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Antibiotic Resistance, Biofilm Formation and Detection of mexA/mexB Efflux-Pump Genes Among Clinical Isolates of *Pseudomonas aeruginosa* in a Tertiary Care Hospital, Nepal

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Efflux-pump system and biofilm formation are two important mechanisms *Pseudomonas aeruginosa* deploys to escape the effects of antibiotics. The current study was undertaken from September 2019 to March 2020 at a tertiary-care hospital in Kathmandu in order to ascertain the burden of *P. aeruginosa* in clinical specimens, examine their biofilm-forming ability and determine their antibiotic susceptibility pattern along with the possession of two efflux-pump genes-*mex*A and *mex*B. Altogether 2820 clinical specimens were collected aseptically from the patients attending the hospital and processed according to standard microbiological procedures. Identification of *P. aeruginosa* was done by Gram stain microscopy and an array of biochemical tests. All the *P. aeruginosa* isolates were subjected to *in vitro* antibiotic susceptibility testing and their biofilm-forming ability was also examined. Presence of *mex*A and *mex*B efflux-pump genes was analyzed by Polymerase Chain Reaction (PCR) using specific primers. Out of 603 culture positive isolates, 31 (5.14%) were found to be *P. aeruginosa*, of which 55% were multi-drug resistant (MDR). Out of 13 commonly used antibiotics tested by Kirby-Bauer disc diffusion method, greatest resistance was shown against piperacillin-tazobactam 15 (48.4%) and ceftazidime 15 (48.4%), and least against meropenem 6 (19.4%) and ofloxacin 5 (16.2%). Of all 17 MDR isolates subjected to biofilm detection, strong biofilm formation was exhibited by 11 (65%) and 14 (82%) isolates with microtiter plate method and tube method respectively. Out of 17 isolates tested, 12 (70.6%) isolates possessed *mex*A and *mex*B genes indicating the presence of active efflux-pump system.

Higher number of the isolates recovered from sputum 7 (58.3%) and pus 5 (41.7%) possessed *mexA*/*mex*B genes while the genes were not detected at all in the isolates recovered from the urine ($p<0.05$). This study assessed no significant association between biofilm production and multi-drug resistance ($p>0.05$). Adoption of stern measures by the concerned authorities to curb the incidence of multi-drug resistant and biofilm-forming isolates is recommended to prevent their dissemination in the hospital settings.

Keywords: *mex*A and *mex*B gene, MDR, efflux pump, PCR, biofilm

INTRODUCTION

Pseudomonas aeruginosa is extensively spread opportunistic human pathogen associated with numerous acute and chronic human infections, such as respiratory and urinary tract infection, pneumonia and bacteremia (1). It can thrive in a broad range of different natural and artificial environments including medical surfaces, due to its suitability and its strong intrinsic antibiotic resistance (2, 3). *P. aeruginosa* is the sixth most common nosocomial pathogen that causes health care associated infections from 2011 to 2014 and the second most common pathogen in ventilator-associated pneumonia in hospitals of USA (4).

In recent years, increasing antibiotic resistance is becoming a global problem, and drug resistance in *P. aeruginosa* has become a serious concern due to its potential to cause numerous nosocomial and chronic infections (2). It is naturally resistant to a wide range of antimicrobial drugs and it may also evolve resistance to other antibiotic classes by mutating or acquiring foreign resistance genes (5). Besides, several resistance mechanisms include efflux-mediated, beta-lactamase production, biofilm production, pori-related resistance and target site modification (6–8). Efflux pumps are membrane proteins that are involved in the export of noxious substances from the bacterial cell into the external environment. According to their composition, the number of transmembrane spanning regions, energy sources and substrates, bacterial efflux pumps is classified into six families: ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) superfamily, the drug metabolite transporter (DMT) superfamily and the multidrug and toxic compound extrusion (MATE) family (9, 10). *P. aeruginosa* has a significant number of efflux pumps, including four potent RND-type multidrug resistance efflux pumps (Mex) that remove harmful chemicals from the periplasm and cytoplasm (6). In particular, the MexAB-oprM efflux pump system was reported to contribute in conferring inherent resistance to chloramphenicol, β-lactams, quinolones, macrolides novobiocin and tetracyclines (11). Overproduction of such types of efflux systems plays a significant role in development of multidrug-resistant (MDR) bacteria (12).

