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BIOFILM AND METTALO BETA-LACTAMASE PRODUCTION IN ASSOCIATION WITH SERUM RESISTANT ACTIVITY AMONG CLINICAL STRAINS OF *PSEUDOMONAS AERUGINOSA*

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Keywords:

Biofilm, Serum Resistance, MBL production, Pseudomonas sp.

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ABSTRACT: Biofilms are communities of bacteria that are append to a surface and play significant role in the persistence diligence of bacterial infections. Bacteria with a biofilm are more defiant to antibiotics compared with planktonic bacteria. Biofilm producing bacteria are able to take possession on medical devices such as catheters and implants. The incidence of Biofilm producing microorganism runs around 80% of infections. These kinds of infections are difficult to make a diagnosis and treat. The present investigation has been done to analyze the correlation of MDR *Pseudomonas sp* with production of Biofilm, MBL production and Serum resistant activity. 63 (12.6%) *Pseudomonas* isolates were incurred from a total of 500 clinical samples and were speciated based on both phenotypic and genotypic methods. Among the total isolates detected 56.2% were positive for both Biofilm production and Serum resistant activity. MBL productions were observed in 70.83% of the isolates. The significance of the result also relates that all the isolates expressed high MAR index values that confirm the association between the virulence and its drug resistant traits.

INTRODUCTION: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the most significant opportunistic pathogen in hospitals. The organism has been reported in cases of rigorous acute and chronic infections in hospitalized, immune compromised hosts [1]. A major cause of nosocomial infections, particularly pneumonia and infections of the urinary tract, skin and soft tissue were caused by these gram-negative, non-fermenting bacteria.

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In addition, patients with chronic lung infections, including cystic fibrosis, with high rates of associated morbidity and mortality have been isolated which is commonly prevalent [2, 3].

The predisposition of *P. aeruginosa* for the development of resistance to antibiotics and expression of multiple virulence factors bestows to the recurrent incompetence of existing therapies. The pathogenicity of *P. aeruginosa* has been unified with multiple bacterial virulence factors, counting biofilm formation and the expression of adhesions, endotoxin and hydrolytic exotoxins, which cause tissue destruction. The resistance to serum bactericidal consequence is one of the foremost virulence factors of *P. aeruginosa* [3]. The host inherent immune system comprises serum

components, such as antibodies and proteins of the complement system that mediate the bactericidal effect of serum. This phenomenon is being professed with a higher frequency of serum resistance among *P. aeruginosa* strains isolated from blood, wounds, urine than among strains isolated from the sputum of asymptomatic patients with cystic fibrosis [4, 5]. Serum resistance might be an important microbial phenotype, which could feasibly differentiate between invasive and non-invasive strains and isolates [6, 7].

Therapy is obscured by the organism's potent ability for adaptation, mutation, and gene acquisition. This diversity of *P. aeruginosa* infections is owing to the development of various adaptive mechanisms such as the nutritional and metabolic pathways, besides the regulation of gene expression. *P. aeruginosa* can form bacterial biofilm that defends the organism from host defenses and antimicrobial therapy [8].

P. aeruginosa biofilm is difficult to eradicate and it causes bacterial persistence, leading to infection chronicity and morbidity [9]. In addition, its capability to form biofilm provides greater protection against host immune defense systems and receptiveness to various antimicrobial agents [10]. *P. aeruginosa* is a multidrug resistant (MDR) organism and is painstaking a phenomenon of bacterial resistance. This is reputable by different types of antibiotic resistance. It is also commonly assumed that in MDR *P. aeruginosa* isolates, reduced virulence may be due to decreased biofilm. However, recent data suggest the other way, and MDR *P. aeruginosa* may remain fully pathogenic [11].

Carbapenem hydrolyzing enzymes belong to classes A, B, and D according to molecular Ambler classification and are called carbapenemases [12]. These in class B (carbapenemases) needs one or more zinc ions for their full catalytic activity and these enzymes are termed as Metallo beta Lactamases [13]. MBL production in bacteria are considered more crucial than any other resistance mechanisms because they can almost hydrolyze all beta lactam antibiotics making these enzymes a serious threat to human health [14]. Emergence of MBL producing *P. aeruginosa* in hospitals alarming and reflects overuse of carbapenems. There is intense selection pressure, due to high usage of broad spectrum antibiotics in hospitals. Notably, high morbidity and mortality rates ranges between 27% to 48% have been observed in critically ill patients. Furthermore, mortality rates are significantly higher in MBL producing *P. aeruginosa* compared to non-MBL *P. aeruginosa*.

