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Biofilm and MBL production among imipenem resistant *Pseudomonas aeruginosa* and *Acinetobacter* species

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ABSTRACT: *Pseudomonas aeruginosa* and *Acinetobacter* species are the primary cause of nosocomial infections. The advent of Metallo-beta-lactamase (MBL) and biofilm-producing bacterial strains poses a serious threat to reserve drugs such as carbapenem. The objective of this study was to determine the rate of MBL and biofilm production among imipenem resistant *P. aeruginosa* (IRPA) and imipenem resistant *Acinetobacter* spp. (IRAS) isolates. A total of 79 *P. aeruginosa* and 117 *Acinetobacter* spp. were isolated from various clinical specimens of patients from July 2016 to January 2017 at Manipal Teaching Hospital, Pokhara. MBL in IRPA and IRAS isolates were detected by Combined disc test and E-test. Biofilm production in imipenem resistant isolates was carried out by Microtitre plate assay. Fifteen (19%) *P. aeruginosa* and 57 (48.7%) *Acinetobacter* spp. were imipenem resistant isolates. MBL producers were found among 53.3% of IRPA and 38.6% of IRAS, whereas 100% of IRPA and 82.5% of IRAS were biofilm producers. All the biofilm producer IRPA isolates were Extensively Drug-Resistant (XDR), and a larger proportion of XDR IRAS strains were of high biofilm-producing phenotype. However, the majority of imipenem resistant (80% of IRPA and 49.1% of IRAS) and MBL producing (63%) isolates were weak biofilm formers. The study demonstrated the high capability of IRPA and IRAS to form a biofilm, which was strongly related to higher drug resistance. Nonetheless, imipenem resistant and MBL producer isolates showed an analogous association with the degree of biofilm formation. These MBL cum biofilm producer isolates were better susceptible to polymyxin B and ampicillin-sulbactam.

Keywords: *Pseudomonas aeruginosa*; *Acinetobacter* spp.; Imipenem-resistance; MBL; Biofilm.

1. INTRODUCTION

Carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species are an emerging cause of the hospital-acquired infection that presents a serious public health threat [1]. High carbapenem resistance in these organisms can be mediated by porin loss and efflux pumps or via carbapenemases [2]. Among various mechanisms of carbapenem resistance in *P. aeruginosa*, the production of MBLs is of particular concern due

to their rapid spread, the effectiveness of carbapenemase, resistance to β-lactamase inhibitors and the ability to hydrolyze all β-lactam antibiotics except aztreonam [3].

P. aeruginosa and *Acinetobacter* spp. are more resistant to antibiotics than other Gram-negative bacteria because of their high ability to form biofilm [4]. Extracellular polymeric substance (EPS) of biofilm acts as a defensive armor that prevents the diffusion of antibiotics [5]. Moreover, the frequent transfer of antibiotic resistance genes takes place among the biofilm cells. Therefore, MBL activity with biofilm formation in *P. aeruginosa* and *Acinetobacte*r spp. can lead to ineffective antimicrobial therapy [6].

Though it is of utmost importance to screen biofilm production and antimicrobial resistance pattern among these bacteria, limited information is available on this issue from Nepal. The objective of this study was to determine the rates of biofilm and MBL production among imipenem resistant *P. aeruginosa* and *Acinetobacter* spp. Further, we determined the rate of MDR and XDR among the strains of. *P. aeruginosa* and *Acinetobacter* spp.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The study was conducted in the Manipal Teaching Hospital (MTH), Pokhara, a tertiary care hospital in western Nepal from July 2016 to January 2017. The study included inpatient and outpatient departments of all age groups and both genders visiting MTH. *P. aeruginosa* and *Acinetobacter* spp. were isolated from various clinical specimens like respiratory specimens, blood, and other body fluids, urine, pus, and indwelling catheters. The bacterial isolates were identified as per standard microbiological techniques [7].

2.2. Antibiotic susceptibility test

Antimicrobial susceptibility testing of the isolates was performed on Mueller Hinton Agar (HiMedia, Mumbai, India) by Kirby-Bauer disc-diffusion method and interpreted following Clinical and Laboratory Standard Institute (CLSI, 2014) guidelines [8]. The isolates resistant to at least one agent in three or more antimicrobial categories were labeled as Multidrug-Resistant (MDR). The isolates resistant to at least one agent in all but susceptible to only one or two categories were defined as Extensively Drug-Resistant (XDR) [9].