P. aeruginosa adherence to host cells *via* type IV pili, along with the secretion of extracellular polysaccharide mediated by polysaccharide synthesis locus (*psl*), enhances biofilm development and conceals the pathogen from the immune system of the host (13, 14). The biofilm formation is widely known for promoting antimicrobial resistance, and its presence during the infection process has been regarded as a limiting factor for treatment effectiveness (15).

In developing countries like Nepal, antibiotic resistances are usually detected merely by phenotypic methods (16). In fact, interpretations by such methods are not sufficient and uncommon resistant patterns are often complicated by false-positive or false-negative results which may lead to inappropriate antibiotic prescriptions and treatment failure. Furthermore, a phenotypic test does not allow detailed molecular identification of antimicrobial resistance which is needed for epidemiological and infection control purposes. The molecular methods can also aid in quick, accurate and sensitive determination of resistance status among such pathogens. Additionally, drug-resistant isolates are becoming more common in Nepal's hospitals and clinics (16–20). The data available in the country for identifying resistant patterns is insufficient to define the antimicrobial resistance trend. Therefore, this study attempts to investigate the presence of two efflux-pump genes in clinical isolates of *P. aeruginosa* besides phenotypic detection of antibiotic resistance and biofilm production.

MATERIALS AND METHODS

Study Period, Design and Sample Size

This was a hospital-based cross-sectional study conducted in Kist Medical College and Teaching Hospital, Lalitpur, Nepal from September 2019 to March 2020. A total of 2820 clinical specimens (pus, urine, sputum, urethral swab, CSF and pleural fluid) were collected during the study period. The samples were collected in sterile, leak-proof, clearly-labelled containers and processed immediately in the Microbiology laboratory of the hospital.

Sample Processing and Identification of Isolates

The clinical specimens were cultivated on Blood agar, CLED Agar (Cystine-Lactose-Electrolyte-Deficient Agar), Nutrient agar, MacConkey agar and brain heart infusion broth as well as bile broth (HiMedia, Mumbai, India)) depending on requirements and the isolated colonies were identified following standard microbiological procedures that involved studying the colonial morphology, Gram staining and various biochemical tests (21).

Screening of Biofilm-Producing *P. aeruginosa* Isolates

Screening of biofilm producing *P. aeruginosa* was done using tube method and tissue culture plate method (TCP). The method standardised by Mathur et al. (22) was followed to develop biofilms using tube method. In brief, a loopful of the overnight bacterial culture was inoculated in glass tube containing 10 mL of trypticase soy broth with 1% glucose. The inoculated tubes were then incubated at 37°C for 24 hours. After incubation, the tubes were emptied, washed with phosphate buffer saline and left to dry. Crystal violet (0.1%) was used to stain the dried tubes for 15 minutes. Excess stain was removed by washing the tubes with deionized water. The tubes were then dried in inverted position and examined for biofilm production. Formation of a stained ring at the air-liquid interface indicated a negative result for biofilm creation, whereas the presence of a visible film along the bottom and wall of the tube indicated a positive result for biofilm production.

Tissue culture plate method was executed following protocols described by Mathur et al. (22) in 96 flat-bottom wells. Briefly, a loopful of freshly cultured isolates was inoculated in 10 ml of trypticase soy broth with 1% glucose and kept in the incubator at 37°C for 24 hours. Bacterial suspension was further diluted (1:100) with fresh medium. Separate wells of a sterile polystyrene tissue culture plate, composed of 96 flat bottom wells, were filled by 200 µL of the prepared bacterial suspension and incubated at 37°C for 24 hours. The plate was gently tapped to remove the content of the wells followed by washing with 200 µL of phosphate buffer saline for four times. Sodium acetate (2%) was added to the wells and kept for 30 minutes to fix the biofilms formed by bacteria attached to the wells. Staining of the fixed biofilms was conducted using crystal violet (0.1%). After 30 minutes, the wells were thoroughly washed by deionized water. After drying, a micro-ELISA reader (at 570 nm wave length) was used to measure the optical densities (OD) of stained bacterial biofilms. The experiment was carried out in triplicate and three times, after which the data were averaged and the standard deviation was determined. To account for background absorbance, OD measurements from the sterile medium, fixative, and dye were averaged and subtracted from all test results. The mean OD value obtained from the medium control well was subtracted from all of the test OD readings.

