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Prevalence of Pseudomonas aeruginosa isolated from clinical specimen with reference to biofilm formation and detection of metallo-β-lactamase in a tertiary care rural hospital

Aparna Singh, Final MBBS part-1, Jawaharlal Nehru Medical College (DMIMS DU) Sawangi, Wardha, Maharashtra

Dr. Suvarna Sande (Tathe), Professor, Department of Microbiology Jawaharlal Nehru Medical College (DMIMS DU) Sawangi (Meghe), Wardha, Maharashtra

Corresponding Author: Dr. Suvarna Sande (Tathe), Professor, Department of Microbiology Jawaharlal Nehru Medical College (DMIMS DU) Sawangi (Meghe), Wardha, Maharashtra

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Abstract

Background: Pseudomonas aeruginosa is aerobic, gram-negative bacilli. It can survive with low levels of nutrients and grow in temperatures ranging from 4-42°C and has an ability to colonized many natural and artificial environments and has important property of biofilm formation. It is a paragon of opportunistic nosocomial pathogen, fabled for its multidrug resistance (MDR) and critical life threatening infections.

Objective: To determine prevalence, biofilm production and antibiotic susceptibility pattern of P.aeruginosa isolated from clinically significant samples.

Material And Method: The cross sectional study was carried for period of 3 month(ICMRSTS) in the microbiology department and included total of 100 P.aeruginosa isolated from clinically significant samples. These isolates were identified by standard procedures and were tested for phenotypic detection of biofilm formation and antibiotic resistance pattern.

Result: Maximum prevalence was seen in age group below 20 years(31 isolates), in males(75 isolates) and specimen wise in pus sample(30 isolates),department wise from surgery department(32 isolates). 81 isolates showed biofilm production by standardtube method. P.aeruginosa was highly resistant to Ceftazidime(74%) and least resistant to colistin(2%). 34% strains were MBL producers, 19% strains were AMPc producer, and both AMPc and MBL production were seen in 87 isolates and 56 were MDR P.aeruginosa.

Conclusion: Present study showed high prevalence of biofilm production and antibiotic resistance in P aeruginosa with MBL and AMPc production. Hence it is necessary to have regular surveillance of drug resistance in P aeruginosa which will be useful for selecting an appropriate antibiotic to know changing trends in antibiotic susceptibility pattern and for limiting the use of colistin and save it for the treatment of resistant and life threatening P. aeruginosa.

Keyword: Pseudomonas aeruginosa, MDR, AMPc. Metallo-β-lactamases

Introduction

Pseudomonas aeruginosa is highly versatile microorganism able to tolerate low oxygen conditions. It can survive with low levels of nutrients and grow in temperatures ranging from 4-42°C [1]and P. aeruginosa is an ubiquitous organism and they are found in soil, water, skin flora, and most man-made environments with worldwide distribution. It is present not only in normal environmental condition, but also in low-oxygen condition, thus has an ability to colonize many

natural and artificial environments. These characteristics allow it to attach itself and survive on medical equipment and on other hospital surfaces, which favors the beginning of infections in immuno compromised patients.[2]

It causes infections in hospitalized patients particularly in Immuno compromised patients, burn patient , orthopedic related infections, respiratory diseases, catheterized patients. It is a paragon of opportunistic nosocomial pathogen, fabled for its multidrug resistance (MDR) and critical life threatening infections.[3]This organism is often hard to treat because of both the intrinsic resistance and acquired resistance i.e. mutations in chromosomal genes, to multiple groups of antimicrobial agents, including β-lactams, aminoglycosides and fluoroquinolones and their property of biofilm formation.[4]Biofilm formation is an endless cycle, in which organized communities of bacteria are encased in a matrix of extracellular polymeric substances (EPS) that hold microbial cells together to a surface.[5]

