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RESEARCH ARTICLE

STUDY OF EXTENDED SPECTRUM BETA LACTAMASES & BIOFILM IN PSEUDOMONAS SPECIES ISOLATED FROM DIFFERENT CLINICAL SAMPLES

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Abstract

Background:-Pseudomonas species are Gram negative bacteria which are resistant to commonly used antibiotics. Due to the formation of biofilm antimicrobial therapy is reduced & leads to chronic bacterial infections. Therefore, we need to isolate species of Pseudomonas from different clinical samples, detect ESBL & biofilm production in them.

Objective:-1. To determine percentage of Pseudomonas species isolated from different clinical samples.
2. To determine the antibiogram of Pseudomonas species isolated from the samples.
3. To detect ESBL & biofilm production in Pseudomonas species.
4. To correlate biofilm & ESBL production.

Method:-The susceptibility of different clinical samples of Pseudomonas spp.was determined by using antibiotics on Muller Hinton Agar by the Kirby -Bauer disk diffusion method using Clinical & Laboratory Standards Institute (CLSI) standards. Confirmatory test for ESBL production was done as per Clinical and Laboratory Standards Institute (CLSI) 2021guidelines. For the detection of biofilm Tissue Culture Plate (TCP) method was used. TCP is considered as the gold-standard method for biofilm detection.

Result:-Out of total 171 Pseudomonas aeruginosa samples 30 were ESBL producers (17.5%) & 141 were Non ESBL Producers (82.5%), 12.8% were ESBL Producers & Strong Biofilm producers, 82.45% were Strong biofilm producers & Non ESBL producers.

Conclusion:-Phenotypic detection of ESBL & antibiotic resistance in P.aeruginosa should be done & both the results should be taken in consideration for better representation of resistant strains, this will help clinicians to give appropriate antibiotics so that they can treat the infections timely.

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Introduction:-

Pseudomonas species are oxidase positive, gram negative bacilli which shows innate resistance towards many antibiotics & disinfectants. They are saprophytes those grow in warm moist situations in the human environment (e.g. sinks, drains, respirators, humidifiers & disinfectant solutions)[1]. Infection of Pseudomonas spp. in the last two decades has become increasingly recognized as the aetiological agent, in a variety of serious infections in patients with impaired immune defenses (Neu 1993) including human immunodeficiency virus infection (O Donnell et al 1993, Hickey &Shanson 1993). Pseudomonas spp. shows exceptional resistance to antibacterial agents & is an important contaminant of pharmaceutical & cosmetic preparations. It is an oxidase positive, pigment producing, non-fermenting, Gram negative bacilli. It is a major pathogen among the hospitalized patients & in patients with cystic fibrosis [2]. It possess genes coding for resistance to several antimicrobial agents; therefore, helping the bacilli to survive under antibiotic pressure especially in the hospital environment. Pseudomonas spp. forms biofilm on the biotic or abiotic surface as it has minimal nutritional requirements & tolerates a wide variety of physical conditions. It has an important array of cell-associated and secreted virulence factors that takes part in its pathogenesis. Key among these are Type IV pili (the major bacterial adhesion factor), the Type III secretion system (secreted exotoxins)[3].

In Pseudomonas spp., biofilm is an important virulence factor and has an important role in antibiotic resistance. Therefore, new therapeutic agents, degrading biofilms, improved efficacy of current antimicrobial agents, are required against Pseudomonas spp., it has many drug resistant plasmids which confer resistance to several antibiotics[4]. Many strains are producers of beta lactamases, such as ESBL, carbapenemases&AmpC& many strains are resistant to aminoglycosides &quinolones[5]. There are many phenotypic detection tests on the basis of synergy between third-generation cephalosporin & clavulanate like the double -disk synergy test (DDST), ESBL E-tests & the combination disk method [6]. For the detection of ESBL these tests are need to be refined as some bacteria produce more cephalosporinase. We have to reduce the distance between disks of cephalosporins & clavulanate so that the sensitivity of the DDST can be improved. A fourth -generation cephalosporin, Cefepime is less rapidly inactivated by cephalosporinase than by ESBL.

Simultaneous hyperproduction of cephalosporiase are inactivated by phenotypic tests performed on a cloxacillin-containing agar. Third -generation cephalosporins and carbapenems (e.g.metallo -beta -lactamases) are hydrolysed by beta -lactamases, these are not inhibited by clavulanate, but are inhibited by EDTA. ESBL covered by metallo-beta -lactamase are detected by double inhibition of EDTA and clavulanate. Clavulanate weakly inhibit Extended-spectrum Ambler class D Oxacillinases Detection of Extended - spectrum Ambler class D Oxacillinasesis difficult in laboratory because it is not inhibited by EDTA[6].