The existing investigation is to decide the relationship of the organism between the biofilm, MBL production and serum resistance activity and to correlate with MDR characteristics of the *Pseudomonas* species from clinical samples [15].

MATERIALS AND METHODS:

Study Design: This cross sectional laboratory based retrospective study examined 500 clinical samples for a period of 10 months duration (from July 2016 to April 2017) which includes Burns (8), Pus (160), Wound swabs (108), Urine (94) and Sputum (130) obtained from various tertiary hospitals at Pondicherry, India. Bacteriological data were recorded from the clinical sources after following standard microbiology procedures for isolation. The suspected Pseudomonas isolates were further recovered upon culture on Cetrimide agar and Pseudomonas isolation agar with an incubation time of 24 h at 37 °C. They were presumptively identified to the species level by using standard microbiology and molecular procedures.

Molecular Identification of *P. aeruginosa* Isolates and Phylogeny Reconstruction: The isolated Pseudomonas culture was subjected to 16srRNA sequencing and identification using Universal 518 Forward primer 5' CCAGCAGC CGCGGTAATACG 3' and 800 Reverse 5' TACCAGGGTATCTAATCC 3' primers. The resultant amplified product (1400 bp) were purified and re-subjected to further sequencing using specific primers (785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT 3') [16]. Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) and the sequenced products have been resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Bio systems, USA). Sequencing results were subjected to BLAST analysis and the phylogenetic was constructed

using MEGA 6 [17]. In the tree the numbers indicates the levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support] based on a neighbor joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% had not indicated. Bar 0.005 substitutions per site.

Evaluation of Multiple Antibiotic Resistances: Determination of Multiple Antibiotic Resistance was done by using Kirby Bauer disc diffusion method in Mueller Hinton Agar (MHA) [18]. The following antibiotic discs (Hi Media) were used in this study: Amikacin (30µg), Gentamicin (10µg), Kanamycin (30µg), Neomycin (30µg), Netillin (30µg), Meropenem (10mg), Cephadroxil (30µg), Ceftriaxone (30µg), Cefoxitin (30µg), Nitro-furantoin (300µg), Ampicillin (10µg), Carbenicillin (100µg), Penicillin (10µg), Ciprofloxacin (5µg), Nalidixic acid (30µg), Tetracycline (30µg), Chlo-ramphenicol (30µg), Streptomycin (10µg), Azi-thromycin (15µg) Norfloxacin (10µg), Clindamycin (2µg), Polymyxin B (300U) and Bacitracin (0.04U).

Multiple Antibiotic Resistances (MAR) Index of *Pseudomonas* species: The MAR index to a single isolate was defined as a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the number of antibiotics to which the isolate was exposed. MAR index value higher than 0.2 is considered to have originated from high risk sources of contamination [19].

Metallo Beta Lactamase Production Assay: Combined Disk Test method was used for the phenotypic detection of Metallo Beta Lactamase in carbapenem resistant Gram negative bacteria. An EDTA solution of 0.5M concentration was prepared by dissolving 46.53 g of disodium EDTA.$2H_2O$ in 250 mL of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10 µg imipenem disks were placed on MH agar and 10 µL of an EDTA solution was added to one of them to obtain the desired concentration. After 16 to 18 h of incubation at 35 °C the zones of inhibition of imipenem and imipenem EDTA disks were compared and the inhibition zone greater than 7 mm with imipenem-EDTA disk was compared to the imipenem disk alone and was considered as MBL positive [20, 21].

Slime Production Assay: [22] BHA plates were prepared containing 0.8g/L Congo red along with 5mL of sterile human blood. All the identified Pseudomonas isolates were inoculated in the surface of the medium and the plates were incubated at 37 ºC for 24 h. After incubation colonies appearing black colored were considered positive for slime production and non-slime producers remained non-pigmented.

Serum Susceptibility Assay: [23] Group 'O' blood were obtained by vein puncture from healthy individuals with no recent history of infection; Pooled sera were separated and used immediately or stored at 7 ºC, Fresh or thawed normal human serum (NHS) were used unaltered. All the Pseudomonas strains were challenged against 65% NHS in a micro colorimetric assay. The strains tested were transferred to micro dilution well containing 100 µL of peptone 1% (v/v) and glucose (1% w/v) broth (PGB). After overnight incubation at 37 ºC, 20 µL of each PGB culture was transferred to the 200 µL of fresh PGB and incubated at 37 ºC for 2 h. Then log phase bacteria was inoculated (20 µL, 107 bacteria) into 100 µL PGB containing 65% NHS and 0.5% of 1.5 µL of stock solution of bromothymol blue (Final concentration 0.0075%) serum resistance was analyzed by visible color change from green (inhibition) to yellow (growth) of the PGB containing NHS. Control consisted of PGB with 65% heat- inactivated serum 56 ºC for 4 h.

