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Article

In vitro biofilm formation by multidrug resistant clinical isolates of *Pseudomonas aeruginosa*

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Abstract: In this study, 15 isolates of *Pseudomonas aeruginosa* were recovered in Cetrimide agar medium from aseptically collected swab samples. Antibiotic susceptibility test revealed highest resistance against Rifampicin (100%) followed by Penicillin (80%), Erythromycin (73.33%), Cephalosporin group (13.33-60%) and Aminoglycoside group (26.67%). The most effective group of antibiotic was Carbapenem with 6.67% resistance. Among 15 isolates, 3 were having highest Multiple Antibiotic Resistance (MAR) index were identified as *Pseudomonas aeruginosa* (P_1, P_2 and P_3) by API®20NE microbial identification kit. Minimum inhibitory concentration (MIC) of the isolates P_1, P_2 and P_3 was 3.05µg/mL, 0.76µg/mL and 3.05µg/mL for Meropenem whereas for Ceftriaxone it was 12.207µg/mL, 12.207µg/mL and 781.25µg/mL, respectively. Minimum bactericidal concentration (MBC) of Meropenem and Ceftriaxone was same for the isolates P_1 and P_2 i.e., 48.83µg/mL and 195.313µg/mL, respectively but in case of P_3 it was 781.25µg/mL for both antibiotics. In case of 70% ethanol, the MIC and MBC was 1:4 dilutions (for isolate P_3, MBC was 1:2 dilutions) whereas for Savlon®, MIC and MBC was 1% and 2% solution, respectively. All of the three isolates were biofilm former according to test tube assay and microtitre plate assay whereas modified congo red agar assay indicated only one isolate as biofilm former. The results suggest that post-operative wound infection may serve as a reservoir for multidrug resistant biofilm forming *P. aeruginosa* which may complicate the treatment regime unless proper treatment ensured based on antibiotic/antiseptic susceptibility test.

Keywords: wound infection; *Pseudomonas aeruginosa*; multidrug resistance; MIC; MBC; biofilm

1. Introduction

Post operative wound infections by multi-drug resistant (MDR) microorganisms are a global threat among the nosocomial infections leading to higher treatment expenditure, longer hospital stay, morbidity and mortality (Holzheimer *et al.*, 2000; Pruitt *et al.*, 1998; Naeem *et al.*, 2006). As the skin constitute the first line of defense in human body, an injury to the skin i.e. any wound can act as a portal of entry of pathogenic as well as opportunistic pathogens. The development of wound infection depends on the protective function of the skin which is a barrier of wound healing. Being most favorable site for biofilm formation, the wounds are considered as very high risk point for MDR microorganism infections. Post-operative wound infection is universal and the bacterial types present vary with geographic location, bacteria residing on the skin, clothing at the site of wound, time between wound and examination (Trilla, 1994). Generally, the most commonly isolated MDR

microorganisms from wounds are *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *E. coli* and *Acinetobacter baumannii (*Hartemann-Heurtier, 2006).

Among these pathogenic microorganisms, *P. aeruginosa* is considered as the second most common microorganism isolated in nosocomial pneumonia (17% of cases), the third most common organism isolated in urinary tract infection (UTI) and surgical site infection (11% of cases) and the fifth most common organism isolated from all sites of nosocomial infection (9% of cases) (Hartemann-Heurtier, 2006). Though *P. aeruginosa* is an opportunistic pathogen, it is one of the most clinically significant organisms because of its multiple drug resistance properties, biofilm formation and production of several virulence factors such as exotoxin A, protease, leukocidin, lipopolysaccharide (LPS), phospholipase and other enzymes. The deadliness of *P. aeruginosa* is observed in burn wounds, post-operative wounds, chronic wounds and cystic fibrosis patients (Richards, 1999).

Biofilm is the aggregation of multilayered cell clusters covered with exo-polymeric substance (EPS) facilitating adherence of the microorganisms to the wound surfaces which provides a moist, warm and nutritious location for biofilm formation. Although several reports described a higher level of biofilm production by MDR organisms, the correlation between biofilm formation and the acquisition of antimicrobial resistance is still debated (Richards *et al.,* 1999; Reiter *et al.,* 2011; Kwon *et al.,* 2008; Rao *et al.,* 2008; Sanchez *et al.,* 2013). The possible reasons responsible for multiple antibiotic resistances may be the induced expression of genes to produce efflux pump, limited diffusion of the antibiotics through the dense exo-polymeric slime layer, quorum sensing etc (Nikolaev and Plakunov, 2007; Haagensen *et al.,* 2007).

