T. Mullai et al /J. Pharm. Sci. & Res. Vol. 11(8), 2019, 2963-2970

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Isolation and characterization of *Pseudomonas aeruginosa* from exudates and determination of phenotypic and genotypic makers of drug resistance

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Abstract:

Pseudomonas aeruginosa is an environmentally ubiquitous Gram-negative bacterium and is an important opportunistic human pathogen that often causes nosocomial infections which can be serious and often life-threatening. Emergence of Multidrug-Resistant (MDR) Gram-negative bacteria pose a serious crisis across the globe especially the Carbapenem resistance and the detection of the underlying resistance mechanisms is critical for the better management of this threat. The Samples collected were subjected to Microscopy, biochemical identification and Antibiotic susceptibility testing and among the 75 *Pseudomonas aeruginosa* isolates obtained, 32 were positive for ESBL and 30 for MBL. These isolates were then subjected to Molecular detection of SHV and IMP gene by employing uniplex PCR.78% of the ESBL positive isolates were positive for SHV gene and 73% of the MBL producers were positive for IMP gene by genotypic method. Hence the combination of genotypic and phenotypic detection for Carbapenem resistance can aid in the surveillance and treatment of the *Pseudomonas aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa,* Carbapenem resistance, Cystic fibrosis, Urinary tract infections, MBL, ESBL, PCR.

INTRODUCTION:

Pseudomonas aeruginosa is typically an opportunistic pathogen. Normally, for an infection to occur, some disruption of the physical barriers (skin or mucous membranes), or by-passing of them (e.g., by urinary catheters (1) endotracheal tubes or other invasive devices), and or an underlying dysfunction of the immune defence mechanisms, such as neutropenia, is necessary. *P. aeruginosa* is mostly a nosocomial pathogen. According to data from the Centers for Disease Control and Prevention and National Nosocomial Infection Surveillance System, in the USA, *P. aeruginosa* was the second most common cause of nosocomial pneumonia,the third most common cause of nosocomial Urinary Tract Infections, and the Seventh most common cause of nosocomial bacteraemia (2). *P. aeruginosa* can, in rare circumstances, cause pneumonias, as well as ventilator-Associated pneumonias, being one of the most common agents isolated in several Studies. Pyocyanin is a virulence factor of the bacteria. However, salicylic acid can inhibit pyocyanin production. One in ten hospital-acquired infections is from *P. aeruginosa*. Cystic fibrosis patients are also predisposed to *P. aeruginosa* infection of the lungs. *P. aeruginosa* is also a common cause of post operative infection in radial skeratotomy surgery patients.

The organism is also associated with the skin lesion Ecthyma gangrenosum. *P. aeruginosa* is frequently associated with osteomylities involving puncture wounds of the foot, believed to be a result from the direct inoculation of *P. aeruginosa* via the Foam padding found in tennis shoes, with diabetic patients at a higher risk (3). Infections due to multidrug-resistant (MDR) Gram-negative bacteria have emerged as a health care crisis across the globe. Carbapenems are the last line of defense against many drug-resistant bacterial infections. Unfortunately, infections due to Carbapenem-resistant (CR) pathogens are on the rise. Carbapenem-resistant Enterobacteriaceae (CRE) has garnered particular attention in the popular press and scientific literature, in part due to several high-profile outbreaks of infection. At our center and others, however, CR *P. aeruginosa* is recovered more commonly from patients than CRE. Mechanisms of carbapenem resistance vary considerably between CRE and CR *P. aeruginosa*. CR in CRE is mediated by carbapenem-hydrolyzing enzymes, including Ambler class A (e.g., KPC), B (e.g., IMP, VIM), and D (e.g., OXA) beta-lactamases. Plasmid-Mediated -lactamases typically are present along with aminoglycoside modifying Enzymes (AMEs) and other resistance determinants.

