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DETECTION OF NEW DELHI METALLO BETA LACTAMASE-1 (NDM-1) CARBAPENEMASE IN *Pseudomonas aeruginosa*IN A TERTIARY CARE CENTRE IN NORTH INDIA

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ABSTRACT

New Delhi metallo-beta-lactamase 1 (NDM-1) is a newly-described metallo-beta-lactamase (MBL).NDM-1 hydrolyses all beta-lactam antibiotics except for aztreonam, which is usually inactivated by co-produced extended-spectrum or AmpC beta-lactamases. Sixty twocarbapenem resistant clinical isolates of a total of 200*P. aeruginosa*isolates culturedduring the study period were screened for the presence of NDM-1by PCR.Results: Of the 62 isolates, NDM-1 was detected in two isolates only. These were isolated from patients inthe intensive care units and chest medicine ward. The source specimens were pus and endotracheal tube. TheNDM-1 producers were susceptible only to polymyxin B and colistin.Our study showed the presence of NDM-1 in clinical isolates. These isolates harbour plasmid mediated multiple drug resistant determinants and can disseminate easily across several unrelated genera. To halt their spread, early identification of these isolates is important.

KEYWORDS-bla NDM, Pseudomonas aeruginosa, MBL: Metallo beta lactamase, E-Test: Epsilon Test

The New Delhi metallo-beta-lactamase (NDM-1) is a novel type of MBL named after the city of origin, which hasbeen recently criticized, following a usual practice with transferable MBLs since VIM-1 was named after Verona, Italy [Lauretti L etal., 1989]. NDM-1 was first reported in 2009 in a Swedish patient of Indian origin, who travelled to New Delhi and acquired a urinary tract infection due to a carbapenem-resistant K. pneumoniae strain resistant to all antibiotics tested except colistin[Lauretti L etal., 1989].

New Delhi metallo beta lactamase-1 (NDM-1) belongs to the metallo-b-lactamase (MBL; class B) family, which contains Zn2+ and other divalent cations as cofactors. The strain inactivates approximately all classes of β-lactam antibiotics, including carbapenems, by catalyzing the hydrolytic cleavage of the substrate amide bond[2],that confers resistance to all β-lactam antibiotics with the exception of aztreonam[Yong D etal., 2009]. However, many strains that port*bla*NDM-1 are also aztreonam resistant, presumably by a different resistance mechanism. The *bla*NDM-1 gene is located on plasmids harbouring multiple resistant determinants, Moreover, most NDM-1-positive bacteria are resistant to a wide variety of other antimicrobial classes and carry several additional resistance mechanisms for example to aminoglycosides, fluoroquinolones, macrolides and sulfonamides, leaving few or no therapeutic options. NDM-1 has been identified mostly in *Escherichia coli*, *Klebsiellapneumoniae*and to a lesser extent in *Pseudomonas* and *Acinetobacter*.[Castanheiraetal., 2011]

Till date, reports of NDM-1 in *Pseudomonas aeruginosa*are scarce, although reports are limited and periodic, knowledge of its prevalence is essential because *P. aeruginosa*is an environmental pathogen with intense colonization capacity and ability to persist for indefinite periods in the hospital environment[Lister PD etal., 2009]. The aim of this study was to determine the occurrence of NDM-1 in clinical isolates of carbapenem resistant Pseudomonas aeruginosa in a tertiary care hospital setting in north India.

MATERIALS AND METHODS

The study was conducted in the Department ofMicrobiology, Rama Medical College andResearch Institute, Kanpur. During the period, a total of 200 clinical isolatesof *P. aeruginosa*were collected, of which 62 werecarbapenem resistant. These were isolated from clinical specimens such as pus (22), urine (18), blood (1), sputum(3), endotracheal tube(12), folley's catheter tip (2), ascitic fluid (1) and throat swab (3). The organisms were identified by their colony characteristics, staining procedures, pigment production, motility and other relevant biochemical

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reactions as per standard laboratory methods for identification of bacteria.

The study protocol was approved by the institutionalethics committee.

Antimicrobial Susceptibility Testing

Susceptibility to various classes of antimicrobial agents was determined by disc diffusion method in accordance with Clinical Laboratory Standard Institute (CLSI) guidelines,2016. [9]The antibiotics tested were amikacin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), aztreonam (30 µg), piperacillin-tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), colistin (10 µg) and polymyxin B (300 units) (Hi-media Laboratories, Mumbai, India).Isolates resistant to imipenem and meropenem were considered as screening positive.

