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TO STUDY THE ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF PSEUDOMONAS AERUGINOSA AND DETECTION OF METALLO BETA LACTAMASE PRODUCING STRAIN WITH SPECIAL REFERENCE TO blaNDM-1 AND blaVIM

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ABSTRACT

The present study was 100 isolates of Pseudomonas aeruginosa from various clinical samples were included in the Study. Antimicrobial susceptibility testing by Disc diffusion method was performed in accordance with CLSI guidelines. Strains that were resistant to meropenem/imipenem are considered as carbapenem resistant strains. All the carbapenem resistant strains were further subjected to various phenotypic methods to detect MBL production. The phenotypic methods employed were Modified Hodge Test, Combined disc diffusion test and Etest. E-test gave better results than Modified Hodge test and Combined disc diffusion test. Conventional PCR technique is employed to detect blaNDM and blaVIM. blaVIM was present in 45.8%(n=11).

Keywords: Pseudomonas aeruginosa, metallo beta lactamase, blaNDM and blaVIM

Introduction

Carbapenems are often used as antibiotics of last resort for treating infections due to multidrug-resistant Gram-negative bacilli, because they are stable even in response to extended spectrum and AmpC-β-lactamases. However, Gram-negative bacilli producing the acquired metallo-β-lactamases (MBLs) have been increasingly reported in Asia and Europe and more recently, they have been detected in Canada and the United States [1].

Infection with the metallo-beta-lactamase (MBL) producing organisms is associated with higher rates of mortality, morbidity, and health care costs. MBL producing Pseudomonas aeruginosa was first reported in Japan in 1991 and since then has been found in various parts of the world [2]. Based on molecular studies, carbapenemhydrolyzing enzymes are classified into four groups A, B, C and D. The metallobetalactamases (MBLs) belong to group B and they require divalent cations as cofactors for enzyme activity, being inhibited by the action of a metal ion chelator. The common form of resistance is mediated by lack of drug penetration (i.e., porin mutations and efflux pumps) and/or carbapenem-hydrolyzing b-lactamases [3].

The MBLs efficiently hydrolyze all beta lactams, except for aztreonam, in vitro [4] therefore, detection of MBL-producing gram-negative bacilli is crucial for the optimal treatment of patients and to control the spread of resistance [5]. However, NCCLS (National Committee on Clinical Laboratory Standards.) documents [6] do not yet contain a method for detection of MBL-producing isolates.

P. aeruginosa is a common Gram-negative bacillus associated with hospital infections and is often difficult to eradicate due to its resistant drug profile. Therefore, detection of MBL-producing Gram-negative bacilli especially P. aeruginosa is crucial for the optimal treatment of patients particularly in critically ill and hospitalized patients, and to control the spread of resistance [7]. In some countries, P. aeruginosa possessing MBLs constitute nearly 20% of all nosocomial isolates [8]. In India, published reports indicate the prevalence of MBLs to range from 7-65 % [9] with a recent study reporting 34% occurrence [10]. The most common MBLs

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include the VIM, IMP, GIM, SPM, SIM enzymes and the recently identified NDM-1. In particular, bla VIM -2 has emerged as a dominant MBL variant worldwide [11, 12].
This study is being done to detect metallobetalactamase producing isolate of Pseudomonas aeruginosa and identify NDM-1 and VIM gene in such isolate.

MATERIALS AND METHODS

Antibiotic used (Source-Himedia)

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)

Methods Inclusion Criteria

P.aeruginosa from all the routine clinical samples received in our central laboratory (Blood, Urine, Pus, Wound swab, Sputum, Endotracheal aspirate) for investigation were included in this study. The study duration is for 15 months Patients belonging to all age groups and both the sexes were included in the study.

Exclusion Criteria

Pseudomonas species other than P. aeruginosa were excluded from this study.

Identification of the Organism

All the Organisms were subjected to standard culture technique and the organisms thus identified were based on the colony characters on nutrient agar, MacConkey agar, Blood agar and cetrimide agar (Fig 2). Gram staining was done to confirm the morphology and hanging drop was done to find out motile and non-motile bacilli. They were further subjected to biochemical reactions such as slide and tube catalase, Oxidase disc test (Fig 4), indole test, MR, VP, citrate utilization test, urease test, TSI, OF test (Fig 3) and sugar fermentation tests.

