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Microbiology

DETECTION OF BIOFILM AND BETA LACTAMASES PRODUCTION IN CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA.

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ABSTRACT Pseudomonas infections are difficult to eradicate because of their intrinsic resistance as well as their capacity to acquire resistance to different antibiotics. MBL producing isolates are associated with higher morbidity and mortality. Biofilm production is an inherent ability of bacteria which can lead to enhanced virulence.

A total of 71 Pseudomonas aeruginosa were isolated from clinical samples like pus, urine, sputum, catheter tip etc.All the isolates were tested for ESBL and AmpC production by Camilla Rodrigues disk placement method. Modified hodge test was used for detection of MBL producing isolates of pseudomonas. Biofilm production was detected by tube method as mentioned by Basu et al.

Out of 71 pseudomonas aeruginosa isolated from various clinical samples, 92% (n=65) are biofilm producer. MBL production in our study was 5%. our study indicates that P. aeruginosa is becoming resistant to commonly used antibiotics and mostly biofilm producers. These strains should be routinely tested for ESBL production for better patient care.

KEYWORDS :

INTRODUCTION

Pseudomonas is an opportunistic, nosocomial pathogens with innate resistance to many antibiotic [1]. It is a frequent cause of respiratory, surgical site and urinary tract infection, especially in patients admitted to intensive care unit (ICU). Infections are difficult to eradicate because of their intrinsic resistance as well as their capacity to acquire resistance to different antibiotics like beta lactams, aminoglycosides & flouroquinolones[2,3].

In recent years there has been an increased incidence and prevalence of extended spectrum beta lactamases (ESBLs), enzymes that hydrolyze and cause resistance to oxymino-cephalosporins and aztreonam [4].

Production of these enzymes is either chromosomally mediated or plasmid mediated. The chromosomally mediated beta lactamases production is mainly through expression of AmpC gene which is either constitutive or inducible [5,6].

MBL producing isolates are associated with higher morbidity and mortality. It will hydrolyse virtually all classes of beta lactams agents including the carbapenems and their continued spread will be a clinical disaster [7].

Another important factor contributing to the pathogenesis of P.aeruginosa in causing fatal infections is its potential to form biofilm on biotic and abiotic surfaces [8]. Biofilm production is an inherent ability of bacteria which can lead to enhanced virulence due to gain of some additional phenotypic characters such as drug resistance [9]. According to a research, more than 60% of all infections are caused by bacteria growing in biofilm [10].

In view of these observation, and increasing reports on emergence and spread of multidrug resistant pseudomonas aeruginosa all over the world, present study was undertaken to find out incidence of beta lactamases and biofilm producing pseudomonas aeruginosa in our hospital and to study their antimicrobial susceptibility features.

MATERIALS AND METHODS

The prospective cross sectional study was carried out in microbiology department of tertiary care hospital for a period of 3 months after obtaining permission from Ethics committee.

A total of 71 Pseudomonas aeruginosa were isolated from clinical samples like pus, urine, sputum, catheter tip etc. The maximum numbers of isolates were obtained from pus followed by urine samples. These isolates were identified by standard techniques [11]. They were further tested for antibiotic susceptibility testing by Kirby-Bauer disc diffusion method as per CLSI guidelines [12].

Biofilm production was detected by tube method as mentioned by Basu et al. TSBglu (10ml) was inoculated with loopful of isolates from overnight culture plates and incubated for 24 hours at 37°c. The tubes were decanted and washed with PBS (pH 7.3) and dried. Dried tubes were stained with safranin (1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm production was scored as negative, weak positive (+1), moderate positive (+2) and strong positive (+3) [13].