PHENOTYPIC CONFIRMATORY TEST

Modified Hodge Test

The modified Hodge Test (MHT) detects carbepenemase production in gram negative isolates. An overnight culture suspension of Escherichia coli ATCC 25922 adjusted to 0.5 McFarland was inoculated using a sterile cotton swab on the surface of Muller-Hinton agar(MHA). After drying, 10 µgimipenemdisk(Hi-Media, Mumbai, India) was kept at the centre of the MHA plate and the test strains suspespension was inoculated by streaking method from the edge of the imipenem disc to the periphery of the petriplate in four different directions. The plates were incubated overnight at optimum temperature. Carbenemase producing strains produced "cloverleaf shaped" zone of inhibition. The test organism was considered as Metallo-beta lactamase (MBL) positive.[Amjad A etal., 2011]

MBL E-test

The E-test MBL Strip contains a double sided seven-dilution range of IP(Imipenem) (4 to 256 µg/ml) and Imipenem (1 to 64µg/ml) in combination with a fixed concentration of EDTA is considered as the most sensitive method for MBL detection]. The E-test was done according to manufacturer's instructions. MIC ratio of IP/ IPI(Imipenem+EDTA) of >8 or >3 log dilutions indicates MBL production. [Walsh T etal., 2002]

VITEK automated identification and susceptibility testing (bioMe´rieux)

Vitek 2 (bioMérieux, Inc., Durham, NC, USA) testing was performed using software version 5.04 and the ID-GN and AST-281 cards, according to the manufacturer's instructions. Four morphologically similar colonies from an overnight agar plate culture were suspended in 3 mL of 0.45% saline and adjusted to a turbidity equivalent to that of a 0.5 McFarland standard using VITEK 2 DensiChek densitometer. 145 µl of suspension was added into 3 ml of normal saline. The antibiotic susceptibility cards were placed into dilution. The dilution was loaded into VITEK (bioMe´rieux, Inc., Durham, NC) and the test was performed. The test results were rapid and time taken was 8 – 10 hours for identification and sensitivity of the test strain.
The results are reported relative to a BSAC breakpoint for Meropenem and imipenem if the MIC is ≥16 mg/L. The test strain is resistant to Imipenem and Meropenem.

Polymerase Chain Reaction (PCR)

All study isolateswere subjected to PCR using primers targeting*bla*NDM-1.[13]The primers (Chromous biotech DNA, India) used in the study were NDM-Fm (5'-GGTTTGGCGATCTGGTTTTC-3') and NDM-Rm (5'- CGGAATGGCTCATCACGATC-3') which amplified an internal fragment of 264 bp of blaNDM-1 gene. Co-existence of other MBL encodinggenes namely *bla*VIM and *bla*IMP was observed by usingprimer.[12] For optimization of PCR, strainspreviously confirmed by PCR were used as positive control and *P. aeruginosa*ATCC27853 was used as negative control. All isolates weresubjected to PCR for the detection of MBL genes: VIM, IMP and NDM.

Nucleotide Sequencing

PCR products of two isolates that carried the *bla*NDM-1 gene were extracted using the PCR DNA purification kit (Chromous biotech DNA, India) and sequenced. The aligned sequences were analyzed with the Bioedit sequence program and similarity searches for the nucleotide sequences

wereperformed with the BLAST program. (*http://www.ncbi.nlm.nih.gov*).

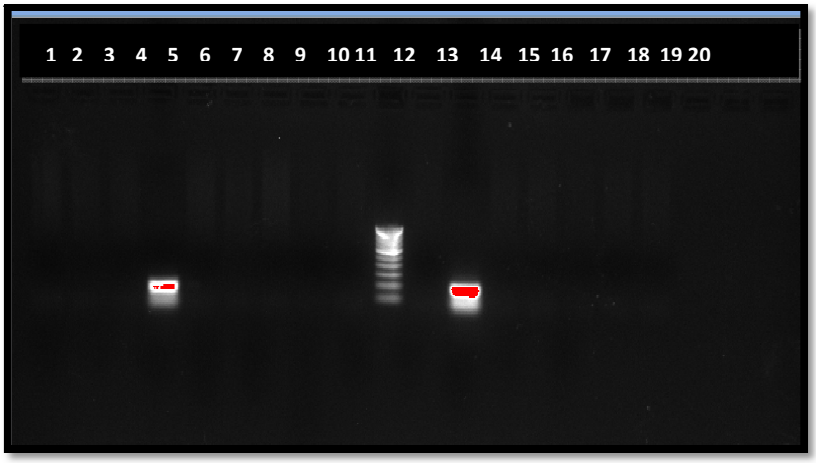

Image 1: Detection of bla NDM gene. 10 th lane is DNA ladder,1 st lane is Negative control and 4th and 12 th lane is sample positive for NDM gene.

During the study period, out of 200 Pseudomonas aeruginosa isolates, a total of 62 isolates were screeningtest positive on the basis of their reduced susceptibility to meropenem or imipenem. Out of these 62 isolates, 22 were from pus, 10 were urine samples, 3 were sputum, 12 endotracheal tube, 1 blood, 2 were folley's catheter tip, 1 ascitic fluid and 3 throat swab. Screening positive 62 isolates were alsotested for Modified hodge test, VITEK 2 system and MBL E-Test. 21/62 showed metallo beta lactamase test positive by phenotypic methods.