2.3. Detection of MBL production

The imipenem-ethylenediamine tetra-acetic acid (EDTA) disk diffusion test [10] and imipenem/imipenem + EDTA E-test according to manufacturer's instruction [8] were used to detect MBL production among all the isolates that showed reduced susceptibility to imipenem. EDTA and E-test strips were obtained from Hi-media, India. Quality control of E-test strips was carried out by testing the strips with *P. aeruginosa* ATCC 27853 recommended by CLSI, 2014 [8].

2.4. Biofilm detection

Biofilm production (biomass) was detected among imipenem resistant isolates by microtiter plate (MTP) assay in accordance with the Christensen et al. [11] technique improvised by Stepanovic et al. [12]. Overnight cultures of bacteria in Brain Heart Infusion (BHI) broth with 1% glucose were standardized to 0.5 McFarland standard (~10^8 CFU/ml). Each well of commercially procurable pre-sterilized polystyrene 96 wells tissue culture plates (HiMedia, Mumbai, India) were filled with 180 µl of sterile BHI broth supplemented with 1% glucose. After that, a thorough vortexed 20 µl of already prepared bacterial suspensions were added to

each well resulting in the final testing inoculums of ~10^6 CFU/ml. Ten different wells containing sterilized 200 µl BHI broth with 1% glucose supplied as controls. The inoculated plate was covered with a lid and incubated at 37°C for 24 hours aerobically. The wells were washed four times with sterile phosphate buffer saline (pH 7.2), fixed with Bouin's fluid (saturated solution of picric acid 75 parts, formaldehyde 25 parts, and glacial acetic acid five parts) followed by washing once with sterile PBS, drying and then stained with 0.1% crystal violet solution. Excess stain was removed by tapping the plate. The plate was then left at room temperature for 15 min, followed by washing three times. Henceforth, 200 µl of 95% ethanol was added to each well. 150 µl of the solution from each well was transferred to another microtiter plate. The absorbance was measured at 630 nm using ELISA reader (HumaReader HS, Human, Germany). Biofilm producer and biofilm non-producer standard strains of *P. aeruginosa* served as positive and negative controls, respectively. The cut off absorbance (OD_C) value was considered as three times the standard deviation above the mean optical density (OD) of the control. Biofilm positive isolates were characterized as strong, moderate, and weak. The categories were based on the following criteria; non-biofilm producer if OD ≤ ODc, weak biofilm producer if ODc < OD ≤ 2ODc, moderate biofilm producer if 2 ODc < OD ≤ 4 ODc, and strong biofilm producer if 4 ODc < OD [12].

3. RESULTS

P. aeruginosa strains exhibited very high resistance rates towards piperacillin-tazobactam (89.9%), followed by ceftazidime (73.4%). The percentage resistance of *P. aeruginosa* isolates towards meropenem and imipenem was 27.8% and 19.0%, respectively (Table 1). Among 79 *P. aeruginosa* evaluated, 9 (11.4%) were MDR, 22 (27.8%) were XDR, and 48 (60.8%) were NMDR (Non-Multidrug-Resistant). All the imipenem resistant strains were XDR. The isolates exhibited discrepant carbapenem susceptibility profile; 8 isolates of *P. aeruginosa* were imipenem susceptible meropenem resistant, 1 was imipenem resistant meropenem susceptible, and 14 were both imipenem and meropenem resistant. All *P. aeruginosa* isolates were susceptible to polymyxin B (Table 1).

Table 1. Antibiotic resistance pattern of *P. aeruginosa* and *Acinetobacter* spp. isolated from different growth positive specimens.

	Number (%) of resistant organisms	
Antibiotics used	***P. aeruginosa* (n=79)**	***Acinetobacter* spp. (n=117)**
Aminoglycosides		
Amikacin (30 µg)	20 (25.3)	62 (53)
Gentamicin (10 µg)	24 (30.4)	67 (57.3)
Tobramycin (10 µg)	23 (29.1)	65 (55.6)
Penicillins with β-lactamase inhibitors		
Piperacillin-tazobactam (100/10 µg)	71 (89.9)	88 (75.2)
Ampicillin-sulbactam (10/10 µg)	-	23 (19.7)
Carbapenems		
Meropenem (10 µg)	22 (27.8)	57 (48.7)
Imipenem (10 µg)	15 (19)	57 (48.7)
Cephems		
Ceftazidime (30 µg)	58 (73.4)	92 (78.6)
Cefepime (30 µg)	54 (68.4)	89 (76.1)
Cefotaxime (30 µg)	-	96 (82.1)

