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Efficacy of Benzylkonium and Clorhexidine against Clinical Isolates of *Pseudomonas* Sp. from Dhaka, Bangladesh

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ABSTRACT

The opportunistic pathogen Pseudomonas aeruginosa has been recognized as a major cause of nosocomial infection due to resistance to multiple classes of antibiotics. This study reports presence of carbapenem resistance genes in clinical Pseudomonas isolates from Dhaka, Bangladesh, as well as their resistance to some common disinfectants (benzylkonium chloride and chlorhexidine gluconate).

Twenty three samples were collected from two hospitals and one diagnostic center from different locations in Dhaka city. Among these, 16 isolates were identified as Pseudomonas sp. from growth on Cetrimide Agar, standard biochemical tests (catalase, oxidase and triple sugar iron, urea, Sulphur Indole Motility medium, Lysine Iron Agar, Methyl Red-Voges Proskaeur medium, nitrate, citrate) and IasL/R-gene targeted PCR. Molecular characterization revealed three of the isolates contained 2.3kb to 2.5 kb plasmids. The genome of 6 isolates contained blaGIM gene encoding metallo-beta-lactamase variant (German), but none of isolates were resistant against imipenem group of antibiotics (meropenem, gentamicin, cefixime, imipenem). Therefore the isolates are genetically potent to resist antibiotics belonging to imipenem group, but are still sensitive to the antibiotics in culture.

3% Benzylkonium chloride and 0.5% chlorhexidine gluconate has slightly synergistic effect in inhibiting Pseudomonas sp. in representative isolates as their Fractional Inhibitory Concentration Index value ranges between 0.22 and 0.5. Taken together, *Pseudomonas* sp. isolates harboring potential virulent genes and imipenem-resistance genes could be inhibited more effectively *in vitro* if more than one suitable disinfectants are applied combinedly.

Keywords: blaIMP, disinfectant, metallo-β-lactamase, *pseudomonas.*

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I. Introduction

Pseudomonas sp. is a non-fermentative, aerobic, motile, invasive, Gram negative bacilli from family Pseudomonadaceae (Golia *et al.,* 2016). A high degree of nutritional versatility and adaptability ensure that *Pseudomonas spp.* is able to colonize a wide range of natural and man-made habitats (Balasubramanian *et al.,* 2014). *Pseudomonas* sp. is the predominant opportunistic pathogen leading to nosocomial infection and outbreak in hospitals (Hu *et al.,* 2017). *Pseudomonas* sp. contributes to 11% of all nosocomial infections, which result in high mortality and morbidity rates (Lila *et al.,* 2018). Pulmonary disease due to chronic infection is the major cause of morbidity and mortality in patients with cystic fibrosis (CF) (Lila *et al.,* 2018). The high mortality associated with these infections is due to a combination of weakened host immunity, bacterial resistance to antibiotics, and the production of extracellular bacterial enzymes and toxins (Iglewski, 1996).

Eradication of *P. aeruginosa* has become increasingly difficult due to its remarkable capacity to resist antibiotics. Strains of *Pseudomonas aeruginosa* are known to utilize their high levels of intrinsic and acquired resistance mechanisms to counter most antibiotics. Carbapenems are often used as "last-line" agents for treating serious infections caused by multidrug resistant (MDR) *P. aeruginosa* (Nagaveni *et al.,* 2011). However, data from European countries, covered by the European Antimicrobial Resistance Surveillance System (EARSS, now EARS-Net), showed an increasing trend in the prevalence of carbapenem-resistant *P. aeruginosa* strains varying between 4.4% and 58.5% among different countries (National Center for Emerging and Zoonotic Infectious Diseases, 2018). Besides, the acquisition of carbapenem-hydrolyzing enzymes (also known as metallo-β-lactamases, MBL) is considered as the most significantly mechanism of

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carbapenem resistance in this bacterium due to an increase in carbapenem use. These enzymes are known to hydrolyze all classes of β-lactams except monobactams (Wickham Laboratories).