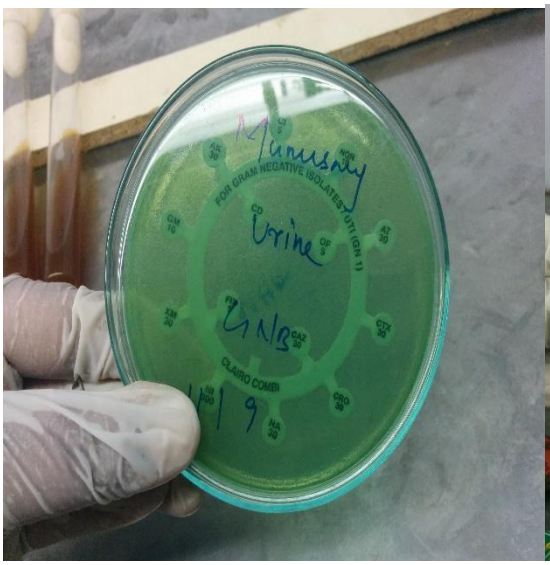

Fig 1: Resistant strains of P.aerugino

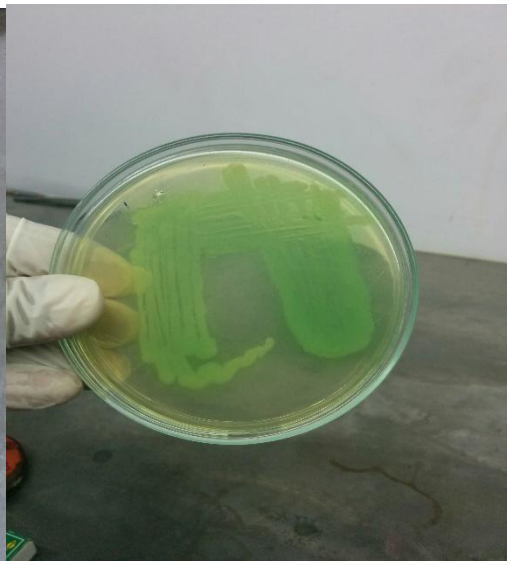

Fig 2: Growth on Cetrimide media

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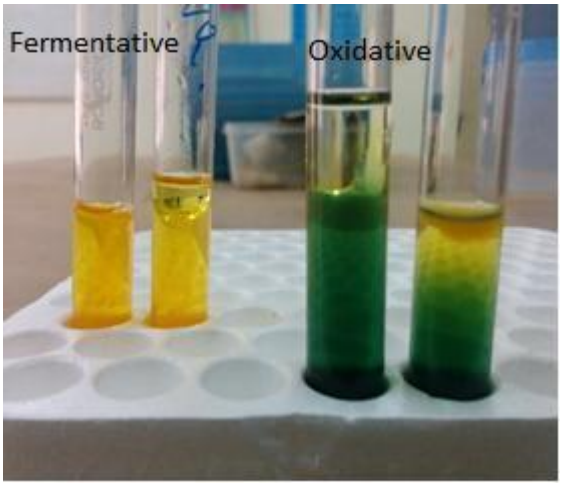

Fig 3: Oxidation/Fermentation test

Fig 4: Oxidase test Escherichia coli Negative control Pseudomonas aeruginosa: Positive control

Antimicrobial susceptibility testing

The antibiotic storage container was refrigerated at 4°C to 10°C and the discs are brought to room temperature before use.

Turbidity standard for inoculum preparation

A 0.5 McFarlands turbidity standard was used to standardize the inoculum density for the susceptibility test. This corresponds to 1.5 x 10^8 CFU/ml.

Preparation of inoculum

Well-Isolated colonies were picked up and emulsified in 2ml of peptone water. It was incubated for 2 hours at 37°C.The turbidity was compared and adjusted to 0.5 McFarland turbidity.

Kirby-Bauer's disc diffusion method

A sterile cotton swab was dipped in to peptone broth and pressed firmly on the side walls of the test tube in order to remove excess broth. Using this inoculum, lawn culture was made on MHA plate with a sterile cotton swab.

Application of the discs to the inoculated agar plate

The antibiotic discs used were as follows Amikacin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), aztreonam (30 µg), piperacillin-tazobactam (100/10 µg), imipenem (10 µg), meropenem (10

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µg), colistin (10 µg) and polymyxin B (300 units). The discs were carefully placed on the agar plates such that there is complete contact with the agar surface. Plates were inverted and incubated at 37°C for 18-24hrs.

Reading Plates and interpretation of results

After 18-24 hrs.of incubation, plates were examined for a confluent lawn of growth and diameter of zones of complete inhibition was measured on the back of inverted petri dish, including the diameter of the disc. The zone diameter was recorded and interpreted as susceptible, intermediate and resistant as per the CLSI zone interpretation criteria.

Controls used with each batch

- Escherichia coli ATCC 25922
- Pseudomonas aeruginosa ATCC 27853

Selection of resistant strains

Isolates found to be resistant or with decreased susceptibility to meropenem by disc diffusion were tested for carbapenamases.