Antibiotic-Susceptibility Testing

The antibiotic-susceptibility testing was performed on Muller Hinton agar (MHA) by the modified Kirby-Bauer disc diffusion method following Clinical and Laboratory Standard guidelines (CLSI) 2018 (23). The antibiotic disks used in this study were procured from HiMedia Laboratories, Mumbai, India and included amikacin (30 µg), levofloxacin (5 µg), cefepime (50 µg), ceftazidime (30 µg), meropenem (10 µg), ciprofloxacin (5 µg), imipenem (10 µg), tobramycin (10 µg), piperacillin (100 µg), piperacillin-tazobactam (100/10 µg), gentamicin (10 µg), ofloxacin (5 µg) and aztreonam (30 µg). A suspension of bacteria adjusted to the 0.5 McFarland standards was swabbed using sterile swabs on MHA plates, and antibiotic discs were placed on top. The plates were incubated for 24 h at 37°C. The diameter of the zone of inhibition for each antibiotic was measured and interpreted as recommended by CLSI guidelines (23). Strains were considered multidrug-resistant (MDR) if they were resistant to at least one agent in three or more antimicrobial categories (24).

Extraction of Chromosomal DNA and Amplification of *mexAB* Gene by PCR

For the amplification of *mexA* and *mexB* gene, chromosomal DNA was extracted from each *P. aeruginosa* isolates following chromosomal DNA extraction method (25, 26). Briefly, the bacterial cells were lysed with 0.5 mg/mL lysozyme in the presence of 1/10 volume of 10% sodium dodecyl sulfate (SDS) at high pH and the lysate was then neutralized. Subsequent deproteinization with 1:1 phenol: chloroform, chromosomal DNA was precipitated with ethanol by spinning at high speed and extracted DNA was stored at -20°C. The primers used to detect the *mexA* and *mexB* gene have been enlisted in **Table 1**.

The polymerase chain reaction targeting *mexAB* gene was done in 25 µL reaction mixture consisting of 21 µL of 1X Qiagen master mix, 0.5 µL each of forward and reverse primers and 3 µL of extracted DNA template. Amplification was performed following conditions for *mexAB* gene which comprised of initial heating at 94°C for 3 min, then 32 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR amplification products and 100bp DNA ladder were fractionated by electrophoresis through 1.5% (for 280bp) and 1% (for 503bp) agarose gel visualized by staining with ethidium bromide (1µg/mL).

Quality Control

The efficiency of laboratory equipments such as an incubator, autoclave, hot air oven, and refrigerator was frequently evaluated. The expiry date and storage conditions of reagents, media, and antibiotic discs were thoroughly examined. Likewise, the quality of each batch of culture and biochemical media was assured by incubating a randomly sampled medium at 37°C for 24 h. A reference strain of *Pseudomonas aeruginosa* ATCC 27853

TABLE 1 | Nucleotide sequence of the primer used in the detection of *mexA* and *mexB* gene.

Gene	Primers	Amplicon Size (bp)	Reference
mexA	F: 5'-CTCGACCCGATCTACGTC-3' R: 5'-GTCTTCACCTCGACACCC-3'	503	(26)
mexB	F: 5'-TGTCGAAGTTTTTCATTGATAG-3' R: 5'-AAGGTCACGGTGATGGT-3'	280	(25)

was used to maintain the quality control of AST. Similarly, the thickness and pH of MHA were maintained at 4 mm and 7.2-7.4 respectively.

Data Analysis

All the data obtained were entered into SPSS and analyzed using SPSS (Version 22.0). Chi square test was performed to draw association between the related variables and a value of $p \leq 0.05$ was considered significant wherever applicable.

RESULTS

Distribution of Bacterial Growth in Different Clinical Specimens

A total of 2820 clinical specimen were collected and processed for microbial analysis of which 603 (21.4%) were culture positive. The highest percentage of microbial growth was reported in urine 313 (51.9%) followed by sputum 117 (28.4%) and body fluids 119 (19.7%). Bacterial growth was not reported in pleural fluid and CSF (**Figure 1**).