Antimicrobial resistance is an innate feature of bacterial biofilms. Organism producing biofilm are far more resistant to antimicrobial agent than non producers. Bacterial biofilm cause chronic infection because they show increased tolerance to antibiotics and disinfectant as well as resisting phagocytosis and other component of body's defense**.** Many studies have shown that biofilm formation is higher in MDR strains [6]**.**Multidrug resistant (MDR) bacteria are well-recognized to be one of the most important current public health problems. The misuse and abuse of antibiotics are recognized to create selective pressure, resulting in the widespread development of resistant bacterial strains**.** Antibiotic resistance has been referred to as "*the silent tsunami facing modern medicine*"[7].Antibiotics have been found to be less effective in biofilm-growing bacteria. The prevalence of multidrug-resistant *P. aeruginosa* in a recreational environment may be important for immune-suppressed or other at-risk individuals, for whom treatment difficulties have greater implications. In P. aeruginosa , resistance to multiple drugs is usually the result of combination of different mechanism in a single isolate.

There is variety of mechanisms involved in the resistance of P. aeruginosa, among them over expression of efflux pump, acquisition of Extended-Spectrum β-Lactamases (ESBLs) and Metallo-β-Lactamases (MBLs); target site or outer membrane modification, porin mutations, plasmid enzymatic modification.[8]Generally, infections with multi drug-resistant *P. aeruginosa* often result in increased cost of treatment, lengthy stay, and overall morbidity and mortality.Hence the present study was under taken with aim to find out the prevalence of Pseudomonas aeruginosa isolated from clinical specimen , biofilm formation and antimicrobial susceptibility with special reference to detection of Metallo-β-Lactamase and AMPc production in a tertiary care rural hospital.

Aim

To find out the prevalence of Pseudomonas aeruginosa isolated from clinical specimen with reference to biofilm formation and detection of metallo-β-Lactamase in a tertiary care rural hospital.

Objective

The objectives of present study are:

1. To identify P.aeruginosa in various clinical specimen by conventional methods.
2. To demonstrate biofilm production in P.aeruginosa isolates by Standard Tube method.
3. To demonstrate, antimicrobial susceptibility of isolated P.aeruginosa using Kirby-Bauer disc diffusion method.
4. Co-relation and antibiotic resistant pattern of biofilm producer & non biofilm producer.

5. Prevalence of metallo-β-lactamase (MBLs) and inducible AMPc production.
6. Co-relation between production of metallo-β-lactamase (MBLs) & biofilm producer.

Material and methods

This cross sectional study was conducted after obtaining approval from Institutional Ethics Committee,in department of Microbiology in a tertiary care rural hospital for 3 months from June 2018 to august 2018(as this was a ICMR STS project).Since duration of study was 3 month out of which data collection was done in 2 months duration. 100 P aeruginosa isolated from various clinically significant samples were included in this study.

Inclusion criteria: P.aeruginosa isolates from various clinically significant sample like blood, urine, indwelling catheter, pus (infected bone and joint prosthetic implants, surgical site infectionsand body fluids).

Exclusion criteria: P.aeruginosa isolated from sample of OPD patient and sample from various environment sources were excluded**.**

Method: Direct microscopy which includes wet mount and gram staining were carried out on appropriate samples. Samples were cultured on Nutrient agar, Blood agar and MacConkey agar and incubated over night at 37^0C.Organisms isolated were identified according to the guidelines of Koneman (1975)[9] on the basis of colony characters, gram staining and biochemical test.

Slime production was detected by Qualitative method that is Standard Tube method [10] **.**Slime production in test strains were compared with reference strains:

P. aeruginosa ATCC 47085(Biofilm producer)

P. aeruginosa ATCC 27853 (Non biofilm producer)

In this method, test strains of P aeruginosa along with positive and negative control strains were inoculated into 5 ml of Trypticase Soya Broth medium taken in the sterile test tubes and incubated at 37ºC for 48 h.At the end of this period, the tubes were emptied without shaking and by using phosphate buffer saline (pH 7.3) the tubes were washed and then allowed to dry.The tubes were stained by using 5 – 6 drops of 0.25% saphranine and deionized . water was used to remove excess stain.Tubes were kept in inverted position and allowed to dry.