The infection of *P. aeruginosa* in post-operative wound infection is becoming more severe in developing countries due to absence of common hygienic practices, mass production and availability of low quality drugs, antiseptics and medicinal solutions for treatment, and ignorance towards proper responsibilities of the hospital staff (Bertrand *et al.,* 2002). In perspective of Bangladesh, there had been a limited report on the study of multi-drug resistant *P. aeruginosa* and biofilm formation associated with post-operative wound infection. Therefore, the present study was designed to investigate the susceptibility pattern of commonly used antibiotics and antiseptics against *P. aeruginosa,* isolated from post-operative wound infection and to demonstrate the biofilm formation potential of MDR *P. aeruginosa* isolates as a measure of their virulence property.

2. Materials and Methods

2.1. Study area

The study was performed at the Microbiological Laboratory, Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh. All the specimens were collected aseptically from patients with post-operative wound infection from Chittagong Medical College Hospital (CMCH), Chittagong, Bangladesh, brought to the laboratory and immediately, processed for isolation and identification of *Pseudomonas aeruginosa* following standard microbiological technique (Forbes, 2000). Verbal informed consent was obtained from the hospital's authority and all patients prior to sample collection.

2.2. Chemicals and media

All the chemicals used in the study were of analytical grade with desired purity and procured from Merck, Germany and Sigma-Aldrich, USA. Cetrimide agar (Hi-Media, India) was used for selective isolation of *Pseudomonas aeruginosa* and Mueller Hinton agar (Hi-Media, India) was used for antibiotic susceptibility test. Commercially prepared standard antibiotic disks were purchased from Oxoid Ltd., UK.

2.3. Specimen collection and bacterial isolation

20 patients of any age with both sexes suffered from post operative wound infections were selected as study population. Swabs from infected sites were collected using sterile cotton swab with aseptic precautions and directly inoculated into 9 ml sterile buffer peptone water (BPW) and mixed homogenously. For enrichment, 1 ml from the BPW was transferred to the sterile 9 ml Brain Heart Infusion Broth and incubated for 24 hours at 37°C. After incubation, 1 loopful culture from the enrichment culture of each sample was streaked on solidified Cetrimide agar plate and incubated for 24 hours at 37°C. Following incubation, selected pure colonies were subjected to further analysis.

2.4. Antibiogram profiling and MAR index calculation

Antibiotic resistance pattern of the isolates were done by Kirby-Bauer disc diffusion method according to Clinical Laboratory and Standards Institute (CLSI) guidelines (CLSI, 2018). The most important anti-pseudomonal drugs are some β-lactams (Ticarcillin, Ureidopenicillins, Piperacillin), Carbapenem (Imipenem

and Meropenem) and Aminoglycosides (Gentamicin, Tobramycin, Netilmicin and Amikacin) (Giamarellou, 2002). Hence, in this study, 17 commonly prescribed antibiotics were used *viz.* Amoxillin-Clavulanic acid (AMC), Amikacin (AK), Tetracycline (T), Gentamicin (CN), Cephalexin (CL), Sulfamethoxazole-Trimethoprim (S*T), Ampicillin (AMP), Ceftriaxone (CRO), Meropenem (MEM), Imipenem (IPM), Chloramphenicol (C), Ciprofloxacin (CIP), Ceftazamide (CAZ), Amoxicillin (AMX), Erythromycin (E), Cefixime (CFM) and Rifampicin (RD). Reference strain of *P. aeruginosa* ATCC 27853 was used as a control strain for interpretation of antibiotic susceptibility test results. The multiple antibiotic resistances (MAR) index was calculated for each isolate by dividing the number of antibiotics against which the isolate showed resistance over the total number of antibiotics tested (Krumpernam, 1983).

$$\text{MAR Index} = \frac{\text{No. of antibiotics against which organisms show resistance}}{\text{Total number of antibiotics used}}$$

MAR index higher than 0.2 indicates wide use of this antibiotic in the originating environment of this isolate (Krumpernam, 1983).

2.5. Bacterial identification

The selected bacterial isolates were identified on the basis of their morphological, biochemical and physiological characteristics according to Bergey's Manual of Determinative Bacteriology, 8th edition (Buchanan and Gibbons, 1974). API®20NE microbial identification kit (BioMerieux, USA) was used for biochemical characterization of the selected bacterial isolates.