Materials &Method:-

1. **Direct Microscopy:**
2. Direct Microscopy was done by Gram staining method.
3. **Culture:**
4. Culture was performed on Blood agar and MacConkey agar as per standard methods.

3. Biochemical Tests:

The biochemical tests included - Citrate reduction test, Indole test, Triple Sugar Iron Test, urease test, oxidase test, motility test, etc. as per standard methods.

Antimicrobial Susceptibility Testing (AST) for ESBL detection:

The susceptibility of different clinical samples of Pseudomonas spp.was determined by using antibiotics on Muller Hinton agar by the Kirby-Bauer disk diffusion method using Clinical & Laboratory Standards Institute (CLSI) standards in which the antibiotics were tested as per organism isolated[7][10].

Disc approximation method –

Isolates are resistant or with decreased susceptibility to Ceftazidime (30g) third generation cephalosporin antibiotics which are subjected to disc approximation method, a phenotypic test for detection of ESBL production. A disc of Ceftazidimeclavulanic acid and second disc containing Ceftazidime alone was placed on Mueller Hinton agar plate which was inoculated with the test strain, at a distance of 15mm from each other. If zone of inhibition around ceftazidime-clavulanic acid disc is 5 mm larger than that around the ceftazidime disc alone than it was interpreted as confirmatory for ESBL production as per Clinical and Laboratory Standards Institute (CLSI) 2021guidelines[7].

Biofilm Detection Method:

For the detection of biofilm Tissue Culture Plate (TCP) method was used. TCP is considered as the gold-standard method for biofilm detection. From the fresh agar plates, organism was isolated & inoculated in 10 ml of trypticase soy broth with 1% glucose at 37°C for 24 hrs. These cultures are then diluted 1:100 in fresh medium. 96 well-flat bottom sterile culture treated plates are than filled with 200µl of the diluted cultures & than incubated at 37°C for 24 hrs. After incubation, we removed contents of each well by gentle tapping. The wells are than washed with 0.2µl of phosphate buffer saline (pH 7.2) four times. Biofilm formed by bacteria were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Optical density (OD) of stained biofilm is then obtained by using ELISA autoreader at wavelength using filters[8][9].

The interpretation of biofilm production was done according to the given table below: -

OD	Value of Biofilm formation
0.120	Non -biofilm producer
0.120 -0.240	Moderate biofilm producer
0.240	Strong biofilm producer

Results:-

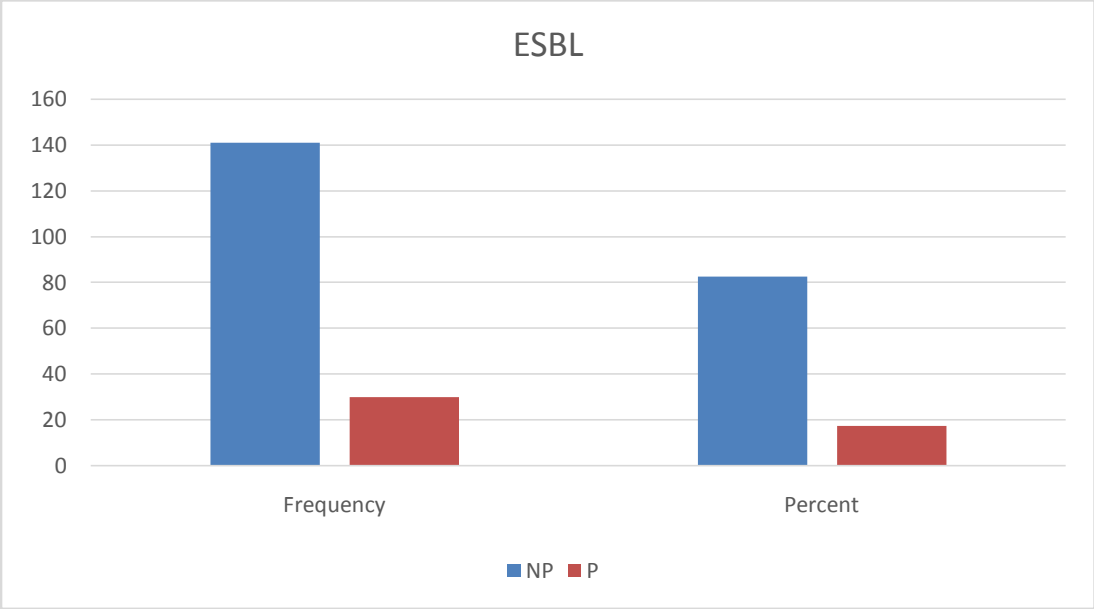
This observational&cross sectional study was conducted to detect theExtended spectrum beta lactamases &biofilm in Pseudomonas aeruginosa isolated from different clinical samples. A total number of 171 samples are collected for this study, Pseudomonas species isolated from all the samples sent to the Microbiology bacteriology laboratory of People's College of Medical Sciences&Research Centre, Bhopal (M.P.) were included in study.