Accordingly, CRE are generally resistant to most of the classes of antimicrobials except for salvage agents such as the polymyxins (4) and tigecycline; Unfortunately, the use of polymyxins and tigecycline is limited by high rates of toxicity and suboptimal pharmacokinetics, respectively. Ceftazidime-avibactam has activity against CRE, but the clinical experience is limited. In addition, recent studies have demonstrated that resistance to ceftazidime-avibactam can develop during or after treatment with this agent. Carbapenem resistance in *P. aeruginosa* stems from a combination of -lactamases (especially AmpC), porin mutations, MexA-MexB-OprM efflux pump Overexpression, and/or penicillin-binding protein alterations. Combinations of mechanisms confer reduced susceptibility to carbapenems, but other -lactam agents and aminoglycosides may retain in vitro activity (5). *P. aeruginosa* is an opportunistic, hospital-acquired pathogen that causes severe diseases in immuno-compromised individuals including urinary Tract infection. Urinary tract infections (UTIs) are some of the most frequent Bacterial infections, affecting 150 million people annually worldwide. Despite advances in anti-microbial therapy, the mortality and morbidity associated with *P. aeruginosa*

induced UTIs remain significantly high. One key reason for therapy failure is the increased level of antibiotic resistance among clinical *P. aeruginosa* isolates (6). Thus, the detection of the underlying resistance mechanisms is critical for better management of this problem. Therefore the aim of the present study was to isolate and characterize *P. aeruginosa* from exudates and determination of drug resistance profile of the isolates by phenotypic and genotypic methods.

MATERIALS AND METHODS:

Study population:

This study was done as a descriptive study during a time period of three months (June 2018 to August 2018) at Saveetha Medical College and Hospital, Thandalam, Chennai, Tamil Nadu. Samples collected from outpatient and in- patient departments of Saveetha Medical College and Hospital were processed and the isolates were used for the study. The study protocol was approved by the Institutional Human Ethical Committee (No: SMC/IEC/2018/04/077).

The samples collected were inoculated in media like Blood agar, Mac conkey agar chocolate agar and was incubated at 370C for 18 hours. Bacterial colonies were observed and identified using standard protocol. The genus Pseudomonas aeruginosa was confirmed by Gram stain, Oxidase and Catalase and biochemical tests (Indole, TSI, citrate, Urease, Mannitol motility). Susceptibility pattern was determined by Kirby- Bauer disk diffusion method for the standard panel of antibiotics and the isolates resistant to Ceftazidime (30µg), Carbapenem were subjected to (include the tests which u have done for phenotypic) .The genotypic analysis was done to confirm the Carbapenem resistance

Sample collection and processing:

Collected exudates (Pus, wound swab, Ear swab, body fluids) samples were processed in microbiology laboratory.Sample processing was done in three steps;

1. Microscopy
2. Identification of bacteria by appropriate biochemical tests.
3. Antibiotic susceptibility testing

Identification by biochemical tests:

Catalase test:

Slide test: pure growth from nutrient agar was transferred to a clean slide by a wooden stick and immediately a drop of Hydrogen Peroxide was added to the growth and the effervescence was noted.

Oxidase test:

A streak of the pure culture was made onto a filter disk infused with the substance tetra methyl para phenylene diamine dihydrochloride which was oxidized to indo phenol, a dark purple colored end product in the presence of oxidase.

Biochemical characteristics of important oxidase positive, motile Pseudomonads

	Pyoverdin	Glucose	Maltose	Lactose	Arginine	Lysine	Urea	ONPG	DNAase
P. aeruginosa	+	+	V	-	+	-	V	-	-
P. fluorescens	+	+	V	-	+	-	-	-	-
P. putida	+	+	V	-	+	-	V	-	-
P. stutzeri	-	+	+	-	-	-	V	-	-
P. cepacia	-	+	+	+	-	+	V	V	-

Antibiotic sensitivity testing:

Antibiotic susceptibility testing was done by Kirby Bauer Disc Diffusion method according to 2018 CLSI guidelines. The antibiotics used were Amikacin Gentamicin, Ciprofloxacin, Ceftazidime, Piperacillin-tazobactum, Cefeperazone–Sulbactum, Imipenem, Meropenem (7).

Mueller Hinton Agar:

Lawn culture of the organism was made on a sterilized Mueller Hinton agar from standard inoculums. Readymade antibiotic discs were placed on the surface of inoculated plates. Then the plates were incubated at 37°C for 24 hrs. After incubation, the plates were observed for the zone of inhibition. An inhibitory zone around the disc indicated that the growth of the organism was inhibited by the antibiotics. The results were compared with the standard charts and were tabulated.

Phenotypic confirmatory disc diffusion test (PCDDT) for ESBL detection:

A Mueller Hinton agar plate was taken and a lawn culture of Pseudomonas aeruginosa was made. Then ceftazidime (30µg) disc along with ceftazidime clavulanic acid (10µg) were placed at a distance of 2 cm from each other on the plate and incubated aerobically at 37°C overnight. A ≥ 5mm increase in zone diameter for Ceftazidime clavulanic acid than the ceftazidime confirms that the organism was a ESBL producer by PCDDT.