RESULTS AND DISCUSSION: From this retrospective cross study 63 (12.6%) isolates of *Pseudomonas* were retrieved from clinical sources in association with other bacterial genera including *Staphylococcus* sp (22%), *Acinetobacter* sp (18.6%), *Streptococcus* sp (11.8%), *Escherichia coli* (14.4%), *Proteus* sp (8.8%), *Klebsiella* sp (4.8%), *Serratia* sp (1.4%), *Shigella* sp (2.2%) and *Salmonella* sp (2%) were recorded during the study. The distribution of these bacterial pathogens among the clinical sources were articulated in earlier reports as predominant nosocomial pathogens is in agreement with present report [24]. The prevalence of these bacterial species has been related to a number of factors, including long-term antimicrobial therapy, cross-transmission, length of hospital stay, invasive procedures and poor immunity [25].

Out of 63 (12.6%) Pseudomonas isolates the most common samples expressing high density of positive growth were Pus, 30 (47.61%), wound swabs 16 (25.39%) sputum 8 (12.69%) and Burns 8 (12.69%) and Urine 1 (1.58%) as shown in **Fig. 3**.

In our present study more number of isolates was obtained from pus followed by other clinical samples. The gender wise distribution of the bacterial pathogen also revealed the same pattern of results and previous reports have been documented from various parts of the country worldwide [26, 27]. The gender-wise prevalence of isolates shows that infections caused by *P. aeruginosa* are more common in males than females. The reason for high incidence in male might be due to habits of smoking and consumption of alcohol and use of narcotic drugs which has a direct correlation with poor immune system. Frequent hospital visit, use of extensive antibiotics and personal hygiene may also contribute the prevalence of higher bacterial density in males than females.

Molecular Identification of *P. aeruginosa* Strains: Based on 16srRNA sequencing and phylogenetic analysis among the identified *Pseudomonas* isolates *Pseudomonas otitidis*, *Pseudomonas taiwanensis* and *Pseudomonas mosselii* were recorded to be different and the remaining strains were presumably identified as *P. aeruginosa* **Fig. 1**. However, a molecular identification was under taken to confirm the identification and further the sequences were deposited in GenBank and the following accessions were obtained MK598324, MK595791, MK598327, MK598329, MK598330, MK598331, MK598332, MK598333, MK598334, MK598335, MK598336 and KX881765 for the selected strains.

