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RESEARCH ARTICLE

A STUDY OF BIOFILM FORMATION & EXTENDED SPECTRUM-β-LACTAMASES PRODUCTION IN *PSEUDOMONAS AERUGINOSA* AT A TERTIARY CARE HOSPITAL IN HARYANA.

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Abstract

Introduction: Emergence of antibiotic resistance and biofilm formation among the nosocomial pathogens is of serious concern due to recurrence and chronicity of infections. The production of the extended spectrum-β-lactamases and their spread among bacterial pathogens is a matter of concern with regard to the future of antimicrobial therapy. Antimicrobial resistance is an innate feature of bacterial biofilms. Biofilm formation is higher in ESBL producing and MDR strains**.** The present study was undertaken with the aim to determine antibiotic resistance pattern of the isolates, to find prevalence of ESBL among isolates and their association with biofilm production.

Material and Methods: The study was carried out in the tertiary care hospital for the period of 1yr, from November 2014 to October 2015. A total of 100 isolates of *P. aeruginosa* from various clinical specimens were taken and identified using standard microbiological procedures. The isolates were subjected to antimicrobial susceptibility testing and ESBL production as per CLSI method. Biofilm formation was achieved according to the Tissue Culture Plate (TCP) assay.

Results: Out of these 100 isolates, 58 were multi drug resistant and 20 were extreme drug resistant. Forty nine (49.0%) isolates were ESBL producers, while 51(51.0%) isolates were non-ESBL producers. Seventy-eight isolates produced biofilm, out of which, 43 were found in ESBL producers and 34 in non-ESBL producers.

Conclusion: ESBL and biofilm producing nosocomial pathogens are of significant concern as they can cause chronic relapses and dissemination of drug resistant isolates. Both ESBL and biofilm production in the isolates further increase the morbidity and mortality in hospitalized patients. Hence it is necessary to detect the biofilm forming bacteria, conduct surveillance of the antibiotic susceptibility and device effective infection control measures.

Introduction:-

Pseudomonas aeruginosa is an epitome of opportunistic nosocomial pathogen which can tolerate low oxygen conditions, survive low levels of nutrients and grow in temperatures ranging from 4 to 42°C. [1] These characteristics allow it to attach itself and survive on medical equipment and on other hospital surfaces.[1, 2] It can cause pneumonias,

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urinary tract infections, bacteraemia and is a cause of high morbidity and mortality in patients with cystic fibrosis. Infections due to *P. aeruginosa* are difficult to eradicate because of their elevated intrinsic resistance as well as their capacity to acquire resistance to different antibiotics.[3]

P. aeruginosa produces biofilm which has an enormous impact on healthcare, and are estimated to be associated with 65% of nosocomial infections.[4] A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide (EPS), protein and DNA. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defence system. Biofilm growth is associated with an increased level of mutations as well as with quorum-sensing-regulated mechanisms. Biofilms can be prevented by early aggressive antibiotic prophylaxis or therapy and they can be treated by chronic suppressive therapy**.** [5]

The development of biofilm is a 5 stage process – 1) reversible attachment 2) irreversible attachment 3) early development 4) maturation 5) detachment or dispersal of cells. [6]

The factors controlling biofilm formation are: i) recognition of attachment sites on a surface ii) nutritional cues iii) change of pH and temperature iv) exposure to antibiotics, chemical biocides, and host defense mechanisms e.g. complement system etc.[7]

Antimicrobial resistance is an innate feature of bacterial biofilms.[8] Many studies have shown that biofilm formation is higher in MDR strains.[9]

There is variety of mechanisms involved in the resistance of *P. aeruginosa*, such as; overexpression of efflux pump, acquisition of ESBLs and Metallo-β-Lactamases (MBLs); target site or outer membrane modification, porin mutations, plasmid enzymatic modification. Resistance to multiple drugs is usually the result of combination of different mechanism in a single isolate.[10]

Material and Methods:-

A total of 100 isolates of *P. aeruginosa* from various clinical specimens like urine, pus, blood, body fluids and sputum, were collected from both indoor and outdoor patients, irrespective of age and sex, and identified by standard microbiological procedures.[11]

The isolates were subjected to antimicrobial susceptibility testing performed by Kirby-Bauer disc diffusion method using Clinical and Laboratory Standard Institute (CLSI) criteria. Mueller-Hinton Agar (MHA) was used for antibiotic sensitivity testing. Various antibiotics were put up and plates incubated at 37^{o}C for 24 hours.