Phenotypic test

Carbapenemase production was screened by the Modified Hodge test (MHT) and MBL Production by inhibitor potentiated disk diffusion test with ethylene diamine tetra acetic acid (EDTA). Though CLSI does not advocate the use of MHT for the detection of carbapenemases production in P.aeruginosa.

1.Modified Hodge test (MHT)

Mueller-Hinton agar was inoculated with a standard suspension (0.5 McFarland standard) of E. coli ATCC 25922.After drying the plate, a meropenem disc (10 µg) was placed on the center of the plate. The test strain was inoculated from the edge of the disc as straight line of about 2-2.5 cm long. The plate was incubated at 37°C for 18-24 hours. The plate was observed for growth around the test streak (at the intersection of test strain and zone of inhibition) (Fig 5)

MHT Positive: Test has a clover leaf-like indentation of the E. coli 25922 growing along the test organism growth streak within the disk diffusion zone.

MHT Negative: Test has no growth of the E. coli 25922 along the test organism growth streak within the disc diffusion.

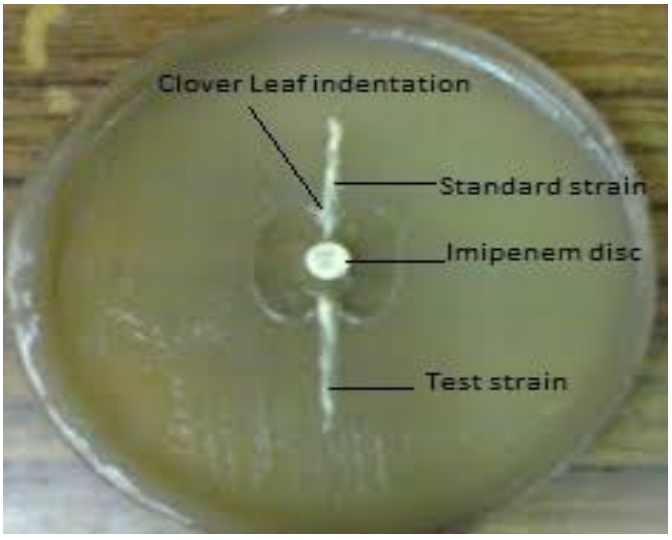

Figure 5: Modified Hodge test showing clover-leaf type indentation at the intersection of the test strain and the standard strain

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Combined Disc Test

The test strain (equivalent to 0.5 M cFarland standard) was inoculated on the surface of MHA plate. The two discs, one containing imipenem and the other with imipenem plus EDTA (Himedia) was placed on the surface of MHA. The plate was incubated at 37°C 18-24 hours.An increase in zone diameter of ≥ 7 mm around the imipenem- EDTA when compared to the meropenemdic alone is considered to be positive for MBL production (Fig 6)

Figure 6: Imipenem-EDTA double disc diffusion test showing difference in the inhibition zones between the two discs is >7 mm represents metallobetalactamase production

E-test (EZY MICTM Strips Himedia-India)

The E-test MBL strip consists of double-sided dilution range of IM in one side and IM-EDTA dilution on the other side. The E-test strip determines the minimum inhibitory concentration (MIC) of the antimicrobial agent.

Procedure

- The individual colonies of strain were suspended in liquid broth to attain a turbidity matching to 0.5 McFarland
- With sterile cotton swab a lawn culture was made in the same way as for disc diffusion
- Then E-test strip was placed on the agar with a sterile applicator
- Then plate was incubated for 16-18 h at 37°C and results of MIC of IM and IM-EDTA read directly from the strip

Interpretation

Ratio of IM/IM-EDTA ≥8, presence of the phantom zone, and distortion of ellipse were interpreted as Positive results. This test was taken as the gold standard for detection of a MBL producer. (Fig 7)

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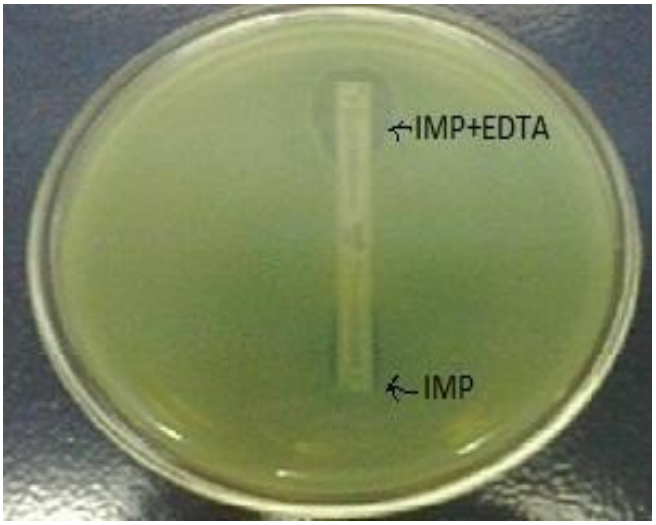

Figure 7: Metallobetalactamase (MBL) E-test showing minimum inhibitory concentration ratio of imipenem (IM)/IM-EDTA of 8 i.e. MBL producing strain of Pseudomonas aeruginosa

Detection of *bla*NDM-1 and *bla*VIM by PCR

Material & Methods: DNA purification kit (PureFast® Bacterial Genomic DNA purification kit), PCR Master Mix,

Agarose gel electrophoresis consumables and Primers (Table 4)

2X Master Mix: It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives.