Combined disc test for Metallo beta lactamase (MBL) production:

The combined disk test Imipenem-EDTA (IMP-EDTA) was performed as the test organisms were inoculated on to plates with Muller Hinton agar as recommended by the CLSI. Two 10 µg imipenem disks (Hi-Media) were placed on the plate, and appropriate amounts of 10 µl of EDTA solution was added to one of them to obtain the desired concentration (750 µg). The ≥ 7 mm inhibition zone size with the IMP-EDTA disc than the Imipenem disc alone, was considered as positive for MBL production.

Isolates which 30 were positive for ESBL and MBL were selected and further subjected to genotypic markers analysis. The gene SHV was amplified for the confirmation of ESBL production and the IMP gene was amplified for the MBL production.

Polymerase Chain Reaction:

Molecular methods to Detect carbapenemase Gene and extended spectrum of beta lactamase Gene:

Uniplex PCR was used for the detection of carbapenemase gene bla SHV for ESBL and IMP gene for MBL (Table-1). The spectroscopy assay to detect hydrolysis of Carbapenem followed by PCR to identify bla SHV and IMP gene is the gold standard to confirm the SHV enzymes and IMP enzymes. However, the limitations of this genotyopic method are that time consuming procedure for a clinical microbiology laboratory and usually requires verification of isolates in a reference laboratory.

Pure culture isolated from clinical specimens was further processed for molecular detection of drug resistance genes targeting. The steps involved in Uniplex PCR were extraction, amplification and gel electrophoresis.

The DNA was extracted as per the manufacturer's protocol (pure Fast R spin column).The bacteria from culture plate was drawn using an inoculation loop and suspended in 180 µl of digestion Buffer and 20µl of Lysozyme was added .The solution was first vortexed and then incubated at 37 °C for 15 min until the cell was completely lysed. Then 1.5 mL micro centrifuge tube was centrifuged to remove the droplets present on the lid. Then 400µl of binding buffer and 5µl of internal control template and 20µl of protein k was added and mixed well by inverting several times. The samples was incubated at 56 ºC for 15 min and then centrifuged. 300µl of Ethanol was added to the sample and mixed by vortexing. The mixture was briefly centrifuged and then the contents were transferred to a pure Fast R spin column. The tubes were centrifuged at 8000 rpm for 30-60 seconds and the flow through was discarded and the column was placed back onto the same collection tube. The pure Fast R spin column was placed in a clean 2ml collection tube. The filtrate was discarded and 500µl 0f buffer was added to the tube, centrifuged at 8000 rpm for 1 mins. The filtrate was discarded and the column was placed in a fresh collection tubes 500µl of wash buffer was added and then centrifuged at 14000 rpm for 3 min. The tube was again centrifuged at full speed for 1min after transferring the column to a new collection tube. The filter was discarded and then 200 µl of buffer was added and the tube was incubated at room temperature for 1 mins. Then step was repeated once again. The filtrate contains DNA.

The primers were designed and used as per requirement of gene to be amplified. The reagents required were 2U of Taq DNA polymerase, 10x Taq, reaction buffer, 2mm, $MgCl_2$, 1 of 10mM, dNTps Mix and red Dye and PCR additives.

Table 1: Sequences of primers used in Uniplex PCR

Gene	Sequence
IMP	5' - GAAGGCGTTTATGTTCATAC -3' 5' - GTATGTTTCAAGAGTGATGC -3'
bla SHV	5'- CCATCTGGCC CCAGTGCTGC -3' 5'- CCCGCAGCCGCTTGAGCAAA -3'

Detection of amplified products:

The Uniplex PCR products were then analyzed by electrophoresis at 100 V on 2% agarose gels stained with Ethidium bromide (0.5 µg/ml). The gel was then visualized in a Gel Docking Apparatus. The base pairs of the product obtained were determined by comparing it to the ladders respective base pair units.

RESULTS:

The present study was conducted at Clinical Microbiology Laboratory of Saveetha Medical College and Hospital during the period of May 2018 to July 2018. A total of 345 exudate samples (pus, wound swabs vaginal swab and ear swabs) were received in the Clinical Microbiology Laboratory and these were included in the study. Out of 345 samples received, 155 (45%) were wound swabs, 52 (15%) were ear swabs, 82 (24%) were pus and 56 (16%) were vaginal swabs. Sample wise distribution is given in figure 1.