In addition, various efflux pump systems are capable of actively removing every antibiotic from the intracellular milieu. Several *Pseudomonas* sp. produce endotoxin, which is a major virulence factor for causing bacteremia and septic shock (Balasubramanian *et al.,* 2014). Antibiotic susceptibility testing of clinical isolates is mandatory because of multiple antibiotic resistance; however, the combination of some antibiotics can be very effective in patients with acute *Pseudomonas* sp. infections (Balasubramanian *et al.,* 2014).

The study was designed to isolate carbapenem-resistant and other drug-resistant *Pseudomonas* sp. from clinical samples from patients with active ailments with cultural methods and disc diffusion, characterizing molecular markers associated with spread of drug-resistance (DNA fragments, plasmids) with conventional polymerase chain reaction (PCR) and assaying the efficacy of two common disinfectant used in clinical settings in controlling pathogens from hospital surfaces with *in vitro* methods. The purpose of the study is to find out what molecular element confers drug-resistant in clinical isolates of *Pseudomonas* sp. and how successfully the routine disinfection regime controls this pathogen. The data generated in this study could set the basis of risk assessment, designing effective disinfection regime to reduce burden of pathogen from healthcare settings. *Pseudomonas sp.* is a convenient organism to be used in such a study due to its propensity to drug- and disinfectant-resistance. *Pseudomonas aeruginosa* has a well-documented mechanism of carbapenem-resistance.

II. Materials and Methods

A. Collection and Processing of Samples

A total of 20 samples including surgical, sputum and urine specimen were collected from two hospital (Asgar Ali Hospital & Dr. Sirajul Islam Medical College and Hospital) and one diagnostic center (Popular Diagnostic Center) in Dhaka area. The samples were collected in a culture media (Nutrient Agar, Hi-Media, India) from sampling locations.

B. Isolation and Identification of Pseudomonas aeruginosa

Culture was pre-enriched in nutrient broth (Oxoid, UK) at 37 °C for 24 h in incubator. Cetrimide Agar Base (CAB) (Oxoid, UK), a selective identification medium for the isolation of *Pseudomonas aeruginosa* was used. The culture was streaked on the media and incubated at 37 °C for 24 h. Development of green colored, sticky, big, circular colony indicated the presence of *pseudomonas aeruginosa* presumptively. The presumptive isolates were transferred into Luria-Bertani Broth (LB broth) and maintained in 20% glycerin for preservation.

The isolates were identified by using conventional biochemical tests such as oxidase test, catalase test, citrate utilization test, MR-VP test, and indole test. Molecular characterization of the selected strains was done by polymerase chain reaction for the presence of bla_{GIM} and bla_{IMP} genes.

C. Antibiotic Disc Diffusion Test

McFarland 0.5 turbidity standards were prepared as per the standard guidelines described (Batel *et al.,* 2017). A volume of 0.5 ml of a 1.175% (w/v) barium chloride dehydrate ($BaCl_2.2H_2O$) solution was added to 99.5% ml of 0.18 mol/L (1% v/v) sulfuric acid with constant stirring to maintain the suspension. The turbidity standard was then aliquoted into test tube, identical to those used to prepare the inoculums suspension. The McFarland standard tubes were sealed with parafilm to prevent evaporation. McFarland standards then stored in the dark at room temperature (22 °C to 25 °C) (Batel *et al.,* 2017).

A sterile cotton swab was dipped into the adjusted suspensions. The swab was rotated several times pressed firmly inside the wall of the tube above the fluid level. This removed excess inoculum from the swab.

The dried surface of a Muller-Hilton agar (Himedia, India) plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60 °C each time to ensure an even distribution of inoculum.

Antimicrobial discs (Table I) were dispensed onto the surface of the inoculated agar plate. A total of 4 discs were placed on one 100 mm plate. Plates were then incubated at 37 °C for 24 h.