2.6. Determination of MIC and MBC of antibiotics

Two commercially available antibiotics Ceftriaxone (500mg; Square Pharmaceuticals Ltd, Bangladesh) and Meropenem (500mg; Popular Pharmaceuticals, Bangladesh) were used for this purpose. The MIC was determined by broth microdilution method following CLSI guidelines (Amsterdam, 1996). Briefly, 50 µL of 2-fold sterile Luria Bertani (LB) broth was added into each well of the 2nd column of a sterile 96-well microtiter plate and 50 µl of 1-fold sterile LB broth (pH 7.2) was added into each other wells of the plate. 50 µl of antibiotic solution was added into the 2nd well of a row that resulted in 2-fold dilution. From this, 50µl suspension was added into the 3rd well of the row and mixed which again produce another 2-fold dilution. In this way, the gradual 2-fold dilution was done upto the 11th well. From the 11th well, 50µl suspension was discarded without further addition to 12th well. The process described above was followed for both antibiotics. Inoculation was done by adding 50µl of inoculum suspension (adjusted to 0.5 McFarland standard) in each well following the direction of 12th well to 1st well of each row. This resulted in another 2-fold dilution. The 1st and 12th wells of each row were considered as positive control for growth of the organism. Following incubation at 37ºC for 24 hours, 10 µl sterile 2, 3, 5-triphenyltetrazolium chloride (0.5% w/v) was added to each well of the microtiter plates and again incubated at the same condition (Sankar *et al.,* 2008). Bacterial growth was observed by changing color from yellow to red and MIC was interpreted by visual observation as the first dilution, which completely inhibited the bacterial growth. In case of MBC, one loopful suspension from each of the three wells containing the three lower concentrations (including MIC) of an antimicrobial agent showing no visual growth was streaked on Mueller-Hinton agar plates, incubated and observed for bacterial growth. The highest dilution which inhibited at least 99% of bacteria was considered as MBC. *P. aeruginosa* ATCC 27853 was used as quality control strain.

2.7. MIC and MBC determination of antiseptics

The bactericidal concentration of the most commonly used two commercial antiseptics *viz.* 70% ethanol and Savlon® (ACI Co. Ltd., Bangladesh) were determined through the classical method of successive dilutions. The inoculum was adjusted to 0.5 McFarland standards as earlier. For quality control, reference strain of *P. aeruginosa* ATCC 27853 was used and all antiseptics were freshly prepared prior to testing.

In a brief, for 70% ethanol, a series of seven test tubes was taken with 1 ml of sterile nutrient broth in each tube, except Tube 1. Then, 1 ml of freshly prepared 70% ethanol was added into Tube 1 and Tube 2. After mixing the contents in Tube 2, 1 ml mixture was transferred into Tube 3 and such serial transfer was repeated upto Tube 5 and 1ml was discarded. Finally, 100 µl of *P. aeruginosa* suspension was inoculated to Tube 1 to Tube 6, except Tube 7. Tube 6 and Tube 7 was considered as positive control (nutrient broth + test organism) and negative control (nutrient broth + distilled water), respectively. After incubation at 37°C for 24 hour, the contents of the

tubes was streaked on cetrimide agar plate and MBC was considered as the concentration of no bacterial growth on cetrimide agar plates (Mazzola *et al.*, 2009).

In case of Savlon®, different concentrations (9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% and 1%) were prepared in 1 ml sterile distilled water and 1 ml sterile nutrient broth was mixed with each concentration. Inoculation was done by adding 100 µl of *P. aeruginosa* suspension. After overnight incubation at 37°C, bacterial growth was observed visually by comparing with the control tubes (positive control and negative control) and MBC was determined after plating on cetrimide agar medium.

2.8. *In vitro* biofilm formation study

2.8.1. Christensen test tube assay

Test tube assay for qualitative detection of biofilm formation was described by Christensen *et al.* (1982). Briefly, 10 ml of sterile trypticase soy broth with 1% glucose were inoculated with *P. aeruginosa* isolates and incubated at 37°C for 24 hours. After incubation, decantation and washing of the test tubes were performed with sterile phosphate buffer (pH-7.2) and dried tubes were then stained with 0.1% crystal violet. After rinsing excess stain with phosphate buffer, tubes were dried in inverted position. Biofilm formation was considered positive when a visible layer of the stained material lined the wall and the bottom of the tube and remarked as weak, moderate and high based on the color intensity. Ring formation at the liquid interface did not indicate biofilm formation.