Agarose gel Electrophoresis: Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide

Table 4: Primers

Primer	**Primer sequence**	**Product size(bp)**
*bla*VIM-F *bla*VIM-R	5'GTGCTTTGACAACTTTCGTT-3' 5'TCCACGCACTTTCATGACGA-3'	442
*bla*NDM-1-F *bla*NDM-1-R	5'GGGCAGTCGCTTCCAACGGT-3' 5'GTAGTGCTCAGTGTCGGCAT-3'	475

PCR Procedure:

[25µl of Master Mix contains: 10X Taq buffer, 2mM Mgcl2, 0.4mM dNTPs mix, and 2U Taq DNA polymerase]

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Components	**Quantity**
In PCR vial Master Mix	10µl
Primer-forward(2pmoles/ µl)	5µl
Primer-reverse(2pmoles/ µl)	5µl
Genomic DNA	5µl
Total volume	25µl

1. Reactions set up as follows;
2. Mixed gently and spin down briefly.
3. Place into PCR machine and program it as follows;

Initial Denaturation: 94ºC for 3 min
Denaturation: 94ºC for 1 min
Annealing: 58ºC for 1min 35 cycles
Extension: 72ºC for 1min
Final extension: 72º C for 5 min
Loading:

1. Prepare 2% agarose gel. [2gm of agarose in 100ml of 1x TAE buffer]
2. Mix 8µl 6X Gel loading dye to each PCR vial and loaded entire PCR product.
3. Run electrophoresis at 50V till the dye reaches three fourth distances and observe the bands in UV Transilluminator.

Agarose gel electrophoresis:

1. Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven)
2. When the agarose gel temperature was around 60ºC, added 5µl of Ethidium bromide.
3. Poured warm agarose solution slowly into the gel platform.
4. Kept the gel set undisturbed till the agarose solidifies.
5. Poured 1XTAE buffer into submarine gel tank.
6. Carefully placed the gel platform into tank. Maintained the tank buffer level 0.5cm above than the gel.
7. PCR Samples are loaded after mixed with gel loading dye along with 10µl HELINI 100bp DNA Ladder. [100bp, 200bp, 300bp, 400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp and 1500bp]
8. Run electrophoresis at 50V till the dye reaches three fourth distance of the gel.
9. Gel viewed in UV Transilluminator and observed the bands pattern.

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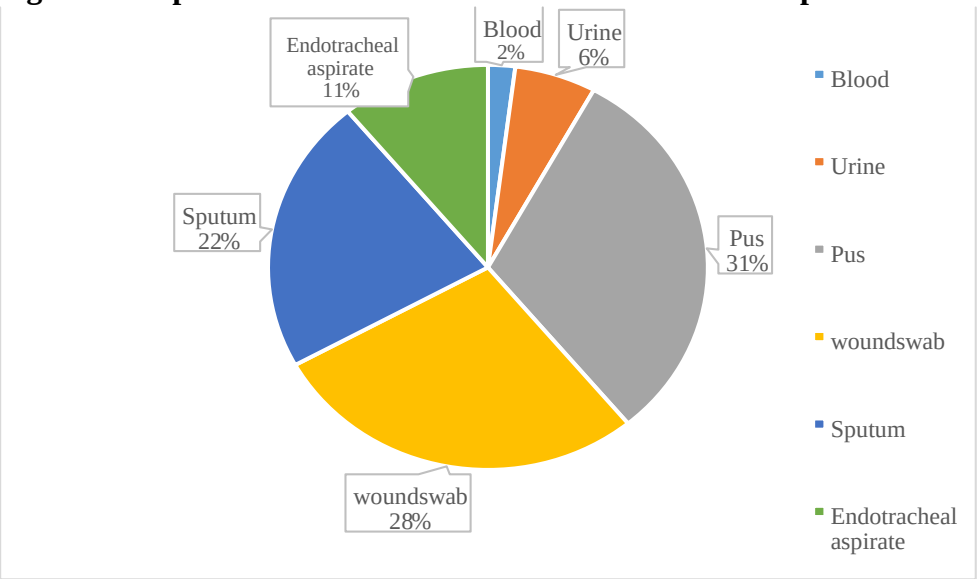
RESULTS