After 24 h of incubation each plate was examined. The sizes of zones of inhibition were interpreted by referring to interpretive standards from zone diameter around the antibiotic disc (Batel *et al.,* 2017). Isolates were reported as susceptible, intermediate or resistant according to standard guidelines from CLSI (Batel *et al.,* 2017).

TABLE I: List of Antibiotic Discs

Antibiotic disc	Concentration	Brand
Imipenem (IMP)	10 µg	Oxoid, UK
Meropenem (MRP)	10µg	Hi-Media, India
Cefixime (CFM)	5µg	Oxoid, UK
Gentamicin (CN)	10µg	Oxoid, UK

D. Disinfection Studies

Representative isolates were cultured in Luria-Bertani (LB) broth, allowed to grow at 37 °C overnight. Next day, each culture was streaked onto the surface of a Muller-Hinton agar media in the form of lawns, 3 wells were bored on the agar media at equal distances and 0.1 ml of 3% Benzylkonium chloride and 0.5% chlorhexidine gluconate were added to the wells. The zones of inhibition were measured in millimeters the next day.

E. PCR Detection of Genus Pseudomonas and BlaGIM Gene Variants

PCR was done using isolated genomic DNA extracted manually with phenol: choloroform: IAA method (Sambrook & Russel, 2006). Briefly, 1.0 ml of an overnight culture of each isolate was added to a 1.5 ml microcentrifuge tube, centrifuged at 3000 rpm for 2 minutes. Then cell pellet was resuspended in 600 µl of Lysis solution and placed at 80 °C for 5 minutes inside water bath.Then it was cooled at room temperature. After that the 30 µl diluted RNase A solution was added into the cell lysate and inverted 2-5 times to mix. It was incubated at 37 °C for 30 minutes to digest RNA. The

samples were cooled at room temperature.Then 200 µl of Protein Precipitation Solution (PPS) was added to the RNase-treated cell lysate. Vortex was done vigorously at high speed for 20 seconds. The samples were then incubated in water slurry for 5 mins. After that the samples were centrifuge at 13,000 rpm for 3 mins. The supernatant was transferred into a clean 1.5 ml microcentrifuge tube containing 600 µl of isopropanol, centrifuged at 13,000 rpm for 2 mins. Then 600 µl of 70% ethanol was added and mixed. Centrifugation was done at 13,000 rpm for 2 mins. The ethanol supernatant was poured off and the tube was inverted on clean absorbent paper to drain. The pellet was allowed to air-dry for 10-15 mins. The DNA pellet was left to completely dry to evaporate as much as of the ethanol as possible. 100 µl micro litre of DNA Rehydration Solution was added to the tube and concentration and purity of DNA was determined by measuring the absorbance at (A260) nm and (A280) nm using a Nano drop-2000 spectrophotometer to estimate the ratio between the absorbance value at 260 and 280 nm to ensure DNA purity.

IasI-F and *IasI-R* primers (5'-ATGATCGTACAAATTGGTCGG-3' and 5'-GTCATGAAACCGCCAGTC-3' respectively) were used to identify Genus *Pseudomonas* with 10 mM PCR buffer solution (RIPA Buffer 2x, Thermo-Fisher, USA), 10µM of each primer and 1 µl of DNA template in a total reaction volume of 20 µl. The PCR reaction consisted of initial denaturation at 94 °C for 10 minutes, cyclic denaturation at 94 °C for 30 seconds, annealing at 66 °C for 40 seconds and extension at 72 °C for 30 seconds. The 600 bp amplicons were resolved in 1% agarose gel (Aghamollaei *et al.,* 2015).