Slime production was determined by the development of a stained coating on the walls and the bottom of the tubes. Strains that developed stains in the form of rings at the air-liquid boundary will be excluded. Tubes were then examined and the amount of biofilm was scored as [10] **(Figure.1)**

- strong (+++)
- moderate (++)
- weak (+)
- absent (0)

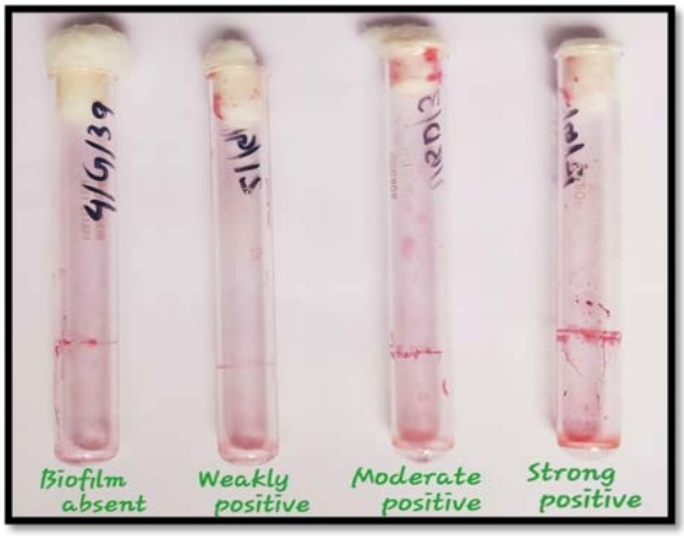


Figure 1: showing biofilm production by P. aeruginosa

Antibiotic Susceptibility profile of 100 P.aeruginosa strains isolated from different clinical samples was studied by Kirby Bauer Disk Diffusion method as per Clinical Laboratory Standard Institute (CLSI) guidelines.[11] Commercially available antibiotics discs (Himedia), Ceftazidime(CAZ)10 µg, Ciprofloxacin(CIP) 5µg,Amikacin(Ak)30µg,Imipenem(IMP)10µg,Piperacillin (PI)100µg,Piperacillin-tazobactum(PIT)10µg,

Netilin(NET) 30µg ,Meropenem (MER) 10 µg, Colistin(CL) 10µg/30 µg,Aztreonam (AT)30µgwere placed aseptically on the surface of Muller Hinton agar plate using sterile forceps and were gently pressed. The plates were incubated at 37^0 C for 16 -18 hours. Next day susceptibility profile of P. aeruginosa to different antibiotics were noted.

Multidrug resistance was defined as resistance to at least three drugs from a variety of antibiotic classes, mainly aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems and fluoroquinolones.[12]**(Figure2)**

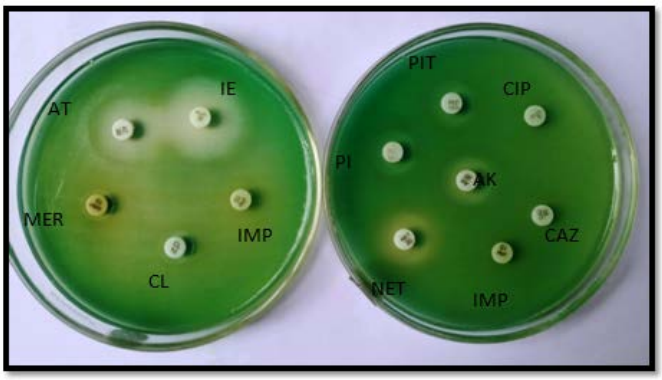


Figure 2: showing Metallobetalactamase (MBL) production and Multidrug resistance(MDR) in P aeruginosa

Detection of metallo beta lactamase was carried out according to CLSI guidelines.[11]. A plain imipenem disc(10µg) and imipenem plus 0.5M EDTA were taken , placed on the surface of agar plates approximately 30mm apart. The plates were incubated overnight at 37°C.An increase in zone diameter of ≥ 7mm around imipenem+EDTA disk in comparison to imipenem disk alone indicated production of MBL.