The study was conducted in the department of Microbiology, SreeBalaji Medical College and Hospital, South India, from January 2014 to August 2015. During this period, a total of 100 clinical isolates of P. aeruginosa were collected, of which 24 % were carbapenem resistant. All of these were from patients hospitalized for 48 h or more. These were cultured from clinical specimens such as blood (2), urine (6), exudative specimens (70) such as pus, wound swabs, and lower respiratory secretions (22) which included endotracheal aspirates (9) and sputum (21) (Fig 8). The organisms were identified by routine culture & biochemical tests.
The study protocol was approved by the Institutional Ethical committee

Table 5: Carbapenem resistant strains among clinical isolates

Clinical specimens	No of P.aeruginosa strains %	Carbapenem Resistant strains %
Blood	2	1(4.16)
Urine	6	2(33.33)
Pus	31	7(22.58)
wound swab	28	6(25)
Sputum	21	5(20.83)
Endotracheal aspirate	9	3(12.5)

Fig 8 :Carbapenem resistant strains from various clinical specimens



Antimicrobial susceptibility testing: Susceptibility to various classes of antimicrobial agents was determined by Kirby bauer disc diffusion method in accordance with Clinical Laboratory Standard Institute (CLSI) guidelines. The antibiotics tested were amikacin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), aztreonam (30 µg), piperacillintazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), colistin (10 µg) and polymyxin B (300 units) (Hi-media Laboratories, Mumbai, India) (Table 6 & Fig 9).

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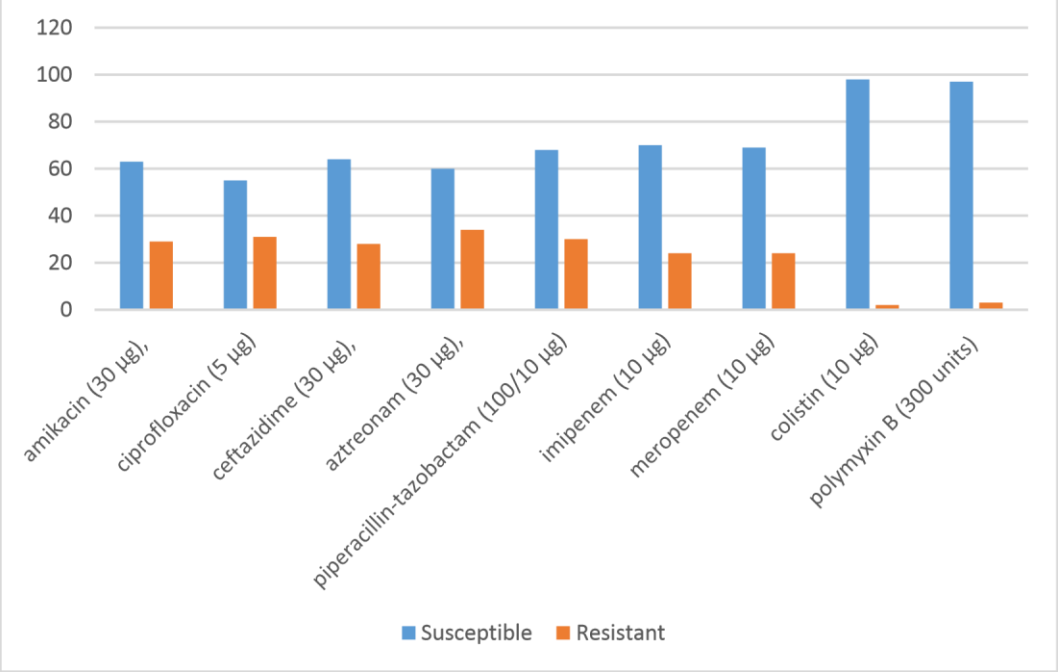
Table 6: Antimicrobial susceptibility pattern of P.aeruginosa by Kirby Bauer technique

Antibiotics	Susceptible	Intermediate	Resistant
amikacin (30 µg),	63	8	29
ciprofloxacin (5 µg)	55	14	31
ceftazidime (30 µg),	64	8	28
aztreonam (30 µg),	60	6	34
piperacillin-tazobactam (100/10 µg)	68	2	30
imipenem (10 µg)	70	6	24
meropenem (10 µg)	69	7	24
colistin (10 µg)	98	*	2
polymyxin B (300 units)	97	*	3

*- No intermediate susceptible guidelines for colistin and Polymyxin B.