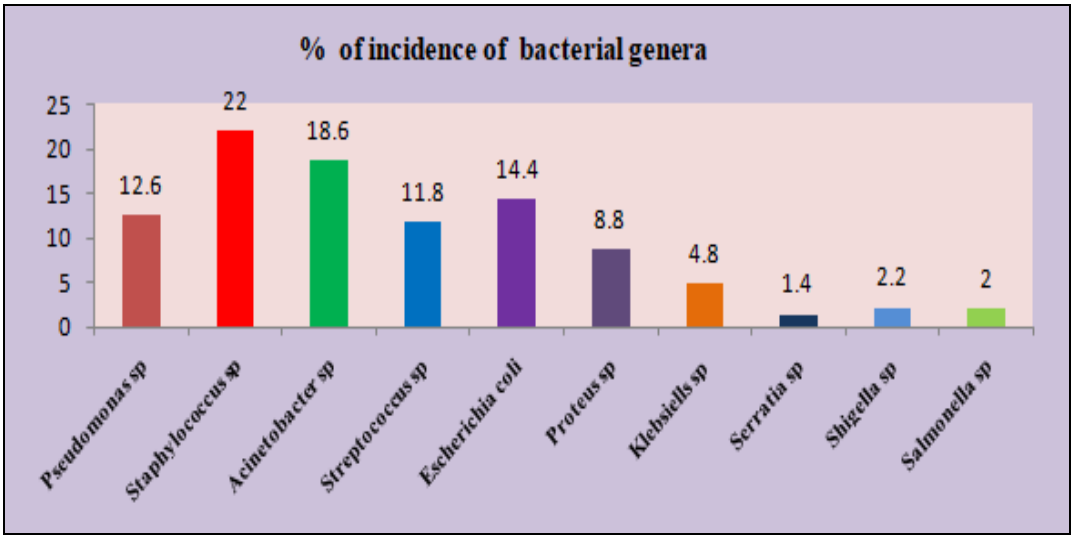

FIG. 1: DISTRIBUTION OF BACTERIAL GENERA IN CLINICAL SAMPLES

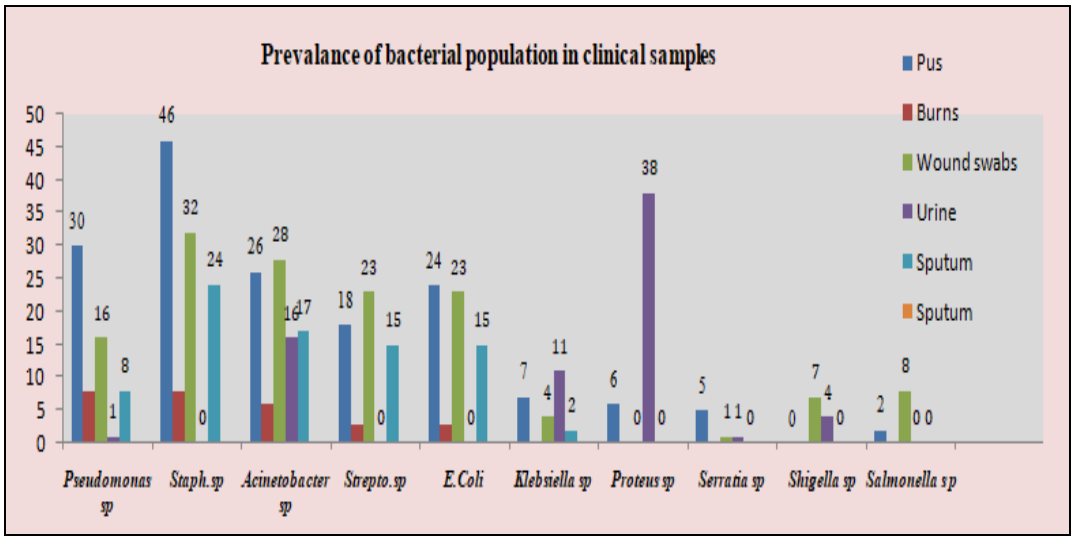

FIG. 2: INCIDENCE OF BACTERIAL GENERA IN CLINICAL SAMPLES

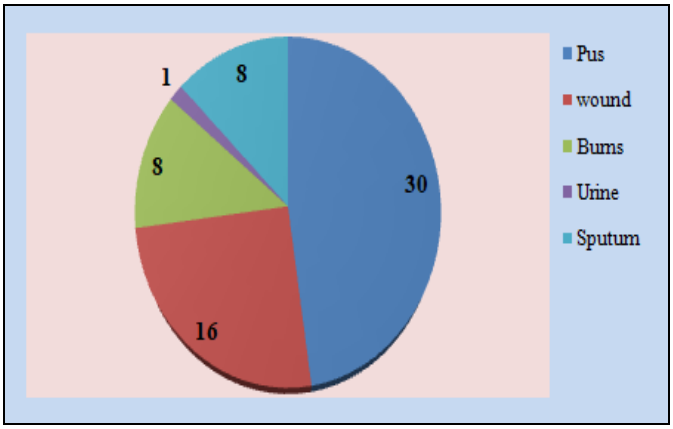

FIG. 3: DISTRIBUTION OF *PSEUDOMONAS* SP ON CLINICAL SAMPLES

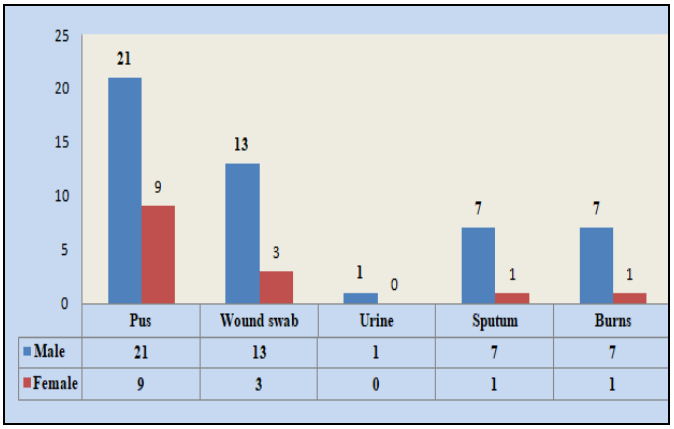

