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PhenotypicandGenotypicDetectionofMetalloBetaLactamase Production in Pseudomonas Isolates at a Tertiary Care Centre.

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ABSTRACT:
Introduction: Pseudomonas aeruginosa is a Gram-negative, multidrug resistant, nosocomial pathogen, frequently found ICUs & is associated with high morbidity and mortality.The most serious infections caused by P.aeruginosa includeendophthalmitis, endocarditis, meningitis, pneumonia, and septicemia. Carbapenems are the antibiotics of choice for severe Pseudomonas infections. However, in recent years, resistance to this novel antibiotic is increasing world-wide. The most common mechanism for carbapenem resistance is the production of metallo-beta-lactamases(MBLs), which can hydrolyze all the β-lactam antibiotics including carbapenems. Therefore, early detection of MBL producing organisms helps in providing optimal treatment for critically ill patients and in initiating strict infection control practices to prevent nosocomial spread.
Materials and Methods: A total of 100 P.aeruginosa isolates were identified from various clinical samples & AST was performed. The isolates which showed resistance to Imipenem(carbapenem), with a zone diameter of <15mm, weresubjected to 3 phenotypic testsi.e MBLE-Test, Combined Disk Test(CDT) and Modified Hodge Test(MHT)for detection of MBL production. MBL E-Test was considered as the phenotypic confirmatory test and those isolates which showed resistance to Imipenem by E-Test, were further subjected to Conventional PCR(Genotypic method), for the detection of the most common MBL genes i.e. blaVIM, blaIMP and blaNDM.
Results: Outof100P.aeruginosa isolates,52(52%)wereImipenem resistant by Disk Diffusion test,outofwhich40 (76.9%) were MBL producers by phenotypic confirmatory E-test. Out of the 40 MBL producers, 27 isolates showed the presence of MBL genes, with blaVIM being the most common (35%), followed by blaNDM(25%) and blaIMP(22.5%).
Conclusion: The present study emphasizes the need for MBL detection by Phenotypic confirmatory E-Test and to periodically evaluate the presence of MBL genes by PCR, to know the varying degree of severity ofinfections & their clinical outcome.
KeyWords:P.aeruginosa,MBLdetection,Carbapenemresistance,Imipenem,E-Test.

I. INTRODUCTION:

Pseudomonas aeruginosa is an opportunistic pathogen, aerobic Gram negative bacillus, belonging to family Pseudomonadaceae and genus Pseudomonas. It flourishes as a saprophyte in warm moist situations in thehuman environments including sinks, drains, respirators, humidifiers and disinfectantsolutions[1].Pseudomonas infection is especially prevalent among patients with burn wounds, cystic fibrosis, acuteleukemia, organ transplants, and intravenous-drug addiction.[2] It can also cause serious infections like meningitis, pneumonia, septicemia and other nosocomial infections with high mortality.[3]

Pseudomonasspeciesareintrinsicallyresistanttomany antibiotics.[4]Carbapenemsaretheantibioticsofchoice for severe Pseudomonas infections. However, in recent years, resistance to this novel antibiotic is increasing world-wide. The most common mechanism for carbapenem resistance is production of metallo-beta- lactamases(MBLs).MBLs are included in Class 2 of Ambler's classification of β-Lactamases and group 3 of Busch Jacoby Mederios classification. They are resistant toall Penicillins, 1st,2nd,3rdgeneration Cephalosporins, Cefamycins and also to Carbapenems.[5,6]

Based on amino acid sequence homology, multiple types of MBL genes have been recognized: IMP (Imipenemase), VIM (Verona

integrin-encoded MBL gene), SPM (Sao Paulo MBL), GIM (German imipenemase),SIM(Seoulimipenemase),NDM-1(NewDelhiMBL),KHM-1andDIM-1(Dutchimipenemase).
[4] These MBL genes are carried on highly mobile genetic elements such as plasmids or transposons, thus enabling widespreaddissemination.Also thespreadof MBLgenesfromP.aeruginosa to Enterobacteriaceaeisa growing concern.[7]Poor therapeutic outcome occurs wheninfections caused by MBL producing organisms are treated with antibiotics to which the organism is resistant. Therefore, early detection of MBL producing organisms helps in initiating optimal treatment and in preventing nosocomial spread.[2,4]

II. MATERIALSAND METHODS:

A cross-sectional study was conducted in the department of Microbiology, Osmania General Hospital, Hyderabad, from August 2019 to December 2020. Institutional Ethics Committee approval was obtained prior to the study. A 100 non-duplicate P.aeruginosa isolates were identified from various clinical samples like pus, wound swabs, blood, urine, and aspirated body fluids. Patients of all ages & both sexes, admitted in ICUs & wards of Osmania General Hospital and Psudomonas isolates which were resistant to Imipenem or Meropenem were included in the study, whereas patients attending OPD and In-patients in whom Pseudomonas was not isolated, were excluded from the study.