Disc placement method

i. All the isolates were tested for ESBL and AmpC production by Camilla Rodrigues disk placement method [14]. The lawn culture of test organisms was made on Muller-Hinton agar (MHA) as done for disc diffusion antimicrobial susceptibility test. In the centre of the plate, imipenem (10 µg) (Inducer) disc was applied. At the distance of 20mm, the disc of cefotaxime (30µg) was placed. From this disc, in a circular manner, clockwise, the discs of cefoxitin (30µg) (Inducer), ceftriaxone (30µg), ceftazidime (30µg), ceftazidime + clavulinic acid (30/10µg), and aztreonam (30µg) were placed such that any two adjacent discs were 20mm apart from centre to centre [Figure 1]. On overnight aerobic incubation at 37°C, the diameters of zones of inhibition were measured and interpreted as follows:

Extended-spectrum β-lactamase

i. Zone diameter for aztreonam ≤27 mm, cefotaxime ≤27 mm, ceftazidime ≤22 mm, and ceftriaxone ≤25 mm [15,16].
ii. Susceptible to cefoxitin [17].
iii. Increase in zone size with addition of inhibitor (ceftazidime+ clavulanic acid) by 5 mm or more [17].

AmpC

a) Inducible
i. Blunting of zone toward inducer [Figure 5]
ii. No increase of zone size with addition of inhibitor
b) Derepressed mutants (DM)
i. Resistant to cefoxitin and cefotaxime
ii. No increase of zone size with addition of inhibitor

Metallo beta -lactamases

Strains showing resistance to imipenem

Multiple mechanisms

(i) Resistant to cefoxitin
(ii) Blunting of zone toward inducer
(iii) Increase of zone size with addition of inhibitor by 5 mm or more.

Modified hodge test

An overnight culture suspension of pseudomonas adjusted to 0.5 McFarland standard was inoculated using a sterile cotton swab on the

surface of a MHA. After drying, 10 µg imipenem disc was placed at the center of the plate and the test strain was streaked heavily from the edge of the disc to the periphery of the plate. The plate was incubated overnight at 37°C. Indentation produced in the zone of inhibition produced by the imipenem indicates a positive test. Maximum four strains can be tested at a time (all four directions) which gives a presence of a "cloverleaf shaped" zone of inhibition if all four test strains are positive for MBL production [18](figure 3)

Results

Out of 71 pseudomonas aeruginosa isolated from various clinical samples, 92% (n=65) are biofilm producer. Of these, 5% (n= 4) isolates showing resistance to imipenem were subjected to Modified Hodge test.

Table 1: specimen wise distribution

Specimen	Number of isolates
Pus	26
Urine	15
Sputum	13
Blood	7
Other	10
Total	71

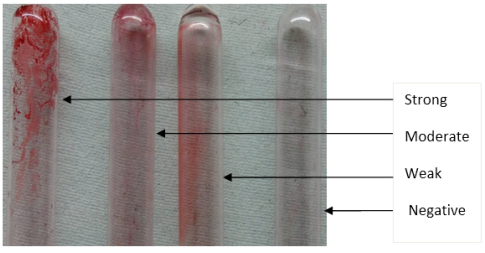


Figure 1: Grading of biofilm formation.

Table 2: No. of isolates forming biofilm

Biofilm formation	No of isolates
Negative	6
Weak	26
Moderate	22
Strong	17
Total	71

A total of 42 (64.6%) isolates produced single enzyme, while 23 (35.3%) strains revealed multiple enzyme production simultaneously.

Only ESBL production was seen in 22 (31%) strains, only AmpC in 16 (23%) and only MBL in 4 (5%) strains. While ESBL and AmpC together were seen in 23 (33%) strains. The distribution of production of ESBL, AmpC, and MBL, either single or in combination is presented in Table 3

Table 3: various mechanism of resistance

N = 71	No. of Isolates	Percentage
ESBL	22	31%
Amp c	16	23%
Both ESBL & Amp c	23	33%
MBL	4	5%
MDR	6	8%

Detection of ESBL and AmpC production by Camilla Rodrigues disc placement method used in this study.

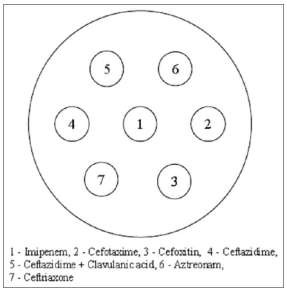


Figure 2: Camilla Rodrigues disk placement method.