All phenotypic test positive 21 isolates were found to be positive for bla_{NDM-1}, bla_{VIM}, bla_{IMP} gene by PCR. bla_{NDM-1} was found intwo isolates only 1 from pus sample and 1 from Endotracheal tube. Furthermore, other 12 of the isolates were carrying bla_{VIM} and 7 were carrying bla_{IMP} gene. None of the isolates were found to be coexisted with multiple MBL genes.bla_{NDM-1}producers were susceptible only to colistin and polymyxin B and resistant to meropenem, amikacin, ciprofloxacin, aztreonam, piperacillin-tazobactum and ceftazidime.

DISCUSSION

Carbapenems have a broad spectrum of antibacterial activity. Hence, they are often used as a last resort in treatment. These are resistant to hydrolysis by most β-lactamases including extended spectrum β-lactamases (ESBL) and AmpC β- lactamases. There has been an increase in reports of Carbapenem resistance in P. *aeruginosa* worldwide.

In India the first report of metallo beta lactamase was published from Bangalore, MS Ramaiah Medical College by Navaneeth et al.12% of the isolates included in the study were resistant to both beta lactamase inhibitors and Carbapenem.100% of these isolates were found to be metallo beta lactamase producer (Navaneeth et al, 2002)

In India the studies done on metallo beta lactamase producing non fermenters are numerous. The prevalence of metallo beta lactamase producers among Carbapenem resistant isolates (Resistant to either or both Imipenem and Meropenem) in the present study was found to be 21/200. The results vary all over the country. The rate of metallo beta lactamase production in our study is much lower

compared to most of the other studies done in India It has been reported as low as 7.5%(Gupta et al, 2006) to as high as 100%(Navaneeth et al, 2002).[14]

In the present study, pus comprised for the majority of specimen followed by urine, sputum endotracheal aspirations, blood, and other sample. This study is similar to the study by Ranjan et al, 2014 where the majority of specimen included was pus (48.28%).This study is different from the study done by Wankhede et al where the majority of specimen was wound swab (44.11%).

The criteria for choosing the isolates for MBL screening are varied. Some studies have chosen Ceftazidime resistant strains for screening MBL(Hemalatha et al,2005). Most of the studies have chosen Imipenem resistant strains for screening of MBL. In the present study strains resistant towards either or both Imipenem and Meropenem were included. However all the isolates included in the study was resistant to Ceftazidime. 62/200(31%) Pseudomonas *aeruginosa*showed screening test positive. The similar finding were seen by Buchunde et al,2012 and Renu et al,2010.

For screening of Metallo beta lactamase in the present study Double disc synergy test, Modified hodge test and Etest was done. Modified hodge test(MHT) shows more positive MBL then that of Imipenem(IMP)- EDTA Combined disc test. Modified hodge test is recommended by CLSI for confirmation of MBL production. One study by Picao et al,2008[19] ,shows that MHT had better sensitivity compared to CDT. Whereas, Buchunde et al,2012 reported Meropenem EDTA CDT to be a better test compared to MHT.

In the present study E-test could detect MBL in the Imipenem resistant isolates correctly. E-test showed high sensitivity as compared to Modified hodge test. Modified Hodge test showed 4 false positive results. This is in accordance with the study done by Behera et al, 2008.

PCR for 3 genes i.e. IMP, VIM, NDM was done in all the 62 screening positive isolates. 12 of the isolates were carrying blaVIM and 7 were carrying blaIMPgene.NDM was detected in only 2 isolates. None of the isolates were found to be carrying multiple MBL genes.

The genes detected in the present study are bla IMP, bla VIM and bla NDM. The study done by Buchunde et al, 2012 from Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra reported all the MBL producing isolates carrying VIM gene. From Christian Medical College, Vellore Manoharan et al,2010 reported 15 VIM producing isolates amongst 20 MBL producer .Purohit et al,2012 from Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra reported bla-VIM MBL gene only in 7 (16.28%) of the 43 screen test positive *Acinetobacter* isolates. No one of the isolates showed presence of bla-IMP gene. Whereas Uma et al, 2009 of Pondicherry University from Puducherry reported only bla-IMP-1 in 42% (23 isolates) of*A. baumannii*. Amudhan et al, 2001 from Chennai reported bla-VIM in 46.55% with and both bla-IMP and bla-VIM in only one isolate of A. baumannii. However all these studies had originate single gene responsible for metallobetalactamase production. A study from Delhi by Niranjan et al,2013 had reported presence of multiple genes responsible for MBL production in *Acinetobacterbaumanii*.They also detected NDM-1 in the isolates. Buchunde et al, 2012 and Renu et al,2010.

CONCLUSION

There is an urgent need to create awareness among health care providers, policy makers and administrators against the danger of emerging antibiotic resistance. National and regional guidelines and policies must not only made but also monitored and implemented against antibiotic resistance and emergence of “super bugs”.

The inceasing incidence of New delhi metallobeta lactamse (NDM) producing Pseudomonas aeruginosa consitutes to a seroius threat to the global health since it is found to be highly resistant even last resort of antimicrobials including

carbapenems, aztreonam and tigecycline leading to the pan rasistant except for polymyxin B therapy.

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