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NDM-1 gene detection from Metallobeta lactamase (MBL) producing *Pseudomonas aeruginosa:* A pilot study from a teritiary care centre

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Abstract

Multidrug resistant bacteria always remain a great challenge. The latest threat being New Delhi Metallobetalactamase-1 (NDM-1) a superbug has brought notoriety to Indian Health care. NDM-1 refers to the transmissible genetic element encoding multiple resistant genes, first isolated from a strain of *Klebsiella* spp. in New Delhi, India, which has the ability to hydrolyse beta lactams and carbapenams. Detection of NDM-1 gene in multidrug resistant Pseudomonas isolates from various clinical samples. 200 Pseudomonas species were isolated in Microbiology laboratory during one year period were included in the study. Samples were processed as per Standard operating procedures. Antibiotic sensitivity testing was done by Kirby-Bauer disc diffusion method. The results were interpreted as per CLSI guidelines. MBL detection was done, by using EDTA Double Disc Synergy Test and Imipenem [I]-EDTA Combined Disc Test. MBL positive isolates were subjected to conventional PCR for genotyping & detection of NDM-1. A cross sectional descriptive study. Out of 12545 samples that were received in microbiology laboratory, 299 Non-Fermenting Gram Negative Bacilli [NFGNB] were isolated of which 200 were speciated as *Pseudomonas aeruginosa*. 20/200 [10%] were resistant to imipenem and 24/200 [12%] to meropenem. 10% of isolates showed MBL positive. NDM-1 gene was not detected in any of the 20 MBL positive isolates. NDM-1 gene since its origin has caused chaos in the health care facility with its ability to cause various infections. Detection is possible only with molecular methods. Thus gene detection plays a pivotal role in patient treatment and reduction of hospital stay. (Pseudomonas, multidrug resistant, New Delhi Metallobetalactamase-1, super bugs).

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Introduction

Pseudomonas aeruginosa a gram-negative bacterium is one of the leading causes of health care associated infections. Multi-drug-resistant *Pseudomonas aeruginosa* is a growing concern. Multi drug resistant bacteria are defined as isolates that show intermediate or resistance to at least three drugs in the following classes: beta-lactams, carbapenems, aminoglycosides, and fluoroquinolones.[1] Reported rates of multi drug resistant *Pseudomonas aeruginosa* varies from 0.6-32% based on geographic location and type of surveillance study.[2] *Pseudomonas aeruginosa* is intrinsically resistant to a wide range of antibiotics like ampicillin, cefuroxime and cefotaxim which is attributed to its production of ß-lactamases.[3] Indiscriminate use of antibiotics, heavy antibiotic pressure further accentuates the mutations in genes coding for ß-lactamase enzymes. This results in the fabrication of new ß-lactamases with wider ranges of activity. The emergence of New Delhi Metallobetalactamase-1 (NDM-1) *Pseudomonas aeruginosa*, a superbug, is a potential threat to human health. Among clinically significant carbapenamases, NDM-1 is the biggest menace. It significantly hydrolyses beta-lactams and carbapenems. NDM-1 producing strains exhibit multidrug resistant profile because they also harbor genes that encode for resistance to aminoglycosides and fluoroquinolones.[4][5]

This mixed bag of rapidly emerging antimicrobial resistant organisms and increasing rates of healthcare infections has always drawn the attention of clinical microbiologists and thus put us under an obligation to detect these resistance mechanism at the earliest. Emerging 'Superbugs or Multi-Drug Resistant (MDR)' pathogens have always been an enduring hitch in the health care settings and also challenges the effectiveness and usefulness of even most potent antibiotics. [6]Knowledge of NDM and its prevalence is essential because *P. aeruginosa* with intrinsic colonization capacity has the ability to persist in the hospital environment for indefinite periods but there is paucity of such reports. Hence the present study was undertaken as a pilot project to detect the NDM-1 gene in multi drug resistant Pseudomonas species, because determination of resistance mechanism helps to formulate efficient antibiotic policy and infection control protocols for holistic health care.

Objectives

1) Identification of pseudomonas species from various clinical isolates
2) Detection of their antimicrobial resistance
3) Detection of MBL production by screening tests
4) Detection of NDM-1 gene in the MBL positive isolates

Materials and methods

Inclusion criteria

Various samples (Pus, Sputum, Urine, ET secretion, Blood, PICC line, Pleural fluid, Ascitic fluid) received to the microbiology laboratory of during one year period were included in the study. Source ranged from cellulitis, diabetic foot, burns, septicemia, RTI, UTI, ear infection, post-operative and post-traumatic infection.