Isolates showing reduced susceptibility to third generation cephalosporins were tested for ESBL production as per CLSI method. The test organism was inoculated on MHA plate. One 30µg disc of ceftazidime and one 30 µg disc of cefotaxime and another 30/10µg disc of ceftazidime/clavulanic acid and 30/10µg disc of cefotaxime/clavulanic acid were placed on surface of agar plate. The plates were incubated at 35^{o}C for 16-18 hours. A ≥5 mm increase in zone diameter for antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was considered positive for ESBL production.[12]

The biofilm formation assay was achieved according to the Tissue Culture Plate (TCP) assay. The results were interpreted depending on the classification of bacterial adherence and biofilm formation by TCP method. [13-14]

Isolates from fresh agar plates were inoculated in Brain heart infusion broth (BHI) with 2% sucrose dispensed in 2 mL amount in the test tubes and incubated for 18-24 hours at 37^{o}C in stationary condition. Then the broth with growth (visible turbidity) was diluted to 1 in 100 with fresh medium. Individual wells of sterile polystyrene, 96 well-flat bottom tissue culture plates were filled with 0.2 mL aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of medium.[15, 16]

These tissue culture plates were incubated for 24 hours at 37^{o}C. After incubation the content of the well was gently removed by tapping the plates. The wells were then washed four times with 0.2 mL of phosphate buffer saline to remove free floating planktonic bacteria. Biofilms formed by adherent sessile organism in plate were fixed with 2%

sodium acetate for half an hour and stained with crystal violet 0.1% w/v for half an hour. Excess stain was rinsed off by thorough washing with de-ionised water and plates were kept for drying. Adherent bacterial cells usually form biofilms on all sides of wells and were uniformly stained with crystal violet. Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA auto-reader at wavelength of 620 nm and were graded as per Christensen's et al. These OD values were considered as an index of bacteria adhering to surface and forming bio-films. [15,16] The detection of biofilms by TCP has been depicted in **Figure 1.**