Detection of Bla_{IMP} and Bla_{GIM} genes for β-lactamase variants were done with primer pair (5'-GGAATAGAGTGGTCTAATTCTC-3' and 5'-CCAAACCACTACGTTATCT-3') and (5'-TCGACACACCTTGGTCTGAA-3' and 5'-AACTTCCAACTTTGCCATGC-3') respectively. The PCR reaction mix composed of 10mM PCR buffer solution (RIPA Buffer 2x, Thermo-Fisher, USA), 10 µM of each primer and 1 µl of DNA template in a total reaction volume of 20 µl. The PCR reaction consisted of initial denaturation at 94 °C for 10 minutes, cyclic denaturation at 94 °C for 30 seconds, annealing at 52 °C for 40 seconds and extension at 72 °C for 30 seconds. The 500 bp amplicons of Bla_{IMP} and 200 bp amplicons of Bla_{GIM} were resolved in 1% agarose gel (Farhan et al. 2018).

F. Plasmid Profiling

Alkaline lysis solution 1:50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0), de-ion water. Alkaline lysis solution 2:0.2 N NaOH, 1% (w/v) SDS, de-ion water. Alkaline lysis solution 3:5 M K-acetate, glacial acetic acid, de-ion water. Ethanol 70% Isopropanol TE-RNAase pH 8.0. The overnight grown culture was poured to 1.5 ml labeled Eppendorf tube. Centrifuge was done at 14,000 rpm for 1 min. The supernatant from the tube was removed. These steps were repeated until leaves bacterial pellet as dry as possible. Then 150 µl of resuspension buffer was added then the bacterial pellet properly resuspended by vortexing. After that 200 µl of lysis solution was added to bacterial suspension, the tube was closed tightly and the contents were mixed thoroughly by inverting the tube 4-6 times until the solution become viscous. Then 300 µl of neutralization solution was added and contents were mixed thoroughly by inverting the tube 4-6 times. Centrifugation was done at 14,000 rpm for 5 mins. Then the supernatant was transferred to a new 1.5 ml Eppendrof tube. Equal volume of isopropanol was added then into the supernatant and it was mixed by inverting the tube couple of times. Then incubated it in -80 °C. Centrifuge was done at 14,000 rpm for 5 mins. Then the supernatant was removed and ethanol 70% was added. After that centrifuge was done at 14,000 rpm for 5 mins. Again, the supernatant was removed and the pellet was dried for 10-30 mins. The pallet was dissolved then in 20-50 µl TE-RNase pH 8.0. The plasmid with 5 µl of DNA solvent was confirmed by Agarose Electrophoresis in 0.8% agarose gel.

III. Results

A. Isolation of Pseudomonas sp. from Clinical Samples

Among 20 samples 17 were selected presumptively as *Pseudomonas aeruginosa* based on their growth characteristics in selective media. The growth characteristics of presumptive *Pseudomonas aeruginosa* in Cetrimide Agar Base (CAB) media were circular, green and sticky. Positive isolates in Cetrimide Agar Base (CAB) were grown in Tryptone Soya Agar (TSA) media for further biochemical confirmation and antibacterial sensitivity test.

B. Identification of Pseudomonas sp.

All the 17 isolates were selected for analysis of gram reaction and was observed under digitally equipped light microscope (Olympus CX41, Olympus Crop; Tokyo, Japan) using oil immersion. All of them were Gram negative and found to be single or pairs and short rods in appearance.

C. Biochemical Test Results

Various biochemical tests of the selected isolates were performed to verify their relatedness of biochemical behaviors with reference *Pseudomonas aeruginosa*. The biochemical results of the isolates were presumptively identified according to standard guideline.

TABLE II: Biochemical Tests of Pseudomonas Aeruginosa

Sample ID	Biochemical Tests Results					
	Catalase test	Oxidase test	Methyl-red	Citrate test	Voges-Proskauer	Indole Test
C-2	+	+	-	+	-	-
C-5	+	+	-	+	-	-
C-6	+	+	-	+	-	-
C-7	+	+	-	+	-	-
C-8	+	+	-	+	-	-
C-11	+	+	-	+	-	-
C-12	+	+	-	+	-	-
C-17	+	+	-	+	-	-