Exclusion criteria

1. Clinical specimens with mixed growth of more than 3 types [probably contaminated samples]
2. Non-NFGNB isolates

Procedure

The samples were immediately processed by standard techniques.[7] Organisms that failed to acidify the butt of Triple Sugar Iron were considered as Non-fermenters and were subjected to a battery of tests(motility, oxidase, indole, citrate, urease, dehydroxylation of arginine, lysine,decarboxylation of ornithine) [8] Among them, *Pseudomonas aeruginosa* was identified and processed further. Antibiotic sensitivity tests were done by Kirby-Bauer disc diffusion method. The following antibiotics were used for susceptibility testing: ceftazidime,

cefepime, ciprofloxacin, ofloxacin, gentamicin, amikacin, tobramycin, piperacillin, piperacillin +tazobactam, meropenem, imipenem, aztreonam, netilmycin, tigecycline, and co-trimoxazole. All the discs were procured from [Hi-media laboratories limited].The diameter of the zone of inhibition was measured and interpreted according to the CLSI guidelines. Imipenem resistant isolates were further subjected to MBL detection. The results were interpreted as per CLSI 2018 guidelines.[9]

EDTA Double Disc Synergy Test [DDST] [10]

Two to three identical colonies from the growth were inoculated into saline and incubated at 37°C for 4 to 6 hours. The optical density of the growth was matched with that of 0.5 McFarland turbidity standards. With a sterile cotton swab, this suspension was plated on to Mueller-Hinton Agar [MHA] by performing lawn culture. Imipenem [10µg] disc and blank disc containing 10µL of 0.5 M EDTA [750µg] were placed 20 mm apart. After incubation at 35°C in ambient air, enhancement of the zone of inhibition in the area between imipenem and the EDTA disc in comparison with the zone of inhibition on the far side of the drug [imipenem] was interpreted as a positive result.

Imipenem [I]-EDTA Combined Disc Test [CDT][11]

Two to three identical colonies from the growth were inoculated into saline and incubated at 37°C for 4 to 6 hours and was matched to 0.5 McFarland turbidity standards. With a sterile cotton swab, this suspension was swabbed on to Mueller Hinton agar [MHA] plates as recommended by the Clinical and Laboratory Standard Institute [CLSI]. Two 10µg imipenem discs are placed on the plate, and 10µL of EDTA solution is poured on one of the disc to obtain the desired concentration [750µg]. The inhibition zones of the imipenem and Imipenem-EDTA discs are compared after 16 to 18 hours of incubation in ambient air at 35°C. In the combined disc test, if the increase in inhibition zone with the Imipenem-EDTA disc is ≥ 7 mm than the imipenem disc alone, it was considered as MBL positive.

NDM-1 gene detection

The MBL positive isolates were subjected to conventional PCR for genotyping and detection of NDM-1 with the following gene primers. (Oligonucleotide Synthesis Order Form, DNA purification kit from Eurofins Genomics India Pvt. Ltd).

BlaNDM-1-F- 5'GGG CAG TCG CTT CCAACG GT 3`– 475bp

BlaNDM-1-R- GTA GTG CTCAGT GTC GGC AT.[12]

PCR products of the isolates that carried the bla gene were purified using the PCR DNA purification kit from Eurofins Genomics India Pvt. Ltd. The purified products were subjected to polymerase chain reaction, thermal cycler (Biorad centex 100 T100™).Later agarose gel electrophoresis was done to identify the NDM-1 gene. The run was done in the presence of known positive control.[13]

Statistical analysis

A cross sectional descriptive study.

Results

12454 clinical samples were received by Microbiology laboratory of Rajarajeswari medical college and hospital, Bengaluru were included in the study. The sample collected were from local infections such as cellulitis, diabetic foot, burns, septicaemia, RTI, UTI, ear infection, post-operative and post-traumatic infection, satisfying the inclusion & exclusion criteria. The percentage distribution of various samples is as shown in table 1.Out of 12545 samples, 299 samples yielded Non-Fermenting Gram Negative Bacilli [NFGNB] of which 200 were speciated as *Pseudomonas aeruginosa*. The distribution of Pseudomonas among various clinical isolates is as shown in Table 2. The cultural characteristic of Pseudomonas on blood agar and nutrient agar is as shown in Fig.-1.