Fig 9: Antimicrobial susceptibility pattern of P. aeruginosa



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Of the 100 isolates of P. aeruginosa, 24 showed resistance to imipenem by the disc diffusion method. A total of 18 isolates were positive for MHT. Furthermore, 17 of these 24 isolates exhibited a significant zone size enhancement with the EDTA impregnated discs when compared with the plain antibiotic discs. Of these, only 14 isolates were positive for Ezy- MIC test (the ratio of the value obtained for Imipenem (IMP): the value of Imipenem + EDTA (IMP+EDTA) was more than 8). The ATCC 27853 P.aeruginosa as expected did not exhibit a zone size enhancement. The results of phenotypic methods are given in (Table 7 and Fig 10& 11).

Table 7: Results of phenotypic methods

Meropenem/imipenem Resistant isolates	Detection of Carbapenamase Production			
	MHT Method (%)	CDT Method (%)	E-test Method (%)	Negative by all the method
24	18(75)	17(70.83)	14(58.33)	6(25)

Fig 10: Comparison chart of Different Phenotypic Methods

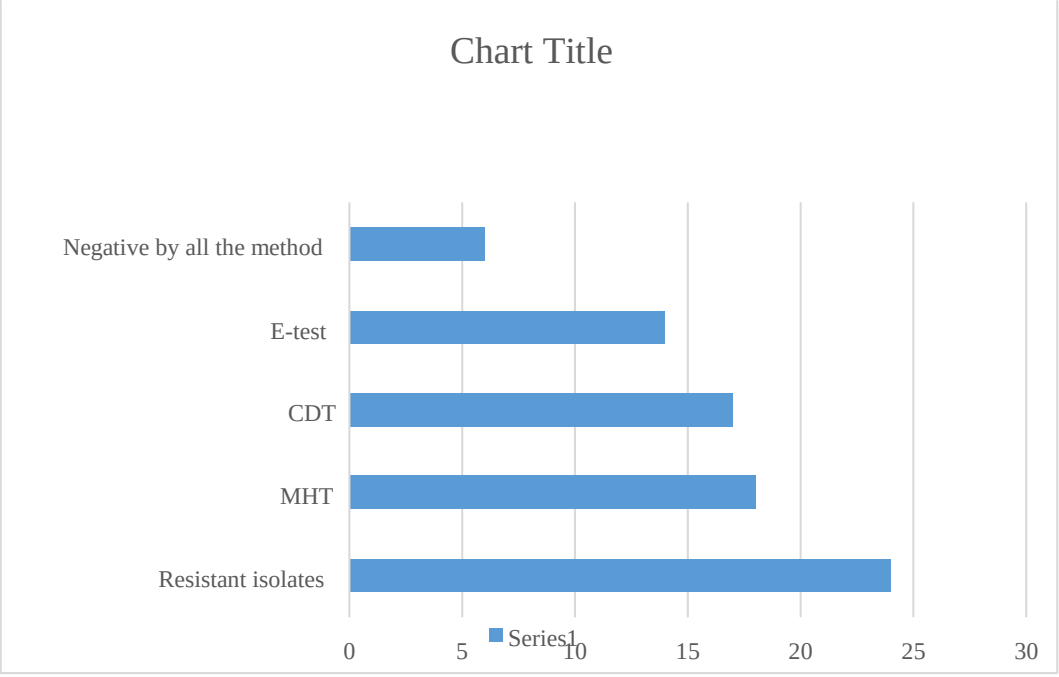

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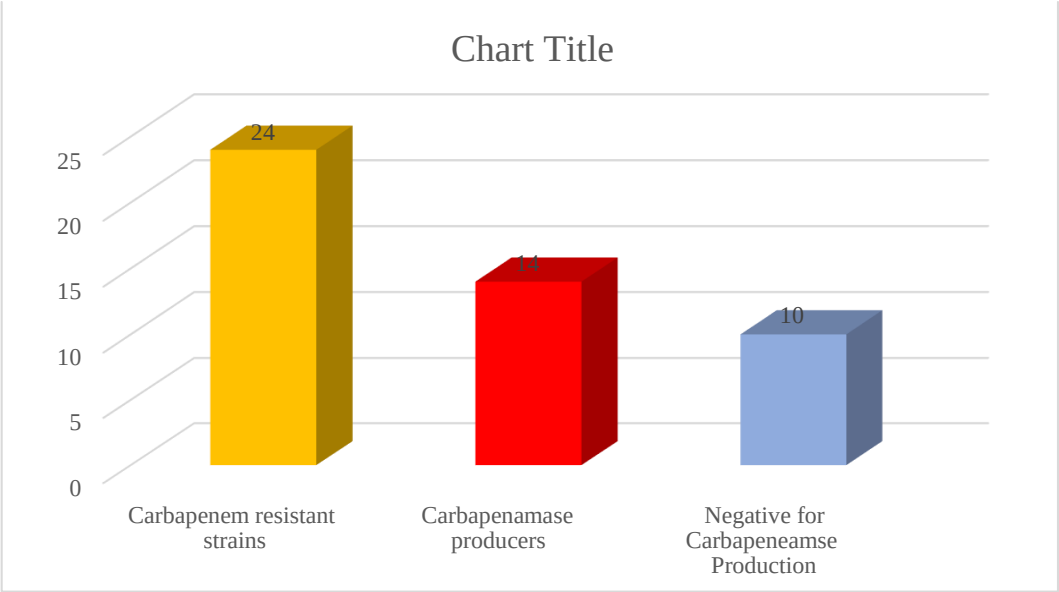
Fig 11: Carbapenamase producers among the Carbapenem resistant strains



