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PHENOTYPIC AND MOLECULAR DETECTION OF CARBAPENEMASE NEW DELHI METALLO BETA LACTAMASE-1 (NDM-1) GENE AMONG *PSEUDOMONAS AERUGINOSA* FROM VARIOUS CLINICAL ISOLATES

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Keywords:

NDM-1 Gene, Combined disc test, E-test, Metallo beta lactamases, Modified Hodge test, *Pseudomonas aeruginosa*

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ABSTRACT: *Pseudomonas aeruginosa* is an opportunistic pathogen which acquires resistance to antibiotics such as carbapenems and cefepime. Information regarding (NDM-1) gene producing *Pseudomonas aeruginosa* is available, but data regarding their degree of infection and percentage in hospital settings are scarce. Hence, this study was carried out to determine the occurrence of blaNDM-1 gene among clinical isolates of multidrug-resistant *Pseudomonas aeruginosa* in a tertiary care hospital in Chennai, Tamil Nadu, India. A total of 126 isolates of *P. aeruginosa* isolated from various clinical samples were evaluated for carbapenem resistance and MBL production. In the present study it was found that majority of isolates (39.7%) were resistant to imipenem followed by ertapenem (35.9%) and doripenem (24.3%). A total of 31/78 (39.7%) isolates of *P. aeruginosa* were resistant to one or both carbapenems (imipenem and ertapenem) used in the screening test. Out of 78 isolates, 27 (34.6%) and 25 (32.1%) isolates were MHT positive and MBL producers by Modified Hodge Test (MHT) and MBL E-Strip test respectively and Out of 25 MBL positive isolates, 7 (28%) isolates were positive, and 18 (72%) were negative for NDM-1 gene production. In this study prevalence of plasmid encoding NDM-1 gene was noted in Multidrug-resistant *Pseudomonas aeruginosa*. Screening of *Pseudomonas aeruginosa* along with routine antimicrobial susceptibility testing, Phenotypic and molecular screening should also be carried out regularly to reflect the proper number of metallo-beta-lactamase producers.

INTRODUCTION: *Pseudomonas aeruginosa* is a leading cause of hospital-acquired infections in patients. Carbapenems, especially imipenem, are used for the treatment of these infections.

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The prevalence of imipenem resistance *P. aeruginosa* has been increasing worldwide. Resistance to carbapenems is due to impermeability *via* the loss of the OprD porin, the up-regulation of an active efflux pump system of the cytoplasmic membrane, or the production of metallo-β-lactamases (MBLs).

These mechanisms can lead to treatment failure in carbapenem therapy of *P. aeruginosa* infections [1]. Resistance to antibiotics has increased over the years among *Pseudomonas aeruginosa* as nearly

most of the strains are now resistant to all commonly used antibiotics. These bacteria produce Metallo-β-lactamases (MBLs). Multidrug resistance among these organisms makes the treatment of infections caused by the very expensive and difficult to treat [2]. Metallo-β-lactamases (MBLs) are metalloenzymes of Ambler class B and are clavulanic acid-resistant enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra-acetic acid (EDTA), as well as other chelating agents of divalent cations [3]. Metallo-β-lactamases producing *P. aeruginosa* isolates were first reported in Japan in 1991 and since then have been detected in various countries [4]. Carbapenem groups of antibiotics play an important role in managing gram negative nosocomial infections because of their broad-spectrum activity and stability against hydrolysis by most of the β-lactamases, including extended-spectrum β-lactamases (ESBLs) [5].

The most potent antibacterial agents used for the treatment of infections caused by multidrug-resistant gram-negative bacilli are carbapenems, including imipenem (IPM or IP) and meropenem [6]. Carbapenems are now frequently used as a reversed drug in treating infections caused by multidrug-resistant gram-negative bacilli. The detection of carbapenemase is difficult. It can be detected by phenotypic as well as genotypic methods. Among phenotypic tests Modified Hodge Test (MHT) is a relatively easy and simple method to be performed in a laboratory [7].