AMPc detection was carried out according to CLSI guidelines [11]. Then disc of 30 µg ceftazidime and 10 µg imipenem were placed at a distance of 20 mm apart .The plate were incubated overnight at 37°C.After overnight incubation, the plate was examined for any obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the imipenem disc. If there was any blunting or flattening of the zone, it was considered as a positive result for AMPc production.**(Figure 3)**

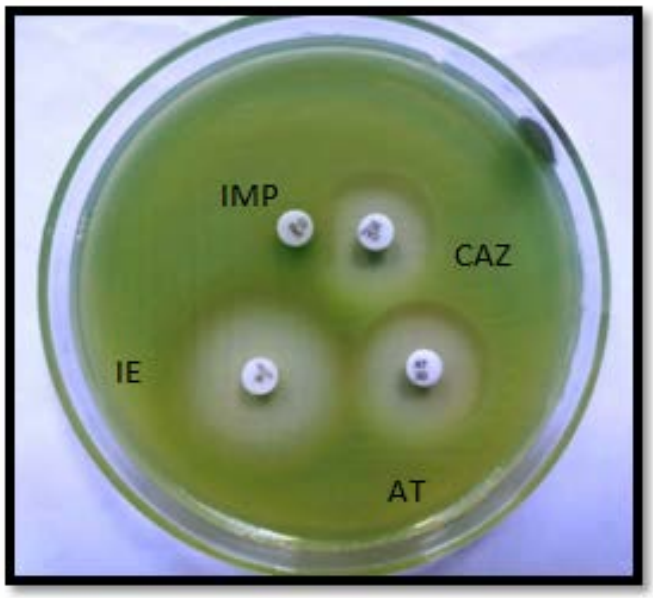


Figure 3: Showing MBL and AMPc production in P aeruginosa

Results

The present study was carried out in Department of Microbiology for period of 3 months from June 2018 to august 2018. Total 100 P aeruginosa isolated from different clinically significant samples were included in the study.

TABLE 1-Age Wise , Gender Wise And Specimen Wise Distribution Of P.Aeruginosa (N=100)			
Age Wise Distribution			
S. No	**Age Group**	**No Of Isolates**	**Percentages (%)**
1	<20	31	31%
2	21-30	12	12%
3	31-40	11	11%
4	41-50	17	17%
5	51-60	13	13%
6	>60	16	16%
	Total	**100**	
Gender Wise Distribution			
	Gender	**No of Isolates**	**Percentage (%)**
1	Male	75	75%
2	Female	25	25%
	Total	100	
Specimen Wise Distribution			
	Specimen	**No Of Isolates**	**Percentage (%)**
1	Pus	30	30%
2	Blood	25	25%
3	Urine	23	23%
4	Sputum	7	7%
5	Misc	15	15%
MISC=Miscellaneous Includes Secretion Like Body Fluid, Asicitic Fluid, Pleural Fluid, Tracheal Secretion And ET			

Table 1: Shows age wise, gender wise and specimen wise distribution of P.aeruginosa isolates. Out of 100 isolates 31 P aeruginosa were isolated from age group of below 20 year followed by 17 isolates from age group between 41-50 year and 13 isolates from age group 51-60 year and 61-70 year each. Gender wise distribution showed 75 isolates were from male patient and 25 isolates were from female patients. Specimen wise distribution showed that 30 isolates were from pus sample followed by 25 from blood sample and 23 isolates were from urine sample.