Of the 100 isolates of P. aeruginosa, 24(16%) showed resistance to imipenem by the disc diffusion method. A total of 18 isolates were positive for MHT (75 %). Furthermore, 17 of these 24 isolates exhibited a significant zone size enhancement with the EDTA impregnated discs when compared with the plain antibiotic discs. Of these, only 14 isolates were positive for Ezy- MIC test (the ratio of the value obtained for Imipenem (IMP) : the value of Imipenem + EDTA (IMP+EDTA) was more than 8). The ATCC 27853 P.aeruginosa as expected did not exhibit a zone size enhancement.Among 24 isolates, blaNDM-1 was detected in none of the isolates. Whereas blaVIM was isolated among 11 isolates.(Fig-3).

PCR RESULTS

Among 24 isolates, *bla*$_{\text{NDM-1}}$ was detected in none of the isolates. Whereas *bla*$_{\text{VIM}}$was isolated among 11 isolates.(Fig-3).

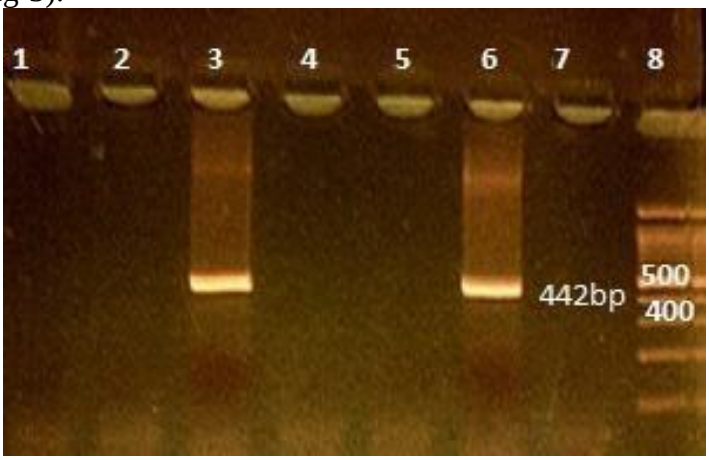


Fig 12: PCR, electrophoresis gel image demonstrating blaVIM-1 gene L- 8: molecular mass marker (100bp DNA ladder), L- 7: Negative control, L- 6: Positive Control, L- 3: Test sample- Positive, L- 1,2, 4 & 5: Test sample- Negative

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DISCUSSION

Pseudomonas aeruginosa is one of the leading nosocomial pathogens worldwide. Nosocomial infections caused by this organism are often hard to treat because of both the intrinsic resistance of the species (it has constitutive expression of AmpC b-lactamase and efflux pumps, combined with a low permeability of the outer membrane), and its remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents, including b-lactams, aminoglycosides and fluoroquinolones [13].

P. aeruginosa represents a phenomenon of bacterial resistance, since practically all known mechanisms of antimicrobial resistance can be seen in it:derepression of chromosomal AmpCcephalosporinase; production of plasmid or integronmediated b-lactamases from different molecular classes (carbenicillinases and extendedspectrum b-lactamases belonging to class A, class D oxacillinases and class B carbapenemhydrolysing enzymes); diminished outer membrane permeability (loss of OprD proteins) [14]; overexpression of active efflux systems with wide substrate profiles; synthesis of aminoglycoside modifying enzymes phosphoryltransferases, acetyltransferases and adenylyl transferases); and structural alterations of topoisomerases II and IV determining quinolone resistance[15,16]. Worryingly, these mechanisms are often present simultaneously, thereby conferring multiresistant phenotypes. This review describes the known resistance mechanisms in P. aeruginosa to the most frequently administrated antipseudomonal antibiotics: b-lactams, aminoglycosides and fluoroquinolones.

The advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections. They have a broad spectrum of activity and are stable to hydrolysis by most of the beta lactamases, including the extended spectrum beta lactamases (ESBL) and the Amp C beta lactamases. In recent years there has been an increase in carbapenem resistance among Gram-negative bacteria in the Indian subcontinent [12,13].