Many multidrug-resistant bacteria produce New Delhi metallo β lactamase-1, a type of carbapenemase which inactivates all β-lactams except aztreonam [3]. Bacterial plasmids encoding the blaNDM-1 gene also encodes for other genes responsible for causing resistance to all amino-glycosides, macrolides, and sulphamethoxazole, thus making these isolates multidrug-resistant or resistant in some cases to all antibiotics [8]. NDM-1 gene producing organisms may spread the multiple drug resistance to non-resistant organism through horizontal gene transfer. The presence of such asymptomatic carriers bearing multidrug-resistant NDM-1 gene organisms in the hospital environment is an alarming situation [9]. Hence this study was carried out for Phenotypic and molecular detection of carbapenemase New Delhi Metallo beta Lactamase-1 (NDM-1) gene among *Pseudomonas aeruginosa* from various clinical isolates and to evaluate different phenotypic methods to guide clinicians in prescribing a proper antibiotic and controlling nosocomial infections.

MATERIALS AND METHODS:

Clinical Isolates: The samples were collected from a tertiary care hospital in Chennai, and the study was carried out for a period of one year from November 2017 to October 2018. A total of 126 isolates of *P. aeruginosa* isolated from various clinical samples were evaluated for carbapenem resistance, MBL production, and molecular detection of the NDM-1 gene. The isolates were identified by standard laboratory techniques. Subculturing was done on a regular basis in order to maintain the fresh cultures for the experiment. *P. aeruginosa* ATCC 27853 was included as a quality control strain [10]. The isolates were confirmed by Grams staining, biochemical tests, pigment production, and growth at 42 °C [10]. Antibiotic susceptibility testing was done on Mueller–Hinton agar by Kirby–Bauer disc-diffusion method, and the results were interpreted as per the CLSI [11]. Screening for MBL production was done by Kirby–Bauer disc diffusion method [12], Imipenem-EDTA Combined Disc Test (CDT), and Modified Hodge Test (MHT) [13, 14]. Confirmation of MBL production was done by the E-test (Himedia Mumbai, India), and molecular detection of the NDM-1 gene was carried out by PCR [12, 15].

Screening of Isolates for MBL Production: The *in-vitro* antibiotic cultural sensitivity of *P. aeruginosa* for MBL production was done by the disc diffusion method [11]. The following antibiotics were used in the screening test: imipenem (10 μg), doripenem (10 μg), and ertapenem (10 μg) (Himedia Mumbai, India). The size of the zone of inhibition was read after overnight incubation at 37 °C and interpreted according to recommended MBL screening criteria as specified in CLSI protocol, M100-S22 [12].

Confirmatory Methods:

Imipenem-EDTA Combined Disc Test (CDT): MBL production was confirmed by IMP-EDTA combined disc test [13]. Imipenem disc (10 μg) and Imipenem/EDTA disc (10 μg/750 μg) (Himedia

Mumbai, India) were placed on the dried plate inoculated with the *P. aeruginosa*. The disc was placed 10 mm apart from edge to edge. After 24 h of incubation at 37 °C, the inhibition zones of IMP and IMP/EDTA discs were compared. For MBL producing organisms, discs with IMP/EDTA increased inhibition zones by 8 to 15 mm, while the increase of such zones for MBL negative isolates were 1 to 5 mm according to CLSI guidelines, 2013 [12].

Modified Hodge Test: The Meropenem and Imipenem resistant strains were subjected to MHT for detection of Carbapenemases. An overnight culture suspension of *E. coli* ATCC 25922, which was adjusted to 1:10 dilution of the 0.5 McFarland standard, was inoculated on the surface of the Muller Hinton agar plate evenly. After the brief drying at room temperature, 10 µg Ertapenem disk was placed in the center of the plate. Imipenem resistant test strain from an overnight culture was streaked heavily from the edge of the disk to the edge of the plate. After 24 h the presence of a distorted or clover leaf-shaped inhibition zone was interpreted as MHT positive due to Carbapenemase production by the test strain [14].

MBL E – Test: MRP/MRP+EDTA E -test strips (Himedia Mumbai, India) consisted of Meropenem (MRP) (4-256 µg/ml) and MRP (1-64 µg/ml) plus a constant level of EDTA. An inoculum (0.5 Mc Farland standards) was prepared from 24 h old culture of the *P. aeruginosa* test strain, inoculated on Muller Hinton Agar plate with the help of sterile cotton swabs.