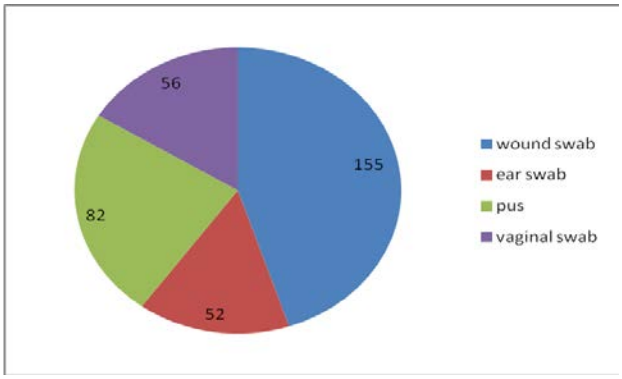

Fig. 1: Sample wise distribution of total samples

Among the 155 wound swabs, 96 (62%) were from males and 59 (38%) were from females. Out of the 52 ear swabs, 34 (65%) were from males and 18 (35%) were from females. Among the 82 pus samples, 47 (57%) were from males and 35 (73%) were from females and 56 (100%) vaginal swabs were from female patients (Table 2).

Table 2: Sex wise distribution of samples

Samples	Male	Female	Total
Wound swab	96	59	155
Ear swab	34	18	52
Pus	47	35	82
Vaginal swab	0	56	56

Among 345 samples, 20 (6%) samples were from persons aged between 0 and 10, 31 (9%)were from persons aged between 11 and 20, 25 (7%) were from persons aged between 21 and 30, 52 (15%) samples were from persons aged between 31 to 40. 78 (23%) were from persons aged between 41 and 50, 74 (21%) were from age group 51-60, 40 (12%) were from persons aged between 61and 70, 20 (6%) samples were from age group 71-80 and 5 (1%) were from age group 81-90. This age wise distribution is given in figure 2.

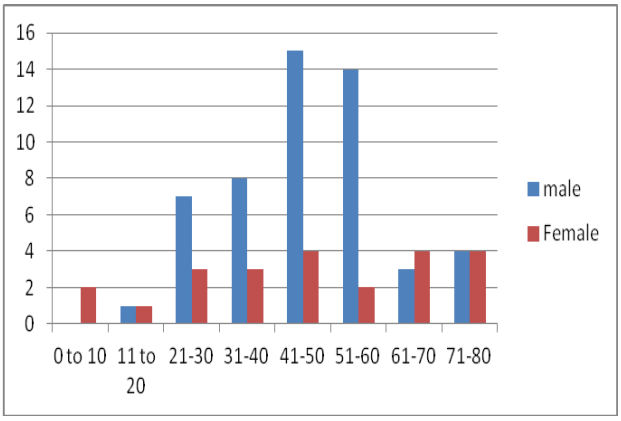

Fig. 2: Age wise distribution of total samples

Among the 345 samples, 168 were from the Surgery department, 17 were from Ortho department, 92 were from General Medicine, 7 were from Obstetrics and Gynaecology, 11 were from ENT, 6 were from ICU, 13 were from Cardiology, 14 were from Emergency, 11 were from Dermatology and 6 were from Special Ward (Table 3).

Table 3: Ward wise distribution of total samples

Department	Total number of samples
Surgery	168
Ortho	17
General medicine	92
Obstetrics and gynaecology	7
ENT	11
ICU	6
Cardiology	13
ER	14
Dermatology	11
Special ward	6

Pesudomonas aeruginosa was isolated from 75 (22%) of the clinical specimens. The distribution of *Pseudomonas aeruginosa* is given in figure 3.

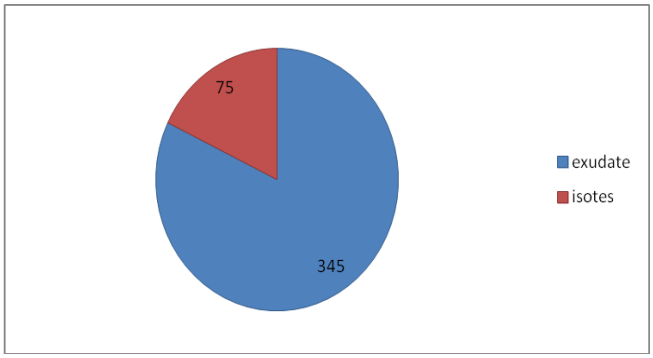

Fig. 3: Prevalence of *Pseudomonas aeruginosa* from total exudates

Number of isolates from wound swabs were 69 in which 51 (74%) were from males and 18 (26%) were from females, 3 were from Ear swab in which 1 (33%) was from male and 2 (67%) were from females, 2 (100%) were pus from female patients and 1 (100%) was a vaginal swab from female patient. Sample wise distribution and sex wise distribution of *Pseudomonas aeruginosa* is given in figure 4.