	Number (%) of resistant organisms	
Antibiotics used	***P. aeruginosa* (n=79)**	***Acinetobacter* spp. (n=117)**
Ceftriaxone (30 µg)	-	95 (81.2)
Fluoroquinolones		
Ciprofloxacin (5 µg)	30 (38)	66 (56.4)
Levofloxacin (5 µg)	24 (30.4)	65 (55.6)
Monobactams		
Aztreonam (30 µg)	52 (65.8)	-
Folate pathway inhibitors		
Trimethoprim-sulphamethoxazole (1.25/23.75 µg)	-	79 (67.5)
Tetracyclines		
Tetracycline (30 µg)	-	70 (59.8)
Lipopeptides		
Polymyxin B (300 Units)	0 (0)	-

Table 2. Distribution of isolates based on specimens and demographic profiles of patients.

Variables	**Number (%) of isolates**		**No. (%) of imipenem resistant**	
	***P. aeruginosa* (n=79)**	***Acinetobacter* spp. (n=117)**	***P. aeruginosa* (n=15)**	***Acinetobacter* spp. (n=57)**
Gender				
Male	52 (65.8)	65 (55.6)	10 (66.6)	34 (59.6)
Female	27 (34.2)	52 (44.4)	5 (33.3)	23 (40.4)
Age group				
Newborn -12 (Children)	4 (5.1)	26 (22.2)	0 (0)	9 (15.8)
13-19 (Adolescent)	6 (7.6)	9 (7.7)	2 (13.3)	5 (8.8)
20-50 (Adult)	20 (25.3)	32 (27.4)	4 (26.7)	12 (21)
>50 (Elderly)	49 (62)	50 (42.7)	9 (60)	31 (54.4)
Specimens				
Respiratory specimens	44 (55.7)	48 (41)	8 (53.3)	33 (57.9)
Pus	17 (21.5)	27 (23.1)	3 (20)	16 (28.1)
Catheters	5 (6.3)	4 (3.4)	3 (20)	3 (5.2)
Urine	10 (12.7)	11 (9.4)	1 (6.7)	0 (0)
Blood and other body fluids	3 (3.8)	27 (23.1)	0 (0)	5 (8.8)
Ward				
Intensive Care Unit (ICU)	29 (36.7)	48 (41)	11 (73.4)	31 (54.3)
Surgery	13 (16.4)	22 (18.8)	2 (13.3)	15 (26.3)
Paediatric	1 (1.3)	6 (5.1)	0 (0)	1 (1.7))
Obstetrics and Gynaecology (OBG)	1 (1.3)	6 (5.1)	0 (0)	0 (0)
Medical	33 (41.8)	32 (27.4)	2 (13.3)	10 (17.5)
Outpatient	2 (2.5)	3 (2.6)	0 (0)	0 (0)

Legend: Respiratory specimens include endotracheal tube tips, endotracheal aspirate, tracheostomy tip, tracheostomy site swab; Other body fluids include pleural fluid and ascitic fluid.

Acinetobacter spp. isolates showed higher resistance towards cefotaxime (82.1%), followed by ceftriaxone (81.2%). Resistance rates of *Acinetobacter* spp. isolates towards meropenem and imipenem were 48.7% each. Fifty-seven strains of *Acinetobacter* spp. were both imipenem and meropenem resistant (Table 1). Of 117 *Acinetobacter* spp. tested, 49 (41.9%) were MDR, 23 (19.7) were XDR, and 45 (38.4%) were NMDR. Among 57 IRAS isolates, 23 were XDR, and 34 were MDR.

A maximum number of imipenem resistant isolates were obtained from respiratory specimens (53.3% *P. aeruginosa* vs. 57.9% *Acinetobacter* spp.) followed by pus (20% *P. aeruginosa* vs. 28.1% *Acinetobacter* spp.). The distribution of IRPA (66.6%) and IRAS (59.6%) was higher among male than female patients. Moreover, the highest percentage of IRPA (60%) and IRAS (54.4%) was recovered from elderly patients. ICU was found to be the most common site for the isolation of IRPA (73.4%) and IRAS (54.3%) (Table 2).

Results of combined disk test and E-test were concordant with all the imipenem resistant isolates showing 8 (53.3%) MBL producers among 15 IRPA and 22 (38.6%) MBL producers among 57 IRAS isolates (Table 3). All the MBL producer IRPA isolates were XDR, 100% resistant to all the tested antibiotics except polymyxin B (0%). In the case of 22 MBL producer IRAS isolates, 9 (40.9%) were XDR, and 13 (59.1%) were MDR. However, MBL producer IRAS isolates were relatively higher susceptible to ampicillin-sulbactam (59%) followed tetracycline, ciprofloxacin, amikacin, and tobramycin (4%) while the rest of the antibiotics were 100% resistant.

