients (25). Resistance to carbapenem is of clinical concern (26,27). *P. aeruginosa* is an opportunistic multidrug-resistant pathogen, which is an increasing problem worldwide (28,29). Kulczycki *et al* (2) revealed a 76.6% prevalence rate, which is high compared with that of the present study (37.9%). Various studies of *P. aeruginosa* infection worldwide have observed percentages of resistance to imipenem and meropenem of 4-70% (5). In the present study, 73.3% isolates were resistant to imipenem and 64.9% to meropenem. These are higher values than usual, which reveals that there is increasing resistance of *P. aeruginosa* towards antimicrobial drugs. MBL expression among *P. aeruginosa* samples was found to be 10-65% across the country from varying clinical samples (15). In the present study, 56.25% *P. aeruginosa* isolates produced MBL, which was a lower percentage than that previously identified in a study group of severe acute respiratory infection, defined by the World Health Organisation as an acute respiratory illness of recent onset (within 7 days) manifested by fever (≥38°C), cough and dypnea requiring overnight hospitalization) (30) and a study by Behara *et al* (31), which observed that 62.5% *P. aeruginosa* isolates produced MBL. The higher rate of MBL production among *P. aeruginosa* suggested that carbapenem resistance in *P. aeruginosa* is mediated by MBL production. Higher morbidity and mortality are associated with *P. aeruginosa* producing MBL (32). In the present study, 36 (57.1%) isolates showed enhancement of the zone surrounding the ceftazidime-EDTA disc, which is lower than the percentage identified by Hemlatha *et al* (33) (87.5%), who also observed a lower percentage of isolates producing MBL.

In the present study, the majority of CF patients recruited were male (62.6%). Among the 217 isolates of *P. aeruginosa* examined, the antibiogram analysis revealed high resistance to ceftazidime (51.6%), which was marginally less compared with that reported by Mayank *et al* (34), who had detected ceftazidime resistance in 63% of isolates. In other studies by Obritsch et al (35) and Arya *et al* (36), 55.4% ceftazidime resistance was observed, which is concordant with the present study. The present study also identified higher resistance to other antibiotics, including tobramycin (51.6%), piperacillin-tazobactam, gatifloxacin, netilmicin (44.2%), ciprofloxacin and gentamicin (35.9%). However, resistance to cefoperazone and cefepime was detected in 28.9% of samples, which is similar to other studies demonstrating reduced susceptibility to commonly used antibiotics (3,4,11,12).

The 159 isolates resistant to imipenem were examined for MIC by agar dilution. A significant four-fold reduction was observed in the MIC of 72 (45.3%) of these samples. In total, MIC ranges of 0.06- to 512-fold were observed, which is in concordance with previous studies by Migliavacca *et al* (37) in 2002, Hemlatha *et al* (33) in 2005, Aggarwal *et al* (38) in 2008 and Jakumar *et al* (39) in 2007.

It is important to confirm the presence of the *blaVIM* and *blaIMP* β-lactamase genes by PCR. In the present study, out of the 217 *P. aeruginosa* strains, the 63 isolates positive for MBL production were selected for PCR analysis. Of the 63 isolates, 53 (84.1%) exhibited the presence of *blaVIM* genes and 48 (76.1%) exhibited the presence of *blaIMP* genes, which corresponds with studies by Mayank et al (34) and Sader *et al* (40).

In conclusion, the present study emphasizes the requirement for clinical microbiology laboratories to analyze MBL production in carbapenem-resistant *P. aeruginosa* strains. As an increase in multi-drug resistance has been identified among Gram-negative bacteria, an uncontrolled increase in MBL production may result in therapeutic complications, which may in turn raise mortality and morbidity. Early and accurate detection of MBLs may control the spread of MDR pathogens in the future. The use of molecular techniques aids in MBL detection in regional laboratories, to provide the appropriate diagnosis and identification of outbreaks by MBL-producing MDR pathogens, particularly in cystic fibrosis patients. Thus, regular surveillance of MBL-producing *P. aeruginosa*, along with judicious use of antibiotics, may prevent the spread of drug resistance.

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