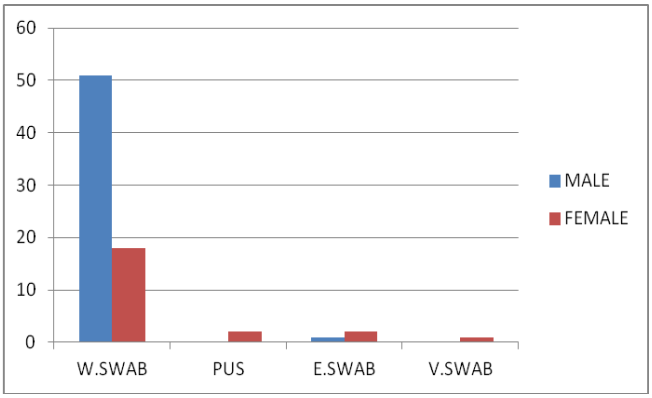

Fig. 4: Sample wise and sex wise distribution of *Pseudomonas aeruginosa*

Among the 75 Pseudomonas isolates, 5 samples were from persons aged between 0 and 10, in which 3 were males and 2 were females, 4 were from persons aged between 11 and 20, in which 1 was male and 3 were females. 13 were from persons aged between 21 and 30, in which 8 were males and 5 were females. 15 samples were from persons aged between 31 to 40, 10 were males and 5 were females. 18 were from persons aged between 41 and 50, where 12 were males and 6 were females. 11 were from age group 51-60, were 9 were males and 2 were females. 9 were from persons aged above 60, in which 3 were males and 6 were females. This age wise and sex wise distribution is given in figure 5.

Among the 75 *P. aeruginosa* isolates, 45 were from the Surgery department in which 37 were males and 8 were females. 7 were from Ortho, in which 5 were males and 2 were females. 10 were from the General Medicine, in which 6 were males and 4 were females. 2 were from Obstetrics and Gynaecology, in which 2 were from females. 5 were from ENT, in which 2 were males and 3 were females. 2 were from ICU, 1 was from male and 1 was from female. 1 was from Cardiology department female patient. 1 was from Emergency, were it was from female. 1 was from Dermatology male patient and 1 was from Special ward female patient (Fig.6)

Out of the 75 *P.aeruginosa* isolates, 3 were sensitive to all the antibiotics that were tested. 32 isolates were resistant to ceftazidime and 30 were resistant to carbapenems 34 isolates were found to be Multi drug resistant. On analysing the antibiotic susceptibility pattern, among 75 isolates, 50 were sensitive to Amikacin and 25 were resistant. 37 were sensitive and 38 were resistant to Gentamicin. 43 were sensitive and 32 were resistant to Ceftazidime. 43 were sensitive and 32 were resistant to Piperacillin tazobactum. 42 were sensitive and 33 were resistant to Cefapearzone sulbactam. 21 were sensitive and 54 were resistant to Ciprofloxacin. 45 were sensitive and 30 were resistant to Imipenem. 53 were sensitive and 23 were resistant to Meropenem (Table 4).