All the clinical samples were processed according to standard microbiological procedures and AST done by modified Kirby Bauer Disk Diffusion method. Pseudomonas isolates which were resistant to Imipenem with a zone diameter of <15mm, were subjected to phenotypic tests for MBL detection.

Currently, no standardized method for MBL detection has been proposed. Various phenotypic methods for MBLdetectionare:CombineddiskTest(CDT),ModifiedHodgeTest(MHT),Doublediskssynergytest(DDST), and E-test.MBL E-test is considered the phenotypic standard method for MBL detection. Polymerase Chain Reaction(PCR) is highly accurate and reliable but its accessibility is often limited to reference laboratories.[8,9,10,11]

PhenotypicmethodsusedinthestudyforMBLdetection:[12,13]

Imipenem-EDTACombinedDiskTest(CDT):

The test organisms were lawn cultured on MHA plates. 10µg of Imipenem disk & 750µg ofImipenem-EDTA combined disk (HiMedia, Ltd,Mumbai)were placed and the plates were incubated at 37°Cfor 16-18 hours.If there is increase in zone diameter of >7mm around the combined disk, then the isolate was considered MBL positive.

ModifiedHodgetest(MHT):

An overnight culture suspension of Escherichia coli ATCC 25922 adjusted to 0.5 McFarland, was inoculated usingasterilecottonswabonthesurfaceofMullerHintonagar(MHA)plate.Afterdrying,10µgImipenemdisk was kept at the centre of the MHA plate and test strain suspensions were inoculated by streaking from the edge of the Imienem disk to the periphery of the plate, in four different directions. The plates were incubated overnight at 37°C.The test strain was considered as MBL producer if there was a clover-leaf shaped zone of inhibition.

MBLE-Test:

Test organisms were lawn cultured on MHA plates and MBL E-strips containing a double sided seven –dilution range of IP(Imipenem) (4 to 256 µg/ml) and Imipenem (1 to 64 µg/ml) in combination with a fixed concentrationofEDTA (HiMedia),wasplaced.Plateswere incubatedat37°Cfor 16-18 hours. An MICratio of IP / IPI (Imipenem + EDTA) of >8 or >3 log dilutions indicates MBL production.

Genotypic detection of MBL production: for detection of most common MBL genes i.eblaVIM, blaNDM&blaIMP.:

Extraction of bacterial DNA followed by Polymerase chain reaction and gel electrophoresis analysis was performed in a step-wise manner.

Bacterial DNA Extraction: All the P.aeruginosa isolates which were MBL positive by phenotypic methods,were freshly subcultured in peptone water broths and incubated at 37°C for 2 hours to obtain a turbidity matching 2.0 McFarland.

300µl of each test inoculum was taken in 1.5ml centrifuge tubes to which 385µl of binding solution (Thermofischer scientific), 15µl of molecular beads solution (Thermofischer scientific) and 15µl of ProteinaseK solution (Thermofischer scientific) was added to each tube and vortexed for 5-10seconds, for the bacterial cells to lyse. These tubes were incubated at room temperature for 10minutes. Then the whole contents of the centrifuge tubes were transferred to deep well plates.

In 3 separate deep well plates, wash

solution 1(ethanol), wash solution 2 (80% ethanol and 20% milli Q water) and elution buffer (80µl in each deep well) were added. All the 4 deep well plates were then placed inautomated DNA extraction machine for extraction of bacterial DNA.

After completion of DNA extraction, 80µl of extracted DNA elute was obtained for each isolate, of which, 5µl was then mixed with the Mastermix (10µl for each sample) & primer mix (1µl each of forward and reverse primers + 8µl of nuclease free water) and subjected to amplification in PCR thermocycler.

Sequenceofprimersusedinthestudy:(procuredfromEurofinsGenomicsIndiaPvt.Ltd.)

Target gene	Primersequence	Amplicon size
blaIMP	F : CTACCGCAGCAGAGTCTTTGC R:GAACAACCAGTTTTGCCTTACC	640bp
blaVIM	F : TTTGGTCGCATATCGCAACG R: CCATTCAGCCAGATCGGCAT	500bp
blaNDM	F:GGTGCATGCCCGGTGAAATC R : ATGCTGGCCTTGGGGAACG	660bp