Table 3. Percentage of MBL producer isolates.

Disc Diffusion Test	E-test			Combined Disc Test
	MIC (Minimum Inhibitory Concentration) of Imipenem (µg/ml)			Number (%) of MBL positive
Imipenem resistant isolates	Sensitive (≤2)	Intermediate (4)	Resistant (≥8)	
IRPA (n=15)	0	4	11	8 (53.3)
IRAS (n=57)	0	0	57	22 (38.6)

Legend: MIC of imipenem; IRPA isolates (7 showed >256 µg/ml, 4 showed 128 µg/ml and 4 showed 6 µg/ml), IRAS isolates (36 showed >256 µg/ml, 12 showed 128 µg/ml and 9 showed 64 µg/ml).

Table 4. Antibiotic resistance pattern of moderate and weak biofilm producer IRPA isolates.

Antibiotics used	Moderate biofilm producer IRPA (n=3)	Weak biofilm producer IRPA (n=12)
Amikacin	3 (100)	10 (83.3)
Gentamicin	3 (100)	11 (91.6)
Tobramycin	3 (100)	12 (100)
Piperacillin-tazobactam	3 (100)	12 (100)
Meropenem	3 (100)	11 (91.6)
Imipenem	3 (100)	12 (100)
Ceftazidime	3 (100)	12 (100)
Cefepime	3 (100)	12 (100)
Ciprofloxacin	3 (100)	12 (100)
Levofloxacin	3 (100)	11 (91.6)
Aztreonam	3 (100)	12 (100)
Polymyxin B	0 (0)	0 (0)

Fifteen (100%) of IRPA isolates were biofilm producers; 3 (20%) were moderate, and 12 (80%) were weak biofilm producers. Though all the biofilm producer IRPA isolates were XDR, the resistance of amikacin, gentamicin, meropenem, and levofloxacin was relatively higher among moderate biofilm producers (100%) than weak biofilm producers (<92%) (Table 4). Among 57 IRAS, 47 (82.5%) were biofilm producers, of which 19 (33.3%) isolates were moderate, 28 (49.1%) strains were weak biofilm producers, and 10 (17.6%) were biofilm non-producers. Resistance to aminoglycosides, tetracycline, and ampicillin-sulbactam was comparatively higher among moderate biofilm producers (100%) than biofilm non-producer (<80%) IRAS isolates (Table 5). None of the imipenem resistant isolates were strong biofilm producers. The population of IRAS isolates that exhibited more stringent biofilm production likely contained a more significant proportion of XDR (Figure 1). The majority of MBL producer IRPA (62.5%) and IRAS (63.6%) isolates belonged to weak biofilm formers (Figure 2).

Table 5. Antibiotic resistance pattern of different levels of biofilm producer and biofilm non-producer IRAS isolates.

Antibiotics used	Moderate biofilm producer (n=19)	Weak biofilm producer (n=28)	Biofilm non- producer (n=10)
Amikacin	19 (100)	27(96.4)	8 (80)
Gentamicin	19 (100)	27 (96.4)	7 (70)
Tobramycin	19 (100)	27 (96.4)	7 (70)
Piperacillin-tazobactam	19 (100)	28 (100)	10 (100)
Ampicillin-sulbactam	12(63.5)	11(39.3)	0 (0)
Meropenem	19 (100)	28 (100)	10 (100)
Imipenem	19 (100)	28 (100)	10 (100)
Cefotaxime	19 (100)	28 (100)	10 (100)
Ceftriaxone	19 (100)	28 (100)	10 (100)
Ceftazidime	19 (100)	28 (100)	10 (100)
Cefepime	19 (100)	28 (100)	10 (100)
Ciprofloxacin	19 (100)	27 (96.4)	10 (100)
Levofloxacin	19 (100)	28 (100)	10 (100)
Trimethoprim-sulphamethoxazole	19 (100)	28 (100)	10 (100)