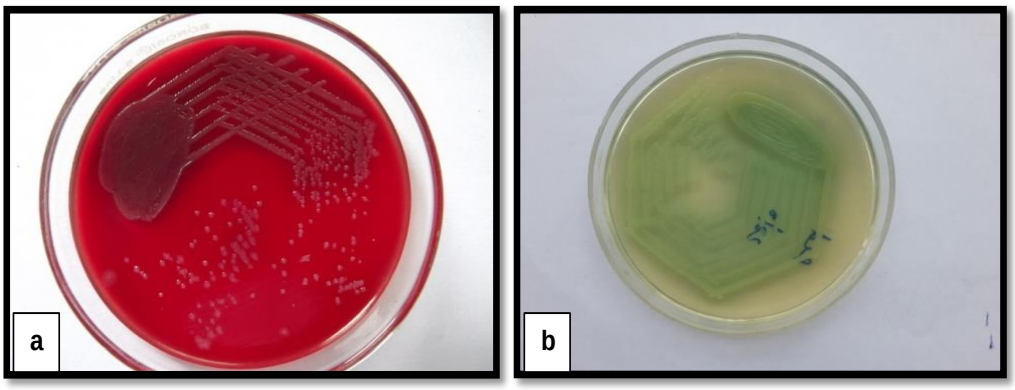
Table 1. Distribution of NFGNB from Various Clinical Samples.

SN	Sample	No of Isolates	Percentage [%]
1	Sputum	110	36.78
2	Pus	73	24.41
3	Urine	42	14.04
4	Et-secretion	40	13.38
5	Blood	28	09.37
6	PICC line	04	01.34
7	Pleural fluid	01	00.34
8	Ascitic fluid	01	00.34
9	Total	299	100.00

Among 200 clinical isolates of *Pseudomonas*, 185 [92.5%] were sensitive to aztreonam, 154 [77%] to cefepime & 151 [75.5%] to ceftazidime, 183 [91.5%] to amikacin, 173 [86.5%] to tobramycin, 158 [79%] to gentamicin, 164 [82%] were sensitive to ofloxacin, 160 [80%] to ciprofloxacin. 170 [85%] to piperacillin+ tazobactam, 136 [68%] to piperacillin. *P. aeruginosa* showed 180 [90%] sensitive to imipenem, 176 [88%] to meropenem. 20[10%] were positive for MBL screening by CDT and DDST test. Antibiotic susceptibility test on muller hinton agar is as shown in fig. 2. Fig. 3 shows the MBL detection by screening test. MBL positive isolates by screening method were subjected to polymerase chain reaction for the detection of NDM-1 gene. NDM-1 gene was not detected in any of the 20 MBL positive isolates. NDM-1 gene detection is as shown in fig. 4.

Table 2. Distribution of *Pseudomonas aeruginosa* from various clinical samples.

Species	Sputum	Urine	Blood	Pus	ET secretion	PICC line	Ascitic fluid	Pleural fluid	Total
Pseudomonas aeruginosa	85	22	04	61	26	01	--	01	200



a) Blood Agar: large irregular, beta-hemolytic colonies with iridescence

b) Nutrient Agar: large, irregular colonies with greenisg diffusible pigment

Fig. 1. Growth of *Pseudomonas aeruginosa* on culture media.

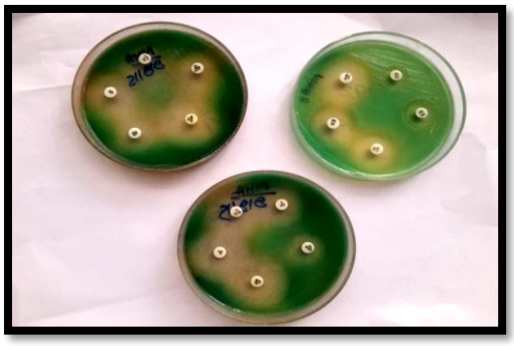


Fig. 2. Antibiotic sensitivity plate: *Pseudomonas aeruginosa.*

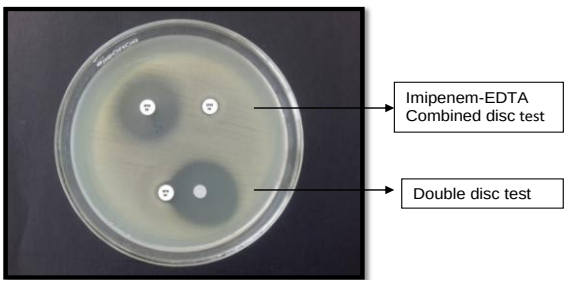


Fig. 3. MBL screening test.