After brief drying, E test strips were applied on MHA plates and incubated for 18-24 h at 37 °C. The MIC endpoints were read where the inhibition ellipses intersected the strips. A ratio of MICs of the Meropenem (MRP) to MRP+EDTA of ≥ 8 was interpreted as MBL positive [12]. S. maltophilia ATCC 13636 and *P. aeruginosa* ATCC 27853 were used as positive and negative control strains, respectively, in the MBL E- test.

Molecular Detection of NDM-1 Gene: All 25 isolates, which were confirmed phenotypically to be positive for MBL production by MBL E-Test, were subjected to PCR using group-specific primers to detect the NDM-1 gene. The primers used in the study are shown in **Table 4**.

DNA was extracted by boiling lysis method. A single colony was inoculated into 1 ml Luria bertani broth and incubated at 37 °C for 24 h. It was subjected to centrifugation at 10,000 rpm, 25 °C for 10 min. The pellet obtained was suspended with 500 µl milli-Q water and boiled at 100 °C for 10 min. It was then stored overnight at -20 °C. The next day it was subjected to centrifugation at 10,000 rpm, 25 °C for 10 min.

The supernatant was collected and used as the template for the PCR. The cycling conditions were as follows: denaturation at 94 °C for 10 min, followed by 35 amplification cycles (94 °C for 30 sec, 58.5 °C for 40 sec, 72 °C for 50 sec) and a final extension cycle (72 °C for 5 min). The DNA band patterns were evaluated by electrophoresis with 1.5% agarose gel in 1X TBE buffer. A 100 bp ladder molecular weight marker was used to measure the molecular weights of tested amplified products. The presence of bands of molecular weight of 621bp suggests the presence of the NDM-1 gene on gel electrophoresis [15].

Results:

Clinical Isolates: The results of the clinical isolates are presented in **Table 1**. A total of 126 clinical isolates were collected from tertiary care hospitals in Chennai, out of which 78 Multidrug-resistant isolates were screened for Carbapenemase and MBL production.

Phenotypic Detection of MBL Producers and their Confirmation:

Screening of Isolates for MBL Production, Imipenem-EDTA Combined Disc Test (CDT), Modified Hodge Test, MBL E – Test: The results of *in-vitro* antibiotic cultural sensitivity to different carbapenems are presented in **Table 2**. Majority of isolates (39.7%) were resistant to imipenem followed by ertapenem (35.9%). However, lower proportions of isolates were recorded resistant to doripenem (24.3%) in **Table 2** / **Fig. 1**.

A total of 31/78 (39.7%) isolates of *P. aeruginosa* were resistant to one or both carbapenems (imipenem and ertapenem) used in the screening test. The Imipenem resistant isolates 31 (39.7%) exhibited a zone size enhancement of ≥ 7 mm in the combined disc test **Table 3/Fig. 1**. Majority (60.3%) isolates were non-MBL producers.

Out of 78 isolates, 27 (34.6%) and 25 (32.1%) isolates were MHT positive and MBL producers by Modified Hodge Test (MHT) and MBL E-Strip test respectively **Table 3**/**Fig. 2** & **3**. *S. maltophilia* ATCC 13636 and *P. aeruginosa* ATCC 27853 were used as positive and negative quality control strains.

Molecular Detection of NDM-1 Gene: The results of the molecular detection of the NDM-1 gene are presented in **Table 5** /**Fig. 4**. PCR for detection of NDM-1 was carried out for 25 isolates. Out of 25 isolates, 7 (28%) isolates were positive, and 18 (72%) were negative for NDM-1 gene production.

FIG. 1: ISOLATES SHOWING RESISTANCE TOWARDS CARBAPENEMS LEFT SIDE OF THE PLATE (IMIPENEM, ERTAPENEM, DORIPENEM). RIGHT SIDE OF THE PLATE (IMIPENEM -IMIPENEM +EDTA) IS POSITIVE FOR MBL BY PHENOTYPIC TEST (COMBINED DISC TEST)