Graph: 1 Department wise distribution of P aeruginosa

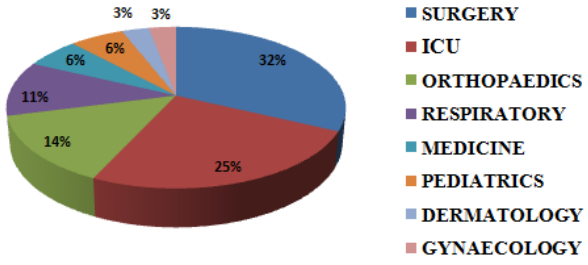
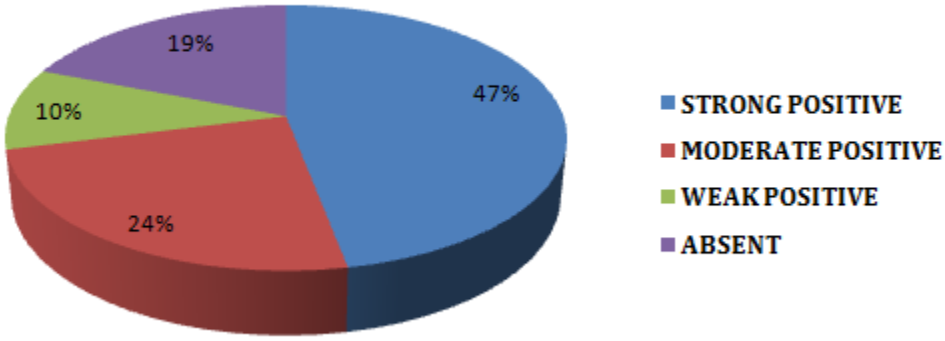


Figure 1 shows department wise distribution of P.aeruginosa. Out of 100 isolates, 32 isolates were from surgery department followed by 25 isolates from ICU and 14 isolates from orthopaedics department.

Graph: 2 Biofilm formations by P. aeruginosa



Graph 2: shows biofilm formation by P.aeruginosa. Out of 100 isolates, 81% showed biofilm production by Standard Tube method. Out of these 81 isolates, 47 were strong biofilm producer,24 were moderate biofilm producer 10 were weak biofilm producer and 19 isolate showed no biofilm formation**.**

Table 2-Antibiotic Resistance Pattern Of Biofilm Producer And Non Biofilm Producer P .Aeruginosa (N=100)

		Resistance				
s.no	**Antibiotics**	**Biofilm positive isolate (n=81)**		**Biofilm negative isolates (n=19)**		**Resistance of all isolates(n=100)**
1	Ceftazidime	67	90%	7	9.45%	74
2	Ciprofloxacin	40	76.92%	12	23.07%	52
3	Amikacin	34	80.95%	8	19.07%	42
4	Imipenem	24	75%	8	25%	32
5	Piperacillin	55	84.61%	10	15.38%	65

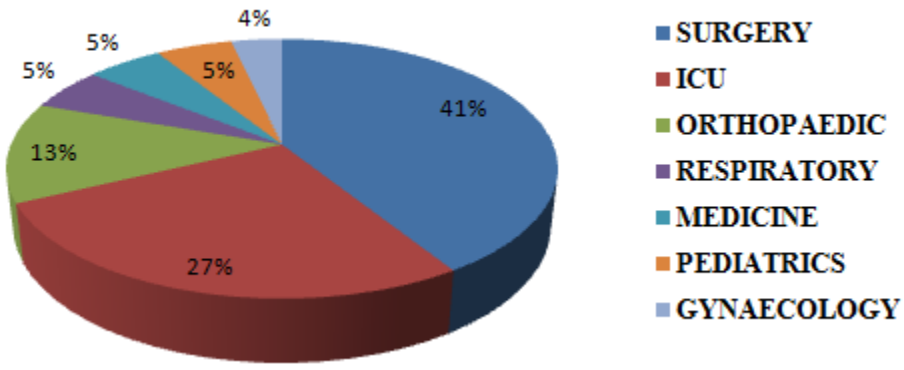
6	Piperacillin-tazobactum	45	84.90%	8	15.09%	53
7	Netilin	21	75%	7	25%	28
8	Meropenem	23	76.66%	7	23.33%	30
9	Colistin	2	100%	0	0%	2
10	Aztreonam	23	65.71%	12	34.28%	35
11	Norfloxacin*	19	95%	1	5%	20
***Norfloxacin was used in urine isolates only**						

Table 2: shows antibiotic resistance pattern of biofilm producer and non biofilm producer. From 81 biofilm producing P.aeruginosa 90% showed resistance to ceftazidime, 84.90% showed resistance to piperacillin tazobactum and 84.61% showed resistance to piperacillin , 80.95% resistance to amikacin ,76.92% were resistant to ciprofloxacin, , 76.66% were resistance to meropenem, 75% were resistance to imipenem and netilin each and 65.71% to aztreonam and 95% of urinary isolates were resistant to norfloxacin .