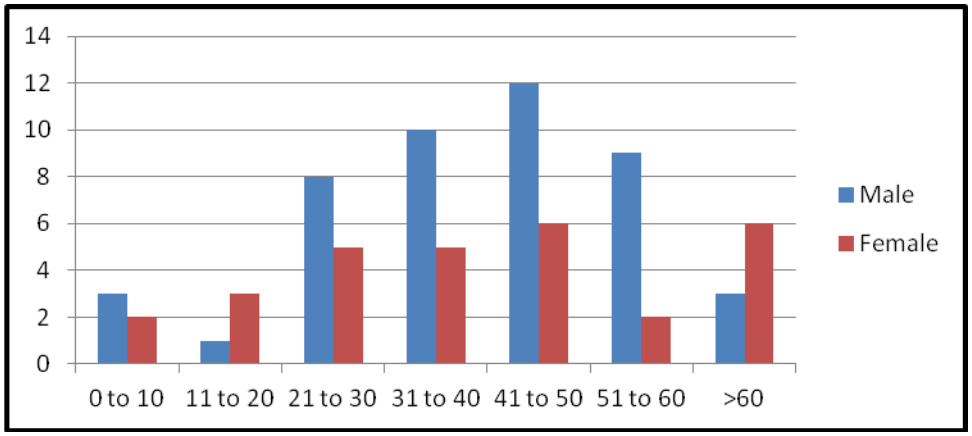

Fig. 5: Age wise and sex wise distribution of isolated *Pseudomonas aeruginosa*

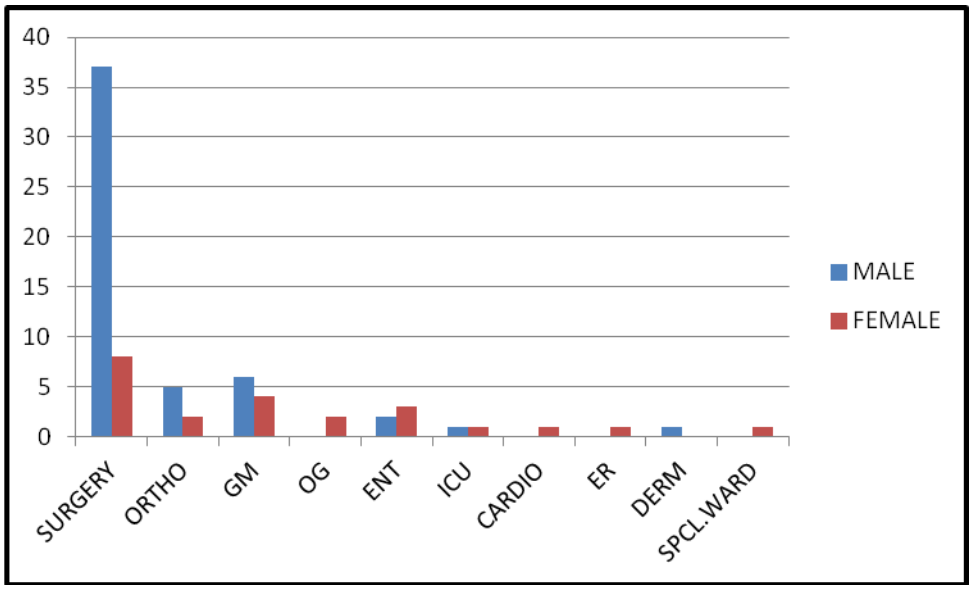

Fig. 6: Department wise distribution

Table 4: Antibiotic sensitivity pattern of *Pseudomonas aeruginosa*

Antibiotic	Sensitive	% Sensitive	Resistant	%. Resistant
Amikacin	50	67	25	33
Gentamicin	37	49	38	51
Ceftazidime	43	57	32	43
Piperacillin tazobactum	43	57	32	43
Cefeperazone sulbactum	42	56	33	44
Ciprofloxacin	21	28	54	72
Imipenem	45	60	30	40
Meropenem	53	71	22	29

Phenotypic Detection:

Out of 75 *Pseudomonas aeruginosa* isolates 32 (43%) were positive for ESBL (Fig.8).

Genotypic Detection of SHV and IMP:

The 32 ESBL producers were subjected to the genotypic detection of SHV. Out of which 25 (78%) were positive for SHV by genotypic method. Whereas 7(22%) of isolates identified as ESBL producer phenotypically were negative for SHV by genotypic method. These 7 isolates may have other gene (TEM, CTX-M) responsible for ESBL production, which is not done in this study (Fig.10).

The 30 MBL producers were subjected for genotypic detection of IMP. Out of which 22(73%) were positive for IMP by genotypic method. Whereas 8 (37%) of isolates identified as MBL producer phenotypically were negative for IMP by genotypic method. These 8 isolates may have other gene (VIM, NDM) responsible for MBL production, which is not targeted in this study (Fig.11). The amplified PCR product showed in Fig. 12 and 13.