Table 1:- Antibiogram of Pseudomonas aeruginosa isolated from different clinical samples.

Antimicrobials	Sensitive	Percent
PiperacillinTazobactam	156	91.22%
Levofloxacin	117	68.42%
Tobramycin	146	85.38%
Gentamicin	139	81.28%
Meropenem	154	90.05%
Amikacin	148	86.54%
Ceftazidime	156	91.22%
CeftazidimeClavulanic Acid	156	91.22%
Total	171	100%

Out of total 171 isolates 91.22% were sensitive towards **PiperacillinTazobactam (**156), 68.42%were sensitive towards **Levofloxacin (**117), 85.38% were sensitive towards **Tobramycin (**146), 81.28% were sensitive towards **Gentamicin (**139), 90.05% were sensitive towards **Meropenem (**154), 86.54% were sensitive towards **Amikacin (**148), 91.22% **Ceftazidime (**156) & 91.22% **CeftazidimeClavulanic Acid (**156).

Graph 1:- Detection of extended-spectrum β-lactamases (ESBL) in Pseudomonas aeruginosa by phenotypicmethods (n=141).



Out of 171 isolates 30 were ESBL producers (17.5%)& 141 were Non ESBL Producers (82.5%).

Table 2:- Results of Biofilm producing Pseudomonas aeruginosastrains isolated from different clinical samples.

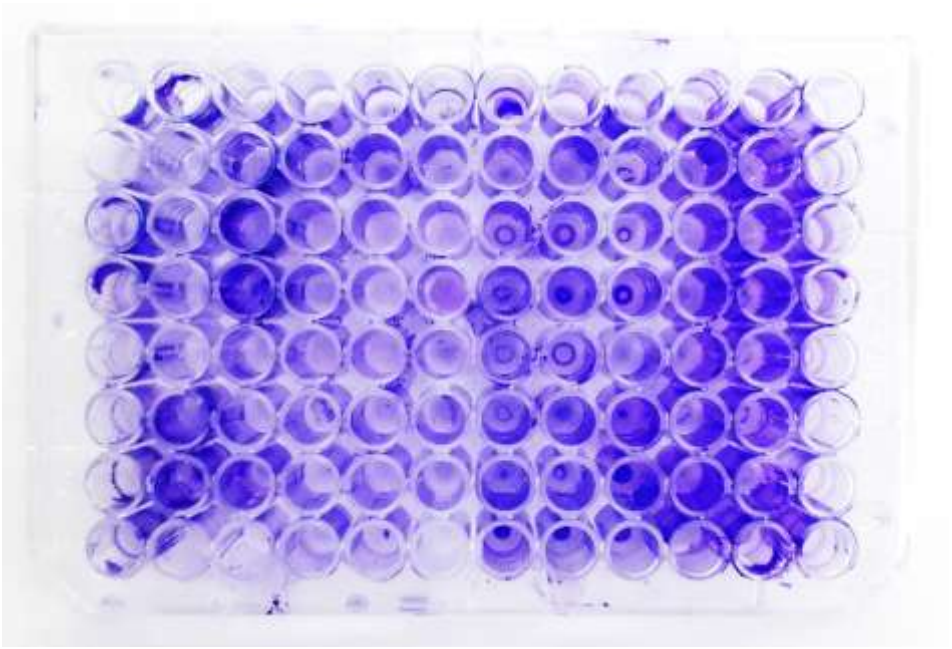
Biofilm	**Number**	**Percent**
Moderate	28	16.4%
Non Producers	16	9.4%
Strong	127	74.3%
Total	171	100%

Out of total 171 Pseudomonas aeruginosa samples 2.33% were ESBL producers (4) & non biofilm producers, 2.33% were Moderate Biofilm producers & ESBL producers (4), 12.8% were ESBL Producers & Strong Biofilm producers, 82.45% were Strong biofilm producers & Non ESBL producers.

Figure1:- Antibiotic Susceptibility Test on Pseudomonas aeruginosa, Pyomelanin&Pyoverdin pigment respectively.