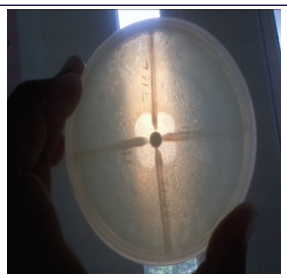


Figure 3: Modified Hodge test

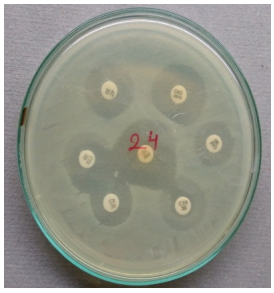


Figure 4: ESBL production

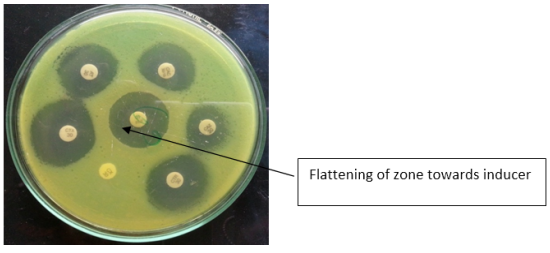
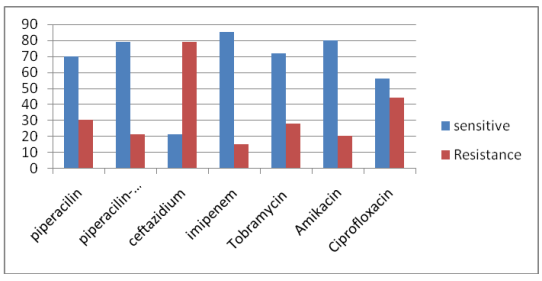


Figure 5: AmpC production



Graph 1: Antibiotic resistance pattern

Discussion

P.aeruginosa has been emerged as a significant pathogen and is the most common dreadful gram negative bacilli found in various health care associated infections all over the world due its potential to form biofilm and beta lactamases. The production of Metallo-beta-lactamase (MBL) is one among various resistance mechanisms found in P. aeruginosa. As MBL producing Pseudomonas species poses a therapeutic problem, it is better to understand the mechanism and spread of such strains which will aid in proper diagnosis and infection control management.

P.aeruginosa were predominantly isolated from pus (36.6%), followed by urine (21.12%) and sputum sample. The same has been reported with Okon et al. (39.2%) [19] & Vijaya Chaudhari et al. (35.3%) [20] Pseudomonas mainly causes wound, urinary tract and respiratory infection.

P.aeruginosa showed resistance too many other classes of antibiotics, including cephalosporins, aminoglycosides and fluoroquinolones. This is due to the coexistence of genes encoding drug resistance to other antibiotics on the plasmids which encode ESBL. This fact has also been observed in our study, Ceftazidium showed 79% highest resistance and Ciprofloxacin showed 44% resistance to P.aeruginosa which shows promising effect in treatment. In various reports on ciprofloxacin resistance to P.aeruginosa was ranged between 0-89percent (Algun et al.) [21].

Presence of different types of β-lactamases including AmpC, ESBL and MBL and the association of some of these enzymes with biofilm formation in P. aeruginosa has been shown in a number of studies [22,23,and 24]. In our study, ESBL+ AmpC, only ESBL, AmpC and MBL production occurred in 33%, 31%, 23% and 5% of the isolates, respectively.

In a recent study, 96% of P. aeruginosa isolates were shown to form biofilm in vitro [25]. In our study 92% pseudomonas are biofilm producers which are slightly close to this study. The MDR P. aeruginosa are strong biofilm producer.

Imipenem was found unaffected by the action of the enzymes in many studies, MBL production in our study was 5% and raised level of carbapenem resistance were reported by Variya et al. (25%) [26].The percentage variation in the resistance mechanism could be due to the study environment where the study was done. These carbapenem agents may be of benefit in the treatment of ESBL infection; however, indiscriminate use of these agents may promote increased resistance to carbapenems.

This study signifies the presence of beta lactamases producing phenotypes with ability to form biofilm, which are emerging pathogens and may be difficult to treat.

Conclusion:

Emergence of multidrug resistant P. aeruginosa is increasing and is a significant clinical challenge, because of limited therapeutic option for this pathogen. Therefore early detection of MDR and β- lactamase producing P. aeruginosa is important to restrict their spread in community. Hence, our study indicates that P. aeruginosa is becoming resistant to commonly used antibiotics and mostly biofilm producers. These strains should be routinely tested for ESBL production for better patient care.

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