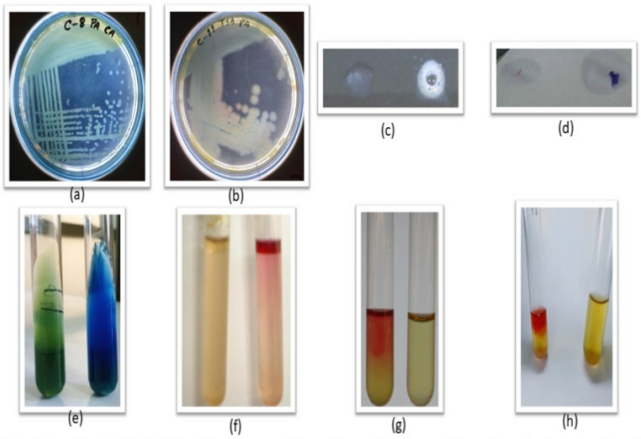

Fig. 1.Biochemical profile of clinical isolates of Pseudomonas. a) growth on cetrimide agar, b) growth on trypticase soy Agar, c) positive catalase test, d) positive oxidase test, e) positive citrate test (right) with negative control (left), f) positive indole test (right) with negative control (left), g) positive methyl red (left) with negative control (right), h) positive Voges-Proskeur test (left) with negative control (right).

D. Molecular Identification

All the 17 isolates showed *IasI* gene amplicon of 600bp, referring to confirmation of *Pseudomonas* genus (Aghamollaei et al 2015) (Fig. 2A).

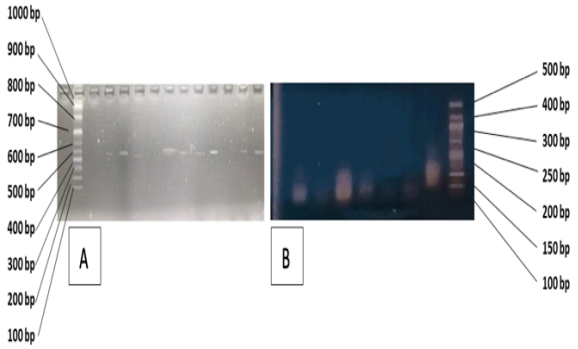

Fig. 2. PCR identification of clinical isolates of *Pseudomonas* with *iasL/iasR* genes with negative control (well 8) (A) and detection of bla_{GIM} in clinical isolates of *Pseudomonas* (B).

1) PCR of bla_{imp} gene

The bla_{imp} gene was not detected in any of the isolates from our study, using the Bla_{IMP} primer pair (result not shown).

2) PCR of bla_{GIM} gene

The isolates C4, C8, C13, C17 and C18 were detected to have bla_{GIM} gene (Fig 2B), indicating the German variant of β-lactamase is circulating in pathogenic strains of *Pseudomonas* from urine samples.

E. Antibiogram

In this study, 17 selected isolates were tested against four commercially available antibiotic discs such as Imipenem (10 µg), Meropenem (10 µg), Gentamicin (10 µg), Cefixime (5 µg). During the period of study, the sizes of zone of inhibition of every antibiotic disc were measured in millimeter and while those zones of inhibition compared with zone diameter interpretive standards from CLSI 2017 (Clinical & Laboratory Standards Institute) for *Pseudomonas aeruginosa.* The isolates showed sensitivity and resistance to the antibiotics. Different isolates produced different sizes of zone of inhibition.