2.8.2. Modified Congo red agar assay

A modified congo red agar (CRA) medium (Composition, g/L: Brain heart infusion broth-37, sucrose-50, NaCl-15, glucose-20, agar-10 and Congo red indicator-8) was used for detection of biofilm formation (Kaiser *et al.*, 2013). Sterilized congo red solution (8 g/L) was added separately with the sterile medium. CRA plates were inoculated with *P. aeruginosa* isolates and incubated overnight at 37°C. The strong biofilm formers produce dry crystalline black colonies whereas weak biofilm formers produce pink colonies with occasional darkening of the centre. Darkening of the colonies with absence of dry crystalline appearance indicates an indeterminate result.

2.8.3. Microtitre plate assay

Microtitre plate assay was done by following the modified method of Stepanovic *et al.* (2000). Briefly, the overnight grown LB broth cultures were diluted (1:100) into fresh medium. 100 µl of the diluted inoculum was triplicately added in each of the 96 well microtitre plate and incubated for 72 hours at 37ºC. Following incubation, cells were dump out by turning the plate over and washed with 200 µl sterile phosphate buffer (pH-7.2) several times. 125 µl of 0.1% crystal violet was added to each well and incubated at room temperature for 10-15 minutes. Again the plate was rinsed with sterile phosphate buffer (pH-7.2) and dried for a few hours. For qualitative assay, the stained microtitre plate was photographed. For quantitative assay, 125µl 95% ethanol was added to each well to solubilize the crystal violet. The plate was covered and incubated at room temperature for about 30 minutes. Absorbance was recorded in a microplate reader at 630 nm using 95% ethanol as control. The biofilm forming ability was categorized into four classes based on OD_{630} values of the isolates and control ($OD_{control}$) as follows:

(1) $OD \leq OD_{control}$: Not a biofilm producer
(2) $OD_{control} < OD \leq 2OD_{control}$: Weak biofilm producer
(3) $2OD_{control} < OD \leq 4OD_{control}$: Moderate biofilm producer
(4) $4OD_{control} < OD$: Strong biofilm producer

2.9. Statistical analysis

All experiments were performed in triplicate. Variation within a set of data was analyzed by GraphPad Prism Software 6 (GPPS 6), and mean ± standard deviation values were expressed.

3. Results and Discussion

3.1. Isolation of *P. aeruginosa* and antibiogram profiling

A total number of 15 *P. aeruginosa* isolates were recovered from post-operative wound infection on cetrimide agar medium following enrichment culture method and subjected to antibiotic susceptibility testing. In general, *P. aeruginosa* is naturally resistant to many antimicrobial agents such as most of the β-lactams, chloramphenicol, tetracycline, quinolones, Trimethoprim/sulfamethoxazole, macrolides and rifampicin because of their lower permeability of the cell wall and chromosomal β-lactamase (Rossolini *et al.*, 2005). Therefore, 17

antibiotics from nine different classes were chosen for antibiogram profiling because of their wide use in hospital as anti-pseudomonal drugs.

The resistance and susceptibility pattern of the selected *P. aeruginosa* are shown in Table 1 and highest resistance was observed against Rifampicin (100%) followed by Ampicillin (80%), Erythromycin (73.33%) and Amoxiclave (53.33%). Previous study in Bangladesh reported 89.5% resistance against Ampicillin and 89.3% resistance against Amoxiclave (Yasmin *et al.*, 2005). Resistance to β lactam antibiotic is due to chromosomal or plasmid mediated β-lactamase enzyme which is responsible for the inactivation or degradation of the antibiotic (Livermore, 1995).

Resistance pattern against carbapenem group i.e., meropenem and imipenem was 6.67% which correlates well with the studies of India, Nepal, Spain and Italy (Ruhil *et al.,* 2009; Chander and Raza, 2013; Bouza *et al.,* 1999; Bonfiglio *et al.,* 1998). All of those studies suggested meropenem and imipenem as the most effective anti-pseudomonal drugs as also in our study. However, several reports indicated increasing resistance towards this antibiotic group day by day (Fatima *et al.,* 2012; Akhtar, 2010). Usually, resistance to carbapenem is often due to loss of porins and up regulation of efflux mechanism or production of the enzyme metallo β lactamase (MBL) (Kohler *et al.,* 1999).