FIG. 4: GENDER WISE DISTRIBUTION OF *PSEUDOMONAS* SP IN CLINICAL SAMPLES

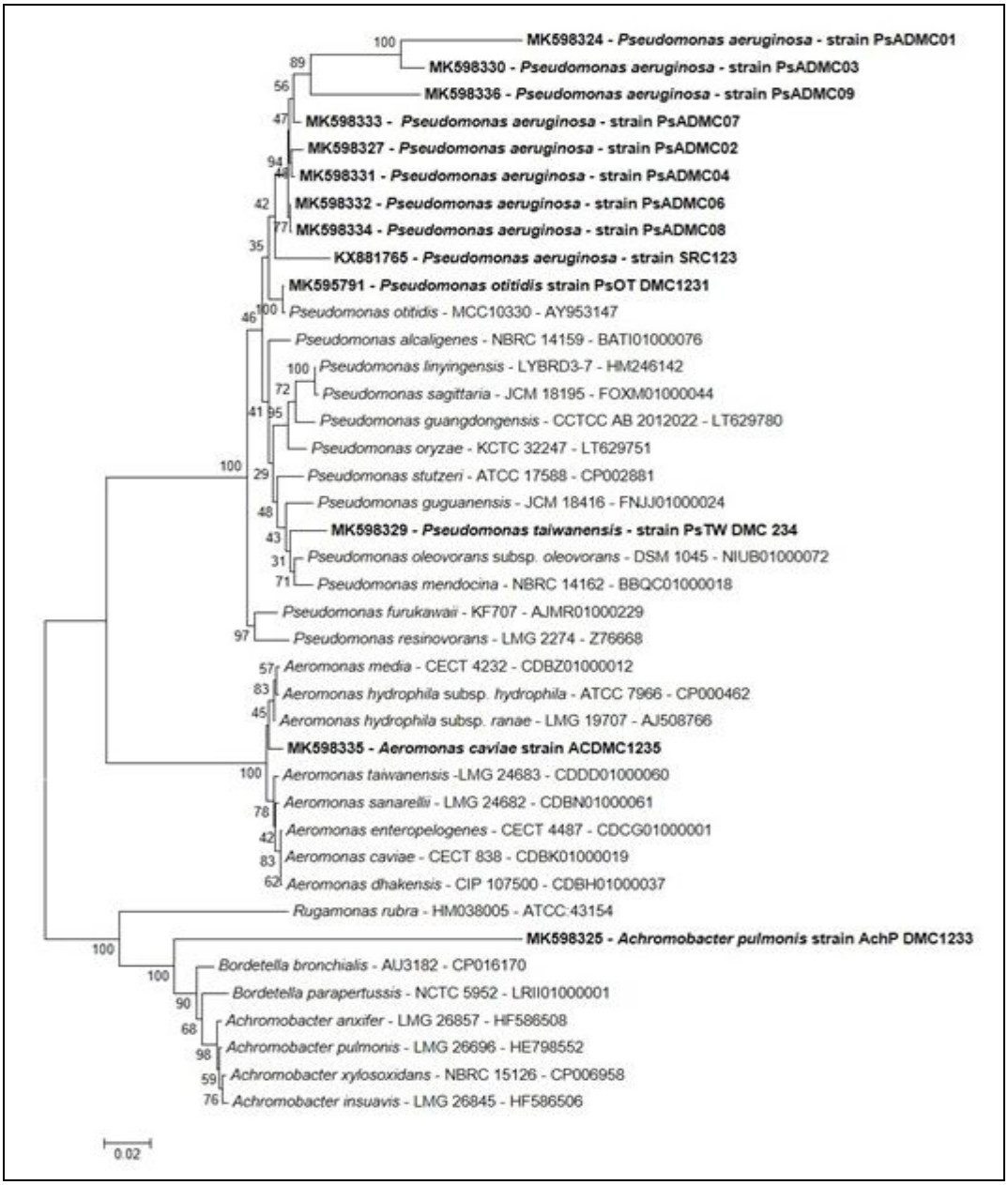

FIG. 5: THE EVOLUTIONARY RELATIONSHIP BETWEEN THE STRAINS RECOVERED FROM CLINICAL SAMPLES. THE PHYLOGENY WAS RECONSTRUCTED USING THE 16SrRNA GENE SEQUENCES. SCALE BAR REPRESENTS 0.02 NUCLEOTIDE SUBSTITUTIONS PER NUCLEOTIDE POSITION