TABLE III: ZONES OF INHIBITION OF GIVEN ANTIBIOTICS AGAINST *PSEUDOMONAS* ISOLATES

Sample ID	Imipenem (10µg)	Meropenem (10µg)	Gentamicin (10µg)	Cefixime (5µg)
	IC (ZI in mm)	IC (ZI in mm)	IC (ZI in mm)	IC (ZI in mm)
C-1	S (23)	S (23)	S (16)	R (6)
C-2	S (24)	S (23)	S (17)	R (6)
C-4	S (23)	S (26)	S (17)	R (6)
C-5	S (26)	S (28)	S (19)	R (6)
C-6	S (26)	S (27)	S (20)	R (6)
C-7	S (26)	S (29)	S (20)	R (6)
C-8	S (21)	S (28)	S (19)	R (6)
C-10	S (19)	S (21)	S (20)	R (6)
C-11	S (25)	S (26)	S (21)	R (6)
C-12	S (24)	S (27)	S (20)	R (6)
C-13	S (22)	S (25)	S (18)	R (6)
C-14	S (25)	S (26)	S (20)	R (6)
C-15	S (28)	S (30)	S (21)	R (6)
C-16	S (25)	S (28)	S (19)	R (6)
C-17	S (32)	S (28)	S (27)	R (6)

F. Disinfection Studies

The gradient diffusion method was used to determine critical concentration of disinfectant making up minimum inhibitory concentration (MIC) of the given disinfectant on selected isolates.

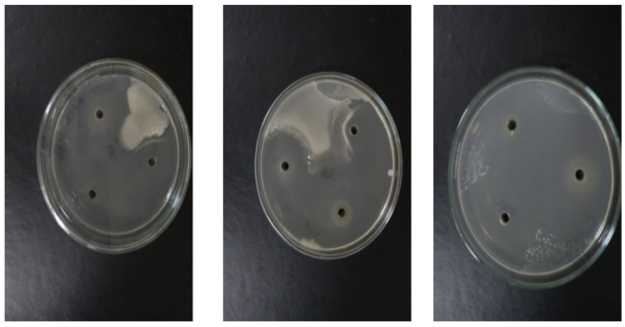

Fig. 3. Representative images of efficacy of common disinfectants benzylkonium chloride, chlorhexidine gluconate on clinical isolates of *Pseuodomonas* sp.

We adopt the calculations given by Mukherjee *et al.,* 2013 to calculate MIC of designated disinfectants:

$A = D/V$

and

$X^2 = 4DT_c \ln(C_0) + F(D, T_c, C_c)$

where,

A = concentration of disinfectant causing MIC
D = Concentration of disinfectant in well
V = Volume absorbed by disc
X = zone of inhibition in mm
D = Diffusion coefficient
T_c = Time of Incubation
C_0 = Concentration of disinfectant
F = Function of diffusion coefficient and concentration
C_c = Lowest concentration of disinfectant required

and

Fractional Inhibitory Concentration Index (FICI) = (zone of inhibition with two agents combined)/(zone of inhibiton by one agent alone)

TABLE IV: INTERACTION BETWEEN COMMON COMMERCIAL DISINFECTANTS IN INHIBITING CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA

Isolate ID	C8				
	Zone of diffusion	Zone of Inhibition with designated agent	Zone of Inhibition with 2 agents	FICI	Interaction
Benzylkonium Chloride	22	18	24	0.222	Synergy
0.5% Chlorhexidine gluconate	20	20	20	1.000	Indifferent
0.3% Chlorhexidine 3% cetrimide	43	9	9	1.000	Indifferent
Isolate ID	C9				
	Zone of diffusion	Zone of Inhibition with designated agent	Zone of Inhibition with 2 agents	FICI	Interaction
Benzylkonium Chloride	22	14	18	0.5714286	Synergy
0.5% Chlorhexidine gluconate	16	10	16	1.6	Indifferent
0.3% Chlorhexidine 3% cetrimide	46	10	10	1	Indifferent
Isolate ID	C16				
	Zone of diffusion	Zone of Inhibition with designated agent	Zone of Inhibition with 2 agents	FICI	Interaction
Benzylkonium Chloride	36	17	18	1.1176471	Indifferent
0.5% Chlorhexidine gluconate	23	13	13	1	Indifferent
0.3% Chlorhexidine 3% cetrimide	42	10	23	2.3	Indifferent
Isolate ID	C18				
	Zone of diffusion	Zone of Inhibition with designated agent	Zone of Inhibition with 2 agents	FICI	Interaction
Benzylkonium Chloride	51	16	16	1	Indifferent
0.5% Chlorhexidine gluconate	39	39	32	0.8205128	Indifferent
0.3% Chlorhexidine 3% cetrimide	69	9	7	0.7777778	Indifferent