L- DNA ladder
NC- Negative control
S- Sample
PC-Positive control

1% Agarose gel of *Pseudomonas aeruginosa* genomic DNA

Fig. 4. NDM-1 gene detection.

Discussion

Pseudomonas aeruginosa has the ability to survive under a wide range of environmental conditions. Their propensity to spread in hospital settings make them unique. Infections caused by *Pseudomonas aeruginosa* includes secondary infections of burns, infections in patients with cystic fibrosis, acute leukemia, organ transplants and intravenous drug abuse.[14] Infections are also seen at sites where moisture tends to accumulate like tracheostomies, indwelling catheters, external ear infections etc.[15] It also causes urinary tract infections, lower respiratory tract infections like pneumonia [especially in immunocompromised patients and patients on ventilators]. Other infections which are rarely caused by *P.aeruginosa* includes endocarditis, meningitis, brain abscess, infections of bones and joints.[16][17]. Metallo beta lactamases (MBLs) producing *P. aeruginosa* was first reported from Japan in 1991[18] later the spread has been reported from various parts of the world, including Asia[19]. Metallo-lactamases belong to Ambler class B and have the ability to hydrolyze a wide variety of β-lactam agents, such as penicillin, cephalosporins, and carbapenems [20] NDM-1, first isolated from a strain of *Klebsiella spp*. in New Delhi, India.[21] It is considered as a major threat as it effectively hydrolyses beta-lactams and carbapenems which are considered last resort antibiotics. It has a transmissible genetic element that encodes multiple resistant genes. [22] Imperilment lies not only in the emergence of NDM-1, but also due to its variants. There have been recent reports of 17 different variants of the NDM-1 gene carried on composite transposon Tn125 in *Acinetobacter baumanii* [23]. Reporting and identification of NDM -1 gene is possible only with molecular methods. Thus gene detection plays a pivotal role in patient treatment and reduction of hospital stay. Metallobeta-lactamase producing *P. aeruginosa* isolates are known for nosocomial outbreaks in tertiary centers. This also illustrates the need for proper infection control practices.

In our study, we identified non fermenters from various clinical samples, isolated *Pseudomonas aeruginosa*, and identified antimicrobial resistance. Out of 200 pseudomonas isolates 20 were MBL positive by screening methods. However, the confirmation of the same was done by the detection of NDM-1 gene by conventional PCR technique. Out of 20 isolates, NDM-1 gene was not present in any of the isolates. Though there is paucity of such studies in southern India, a similar study done by Mariappan Shanthi *et al* at Department of Microbiology, & Sri Ramachandra Medical College & Research Institute, Sri Ramachandra University, Chennai, India showed that of the 61 isolates, four isolates were positive for NDM-1 gene. These isolates are from the intensive care units and chest medicine ward. [5] In our study, the possible operative mechanisms in the isolates that showed resistance by screening tests may be due to hyper production of Amp C or other beta lactamases, and porin defect. Though absence of NDM-1 gene in any of our isolates is a solace, it remains as an enduring hitch. Hence the need for vigilant antimicrobial surveillance and good infection control practices.

The World Health Organization (WHO) is concerned that NDM-1 could spell "the doomsday scenario of a world without antibiotics." [24] The Centers for Disease Control and Prevention (CDC) have classified NDM-1 as an emerging issue in

the field of infectious diseases.[25] At the moment, the only way out is to combat the spread of bacteria expressing NDM-1 is by surveillance, quick identification and isolation of patients with the bacteria, disinfection of hospital equipment and strict following of hand-hygiene procedures in hospitals. A coordinated effort should be made in educating the hospital staff regarding the problem of drug resistance, the prudent use of antimicrobials by physicians, timely reporting and implementation of the appropriate infection control measures also help in curbing the spread of infection right at its source.

Conclusion

Strict vigilance and continuous surveillance and good infection control practices of NDM-1 is essential considering the difficulties in therapeutic management and control. Thus NDM-1 detection should be a routine process to prevent organisms from turning into a superbug. Implementation of antibiotic policy and integrated approach is the need of the hour to dynamite these bad bugs.

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