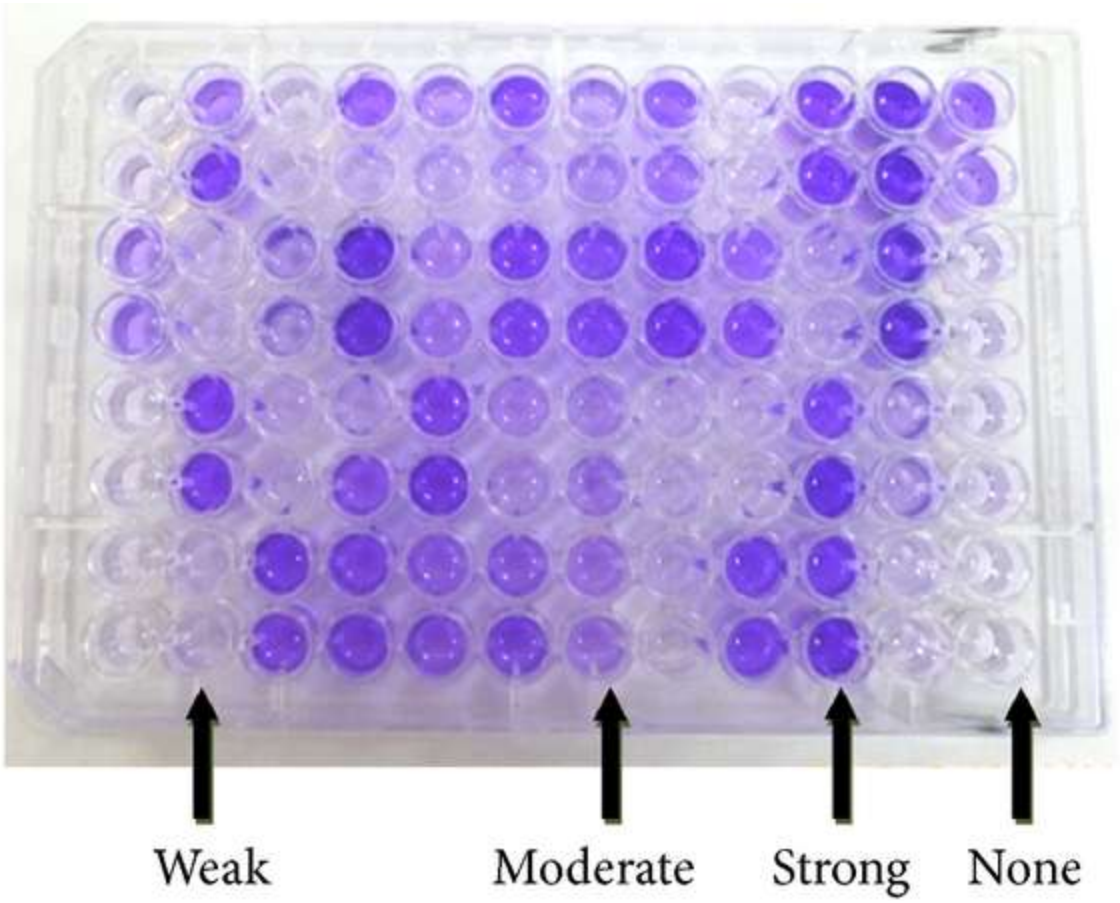


Figure.1:-Detection of biofilm by Tissue Culture Plate (TCP) method.

Classification of bacterial adherence by MTP method

Mean O.D value	Adherence	Biofilm formation
<0. 120	**None**	**None/ Weak**
0.120-0.240	**Moderate**	**Moderate**
≥0.240	**Strong**	**High**

Results:-

Out of these 100 isolates, 58 were multi drug resistant (MDR; isolates showing resistance to at least three antimicrobial drugs among β- lactams, carbapenems, aminoglycosides & fluoroquinolones) and 20 were extreme drug resistant (XDR; isolates showing resistance to all the above mentioned four antimicrobial drugs).

Forty nine (49.0%) isolates were ESBL producers, while 51(51.0%) isolates were non-ESBL producers (**Figure 2).**

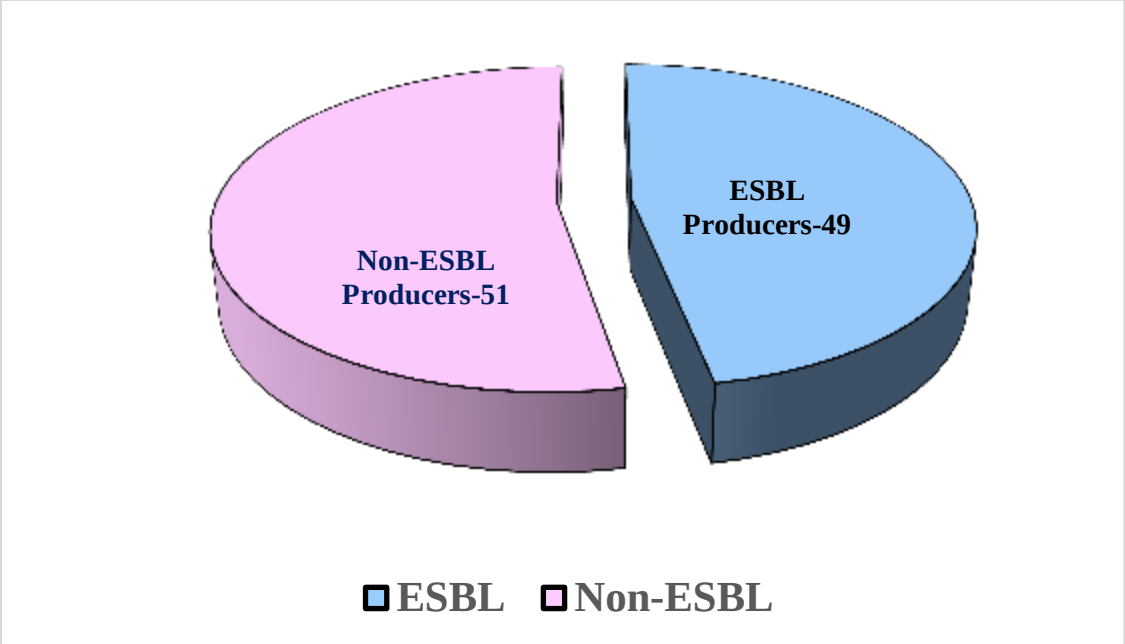


Figure 2:-Distribution of ESBL and non-ESBL producing *P. aeruginosa* isolates.