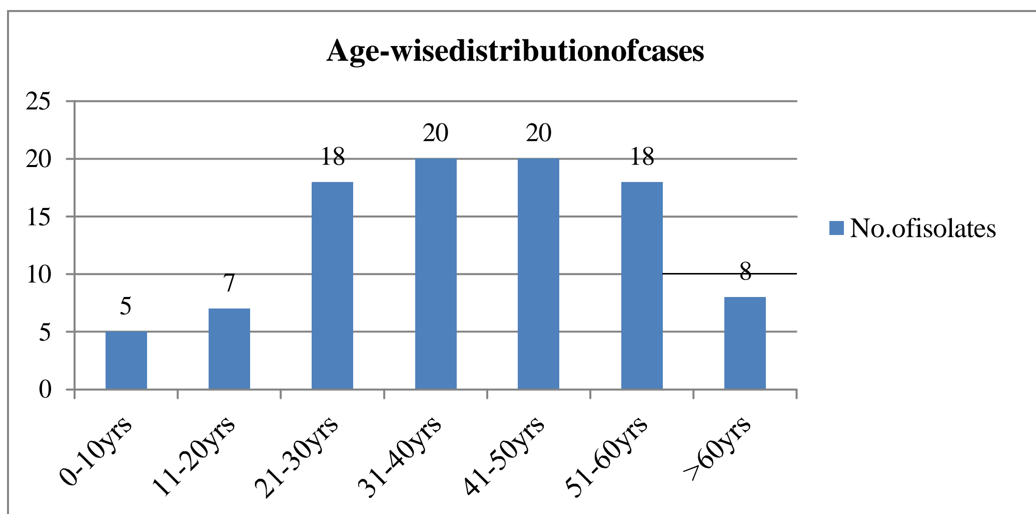
Amplification was carried outusing using the following thermal cycling conditions: Initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing for blaVIM at 58.5°C for 45sec, for blaNDM at 61°C for for 45sec, for blaIMP at 44°C for 45sec, and extension at 72°C for 30sec, followed by final extension at 72°C for 5min. The amplified PCR products (10µl of each isolate) were subjected to gel electrophoresis on 2% agarose in a TAE buffer, after staining with 0.5µg/ml of ethidium bromide and visualizing the amplicons by a Gel Documentation System, they were compared with a 100bp ladder.

III. RESULTS:

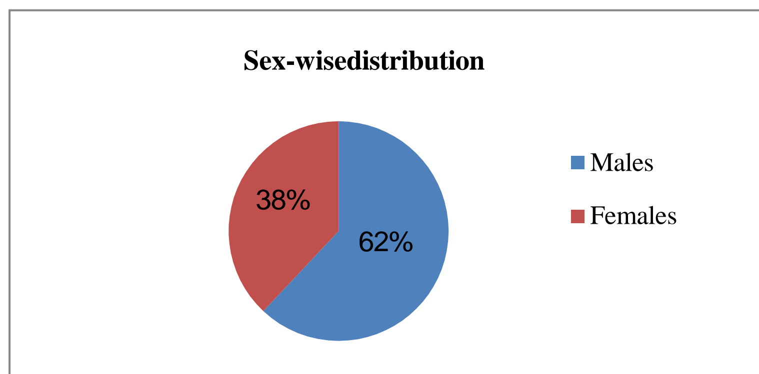
Out of the 100 Pseudomonas isolates, 52 (52%) were resistant to Imipenem by Disk diffusion method, out of which, 40 were MBL positive by phenotypic confirmatory ETest.PCR was done for the 52 isolates (which were resistant to Imipenem), out of which, 27 strains showed 3 types of MBL genes i.eblaVIM, blaIMP&blaNDM.

Out of the 100 cases studied, most of the isolates were obtained from 21 – 60 years age group (76%) and the least number was from age group 0 – 5 years (05%).

Agewisedistributionofcases:

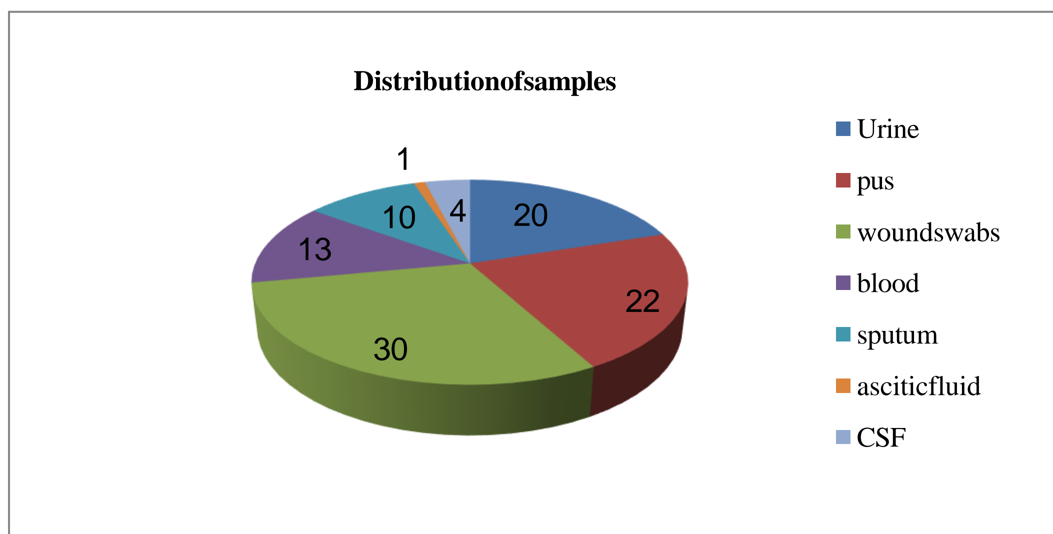


Sex-wisedistributionofcases:



Distributionofvariousspecimensincludedinthestudy:

SampleType	No.ofSamples	Percentage(%)
Urine	20	20%
Sputum	10	10%
Blood	13	13%
Pus	22	22%
WoundSwabs	30	30%
CSF	04	04%
Asciticfluid	01	01%



Susceptibility&ResistancepatternsofPseudomonasisolatesusedinthestudy:

Antibiotic	Susceptibility	Resistance
Ceftazidime(CAZ)	40%	60%
Cefotaxime(CTX)	42%	58%
Gentamycin(GEN)	45%	55%
Imipenem(IMP)	48%	52%
Cefoperazone-sulbactam(CPZ)	88%	12%
Piperacillin-Tazobactam(PTZ)	60%	40%
Cefipime(CPM)	70%	30%
Ciprofloxacin(CIP)	46%	54%
Colistin(CL)	100%	0%

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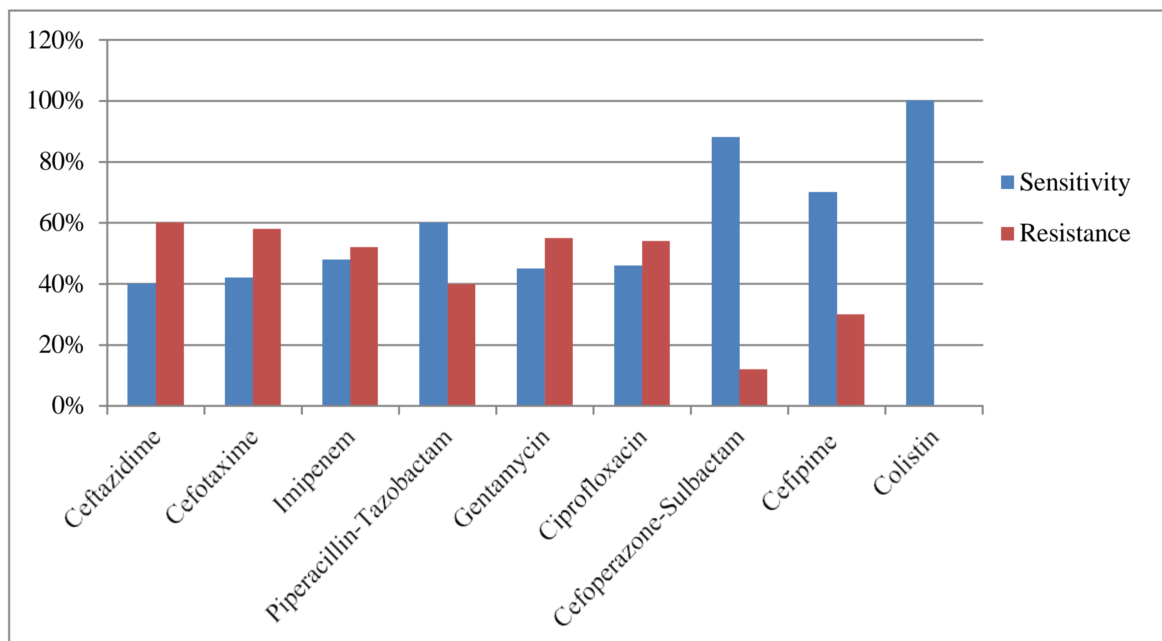
AntibiogramofPseudomonasisolatesinthestudy(n=100):