Table-3:Distribution of Imipenem resistance strains and MBL producing strain of P.aeruginosa	
Total no of P.aeruginosa isolates (n=100)	**Total no of MBL producing strains**
Imipenem resistant strain 32	28
Imipenem sensitive strain 68	6
TOTAL=100	34

Table 3 shows that out of 100 P aeruginosa isolates 32 were Imipenem resistant and out of which 28 were MBL producer. 68 isolates were Imipenem sensitive out of which 6 were MBL producer. Though it was not our objective but we have screened for inducible AMPc producer by using Imipenem 10µg and Ceftazidime 30µg disc placed at distance of 20 mm apart. We got flattening of zone of inhibition between Ceftazidime disc and Imipenem disc in 19 isolates. Both MBL and AMPc were seen in 8 isolates.

Graph 3: Department wise distribution of multidrug resistant (MDR) P aeruginosa (n=56)



Graph 3: shows the department wise distribution of MDR P.aeruginosa. Out of 100 isolates 56 were MDR and among these 56 MDR isolates 23 (41%) isolates were from surgery department followed by 15(27%) isolates

from ICU and 7(13%)isolates from orthopaedic department were MDR.

Table 4-multidrug resistant isolates of p aeruginosa showing biofilm formation			
S.No	**Biofilm**	**No of MDR Isolates**	**%**
1	Strong Positive(47)	33	70.21%
2	Moderate Positive(24)	18	75%
3	Weak Positive(10)	3	30%
4	Negative(19)	2	10.52%
	Total	56	100

Table 4 Shows that out of 47 strong biofilm forming P.aeruginosa 33 isolates were MDR, out of 24 moderate biofilm forming P aeruginosa 18 isolates were MDR, and out of 10 weak positive biofilm forming P aeruginosa 3 isolates were MDR. Out of total 13 non biofilm producer P aeruginosa 2 isolates were MDR.

Table 5: Specimen wise distribution of biofilm formation MBL and Multidrug resistance(MDR) P.aeruginosa

	Specimen	**No of Isolates**	**Biofilm Formation**				**MBL**	**%**	**MDR**	**%**
			SP	**MP**	**WP**	**ABS**				
1	PUS	30	15	8	4	6	15	50%	20	66.66%
2	BLOOD	25	12	7	2	4	8	32%	15	60%
3	URINE	23	11	6	3	3	6	26.08%	12	52.17%
4	SPUTUM	7	3	1	1	1	2	28.57%	3	42.85%
5	MISC	15	6	2	0	5	3	20%	6	40%
	TOTAL	100	47	24	10	19	34	100	56	100

MISC=MISCELLANEOUS includes secretion like body fluid, ascitic fluid, pleural fluid,ear swab,tracheal secretion and ET

- SP=STRONG POSITIVE
- MP=MODERATE POSITIVE
- WP=WEAK POSITIVE
- ABS=ABSENT

Table 5 shows that out of 30 isolates from pus sample 27 isolates were biofilm producer ,15 were MBL producer and 20 were MDR Pseudomonas aeruginosa. Out of total 25 isolates from blood 21 isolates were biofilm producer 8 isolates were MBL producer and 15 were MDR. Out of 23 urine isolates 20 were biofilm producer 6 isolates were MBL producer and 12 were MDR.