On comparing the antibiotic resistance among ESBL producing and non-ESBL producing *P. aeruginosa* isolates, a significant statistical difference (p value <0.05) was observed for all cephalosporins (except cefoxitin), carbepenems, aminoglycosides and ciprofloxacin (among fluoroquinolones) **(Table 1).**

Table 1:-Comparison of antimicrobial resistance pattern of ESBL producing and non-ESBL producing *P. aeruginosa* isolate to various antibiotics

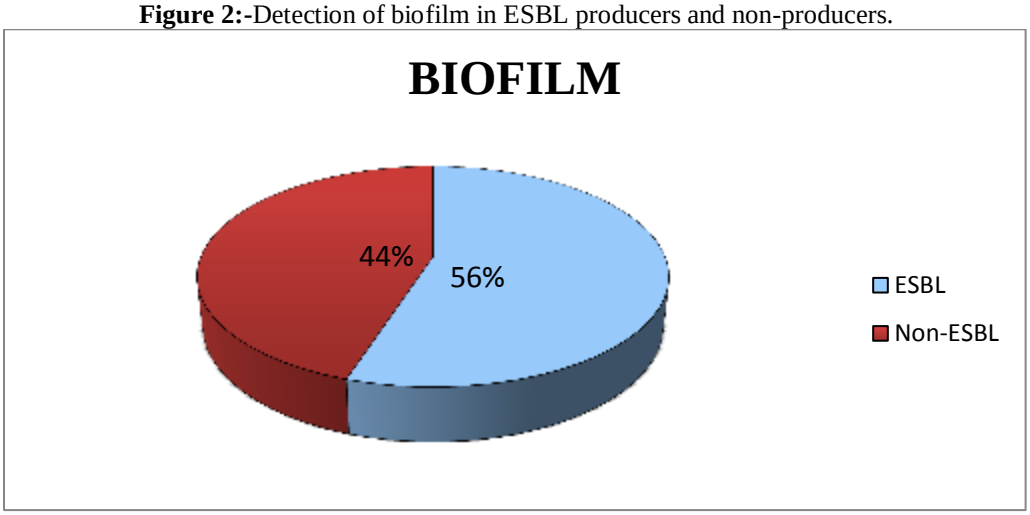
Antimicrobial drugs	ESBL producers (n=49)		Non-ESBL producers (n=51)		P value
	n	%	n	%	
β- lactams					
Ceftazidime	49	100.0	24	47.1	<0.05
Ceftizoxime	45	91.8	5	9.8	<0.05
Ceftriaxone	47	95.9	1	2.0	<0.05
Cefepime	32	65.3	13	35.5	<0.05
Cefoxitin	36	73.5	32	62.7	>0.05
Aztreonam	42	85.72	36	70.6	>0.05
Imipenem	23	47.0	10	19.6	<0.05
Meropenem	24	49	10	19.6	<0.05
Piperacillin /tazobactam	9	18.4	3	5.8	>0.05
Ticarcillin/ clavulanic acid	40	81.6	39	76.5	>0.05
Aminoglycosides					
Gentamicin	27	55.0	14	27.4	<0.05
Amikacin	29	59.2	14	27.4	<0.05
Netilmicin	37	75.5	27	53.0	<0.05
Fluoroquinolones					
Ciprofloxacin	28	57.0	16	31.4	<0.05
Norfloxacin	16	32.7	17	33.3	>0.05
Others					
Polymyxin	0	0.0	2	3.9	>0.05
Colistin	1	2.0	2	3.9	>0.05

In our study, out of 100 isolates tested, 78 were positive for biofilm production. Out of these 78 isolates, 44 were found in ESBL producers and 34 in non-ESBL producers. The finding has been depicted in **Figure 2**.

Table 2:-Detection of biofilm in ESBL producers and non-producers.

Virulence factor (n)	ESBL (n=49)		Non-ESBL (n=51)	
	n	%	n	%
Biofilm (78)	44	56	34	44

Figure 2:-Detection of biofilm in ESBL producers and non-producers.