Resistance Profile and Multiple Antibiotic Index of *Pseudomonas* sp: The anti-biographic profile narrates that all the isolates were completely resistant to penicillin G, Ampicillin, Tetracycline, Chloramphenicol, Bacitracin and Clindamycin (100%) followed by resistant to Nitrofurnatoin and Cefadroxil (95.65%) each respectively. Nalidixic acid (91.3%), Kanamycin, Cefoxitin and Polymyxin B were scored as (82.60%). For Azithromycin and Ceftriaxone the level of resistance were been recorded as 69.56%. Meropenem was found to be 60.86% resistant. Moderate level of resistant been noticed for Netillin (47.82%) Ciprofloxacin and Amikacin (43.47%) respectively. Gentamicin (39.13%) and Streptomycin (26.08%) were also identified during the study.

Resistance to Quinolones like Ciprofloxacin and Azithromycin (50% & 53.8%) were reported [28]. High resistance to polymyxin B was also described [29]. Similarly higher rates of resistance to fluoro-quinolones such as ciprofloxacin (40.5%) had been reported in a study done in North Kerala, India [30] and ciprofloxacin resistance (92%) was shown in a study from Malaysia [31].

Resistance to Amikacin (82%) and Ciprofloxacin (70%) were also demonstrated [32]. A much higher resistance to Ceftriaxone of 75%, 86% and 93.9% had been reported in the studies accomplished in India [26], Bangladesh [33] and Nepal [34]. High rate of resistance to the third generation cephalosporin drug-ceftriaxone (68.96%) had been reported in the studies done in India and Bangladesh [33].

High level of Susceptibility (65.7%) to the Ceftriaxone was observed in the preceding study conducted at Kolkata during 2005 [34] and the study justifies the current report as resulted in the same pattern. The highest Carbapenem resistance rate for Meropenem about 65.52% were demonstrated in earlier report [35]. In a collaborative study carried out during 2013 at Latin America, a very low level resistance pattern was observed for 586 isolates. Similarly, the average resistance for Gentamicin was 32.6%, 24.6% for Amikacin were documented from several countries which supports the present study [36].

Multiple antibiotic resistant (MAR) index is commonly used as a tool for health risk assessment to identify whether isolates are from regions of low or high antibiotic use. A MAR index of more than 0.2 indicates that the organism have originated from an environment where antibiotics are frequently used [37]. In the present study it was observed that none of the isolates showed identical MAR index and it ranged from 0.52 to 0.91 **Table 1** to **Table 4**. This indicates that clinical cases were reported in an environment of high use of antibiotics. High range of MAR index 0.5 to 0.9 and 0.17 to 0.50 was previously described by several researchers around the world [38].

TABLE 1: RESISTANT PROFILE FOR *PSEUDOMONAS* STRAINS FROM PUS

Culture no.	Resistant Profile	MAR index
1	P, A, K, NA, T, C, B, NET, N, CD, AZM	0.6
2	NX, P, CIP, A, K, NA, AK, T, C, B, MRP, NET, N, CTR, CD	0.69
3	NX, P, CIP, A, K, NA, AK, T, C, B, MRP, NIT, NET, N, GEN, CB, CTR, CX, CFR, CD, AZM	0.91
4	NX, P, CP, A, K, NA, AK, T, C, B, MRP, N, CX, CFR, CD	0.69
5	NX, P, CIP, A, K, NA, AK, T, C, B, MRP, N, GEN, CX, CFR, CD, AZM, NIT	0.78
6	NX, P, CIP, R, K, NA, T, C, B, MRP, NIT, N, CTR, CFR, CD, PB, AZM	0.69
7	P, A, K, NA, T, CB, NIT, N, CTR, CX, CFR, CD, AZM	0.6
8	P, A, K, NA, AK, T, C, B, N, CTR, CX, CFR, CD, NIT	0.56
9	P, A, K, NA, AK, T, C, B, NIT, N, CTR, CX, CTR, CD, AZM	0.6
10	P, A, K, NA, T, C, B, NIT, NET, N, CTR, CX, CFR, CD	0.6
11	P, A, K, NA, T, C, B, NIT, N, CTR, CX, CFR, CD	0.56
12	P, A, K, NA, T, C, B, NIT, N, CTR, CX, CFR, CD, AZM	0.6
13	P, A, K, T, C, B, NIT, N, CX, CFR, CD, AZM	0.6
14	P, A, K, T, C, B, NIT, N, CFR	0.6
15	P, A, K, NA, T, C, B, MRPNIT, N, CFR, CD	0.52
16	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
17	P, CIP, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
18	NX, P, CIP, A, K, , NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.91
19	NX, P, CIP, A, K, NA, AK, T, C, B, MRP, NIT, NET, N, CTR, CX, CFR, CD, AZM	0.86
20	P, A, K, NA, T, C, B, MRP, NIT, N, GEN, CX, CFR, CD, AZM	0.63
21	P, K, NA, T, C, B, S, MRPNIT, NET, N, GEN, CTR, CFR, CD, AZM	0.82