Discussion

❖ **Prevalence of P.aeruginosa**

P.aeruginosa is a species of substantial medical importance and is a prototypical multidrug resistance pathogen and is recognized for its ubiquity , and is notorious for being intrinsically advanced antibiotic resistance .Although wide variety of antimicrobial agents

with antipseudomonal activities are available, life threatening infections caused by P. aeruginosa continue to be prevalent. It is reason for high fatality rate as it has arisen as a vital pathogen. [13]

In present study, a total of 100 P.aeruginosa were isolated from various clinically significant samples, from the hospitalized patients and their antimicrobial susceptibility and MBL production were determined. Most of the isolates belonged to younger age group of <20 years (31%) followed by elderly age group of 41-50 years (17%) and older age group of >60 year (16%). This finding was in accordance with the study of Olayinka AT et al [14] with 30.18% below age group of 20 year.

In present study, prevalence of P. aeruginosa infection was found to be more in males (75%).This finding was in accordance with study of Ahmed et al.(77.7%) [15]and Raakhee et al. (66.78%).[16] The reason for male preponderance can be due to more outdoor activity, personal habits, nature of work and exposure to soil, water and other areas which are inhabited by organism. Present

❖ **Antibiotic susceptibility**

Study showed the maximum P aeruginosa was isolated from pus sample(30%) followed by blood sample (25%) and urine sample (23%). Study by Shirisha, K et al [17] reported maximum P. aeruginosa isolates from pus (48%) followed by urine (22%) and blood (10%).

In present study 32% of Pseudomonas aeruginosa isolates were from surgery ward, followed by ICU (26%) and orthopaedics (15%) which was in accordance of other studies done by Shenoy s.et al., 2002[18] .

❖ **Biofilm production**

In present study 47% P. aeruginosa were strong biofilm producer, 24% were moderate biofilm producer 10% were weak biofilm producer and 19% isolate showed no biofilm formation which was similar to study of J. D. Andhale et al [19] which showed 43.33% isolates were biofilm producer and biofilm production was strongly positive for 33.33% isolates and 10% isolates were weakly positive by Tube method.

Table 6: Comparison of overall resistance pattern to various antibiotic in different studies with present study

Various study	CAZ	CIP	AK	IMP	PI	PIT	NET	MER	CL	AZ	NOR
Shenoy et al [18]	56.3%	41%	42%	30%	64%	33.5%	34%	50%	4.6%	53%	-
Khan et al[20]	65%	75%	48%	32%	61%	44%	-	30%	-		35%
Present study	74%	52%	42%	32%	65%	53%	28%	30%	2%	35%	20%

In present study overall maximum resistance (74%) was seen for ceftazidime followed by 65% and 53% of P aeruginosa resistant to piperacillin and piperacillin tazobactum respectively.

In present study 52% of P aeruginosa showed resistance to ciprofloxacin which was in accordance with study of Mohmoud et al. [21] A similar recent study by Dheepa M et al [22] showed significantly higher resistance to ceftazidime ciprofloxacin and piperacillin.In present study impinem and meropenem reistance was 32% and 30% respectively which was in accordance with study by Khan et al.[20] Earlier studies reported that imipenem was the most effective antibiotic against P. aeruginosa. [23] However, recent study by Maria et al [24] demonstrated the evolution of imipenem - resistant strains of P. aeruginosa. 2% of P. aeruginosa were resistant to colistin. We conclude that colistin represent the best treatment option for P aeruginosa infection. Hence, its use should be restricted to severe nosocomial infections However, Colistin is very expensive and this limits its use. The rapid dissemination

of carbapenem resistance is worrisome and calls for the implementation of surveillance studies and the judicious selection of antibiotics in clinical practice. In present study only 2% isolates were resistant to colistin which is in accordance to study by Bimla et al.[25]

In present study high rates of antibiotic resistance were noted among these biofilm producers. Similarly high rates of biofilm formation and antibiotic resistance were observed by Carlos J et al [26] and Saffari et al [27] who reported biofilm formation in 83% of clinical strains of P.aeruginosa.

In the present study, out of 32 imipenem resistant Pseudomonas aeruginosa, 28 (87.5%%) strains were MBL producers. The result was in accordance with the study by Mirbagheri et al [28] with imepenem resistance of (48.5%) and 88.8% being MBL producers, indicating that the production of MBL is one of the main mechanisms for resistance to imipenem in *P. aeruginosa* strains isolated from patients .