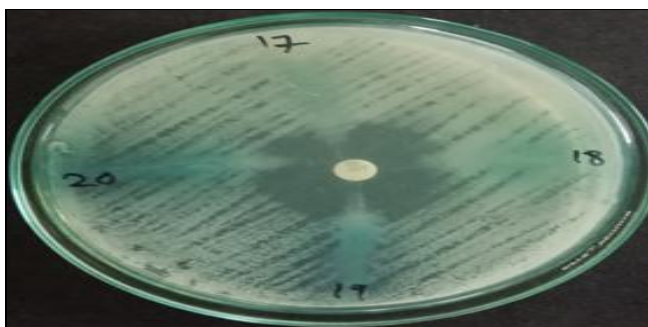

FIG. 2: THE PRESENCE OF A DISTORTED OR CLOVER LEAF SHAPED INHIBITION ZONE IS A MHT POSITIVE DUE TO CARBAPENEMASE PRODUCTION BY THE TEST STRAIN

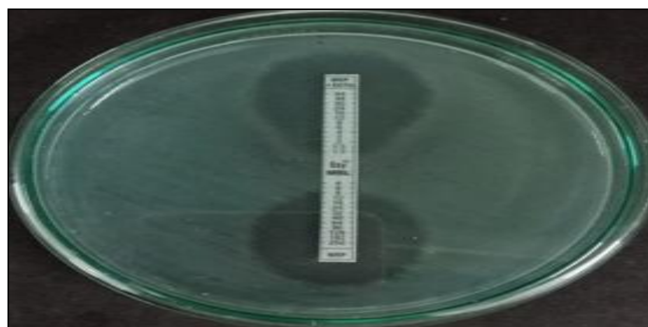

FIG. 3: A RATIO OF MICS OF THE MEROPENEM (MRP) TO MRP+EDTA OF ≥ 8 WAS INTERPRETED AS MBL POSITIVE

TABLE 1: ISOLATION OF *PSEUDOMONAS AERUGINOSA* FROM VARIOUS CLINICAL ISOLATES

S. no.	Clinical sample	Total number of isolates	Total percentage
1	Pus	43	34.1%
2	Wound Swab	21	16.6%
3	Cerebrospinal fluid (CSF)	2	1.6%
4	Bronchoalveolar Lavage (BAL)	4	3.1%
5	Endotracheal Aspirate (ETA)	7	5.6%
6	Sputum	15	12%
7	Blood	9	7.1%
8	Ear swab	13	10.3%
9	Urine	7	5.6%
10	High Vaginal Swab (HVS)	5	4%
	Total	126	100%

TABLE 2: *IN-VITRO* CULTURAL SENSITIVITY ASSAY USING CARBAPENEMS AGAINST *P. AERUGINOSA* ISOLATES

S. no.	Antibiotics	Sensitive	Resistance
1	Imipenem	47 (60.3%)	31 (39.7%)
2	Ertapenem	50 (64.1%)	28 (35.9%)
3	Doripenem	59 (75.7%)	19 (24.3%)

TABLE 3: PHENOTYPIC DETECTION OF METALLO-Β-LACTAMASE PRODUCING *P. AERUGINOSA* ISOLATES

S. no	No. of isolates Multidrug-resistant	No. of isolates resistant to Imipenem	Combined disc test	Modified Hodge Test (MHT) test	E test
1	78	31(39.7%)	31(39.7%)	27 (34.6%)	25 (32.1%)

TABLE 4: PRIMER SEQUENCE

S. no.	Primer	Primer sequence (5'-3')	Amplicon size (bp)
1	bla_{NDM-1}-F	GGTTTGGCGATCTGGTTTTC	621 bp
2	bla_{NDM-1}-R	GGTTTGGCGATCTGGTTTTC	621 bp

TABLE 5: MOLECULAR DETECTION OF NDM-1 GENE

S. no.	Total number of isolates tested for NDM-1 gene	Positive for NDM-1 gene	Negative for NDM-1 gene
1	25	7(28%)	18(72%)