Figure 2:- Detection of ESBL on Pseudomonas aeruginosaby disk approximation method.

Figure 3: - Detection of Biofilm on Pseudomonas aeruginosain 96 well flat bottom culture treated plates by TCP Method.



Discussion:-

Pseudomonas aeruginosapossess genes coding for resistance to several antimicrobial agents; therefore, helping the bacilli to survive under antibiotic pressure especially in the hospital environment.

Broad spectrum β-lactum antibiotics; third & fourth generation cepahlosporins, azetronam, and extended spectrum penicillins are resistant towards plasmid mediated β-lactamases ESBLs [11].

In this study 8.8% of Pseudomonas aeruginosawere resistant towards Ceftazidimewhile 31.6%, 18.7%, 13.5%, 14.61%, 9.9%, 8.8% were resistant towards Levofloxacin, Gentamicin, Amikacin, Meropenem, Tobramycin, PiperacillinTazobactam, respectively. In study by Kaur C et al. revealed (62%) P.aeruginosa isolates were resistant to Ceftazidime while in a previous study by Aggarwal et al resistance to Ceftazidime was 10.35% [12]. In our study

18.7% were resistant towards Gentamicin, while strains resistant to Gentamicin were 48% in study by Kaur C et al. [10] & 45% at Sarkar et al [13] which was not in concordance with our study.

In this study there is prevalence of ESBL among P.aeruginosa from OPD (31.6%) whereas in study by Goel et al. [14] high incidence is observed from ICU. But results by Agarwal et al were different which showed 20.27% of ESBL production [15].

As infections caused by P. aeruginosa are highly pathogenic & shows resistance towards commonly used antibiotics, production of biofilm is an important factor in its pathogenicity [16]. Due to the formation of biofilms persisting bacterial infections are eased which in turn lower the potency of antimicrobial treatment [15], [16], [17]. In this study 74.3% of P.aeruginosa were Strong Biofilm producers, 16.4% were moderate biofilm producers, 9.4% were Non biofilm producers, similarly in study by SomayehAzimiet al 69% of them were strongly biofilm producers, 11% were moderate biofilm producers, 7% were weak biofilmproducers [18].

In thisstudy 13. 5%, 18.7%, 9.9% & 8.8% were resistant towards Amikacin, Gentamicin, Meropenem&Ceftazidime, respectively. In study by Maryam Banar et al. [21] more than 90% of the isolates were resistant to amikacin, gentamicin, meropenem and the rate of resistance to ceftazidime and Imipenem were 61% and 83%, respectively.

In a study conducted by Anvarinejad et al., [19], resistance level to the amikacin, gentamicin, cefepime and meropenem were 90%. In study done by Maryam Banar et al., resistance to ceftazidime and imipenem were higher i.e. 72% and 98%, respectively.Nikokar et al., reviewed resistance rate for 23.30% imipenem, 37.20% gentamicin and 48.80%amikacin [20].

Due to the difference in consumption of antibiotics in different areas, P.aeruginosa shows difference in antibiotic resistance done in different studies. Therefore, deployed on the place of bacterial isolation suitable healing regimen for the therapy of P.aeruginosa infections should be done [21].

In study by Maryam Banar et al. [21] 98.4% of P.aeruginosa isolates formed biofilm, Vasiljević et al. showed 16.25% produced strong biofilm; 33.75% produced moderate biofilm; 33.75% produced weak biofilm & 16.25% of isolates were non-biofilm producers [22], Jabalameli et al. 47% were strong biofilm producers, 26% were moderate and 22.9% were weak biofilm producers, [23]. Whereas, in this study 74.3% of P.aeruginosa were Strong Biofilm producers, 16.4% were moderate biofilm producers, 9.4% were Non biofilm producers, which implies the importance of biofilm formation by P.aeruginosa& its resistance towards commonly used antibiotics.

Conclusion:-

According to this study, Pseudomonas aeruginosa showed resistance towards multiple antibiotics & different strains showed resistivity towards different antibiotics. Resistance of Pseudomonas aeruginosatowards multiple antibiotics is giving clinicians more therapeutic challenges.

Phenotypic detection of ESBL & antibiotic resistance in P.aeruginosa should be done & both the results should be taken in consideration for better representation of resistant strains, this will help clinicians to give appropriate antibiotics so that they can treat the infections timely & can prevent the spreading of resistance.P.aeruginosafound to be more sensitive towardsPiperacillinTazobactam& resistant towards Levofloxacin, Gentamicin &Amikacin.

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