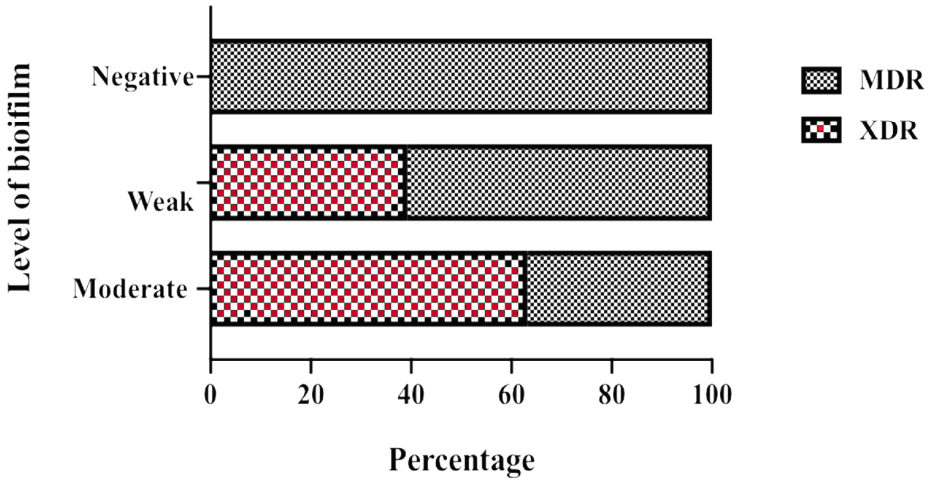

Figure 1. Distribution of resistance phenotypes among different biofilm production abilities of IRAS isolates displayed as a percentage stacked bar graph.

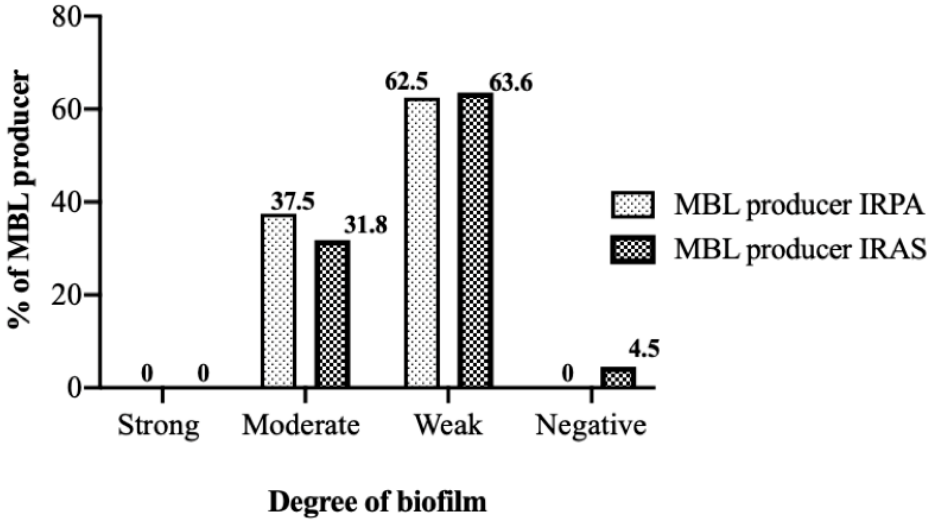

Figure 2. Percentage of MBL production among different biofilm production abilities of IRPA and IRAS isolates.

4. DISCUSSION

In the present study, the resistance rates of *Acinetobacter* spp. isolates towards meropenem and imipenem were 48.7% each, whereas that of *P. aeruginosa* isolates towards meropenem and imipenem were 27.8% and 19.0%, respectively. The disparity between imipenem and meropenem susceptibility in a single isolate of *P. aeruginosa* is attributed to the chromosomal mediated mechanism, such as OprD loss leading to imipenem resistance and overexpression of efflux pumps adding to meropenem resistance [13]. In this study, 27.8% of *P. aeruginosa* and 19.7% of *Acinetobacter* spp. were XDR, whereas 11.4% of *P. aeruginosa* and 41.9% of *Acinetobacter* spp. were MDR. This rate was lower than those documented in the earlier study from Nepal [14,15].

In this study, the highest number of imipenem resistant *P. aeruginosa* (53.3%) and *Acinetobacter* spp. (57.9%) were isolated from respiratory samples. It is well accepted that intubated patients with severe pulmonary disease are usually treated with prophylactic antibiotics, which increase the risk of resistance [15]. Similarly, the highest rate of infection by IRAS (54.4%) and IRPA (60%) strains were found among old age group patients. This might be due to their lowered immune system, which increases the risk of progression to infection. 73.4% of IRPA and 54.3% of IRAS isolates were recovered from ICU. The present finding is alarming as the use of broad-spectrum antibiotics without monitoring properly, and invasive procedures in ICU can be the epicenter of emergence and spread of highly drug-resistant strains. The study showed 53.3% of IRPA and 38.6% of IRAS isolates as MBL producers. The rate was lower than those documented in the previous study from Nepal [16]. In our study, all the MBL producer IRPA isolates were XDR, while 40.9% and 59.1% of MBL producer IRAS isolates were XDR and MDR, respectively.