Resistance mechanisms include lack of drug penetration (i.e., porin mutations and efflux pumps) and/or carbapenemhydrolyzingbetalactamase enzymes [2]. Over the past few years MBL producing isolates have emerged worldwide and are associated with outbreaks in health-care settings. They cause serious infections such as bacteremia and ventilator associated pneumonia, particularly in patients admitted to the ICU. The CLSI has not recommended any standardized phenotypic methods for screening MBL in clinical isolates. We screened for carbapenamases production by Modified Hodge Test and MBL production by inhibitor-based methods using EDTA as the inhibitor. Of the 100 isolates studied, MHT was positive in 18 indicating the production of carbapenamases. The remaining 6 were MHT negative, thereby suggestive of other mechanisms such as loss of porins or upregulation of efflux pumps [17].

Among the 24 study isolates, the MBL screen test was positive in 70.8% (n = 17). PCR detected the MBL genes bla VIM in 45.8% (n = 11) and the MBL screen test was positive in all these isolates. In 6 isolates that were MBL screen test positive, bla VIM/blaNDM were not found, suggesting the presence of other MBL genes such as SPM-1, GIM-1, SIM-1 or IMP-1 [18]. Despite the good performance of inhibitorbased methods for the detection of MBL by using EDTA, it is not a specific test. False positive results have been reported in P. aeruginosa as EDTA acts on the membrane of the bacterial cell and increases the cell permeability. The overall bla VIM/blaIMP production among the study isolates was 51.4%. These results indicate that carbapenem resistance in P. aeruginosa is mainly due to MBL production.

There are reports on MBL production in P.aeruginosa from various countries like Brazil, Korea, Singapore, and France. Metallobetalactamase was first reported as a zinc dependent enzyme in Bacillus cereus in mid 1960s. A few decades later, imipenem-hydrolyzingmetallo enzymes were

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found in Aeromonashydrophila and Bacteroidesfragilis. All these enzymes were produced by chromosomal genes and at first were recovered only from single clinical isolates [20]. In 1991, Japan reported the first Plasmid-mediated metallobetalactamase in P. aeruginosa [21].
This was soon followed by another report of transferable metalloenzyme in B. fragilis. Apart from P. aeruginosa, other bacteria like Serratia, Klebsiellapneumoniae, Escherichia coli, Enterobacteraerogenes, E. cloacae, Citrobacterfreudii, Proteusvulgaris, P. putida, Acinetobacter and Alcaligenesxylosoxidans were also shown to produce MBL. blaNDM was not found in any of the isolates. The common MBL genotype was the bla VIM (n =11). In Asia, blaIMP and blaVIM are prevalent. blaIMP is found mainly in Japan, Korea, China, Taiwan, and Iran [22-24]. The prevalence of MBL in India has ranged from 7% to 65% among carbapenem-resistant P. aeruginosa. In a study from India, the rate of MBL production was 24.5% among 61 P. aeruginosa isolates, and bla VIM type was the most common [25]. Another study from India also reported blaVIM-2 from P. aeruginosa [26]. In a nation-wide survey conducted to characterise 301 MBL producing Pseudomonas species in 10 medical centres from India, the MBL genes were detected in 18.9% of the isolates and 5 VIM variants were reported with VIM-2 being the most common. The others were VIM-6, VIM-11, VIM-5 and VIM-18 [27].

Regarding resistance profiles, all isolates were resistant to other classes of antimicrobial agents such as aminoglycosides and fluoroquinolones. Polymyxins predominate as the mainstay of treatment for P.aeruginosa with susceptibility of 97%. In this study, blaVIM production contributes to 45.8% of carbapenem resistance. Hence early detection of MBL producing organisms is important to guide in the treatment of infections caused by them and also to arrest their spread. In the clinical microbiology laboratory, all clinical isolates that are resistant to carbapenems must be screened for carbapenamase and MBL production by using simple phenotypic tests like E-test. To conclude, carbapenem resistance in P. aeruginosa is chiefly mediated by MBL production. The common MBL gene is blaVIM45.8%(n=11). The development of simple and inexpensive screening methods to detect carbapenamases and MBL production in microbiology Laboratories is crucial for optimal treatment of patients, particularly critically ill and hospitalized patients, and to control the spread of resistance.

CONCLUSION

Carbapenem resistant P. aeruginosa are resistant to almost all beta lactams except monobactams .In addition they are also resistant to fluoroquinolones and Aminoglycosides. Polymyxin B and Colistin are the only available treatment options for carbapenem resistant strains of P.aeruginosacarbapenem resistance in P. aeruginosa is chiefly mediated by MBL production. E-test is gold standard phenotypic method to detect MBL production. The common MBL gene is blaVIM. NDM mediated MBL resistance is rare in P. aeruginosa.

Funding: No funding sources

Ethical approval: The study was approved by the Institutional Ethics Committee

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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