Distribution of *P. aeruginosa* in Culture Positive Samples

Out of 603 culture positive isolates, 31 isolates were reported as *P. aeruginosa*. The prevalence of *P. aeruginosa* among culture positive isolates was 5.14% (**Figure 2**).

Distribution of Bacterial Genera in Different Clinical Specimens

Out of 2820 clinical samples processed, 603 were culture positive, among which *E. coli* (49.6%) was the most frequently isolated bacteria followed by *K. pneumoniae* (14.6%), *Acinetobacter* spp. (9.8%), and *Staphylococcus aureus* (8.4%). The most common bacteria isolated from urine, sputum and body fluids were *E. coli* (78.9%), *K. pneumoniae* (30.9%) and *Staphylococcus aureus* (38.7%) respectively. Highest incidence of *P. aeruginosa* was observed in sputum 14 (45.16%) samples (**Table 2**).

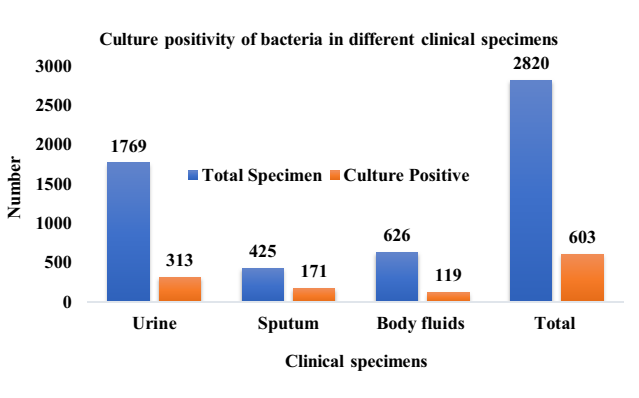

FIGURE 1 | Distribution of microbial growth in different clinical specimens.

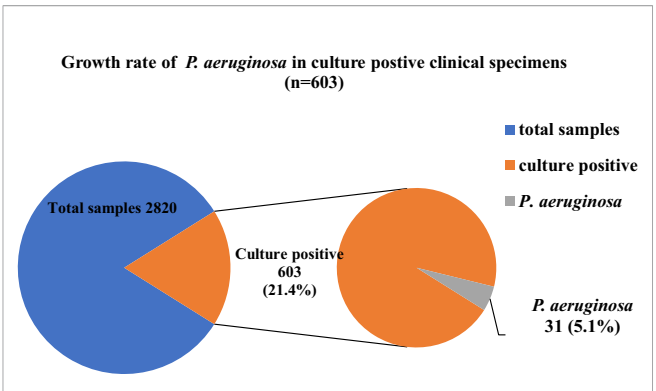

FIGURE 2 | Distribution of *P. aeruginosa* in culture positive clinical specimens.

Antibiotic Susceptibility Pattern of *P. aeruginosa*

The antibiotic susceptibility pattern of *P. aeruginosa* isolates revealed that ofloxacin, meropenem and amikacin were the most effective antibiotics *in vitro* with sensitivities of 83.8%, 80.6% and 74.2% respectively. In contrast, piperacillin-tazobactam and ceftazidime were the least effective antibiotics inhibiting the growth of 48.4% isolates. The antibiotic susceptibility pattern of *P. aeruginosa* isolates is depicted in **Table 3**. Out of 31 P. *aeruginosa* isolates subjected to AST, 17 (54.84%) were identified to be MDR (**Figure 3**).

Biofilm Production and Its Association With Antibiotic Resistance

All seventeen MDR isolates were selected and tested for biofilm production by both tube culture method and microtiter plate method. Out of 17 isolates of *P. aeruginosa* subjected to biofilm detection, 11 (65%) showed strong biofilm with microtiter plate method while 14 (82%) showed strong biofilm production with tube method. There was no significant association between two methods for biofilm formation ($p>0.05$). Among thirteen antibiotics tested, resistance to piperacillin, cefepime, aztreonam, imipenem and meropenem showed significant association with strong biofilm production ($p<0.05$) (**Table 4**).

Distribution of *mex*A and *mex*B Gene in *P. aeruginosa*

Among thirty-one isolates of *P. aeruginosa*, only the MDR isolates (17) were subjected to PCR amplification for the detection of *mex*A and *mex*B genes. Among them, twelve (71%) isolates were found to possess both *mex*A and *mex*B genes. The PCR amplified product size of *mex*A and *mex*B genes were 503 bp and 280 bp respectively (**Figures 4A, B**).