Discussion:-

ESBL producing strains are usually found in those areas of hospitals where antibiotic use is frequent and the patient's condition is critical. In India, prevalence rate of ESBLs ranging from 28% to 84% has been reported from various parts of the country: Bakshi et al reported high prevalence (50%) of ESBL production among *P. aeruginosa* at Patiala (Punjab).[17]Similar incidence of ESBL production among *P. aeruginosa* (42.31%) has been reported by researchers at AIIMS, New Delhi [18] and 40% from Coimbatore. [19]

Our study corroborates these findings. This relatively high rate of ESBL production in our study may be due to selection pressure caused by extensive use of β-lactam antibiotics in our hospital. This ESBL production rate might be much higher but could not be detected due to presence of silent genes which do not express phenotypically because in the present study we have not confirmed the ESBL genes using genotypic methods. [20] High antibiotic resistance in ESBL producing *P. aeruginosa* isolates have also been reported by authors. Tavajjohi et al reported that resistant to antibiotics like piperacillin, cefotaxime, ceftriaxone, ceftazidime, imipenem, aztreonam was observed in 42.9%, 71.4%, 57.1%, 57.1%, 28.5%, 42.9% ESBL producing *P. aeruginosa* isolates. [21, 22]

In this study, the ESBL producing isolates were significantly more resistance to anti-bacterial agents compared with non-ESBL producing isolates ($p < 0.05$). The same results were reported for clinical isolates of enteric bacteria by Mansouri et al and the gram negative bacteria from urinary tract infections by Selvakumar et al and Mashouf et al . [23, 24] This can be explained by the fact that ESBL producing bacteria usually have mobile genetic elements coding gene for resistance to other antibacterial agents. Different efflux pumps in *P. aeruginosa* are able to eject multiple antimicrobials from cell, including beta-lactams and effect penicillin as well as non-beta-lactams drugs such as fluoroquinolones and aminoglycosides.[25]

In our study out of 100 isolates, 78 produced biofilm, of which 43 (55%) were in ESBL producers and 34 (45%) in non-ESBL producers (Table 20, Figure 21). In a study done by Saxena et al on biofilm formation in P. aeruginosa isolates from lower respiratory tract infections (LRTI), out of 80 isolates 64 (80%) produced biofilm by TCP method which is in concordance with our study.[26] In a study done by Mansouri et al on comparison of cell surface hydrophobicity and biofilm formation among 50 ESBL and 50 non-ESBL producing *P. aeruginosa* isolates, 100% of strains produced biofilm. The percentage of isolates producing moderate and strong biofilm was 72% for ESBL and 32% for non-ESBL isolates (p-value < 0.001). The data demonstrate that the biofilm formation was higher in the

ESBL producing compared with non-ESBL producing isolates and this property can render the ESBL positive isolates more pathogenic. [23] In a similar study done by Khalil et al, the amount of biofilm production was 76 % and the incidence was more in ESBL producers than non-ESBL producers similar to our study.[27] Choy et al. compared virulence factors in fifty-five *P. aeruginosa* strains isolated from contact lens and non-contact lens related keratitis. All (100%) the isolates produced biofilm. Twenty eight out of 55 (51%) and 27 out of 55 (49%) were found in contact lens and non-contact lens wearers, respectively. Majority of isolates showed strong biofilm formation (65%), 31% isolates formed moderate biofilms, while weak biofilm formation was found in 4% isolates.[28]

Conclusion:-

Antibiotic resistance among nosocomial pathogens is on the rise leading to prolonged hospitalization and excessive treatment costs. Inappropriate use of antibiotics is an important factor contributing to the emergence of multidrug resistant bacteria. Hence its necessary to conduct surveillance of the antibiotic susceptibility pattern for such bacteria and empiric therapy initiated based on it. This will help in the prevention of the emergence of drug resistant isolate.

The simultaneous detection of ESBL and biofilm production is the contemporary approach for the examination of the microbiological aspects of infections caused by *P. aeruginosa*. Regular antimicrobial susceptibility surveillance and ESBL detection is essential. An effective national and state level area-wise monitoring of the resistance patterns, antibiotic policy and draft guidelines should be introduced to preserve the effectiveness of antibiotics and for better patient management.

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