Among the 75 *Pseudomonas aeruginosa* isolates, 30 (40%) were positive for MBL phenotypically (Fig.9)

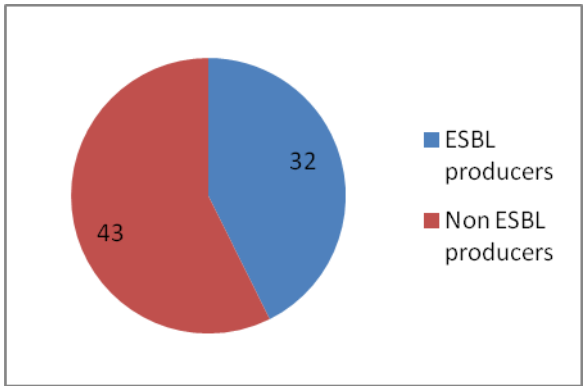

Figure 8: Phenotypic detection of ESBL

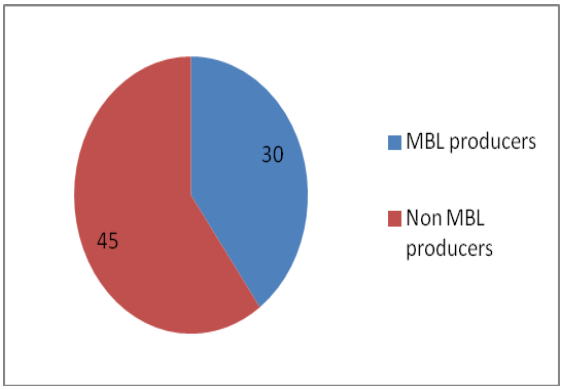

Fig. 9: Phenotypic Detection of MBL

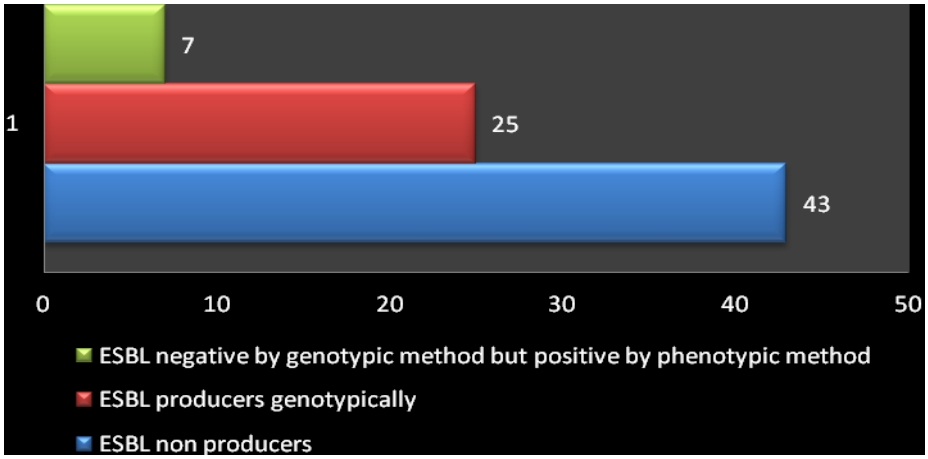

Fig. 10: Genotypic detection

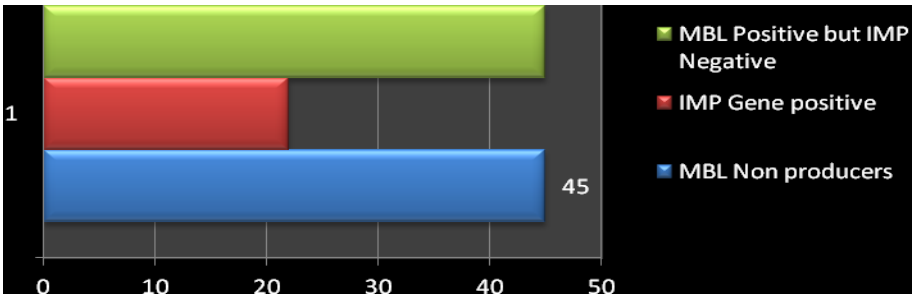

Fig. 11: Genotypic detection of IMP gene

Fig.12: SHV gene **Fig.13: IMP gene**

DISCUSSION:

In recent times, emergence of antibiotic resistance has threatened the effectiveness of many antibiotic agents and it is recognized as a public health threat. *P. aeruginosa* which has particular tendency for drug resistance has been reported to be associated with increased morbidity and mortality. Resistance to Carbapenem is one of the predominant features of *P. aeruginosa*. Infections due to carbapenem resistant *P. aeruginosa* is on rise worldwide (1). In our study a total of 345 exudate samples were collected. Out of which 75 (28%) isolates were *P. aeruginosa*. In a study conducted by Amani *et al.*,(9)33% of total exudates samples showed *P. aeruginosa* growth. The maximum number i.e., 92% of isolates in our study were from wound swabs followed by ear swab (4%), pus (3%) and vaginal swab (1%). In a study conducted by Anuradha *et al*.,(10) in 2014 showed that 39.39% of isolates were from pus samples. Another study by Javiya *et al.*,(11) reported highest number of *P. aeruginosa* from urine followed by pus and sputum. Our results are comparable with the study conducted by Nithyalakshmi *et al.*,(12)where 61% of the samples were from exudates (8). In our study, out of 75 *P. aeruginosa* isolates, 52 (69%) were from male patients and 23 (31%) were from female patients. In a similar study conducted by Manjunath *et al*.,(13)said that out of 156 culture identified *P. aeruginosa*, 72 (46%) were from male and 84 (58%) were from females.