In case of Aminoglycosides, 26.67% of *P. aeruginosa* isolates were resistant to Gentamicin and Amikacin. It was reported in Pakistan that the resistance to Gentamicin was higher than Amikacin which supported a study of India (Akhtar, 2010; Sasirekha *et al.,* 2010). Another research conducted in Bangladesh reported Gentamicin and Amikacin resistance 40% and 36.3% respectively which is notably high resistance pattern observed in the south Asian region (Begum *et al*., 2013).

The isolated bacteria showed moderate resistance rate (13.33-60%) against the antibiotics of Cephalosporin antibiotic group *viz.* Ceftriaxone, Cefixime, Cephalexin and Ceftazidime in the present study. The highest resistance (60%) was recorded against Cephalexin. As Cephalexin is from the first generation Cephalosporin group, resistance to this antibiotic is quite normal. A Bangladeshi research documented 100% resistance to Ceftriaxone and 80% resistance to Ceftazidime which also corroborates with a more recent study pointing out 100% resistance rate to Ceftriaxone but not with the Ceftazidime sensitivity as it was only 13.33% in the present study (Haque *et al.,* 2010; Mengesha *et al.,* 2014).

In the present study, 13.33% resistance was recorded against Ciprofloxacin of Quinolone group. But Corona-Nakamura *et al.* showed that *P. aeruginosa* was absolutely susceptible to Ciprofloxacin (Corona-Nakamura *et al*., 2001). However, resistance to fluoroquinolones has been reported in recent years as well. Many studies correlated with the increased resistance rate against the Ciprofloxacin (Ruhil *et al.,* 2009; Begum *et al.,* 2013; Corona-Nakamura *et al.,* 2001). Decrease amount of quinolones entering cells because of the defects in the function of porin channels and efflux systems in the bacterial membrane contribute to the multi-drug resistance problem (Livermore, 2004).

33.33% of *P. aeruginosa* were resistant against Tetracycline group which is because of the low permeability of the outer membrane of bacteria. The resistance rate was 100% in study of Mahmoud *et al*., 2013. 46.67% of *P. aeruginosa* were resistant against the Co-trimoxazole which is much lower than a recent study (Bessa *et al.,* 2015).

This study shows that these drugs can no more be used as empirical treatment of infections caused by clinical *P. aeruginosa* isolates. Additional studies are required to determine the drug resistance mechanism along with computational biology and to identify potent drug target for designing novel therapeutics against MDR pathogens.

3.2. MAR indexing

The multiple antibiotic resistance (MAR) indices were determined for each isolate by dividing the number of antibiotics to which the isolate is resistant by the total number of antibiotics tested (Krumpernam, 1983). In present investigation, MAR index of all of the isolates were >0.2, indicating that all the isolates were originated from the environment where antibiotics were frequently used (Krumpernam, 1983). MAR analysis has been used to differentiate bacteria from different sources using antibiotics that are commonly used for human therapy. The isolates having identical MAR index were might be from common niche (Kaspar *et al.,* 1990).

The three isolates P_1, P_2 and P_3 having highest MAR Index 0.72, 0.71 and 1.0 respectively were selected as multidrug resistant *P. aeruginosa* and used for further study (Figure 2).

3.3. Bacterial identification

The bacterial isolates were characterized based on their cultural (e.g., colony color, form, margin, surface and elevation); morphological (e.g., cell shape and arrangement, sporulation); physiological (e.g., growth response

at different temperature, pH and salt concentration) and biochemical (e.g., API®20NE microbial identification kit (BioMerieux, USA)) characteristics as described in the Cowan and Steel's manual for the identification of medical bacteria (Barrow and Feltham, 1993). The isolates were then identified as *P. aeruginosa* by comparing the test results with the standard descriptions given in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

3.4. MIC and MBC of antibiotics and antiseptics

Determination of MBC and MIC of antimicrobials for a clinical pathogen has now become very essential in clinical microbiology laboratories as treatment of immunocompromised or other patients require bactericidal rather than bacteriostatic response. In Bangladesh, there are very little literature regarding MIC and MBC of antibiotics and antiseptics against clinical isolates of *P. aeruginosa* from wound infection. Therefore, in present study, MIC and MBC of two commonly prescribed antibiotics (Meropenem and Ceftriaxone) and antiseptics (70% ethanol and Savlon®) against *P. aeruginosa* isolates were determined by broth micro-dilution method.