Exceedingly high, 100% of IRPA and 82.5% of IRAS isolates were biofilm producers. The majority of imipenem resistant strains (80% of IRPA and 49.1% of IRAS) were weak biofilm producers. Similarly, 62.5% of MBL producer IRPA and 63.6% of MBL producer IRAS isolates were weak biofilm-producing phenotype. Only Polymyxin B and ampicillin-sulbactam were found to be effective against biofilm cum MBL producer IRPA, and IRAS isolates, respectively. Similar to the present study, Rodríguez-Baño et al. [17] demonstrated that 63% of biofilm producer *A. baumanii* showed less frequently resistant to imipenem than those that were biofilm non-producers, indicating that degree of biofilm production by these strains are not dependent on

imipenem resistance. Likewise, Perez et al. [18] and Wang et al. [19] presented that carbapenem-resistant *A. baumanii* strains less likely to produce biofilm than carbapenem-susceptible strains. These results suggest that biofilm acts as a mechanism for bacteria to achieve better survival, notably in isolates with an insufficiently high resistance level [20]. In yet another study, Gallant et al. [21] showed that the expression of certain types of beta-lactamases diminished the formation of biofilm in *P. aeruginosa* and *E. coli*.

In contrast, Chakraborty et al. [22] found a higher degree of biofilm formation among MBL producer *P. aeruginosa* isolates. Despite the inverse association between imipenem resistant (or MBL production) and degree of biofilm in our study, all the biofilm producer IRPA isolates were XDR, and more robust biofilm-producing IRAS strains contained a larger proportion of XDR. Correspondingly, several studies [23-25] found that biofilm producer *P. aeruginosa* and *A. baumannii* were of higher drug-resistant. Notwithstanding, Qi et al. [26] accounted that most of *A. baumanii* isolates with a higher level of resistance appeared to form weaker biofilms. Several studies documented controversies between the ability of biofilm production and antimicrobial resistance. Therefore, further molecular analysis of these isolates would confirm the correlation between biofilm formation and antibiotic resistance. Moreover, it would provide new perspectives into the treatment and prevention against *P. aeruginosa* and *Acinetobacter* spp. 'overlapping mechanisms' related infections.

5. CONCLUSION

The study showed that XDR isolates of IRPA and IRAS have a high propensity to form a strong biofilm. However, the majority of imipenem resistant and MBL producer isolates were weak biofilm formers. Ampicillin-sulbactam and polymyxin B showed better susceptibility against such strains. This indicates the need for regular screening of biofilm production and monitoring antimicrobial resistance profiles of these isolates to restrict their uncontrolled spread and infection.

Limitations: Further studies with adequate sample size, including imipenem susceptible strains, would help in testing the hypothesis of inferential statistics. Besides, molecular analysis of these isolates would confirm the correlation between biofilm formation and antimicrobial resistance.

List of abbreviations:

IRPA: Imipenem Resistant *Pseudomonas aeruginosa*

IRAS: Imipenem Resistant *Acinetobacter* spp.

MBL: Metallo-beta-lactamase

XDR: Extensively Drug-Resistant

MDR: Multidrug-Resistant

NMDR: Non-Multidrug- Resistant

MIC: Minimum Inhibitory Concentration

EPS: Extracellular Polymeric Substance

MHA: Mueller Hinton Agar

MTH: Manipal Teaching Hospital

CLSI: Clinical Laboratory Standard Institute

BHI: Brain Heart Infusion Agar

EDTA: Ethylenediamine tetra-acetic acid

Authors' contributions: YM conceived and designed the study, collected specimens, processed the specimen, analyzed data, and wrote the manuscript. SHS contributed towards supervision, manuscript writing, and critical evaluation of the manuscript. UTS, LRR, NA, and NN contributed towards distilling the results, discussion, and manuscript preparation. All authors read and approved the final manuscript.

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Availability of data and materials: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics approval: The research proposal was approved by the Institutional Ethics Committee, Manipal Teaching Hospital, Pokhara, Nepal, Reference number: MEMG/IRC/GA/1269/2016, and their permission were obtained to use the clinical isolates in this study.

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