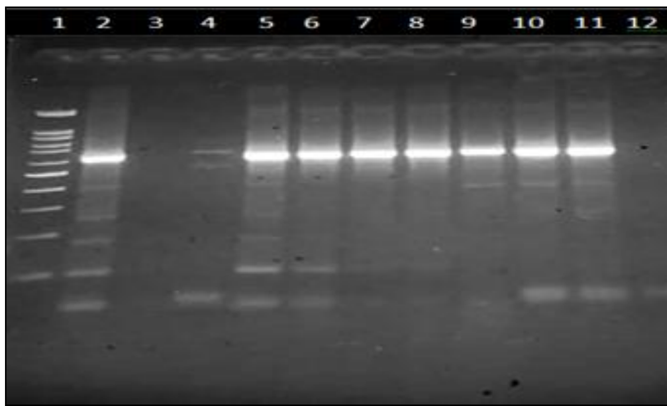

FIG. 4: AGAROSE GEL ELECTROPHORESIS (1.5%) REPRESENTS 621 BP BLANDM-1 GENE IN *PSEUDOMONAS AERUGINOSA*., LANE 1: 100BP DNA LADDER, LANE 2: POSITIVE CONTROL, LANE 3: NEGATIVE CONTROL, LANES 4 & 12: NEGATIVE ISOLATES FOR NDM-1 PRODUCTION (AMPLICON SIZE-621 BP); LANES 5-11: POSITIVE ISOLATES FOR NDM-1 PRODUCTION (AMPLICON SIZE-621 BP)

DISCUSSION: *Pseudomonas aeruginosa* is an opportunistic pathogen that causes serious infection in patients with weakened immune systems and it has become the important cause of nosocomial infections with a high mortality rate [16, 17].

It is predominantly responsible for emerging hospital-acquired infections and poses serious health concerns due to increased levels of multidrug resistance [18]. *P. aeruginosa* is resistant to many classes of antimicrobial agents and can acquire resistance by mutation and horizontal transfer of resistance determinants. The risk of mortality and morbidity in infections caused by *P. aeruginosa* is increased due to wrong or delayed initial antibiotic therapy, especially when the infection is caused by multidrug-resistant pathogens [19, 20].

In our study total of 126 *P. aeruginosa* isolates were isolated from the following clinical samples Pus 43(34.1%), Wound Swab 21(16.6%), Cerebrospinal fluid (CSF) 2 (1.6%), Bronchoalveolar Lavage (BAL) 4 (3.1%), Endotracheal Aspirate (ETA) 7(5.6%), Sputum 15 (12%), Blood 9 (7.1%), Ear swab 13(10.3%), Urine 7(5.6%), High Vaginal Swab (HVS) 5 (4%). The highest numbers of isolates were obtained from the pus sample and the lowest from CSF. In a similar study carried out previously, 110 different organisms were isolated from a total of 170 clinical samples such as pus, urine blood, sputum, and drain fluid, and they reported 15.78% of isolates were *P. aeruginosa* and 57 (22.4%) isolates were isolated from 254 pus samples in a study carried out in Bathinda [21, 22]. The occurrence of multidrug-resistant Metallo-β-lactamase *P. aeruginosa* isolates in a hospital setting is of concern as it poses a problem in therapy and infection control management. In our study, the prevalence of MBL-PA isolates was 39.7%.

Metallo-β-lactamase producing *Pseudomonas aeruginosa* is an important nosocomial pathogen that shows resistance to all β-lactam antibiotics except monobactam. In another study, they isolated 121 (96.1%) *Pseudomonas aeruginosa* out of 126 clinical isolates from the following samples, pus 35 (27.8%), urine 25 (19.84%), endotracheal aspirate 24 (19.04%), blood 14 (11.11%), and sputum 4 (3.17%) [23]. Similarly, 21% of MBL positive *P. aeruginosa* were isolated from urine, wound swabs, sputum, blood, tissue aspirates, ear swab, and CSF samples in a study carried out in Sudan [24]. While in a previous study in Jaipur, it was reported 20% [25]. 18.37% isolates of *P. aeruginosa* were positive for MBL production from Uttarakhand [26]. 41.3% of *Pseudomonas aeruginosa* isolates were screened positive for MBL production, which was reported from Bengaluru [27].

Similar to our study, the prevalence of MBL production was found to be 10% in India, 53.2% in Iran [28]. 12% in Canada, 12.7% in the United Arab Emirates, 13.4% in Russia, 14% in Spain, 38.3% in São Luis of Brazil, 47.3% in Taiwan, and 62% in Greece 29,30. 93.75% isolates were MBL positive in a study carried out in Karim Nagar, and 20.3% were positive for MBL production in a study carried out in 2019 in Thailand [31, 32]. In another study, 13.2% of isolates in Barabanki and 52% of isolates in Bahrain were found to be positive for MBL production [33, 34]. Similarly, [39] isolates were screened by the combined disc and disc diffusion methods, and 64.1% were found to be MBL producers in Egypt [35]. MBL was detected by a modified Hodge, and imipenem-EDTA double-disk synergy test and 14.4% isolates were positive for MBL production in Aligarh [36].