The MIC value of ceftriaxone was 12.207µg/mL, 12.207µg/mL and 781.25µg/mL whereas the MBC value was 195.313µg/mL, 195.313µg/mL and 781.25µg/mL for the isolates P_1, P_2 and P_3, respectively (Table 2). The MIC of meropenem was 3.05µg/mL, 0.76µg/mL and 3.05µg/mL for three isolates whereas MBC was 48.83µg/mL, 48.83µg/mL and 781.25µg/mL for the isolates P_1, P_2 and P_3, respectively.

The isolates P_1 and P_2 were categorized as 'susceptible' whereas P_3 as 'resistant' in the broth micro-dilution method according to the CLSI standards (Table 2). This result also correlates with the result of disc diffusion method. Moreover, disc diffusion method is more appropriate method than MIC determination because of its ease of performance and no requirements of special equipment (Farahani *et al.,* 2013).

70% ethanol is the disinfectant of choice in hospitals and healthcare settings for both hard surfaces and skin antisepsis. The specific mode of action includes membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lysis (McDonnell and Russell, 1999). In present study, 1:4 dilutions of 70% ethanol were found as MIC for all three isolates and also MBC for P_1 and P_2, except for P_3 where 1:2 dilutions were found as MBC (Table 3).

The MIC value for Savlon® was 1% concentration whereas exhibited bactericidal effect at 2% concentration against all three isolates. These findings are comparable to a report of Nigeria where 61% of *P. aeruginosa* isolates were susceptible to 1% Savlon® concentration (Iroha *et al.,* 2011). It was found that the higher concentrations of antiseptics were required by the tested isolates compared to the control strain *P. aeruginosa* ATCC 27853. It is necessary to have knowledge about the MIC and MBC of the antimicrobials before applying in any infection as emergence of antimicrobial resistant microorganism can be concentration dependent (Al-Jailawi *et al.,* 2013).

According to McDonnell and Russell (1999), reduced susceptibility of *P. aeruginosa* to any antiseptics is linked with the biofilm forming potentiality of clinical isolates. The micropopulation within the biofilm shows distinct genetic diversity such as modulation of microenvironment, genetic exchange between the cells etc. which is responsible for tolerance towards the antiseptics (McDonnell and Russell, 1999).

3.5. *In vitro* biofilm formation study

Any kind of wound bed serves as a good place for biofilm formation because of its fibrin network and nutritional status. In present study, the biofilm forming property of the selected *P. aeruginosa* isolates (P_1, P_2 and P_3) were evaluated by three methods *viz.* test tube assay, modified CRA assay and microtitre plate assay.

In test tube assay (Table 4), P_2 isolate was observed as strong biofilm former which also showed positive result in modified CRA assay as it produced dry crystalline black colonies (Figure 5). The modified CRA assay was negative for other two isolates i.e., P_1 and P_3 but in test tube assay, they (P_1 and $P_{3)}$ were categorized as moderate biofilm former (Table 4 and Table 5). All three isolates were biofilm former according to microtitre plate assay. Meanwhile, P_2 categorized as strong biofilm former whereas P_1 and P_3 as moderate biofilm former (Table 6). Despite of growing under same culture conditions, the biofilm formation ability of each isolate from same organism is different because of the failure of primary cell numbers to initiate biofilm formation and the absence of auto inducers i.e., quorum signaling molecules. Addition of 1% glucose increases the biofilm formation potential of microorganisms in both microtitre plate assay and test tube assay (Nagaveni *et al.,* 2010). A correlation was observed with some other studies (Mathur *et al.,* 2006; Bose *et al.,* 2009).

Among three assays, the microtitre plate assay was recommended as the most reliable and sensitive quantitative tool for determining the biofilm formation than that of the two methods, i.e., test tube assay and modified CRA assay (Nagaveni *et al.,* 2010).

Table 1. Antibiotic resistance and susceptibility pattern of *P. aeruginosa* isolates (n=15).