In this study, the rate of isolation of *P. aeruginosa* was from persons aged 41-50 (24%) followed by persons aged between 31-40 (20%). A study done by Rajat *et al*.,(14)in 2012 showed that 29% of isolation rate was in the age group of 31-45. In a similar study done by Chander *et al*.,(15) shows 20% of isolation was from age groups of 21-40 years. In the present study, among the 75 *P. aeruginosa* isolates, 45(60%) were from Surgery department, 10(13%) were from General Medicine, 2(3%) were from Obstetrics and Gynaecology, 5(7%) were from ENT, 2(3%) were from ICU, each (14%) from Cardiology department, Emergency, Dermatology and Special Ward. Harshada *et al.*,(16) in a similar study says that 31.8% were from ICU patients, 23.8% were from surgical wards and 16.4% were from Burn units.

The antibiotic susceptibility pattern of the isolates showed that highest percentage of sensitivity was for Carbapenems with 60% for Imipenem and 71% for Meropenem. In the study by Vidhyarani *et al.*,(12) the susceptibility for Imipenem was 96.6%. In the study by Abbas *et al.,* (9)at Egypt the susceptibility to Imipenem was 90%. Both the studies have higher imipenem susceptibility compared to our study. After carbapenems, higher sensitivity percentage was for Amikacin with 66.6 % in our study. The study by Abbas *et al.,* (9)had a susceptibility percentage of 98% which was high compared to our study (5). In this study, Ceftazidime had susceptibility percentage of 57.3%. In the study by Mohanakrishnan et al.,(19) the susceptibility to Ceftazidime was 24.2% which was less compared to our study. In the study by Abbas *et al.,* all the isolates were resistant to Ceftazidime (5).

The sensitivity percentage of Piperacillin-tazobactum was 57.3%. In the study by Ganiny *et al.*,(20) the susceptibility of Piperacillin –tazobactum was 64% and in the study by Sumita *et al.,* (21)the susceptibility was 86.2%. The sensitive to the quinolone Ciprofloxacin was very low with a percentage of 28%. In contrast in the study by Kamel *et al.*,(22)the sensitive to Ciprofloxacin was 92%. According to the antibiotic sensitive pattern of our isolates, the carbapenems and Amikacin have the highest susceptibility percentage. The highest resistance percentage was for Ciprofloxacin (6). The ESBL percentage in our study was 32 (43%) phenotypically. In the study by Mohankrishnan *et al.*,(19)the percentage of ESBL was 21.96%. Aggarwal *et al.,* (23) and Shaikh *et al.,* (24)had ESBL rate of 20.3% and 25.13% in their studies respectively. High prevalence of ESBL producing *P. aeruginosa* was reported by Vijay Mane *et al.,* (25)Varun Goel *et al.*,(26) and Silpi Bask *et al.,* (12)who observed 57%, 42.3% and 40% respectively. In contrast to our study, Abbas *et al.,* (9)reported 0% ESBL producers. The MBL percentage in our study was 30 (40%) phenotypically. Gupta *et al.,* (27)in their study said that MBL production ranged from 7% to 65%. MBL producers in the studies of Navneeth, Mendiratta and Hemalatha were 12%, 8.62% and 14% respectively. The prevalence of SHV by genotypic method was found to be 25 (33%) in our study. The prevalence of IMP by genotypic method was found to be 22 (29%) in our study.

CONCLUSION:

P. aeruginosa strains harbouring Carbapenem resistance mechanisms compromise severely the selection of appropriate treatments because of the fact that Carbapenem resistance is commonly associated with resistance to other antibiotic classes. In this study ESBL and MBL positivity by phenotypic method has shown to be 78% and 73%. Thus phenotypic method of combined disc test for ESBL and MBL can be adopted for checking the prevalence of ESBL and MBL in hospital surveillance programme. Thereby detection of carbapenem resistant strains and the implementation of strict infection control measure help in controlling the nosocomial infection rate in our set up.

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