22	NX, P, CIP, A, K, NA, T, C, B, S, MRPNIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.86
23	NX, P, CIP, A, K, NA, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.86
24	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
25	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
26	NX, P, CIP, A, K, NA, T, C, B, S, MRPNIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.86
27	P, A, K, NA, T, C, B, NET, N, CD, AZM	0.6
28	P, A, K, NA, T, CB, NIT, N, CTR, CX, CFR, CD, AZM	0.6
29	P, A, K, NA, AK, T, C, B, , N, CTR, CX, CFR, CD, NIT	0.56
30	P, A, K, NA, AK, T, C, B, NIT, N, CTR, CX, CTR, CD, AZM	0.6

TABLE 2: RESISTANT PROFILE FOR *PSEUDOMONAS* STRAINS FROM WOUND

Culture no.	Resistant Profile	MAR index
31	P, A, K, NA, T, C, B, NIT, NET, N, CTR, CX, CFR, CD	0.6
32	P, A, K, NA, T, C, B, NIT, N, CTR, CX, CFR, CD	0.56
33	P, A, K, NA, T, C, B, NIT, N, CTR, CX, CFR, CD, AZM	0.6
34	P, A, K, T, C, B, NIT, N, CX, CFR, CD, AZM	0.6
35	P, A, K, T, C, B, NIT, N, CFR	0.6
36	P, A, K, NA, T, C, B, MRPNIT, N, CFR, CD	0.52
37	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
38	P, CIP, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
39	NX, P, CIP, A, K, , NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.91
40	NX, P, CIP, A, K, NA, AK, T, C, B, MRP, NIT, NET, N, CTR, CX, CFR, CD, AZM	0.86
41	P, A, K, NA, T, C, B, MRP, NIT, N, GEN, CX, CFR, CD, AZM	0.63
42	P, A, K, T, C, B, NIT, N, CFR	0.6
43	P, A, K, NA, T, C, B, MRPNIT, N, CFR, CD	0.52
44	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
45	P, CIP, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
46	NX, P, CIP, A, K, , NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.91

TABLE 3: RESISTANT PROFILE FOR *PSEUDOMONAS* STRAINS FROM BURNS

Culture no.	Resistant Profile	MAR index
47	NX, P, CIP, A, K, NA, AK, T, C, B, MRP, NIT, NET, N, CTR, CX, CFR, CD, AZM	0.86
48	P, A, K, NA, T, C, B, MRP, NIT, N, GEN, CX, CFR, CD, AZM	0.63
49	P, K, NA, T, C, B, S, MRPNIT, NET, N, GEN, CTR, CFR, CD, AZM	0.82
50	NX, P, CIP, A, K, NA, T, C, B, S, MRPNIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.86
51	NX, P, CIP, A, K, NA, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.86
52	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
53	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
54	NX, P, CIP, A, K, NA, T, C, B, S, MRPNIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.86

TABLE 4: RESISTANT PROFILE FOR *PSEUDOMONAS* STRAINS FROM SPUTUM &URINE

Culture no.	Resistant Profile	MAR index
55	P, A, K, NA, T, C, B, NET, N, CD, AZM	0.6
56	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
57	P, CIP, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
58	NX, P, CIP, A, K, , NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.91
59	NX, P, CIP, A, K, NA, AK, T, C, B, MRP, NIT, NET, N, CTR, CX, CFR, CD, AZM	0.86
60	P, A, K, NA, T, C, B, MRP, NIT, N, GEN, CX, CFR, CD, AZM	0.63
61	P, A, K, NA, T, C, B, NET, N, CD, AZM	0.6
62	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82
63	P, A, K, NA, AK, T, C, B, S, MRP, NIT, NET, N, GEN, CTR, CX, CFR, CD, AZM	0.82

Metallo Beta Lactamase Production: 70.83% of the strains including *Pseudomonas otitidis*, *Pseudomonas taiwanesis* and *Pseudomonas mosselii* have been found positive for MBL production. This parameter is more significant than other resistance mechanisms because they can hydrolyze all betalactam antibiotics. The MBL determinant appears to be widespread in the Indian subcontinent and in India 70-90% of the population carry MBL producers were reported [39].