AMPc mediated resistance was observed in 19% of isolates in our study which was comparable to 17.6%observed by Khanal et al. in 2013.[29] In present study both MBLs and AMPc production was seen in 8% of isolates which was in accordance with result of Obritsch et al [30]who reported MBLs together with AMPc mediated resistance in 7.9% isolates. The bacterial strains that show resistance to three or more categories of antibiotics are defined as multidrug resistanct (MDR) strains.[30]Multidrug-resistant (MDR) P. aeruginosa is a growing problem. Altered target sites, bacterial efflux pumps, enzyme production or inhibition, loss of membrane protein, etc. are different mechanisms mediated by MDR P. aeruginosa. In present study 56% isolates of P aeruginosa were MDR and this was in accordance with the study of Jahanzeb et al [31] who reported 55.1% were MDR P. aeruginosa and Saxena et al[32] reported53.5% MDR P. aeruginosa. In present study the department wise distribution of P aeruginosa showed that 41% of MDR P. aeruginosa were isolated from surgery department 27% from ICU and 13% from department of orthopaedics. This result was very much close with study of Chander et al [33] and Anupurbha et al. [34]

In present study 70.21% MDR P aeruginosa were strong biofilm producer 75% were moderate biofilm producer 30% were weak biofilm producer and 10.52% P aeruginosa show no biofilm production which was in accordance with study of J. D. Andhale et al[19] who reported 66.66% of P. aeruginosa producing biofilm formations showed MDR pattern . Similar study by Dardi et al[35]also reported 65% MDR pattern in strong biofilm producer .Present study, showed that out of 30 isolates from pus sample 27 were biofilm producer ,15 were MBL producer and 20 were MDR Pseudomonas aeruginosa. Out of total 25 isolates from blood 21 isolates were biofilm producer 8 isolates were MBL producer and 15 were MDR. And out of 23 urine isolates 20 were biofilm producer 6 isolates were MBL producer and 12 were MDR.The result was in accordance with study of Pittaya Maita et al [36] who reported that the biofilm, produced by P. aeruginosa from clinical specimens, was shown in samples isolated from blood (100%), urine (88.6%), sputum (73.7%) and pus (77.5%) and Dardi et al [35] who also show maximum prevalence of Metallo-β-Lactamases was seen in Pus 41.66%) followed by Urine (33.33%) blood (23.07%), sputum (20%), Miscellaneous (30**%).**

Conclusion

This study showed high prevalence of biofilm formation and multidrug resistant P.aeruginosa with MBL and AMPc production. Maximum strains were sensitive to colistin.Hence it is necessary to have regular surveillance of drug resistance in P aeruginosa which will be useful for selecting an appropriate antibiotic to know changing

trends in antibiotic susceptibility pattern and for limiting the use of colistin and save it for the treatment of resistant and life threatening P. aeruginosa.

Limitation

1. Present study was of short duration(3 months) (ICMR STS PROJECT) and sample size was 100 isolates ,studies involving large sample size and more duration to know about prevalence and changing pattern of antibiogram of P aeruginosa will be more appropriate. Therefore futher studies should be multicentric and include a large sample size.
2. Biofilm production in this study was detected by Standard tube method in which interpretation is subjective. Detection of biofilm production by tissue culture plate method(TCP) will be more appropriate as TCP is considered as Gold Standard test.

Recommendation

- As infection caused by bacteria producing biofilm, are difficult to treat and eradicate, early diagnosis and management is necessary to reduce morbidity and mortality.ST method was established to be simple,easy, economical,general screening method for biofilm detection which can be recommended for early and prompt diagnosis of biofilm production in routine clinical laboratory.
- Nowadays increasing prevalence of P. aeruginosa along with emerging antibiotic resistance pattern emphasizes the detection of antibiotic resistance pattern among patients for better and improved management of such cases and to prevent emergence of drug resistance.
- Screening for MBL and AMPc production should be carried out in routine with simple economic methods to know the exact resistance pattern.

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