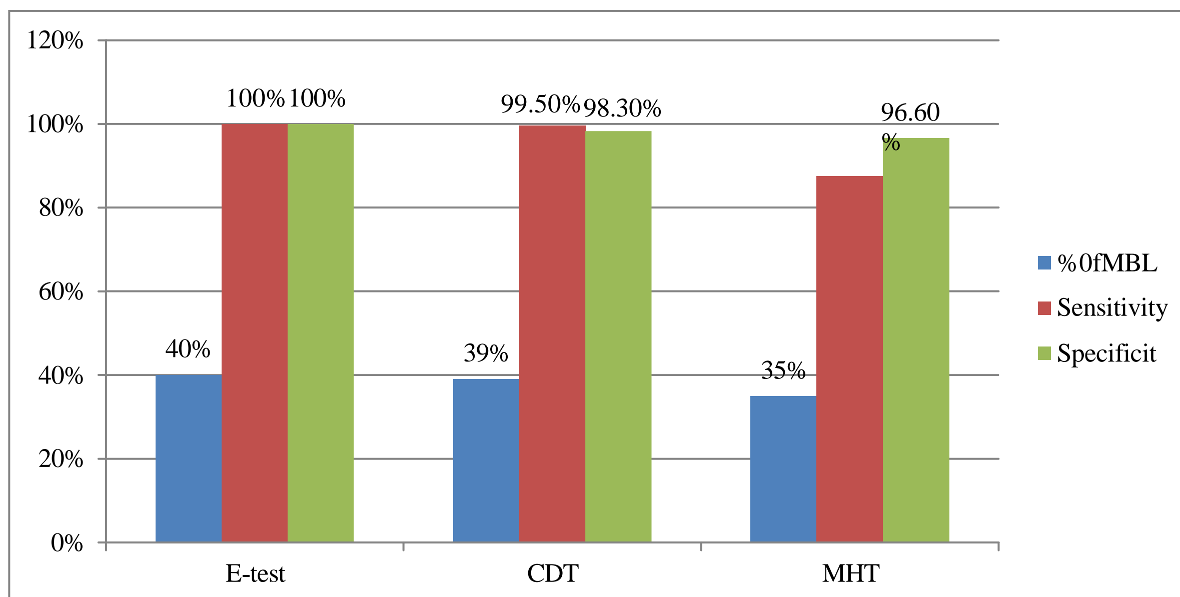


In the present study, most of the Pseudomonas isolates were multi-drug resistant with an MDR prevalence of 44%.The isolates showed 100% sensitivity to Colistin and 88% sensitivity to CFS. 64% of isolates were sensitivetoCefipime,while60%ofthemwere sensitivetoPTZ. Theisolatesshowedhighestresistanceto CAZ (60%),followedbyCefotaxime(58%),GEN(55%),CIP(54%),IMP(52%),PTZ(40%)andCPM(30%). LeastresistancewasseenagainstCFS(12%).

MBLdetectionbyPhenotypictests:

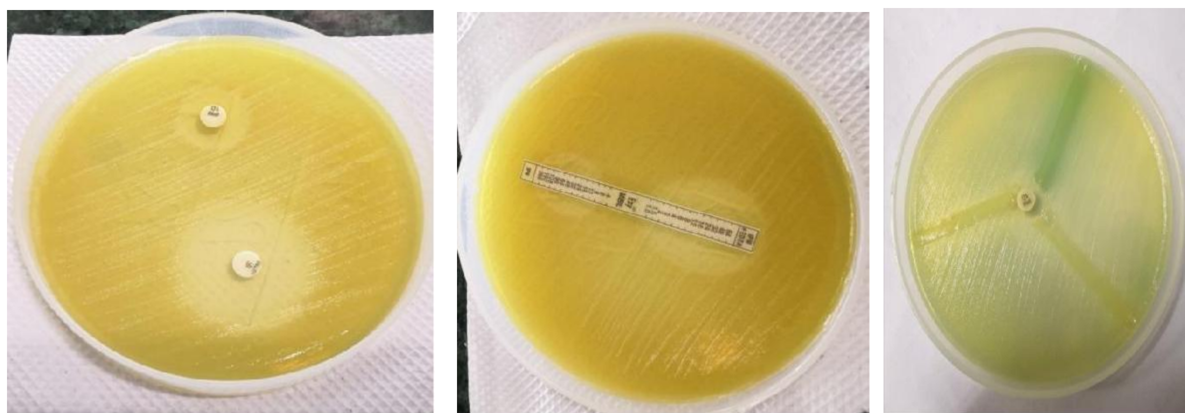
PhenotypicTest	**E-Test**		**Sensitivity& Specificity**
	Positive(n=40)	**Negative(n=60)**	
CDT:			
Positive(n=40)	39	01	Sensitivity=97.5%
Negative(n=60)	01	59	Specificity=98.3%
MHT:			
Positive(n=37)	35	02	Sensitivity=87.5%
Negative(n=63)	05	58	Specificity=96.5%

Sensitivity&SpecificityofPhenotypicTestsusedinthestudy:



The sensitivity and specificity of E-Test was 100% whereas that of CDT was 97.5% & 98.3% respectively, whichwas higher than the sensitivity (87.5%) and specificity (96.6%) of MHT.