FICI $\leq$ 0.5 indicates synergy between 2 disinfectants to kill the pathogen
0.5 $\leq$ FICI $\leq$ 4 indicates indifference of one disinfectant on another
FICI $\geq$ 4 indicates antagonism between 2 disinfectants to kill the pathogen

IV. DISCUSSION

In this study, the samples have been collected from two hospitals and one diagnostic center from August to October 2018. Out of 20 samples, 17 were identified as *Pseudomonas aeruginosa* (by standard bacteriological identification procedures). Here several complex and selective media have been used to isolate the desired microorganism. Nutrient broth and Tryptone Soya Broth (TSB) were used for storage and enrichment growth of samples. Selective Cetrimide Agar Base (Hi-Media, India) was used for selective isolation of *Pseudomonas aeruginosa* based on colony characteristics. Mueller-Hinton Agar (MHA) was used for antimicrobial sensitivity test.

Several standard biochemical tests have been carried out to confirm the presumptive isolates from selective media. Among 17 selected isolates, 16 were catalase, oxidase, citrate positive while methyl red, Voges-Proskauer, indole tested negative. In Gram staining the morphology of the isolated bacteria exhibited Gram-negative, rods, arranged in single or paired and motile.

Pseudomonas aeruginosa is emerging as a deadly nosocomial infectious agent. It has intrinsic as well as acquired resistance to many antimicrobial drugs. Many of them show multi-drug resistance. In order to investigate the sensitivity of *Pseudomonas aeruginosa,* the antimicrobial sensitivity test was performed in this study by Kirby-Bauer disc diffusion method and resulted according to The Clinical & Laboratory Standards Institute, 2017. The result showed that, the isolates were 100% sensitive to carbapenem (imipenem, meropenem) and gentamicin while 100% resistant to cefixime.

Molecular characterization revealed three of the isolates contained plasmids; isolates C4 and C5 contained 2.3 Kb plasmids while C13 had a 2.5 Kb plasmid. Plasmids in *Pseudomonas aeruginosa* range in size from 2 Kb to 1 Mb and carry a wide-range of molecular properties to recipient strain (Yoon & Jeong, 2021). None of the isolates carried Bla_{IMP} genes encoding metallo-beta-lactamase responsible for resistance against imipenem group of antibiotics and 6 isolates contained Bla_{GIM} gene encoding metallo-beta-lactamase variant from Germany. On the contrary, all the isolates showing resistance to Cefixime alone raises question about the state of antibiotic resistance at community level and the aberrant practice of antibiotics in Bangladesh. Resistance against a third-generation cephalosporin is alarming, because it reduces options for antibiotic therapy for clinical cases.

The final results on controlling *Pseudomonas* pathogens *in vitro* indicates that the combination of 3% benzylkonium chloride and 0.5% chlorhexidine gluconate has limited synergistic effect in inhibiting *Pseudomonas sp.* evident from Fig. 3 and Table IV. The Fractional Inhibitory Concentration Index value for benzylkonium chloride and chlorhexidine gluconate was 0.22 and 0.5 in two out of 4 representative isolates, indicating a synergistic effect of the two disinfectants in inhibiting the pathogens. There are growing concerns over slow acquisition to resistance against common disinfectants among clinical pathogens, especially *Pseudomonas*. In such cases, combined use of disinfectants might benefit healthcare facilities and patient management services to control nosocomial spread of resistant pathogens.

V. Conclusion

Taken together, *Pseudomonas* sp. isolates harboring potential virulent genes and imipenem-resistance genes could be inhibited more effectively *in vitro* if more than one suitable disinfectant are applied combinedly.

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