Resistance to carbapenem antibiotics in various Gram-negative bacteria suggests that they may be responsible for increased mortality. The emergence of resistance was associated with a significantly

increased number of adverse outcomes [37]. Carbapenems are the most effective antimicrobial agents against gram-positive and gram-negative bacteria, including *P. aeruginosa*. Carbapenems have the beta-lactam ring, and, like all other beta-lactams, they inhibit bacterial cell wall synthesis by binding to and inactivating Penicillin Binding Proteins (PBPs). This unique molecular structure offers them their exceptional stability to many beta-lactamases, including AmpC and most of the extended-spectrum beta-lactamases (ESBLs) [38]. *Pseudomonas aeruginosa* isolates acquire resistance to carbapenems *via* several mechanisms including overexpression of efflux systems, change or lack of outer membrane proteins (such as OprD porin), chromosomal AmpC beta-lactamase, and production of carbapenemases [39].

A simple screening method using a combined disc diffusion test has been very useful to screen MBL positive isolates. We screened MBL producers among imipenem resistant isolates by three methods, the Combined Disc Test (CDT), Modified Hodge test (MHT), and E - strip test for MBL screening and found that majority of isolates (39.7%) were resistant to imipenem followed by ertapenem (35.9%) and doripenem (24.3%). A total of 31/78 (39.7%) isolates of *P. aeruginosa* were resistant to one or both carbapenems (imipenem and ertapenem) used in the screening test. The Imipenem resistant isolates 31 (39.7%) exhibited a zone size enhancement of ≥ 7mm in the combined disc test.

The majority (60.3%) isolates were non-MBL producers. Out of 78 isolates, 27 (34.6%) and 25 (32.1%) isolates were MHT positive and MBL producers by Modified Hodge Test (MHT) and MBL E-Strip test, respectively. Based on another study carried out previously for the screening of MBL production, 66 isolates were screened positive. The prevalence of MBL producing isolates of *P. aeruginosa* was 15% (24/160) based on the E-test result. MHT showed the highest sensitivity (87.5%), followed by CDT (79.2%), while specificity was highest for DDST (100%), followed by PT (95.2%). Out of 24 MBL producers, 15 isolates (62.5%) were resistant to both imipenem (IPM) and meropenem [27]. It is lower in incidence as compared to our study. Similarly, 94.6% of carbapenemase production in *P. aeruginosa* isolates were reported in Delhi [23]. In a previous study, 28.17% of isolates in Iran and 16.08% of isolates in Barabanki were found to be carbapenem resistant *P. aeruginosa* [40, 33]. While in a previous study in Egypt, it was reported 26.5% 35. In a retrospective cohort study carried out in Pittsburgh Medical Centre, they reported a 19% mortality rate in patients with bacteremia is due to carbapenem-resistant (CR) *Pseudomonas aeruginosa* [41]. In this study, out of 25 isolates, 7 (28%) isolates were positive, and 18 (72%) were negative for the NDM-1 gene. Similarly, 46.06% isolates in Delhi, 3.91% isolates in Barabanki, 2.5% isolates in Bahrain, and 10.4% isolates in Singapore were found to be positive for the production of NDM-1 gene [23, 33, 34, 42]. In another study carried out in Iraq during 2018. They reported the emergence of *Pseudomonas aeruginosa* carrying NDM-1 gene variants, which exhibited resistance to imipenem and meropenem for the first time in Iraq [43].

CONCLUSION: The prevalence of multidrug-resistant *Pseudomonas aeruginosa* infections leads to a worldwide increase in the occurrence of MBL-PA, which is alarming. A high rate of the NDM-1 gene producer was noted among Multidrug-resistant *Pseudomonas aeruginosa*. Apart from performing the only antimicrobial sensitivity tests, phenotypic and molecular screening should also be employed regularly to find out the actual number of the NDM-1 resistance gene and for proper diagnosis and management of all *P. aeruginosa* infections.

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