CombinedDiskTest: **MBLE-Test:** **ModifiedHodgeTest(MHT):**



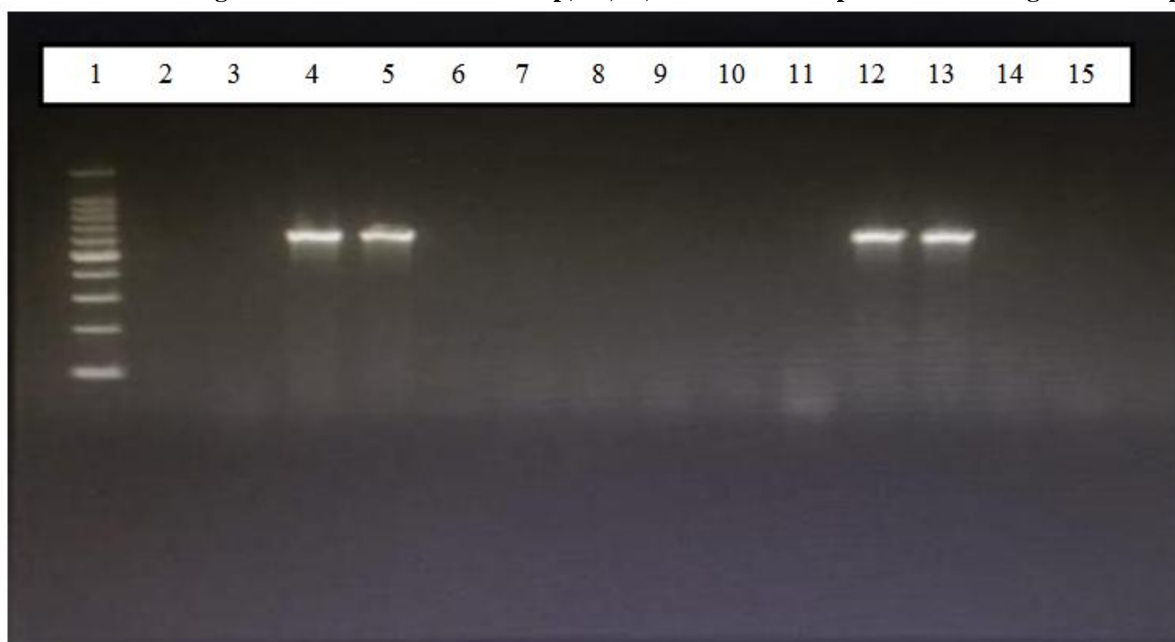
DistributionofMBLgenesinImipenemresistantisolates:(n=52):

Conventional PCR was done for all the Inipenem resistant isolates. Results showed that out of 40 MBL positive isolates, 27 showed the presence of various MBL genes, whose distribution is as follows: 14(35%) showed the presence of blaVIMgene,10(25%) showedthepresenceofblaNDM gene and09(22.5%)isolatesharbouredblaIMP gene. 04 isolates showed the presence of both blaVIM and blaNDM genes and 02 isolates showed the presence of both blaVIM and blaIMP genes. None of the isolates showed the presence of all 3 genes.

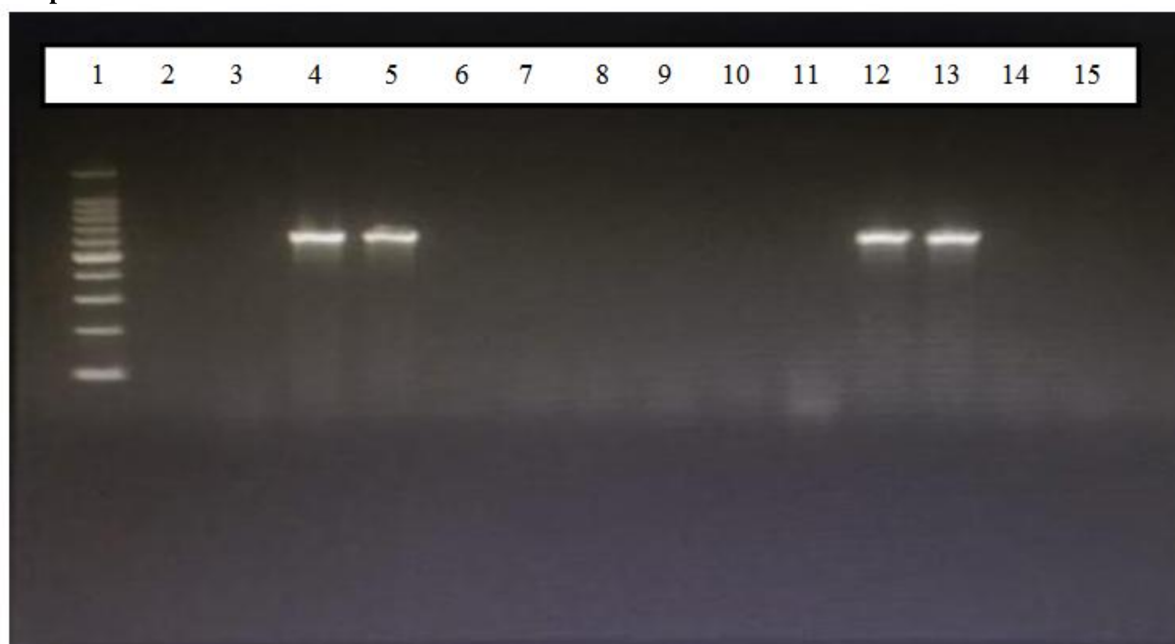
MBLGene	No.ofisolates
blaVIM	O8
blaNDM	07