Classes of antibiotics	Type of antibiotic	No. (n) and percentage of resistant (%)	No. (n) and percentage of susceptible (%)
Penicillin	Ampicillin (10µg)	12 (80)	3 (20)
	Amoxicillin (30 µg)	10 (66.67)	5 (33.33)
Aminoglycoside	Gentamicin (30 µg)	4 (26.67)	11 (73.33)
	Amikacin (30 µg)	3 (26.67)	12 (73.33)
Quinolones	Ciprofloxacin (5 µg)	2 (13.33)	13 (86.67)
Cephalosporin	Ceftriaxone (30 µg)	2 (13.33)	13 (86.67)
	Cefixime (5 µg)	4 (26.67)	11 (73.33)
	Cephalexin (30 µg)	9 (60)	6 (40)
	Ceftazidime (30 µg)	2 (13.33)	13 (86.67)
Macrolide	Erythromycin (15 µg)	11 (73.33)	4 (26.67)
Carbapenem	Meropenem (10 µg)	1 (6.67)	14 (93.33)
	Imipenem (10 µg)	1 (6.67)	14 (93.33)
Sulfonamides	Sulfamethoxazole× Trimethoprim (Co-trimoxazole) (25 µg)	7 (46.67)	8 (53.33)
Tetraycline	Tetracycline (30 µg)	5 (33.33)	10 (66.67)
	Rifampicin (5 µg)	15 (100)	0 (0)
Others	Chloramphenicol (30 µg)	4 (26.67)	11 (73.33)
	Amoxicillin-clavulanic acid (30 µg)	8 (53.33)	7 (46.67)

Table 2. MIC and MBC of antibiotics (Ceftriaxone and Meropenem).

***P. aeruginosa* Isolates**	**Meropenem**		**CLSI Standard[15]**		**Ceftriaxone**		**CLSI Standard[15]**	
	MIC (µg/mL)	**MBC (µg/mL)**	**R**	**S**	**MIC (µg/mL)**	**MBC (µg/mL)**	**R**	**S**
P_1	3.05	48.83	≥16	≤4	12.207	195.313	≥64	≤8
P_2	0.76	48.83			12.207	195.313		
P_3	3.05	781.25			781.25	781.25		
P. aeruginosa ATCC 27853	0.0067	0.0067			0.002	0.011		

Note: R=Resistant; S=Susceptible

Table 3. MIC and MBC of antiseptics (70% ethanol and Savlon®).

***P. aeruginosa* Isolates**	**70 % ethanol**		**Savlon®**	
	MIC (dilution)	**MBC (dilution)**	**MIC (dilution)**	**MBC (dilution)**
P_1	1:4	1:4	2%	1%
P_2	1:4	1:4	2%	1%
P_3	1:4	1:2	2%	1%
P. aeruginosa ATCC 27853	1:4	1:4	2%	2%

Table 4. Test tube assay for *in vitro* biofilm formation study.

Isolates	Visual observation	Remarks
P_1	+	Weak
P_2	+++	Strong
P_3	++	Moderate

Table 5. Modified congo red agar (CRA) assay for *in vitro* biofilm formation study.

Isolates	Remarks
P_1	-
P_2	+
P_3	-

Note: + = Positive; - = Negative

Table 6. Microtitre plate assay for *in vitro* biofilm formation study.

Isolates	Absorbance of de-stained solutions (OD_{630})	Remarks
P_1	0.2427±0.079	Moderate
P_2	0.2950±0.163	Strong
P_3	0.2274±0.005	Moderate
Control	0.06	

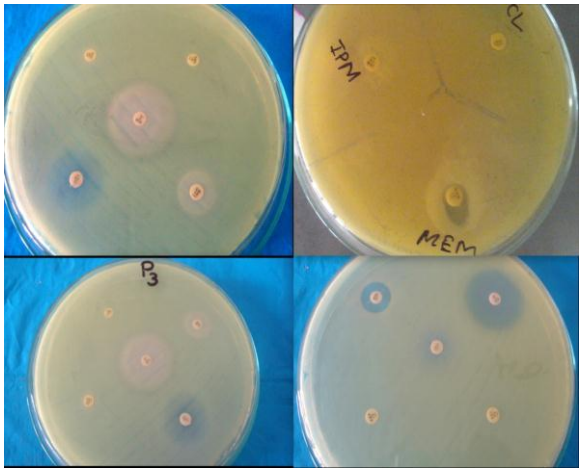

Figure 1. Antimicrobial susceptibility test of *P. aeruginosa* isolates by disc diffusion method.