By the utilization of horizontal gene transfer mechanism MBL encoding genes can be transferred from one bacterium to another. This is an endangered mechanism found in some bacteria [40]. For most of the Pseudomonas infections Carbapenems were chosen for treating severe infections and it was the drug of choice frequently recommended by the clinicians, but resistance to Carbapenems is increasing worldwide is an another worrying factor. MBL production was the main

resistance mechanism in carbapenem resistant *P. aeruginosa* and multidrug resistance were frequently detected [41].

Slime Production: Bacteria producing biofilms are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy. The biofilm production was detected in 56.52% of the Pseudomonas isolates including *Pseudomonas otitidis* and *Pseudomonas taiwanesis*. But *Pseudomonas mosselii* was found negative for biofilm production during the study **Fig. 3** which correlates with earlier reports [42]. Biofilm can reduce the immune response and phagocytic activity, thereby interfering with host defense mechanism. The resistance may be increased due to low penetrations of antimicrobials with biofilms. Finding out the exact relationship between the antibiotic resistance and bacterial pathogenic virulence factors is a significant parameter to understand the pathogenicity of the organism. Many researchers have reported that bacterial biofilm is associated with resistance to a wide range of antimicrobial agents [43].

Biofilms are the cause of persistent infections associated with a variety of medical implants, and are also connected with diseases such as chronic wounds, chronic obstructive pulmonary disease, urinary tract infections, and cystic fibrosis [44]. The main clinical consequence of tolerance of biofilms to antibiotics is that high concentrations of antibiotics are required for treating biofilm infections [45, 46].

Serum Susceptibility: The capacity to resist bactericidal activity of normal human serum (NHS) contributes to the virulence of many gram-negative pathogens. Resistance to the bactericidal activity of normal human serum (NHS) was noticed in 56.2% of the total isolates which includes *Pseudomonas otititdis and Pseudomonas mosselii*. *Pseudomonas taiwaneisis* did not yield positive results for Serum susceptibility test. The present report completely matches with the earlier study of Greta *et al.,* 2014. Serum sensitivity/resistance might be an important microbial phenotype, which could conceivably differentiate between invasive and non-invasive strains [47] High density of serum resistant pseudomonas isolate (72.9%) were accounted in earlier studies [48]. Resistance to the bactericidal activity of NHS is probably connected with the cell surface components of bacteria, including LPS. Further from LPS other bacterial molecules such as outer membrane proteins [49] were also associated with serum resistance. This diversity is also due to development of various adaptive mechanisms such as the nutritional and metabolic pathways, besides the regulation of gene expression [50].

CONCLUSION: On the basis of the present novel investigation, pathogenic multi drug resistant *pseudomonas* with various virulence factors including biofilm, MBL production and serum resistance activity were confirmed from the clinical samples. The antibiotics currently used may decrease the number of bacteria in biofilm formation, but they cannot completely eradicate the biofilms and hence relapses of biofilm infections often do occur. Therefore, removal of infected tissues on the implanted devices, and subsequent long term antimicrobial therapy may be required for treatment of biofilm infections. Providing high antibiotic concentrations through topical administration, combined antimicrobials and sequential therapies or the use of adjuvants to improve the efficacy of antibiotics are the therapeutic strategies that are employed to treat biofilm infections. Though, there is no correlation between biofilm production and serum resistance activity the MAR indices expressed by the isolates confirmed their MDR nature and should be taken into account during treatment of Pseudomonas infections. Similarly, in the present study, no substantive association between biofilm and metallo beta-lactamase production could be established. However, higher rates of drug resistance were seen among biofilm producers in comparison to non-biofilm producers were confirmed. Similarly strong correlations between MBL producers in connection with MDR characteristic were detected.

Most of the MDR Pseudomonas species from the current study were biofilm producers and resist bactericidal activity of NHS.

The concentration of an antimicrobial agent required to destroy a bacterial biofilm should be tested in the laboratory to select the appropriate type and concentration of antibiotics needed to eliminate bacterial biofilms. This may improve the

success rate of treating infectious diseases. Moreover, the ability of bacteria to form biofilms and MBLs has been enhancing the spread of antibiotic resistance and the accumulation of virulence genes. New therapeutic strategies should be aimed at a co-treatment approach that combines traditional antibiotics with a substance that interferes with biofilms and MBLs, and this may render the biofilms and MBLs more susceptible to treatment.

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