blaIMP	06
blaVIM+blaNDM	04
blaVIM+blaIMP	02
Total	27

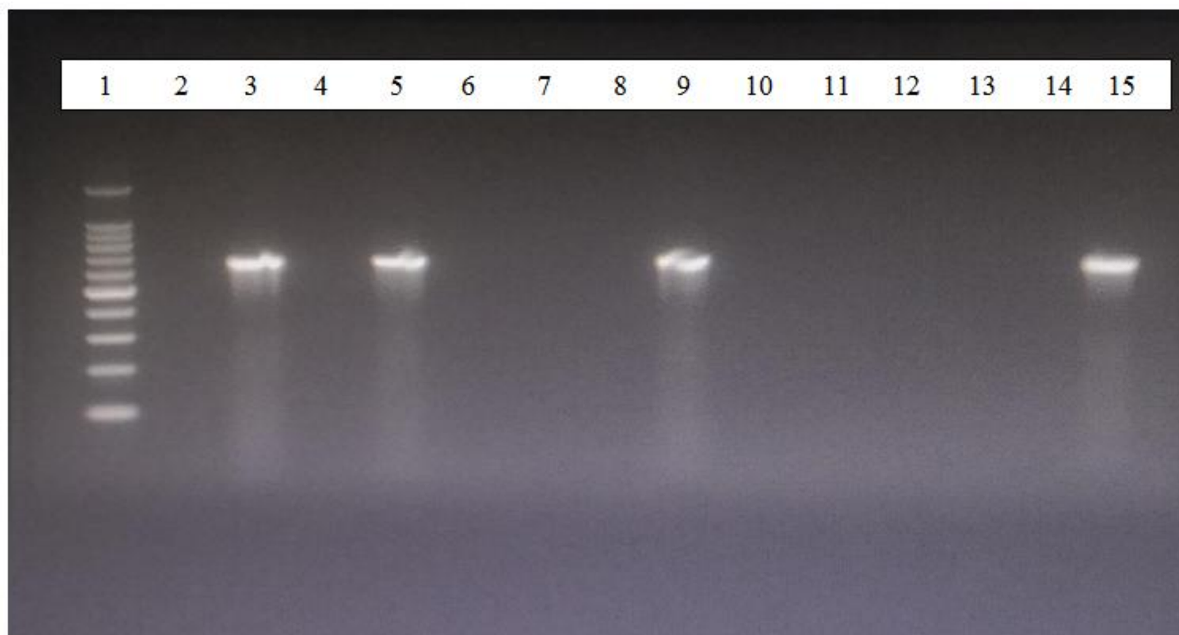
DetectionofblaVIMgene:1stlaneisDNAladder100bp;2nd,6th,13thand14thlanespositivefor VIM gene at 500bp:



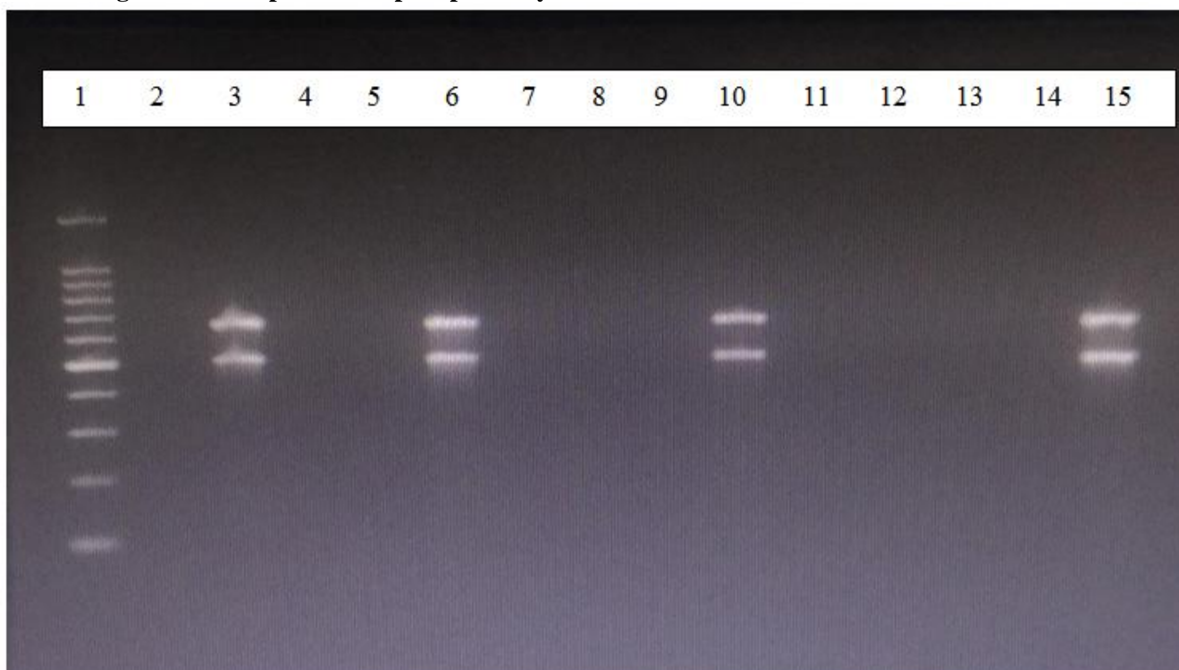
DetectionofblaNDMgene: 1stlaneisDNAladder100bp;4th,5th,12thand13th lanespositivefor NDM gene at 660bp:



DetectionofblaIMPgene:1stlaneisDNAladder100bp;3rd,5th,9thand15thlanespositiveforIMP gene at 640bp:



DetectionofblaVIMandblaNDM gene:1stlaneisDNAladder100bp;3rd,6th,10thand15th lanes positive for VIM and NDM genes at 500bp and 660bp respectively:



IV. DISCUSSION:

The intrinsic and extensive antibiotic resistance of P.aeruginosa has restricted its therapeutic choices. Carbapenems are the drugs of choice in treating infections caused by P.aeruginosa as they are resistant to hydrolysis by most β-lactamases. However, there is an increase in Carbapenem resistance in Pseudomonas isolates worldwide &its reported from various parts of India, as well. In India, the first report of MBL production in Pseudomonas isolates was published by Navneeth et al[14] MS Ramaiah Medical College, Bangalore.

In the present study, Imipenem resistance was seen in 52/100 Pseudomonas isolates (52%), which correlated with a study done by Shyama Sree Nandi et al[15] , whereas in studies done by Wang Wei et al[16]& Ronni Mol et al[17] , the

resistance was much higher, even upto 80%, indicating that P.aeruginosa has become a deadly pathogen with limited therapeutic options. The prevalence of MBL in Imipenem- resistant isolates, in thepresent study was 40%, with an MDR prevalence of 44%. Pus and wound swabs (52%) constituted for the majority of specimens, followed by urine(20%), sputum(10%), & other body fluids(5%). The rate of isolation was more common in males(62%), compared to females(38%).

The present study also focused on detecting the most common MBL genes reported in India, i.eblaVIM,blaIMP&blaNDM. These genotypes accounted for varying severity of infections caused by the organism. blaVIM(35%) was the most common MBL gene, followed by blaNDM(25%) &blaIMP(22.5%). The present study also showed that the patients who harbouredblaNDM gene were more critical & responded poorly to treatment by last resort drug i.e Colistin. This observation indicates that it is necessary to periodically perform genotypic testing to know the correlation between the genotypes & the severity they cause. It is also necessaryto routinelyperformphenotypic tests by affordablemethodslike MBL E-strips or Combined disk tests, to know the presence of MBL genes and to choose the appropriate antibiotic.

V. CONCLUSION:

To conclude, it is crucial to provide information on the current status of P.aeruginosa in the hospitals, especially in view of the multi-drug resistance in the organism. Considering this, it is pivotal to investigate the prevalence as well as the susceptibility pattern of P.aeruginosa in hospitals.The present study emphasizes the need to detect MBL production in P.aeruginosa isolates in view of the increasing prevalence of MBL genes, which are responsible for dissemination of antibiotic resistance through horizontal gene transfer to other Gram negative bacteria, which may contribute to further emergence of Carbapenem resistance.

It is therefore necessary to periodically evaluate the presence of MBL genes by PCR, which helps in knowing the prevalence of the most common MBL genes in a particular geographical area, and the clinical threat they pose in critically-ill patients.Detection of MBL producing isolates helps in early initiation of appropriate antibiotic therapy and thus helps in avoiding further spread of these multi-drug resistant strains.

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