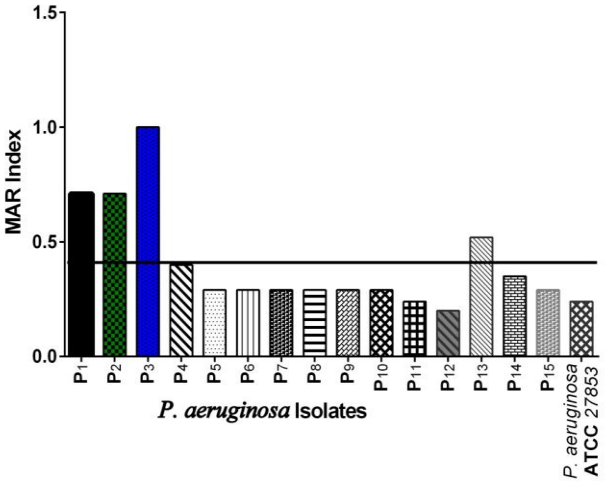

Figure 2. Multiple antibiotic resistance (MAR) index of *Pseudomonas aeruginosa* isolates (P_1-P_{15}).

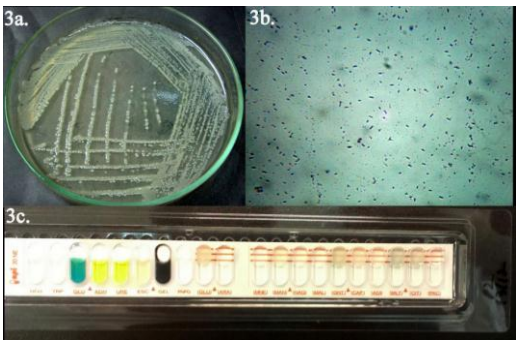

Figure 3. a) Cultural characteristics of *P. aeruginosa* on Cetrimide agar plate; b) Microscopic observation of gram negative rod shaped bacterial cell; c) Biochemical characterization by API®20NE microbial identification kit.

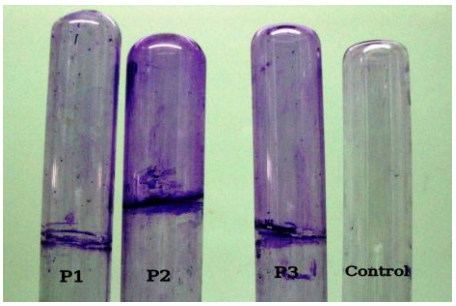

Figure 4. *In vitro* biofilm formation by *P. aeruginosa* isolates (P_1, P_2 and P_3) in test tube assay.

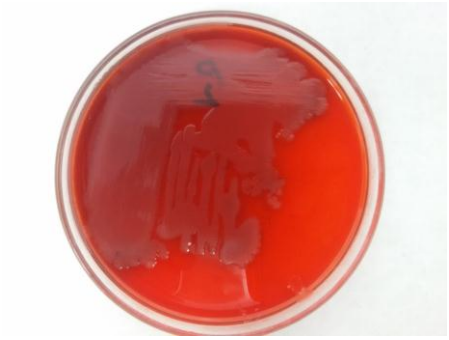

Figure 5. Black colonies of *P. aeruginosa* isolate P_2 on modified congo red agar (CRA) plate.

It has been reported that biofilm formation, particularly by MDROs, may play a relevant role in the pathogenesis of chronic wounds, considering its effects on the antibiotic resistance and the ensuing limitation of therapeutic options (Percival *et al.,* 2015). All of the three isolates which showed higher resistances to antibiotics were biofilm formers, indicating that that the majority of MDR pathogens are biofilm producers but this is still under study. Some research suggested that MDR isolates have more biofilm forming potential than susceptible organisms because of the greater biomass, intrinsic resistance, restricted and delayed penetration of antibiotics into the bacterial cell, the presence of starved cell due to nutrient limitation, exchange of virulence genes and so on (Stewart and Costerton, 2001; Ghotaslou and Salahi, 2013).
Tolerance to multiple classes of antibiotics by the micro-population of biofilm is one of the major virulence determinants of *P. aeruginosa*, which is making the wound infection management a challenging task day by day. Implementation of good sanitation and disinfection practices and proper utilization of antibiotics may play an important role in the prevention of post-operative wound infections due to MDR pathogens.

4. Conclusions

Multidrug resistance in bacterial population is a difficult task for the proper management of wound infections. In this study, the antibiotics of Carbapenem group (Meropenem and Imipenem) were found as the most efficacious drugs against MDR *P. aeruginosa* isolates while other drugs were found virtually useless and all the clinical pseudomonad isolates were biofilm former. To reduce infections by biofilm producing MDR microorganisms, a multidisciplinary approach should be built up involving both clinicians and microbiologists for routine microbiological surveillance. Further analysis should be carried out to investigate the relationship between MDR and biofilm formation.

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Conflict of interest

None to declare.

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