TABLE 2 | Distribution of bacterial genera in various clinical specimens (N=603).

Bacteria isolated/Clinical specimens	Urine (N = 313)	Sputum (N = 171)	Body fluids (N = 119)	Total
	N (%)	N (%)	N (%)	N (%)
E. coli	247 (78.9)	21 (12.3)	31(27.1)	299 (49.6)
K. pneumoniae	32 (10.2)	53 (30.9)	3 (2.5)	88(14.6)
P. aeruginosa	7 (2.2)	14 (8.2)	10 (8.4)	31 (5.1)
Candida albicans	8 (2.6)	25 (14.6)	7 (5.9)	40 (6.6)
Enterococcus spp.	15 (4.7)	6 (3.5)	0 (0)	21(3.5)
Proteus spp.	4 (1.3)	3 (1.8)	0 (0)	7 (1.2)
Staphylococcus aureus	0 (0)	5 (2.9)	46 (38.7)	51 (8.4)
Acinetobacter spp.	0 (0)	42 (24.6)	17 (14.3)	59 (9.8)
Citrobacter spp.	0 (0)	2 (1.2)	5 (4.2)	7(1.2)

TABLE 3 | Antibiotic Susceptibility Pattern of *P. aeruginosa* (N=31).

Antibiotics	Susceptible N (%)	Resistant N (%)
Piperacillin (PI)	23 (74.2)	8 (25.8)
Piperacillin-tazobactam (PIT)	16 (51.6)	15 (48.4%)
Cefepime (CPM)	19 (61.2)	12 (38.8)
Ceftazidime (CAZ)	16 (51.6)	15(48.4)
Aztreonam (AT)	18 (58)	13 (42)
Imipenem (IMP)	17 (54.8)	14(45.2)
Meropenem (MRP)	25 (80.6)	6 (19.4)
Gentamicin (GEN)	20 (64.5)	11 (35.5)
Tobramycin (TOB)	18 (58)	13 (42)
Amikacin (AK)	23 (74.2)	8 (25.8)
Ciprofloxacin (CIP)	22 (70.9)	9(29.1)
Levofloxacin (LEV)	21 (67.7)	10 (32.3)
Ofloxacin (OF)	26 (83.8)	5 (16.2)

Association of Occurrence of *mex*A and *mex*B Genes With Few Variables

A substantial number of *P. aeruginosa* isolates recovered from sputum 7 (58.3%) and body fluids 5 (41.7%) possessed both *mex*A and *mex*B genes ($p<0.05$). The occurrence of *mex*A and *mex*B genes was found more in the isolates that were resistant to levofloxacin ($p>0.05$). Majority of the MDR isolates possessed both *mex*A and *mex*B genes ($p>0.05$). Moreover, the prevalence of the *mex*A and *mex*B gene was found more in the isolates that were resistant to beta-lactam antibiotics ($p>0.05$) (**Table 5**).

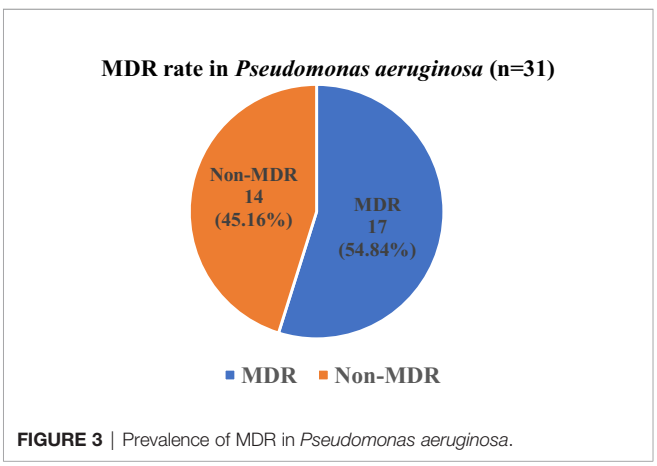

FIGURE 3 | Prevalence of MDR in *Pseudomonas aeruginosa*.

DISCUSSION

P. aeruginosa is regarded as a diverse opportunistic human pathogen, with final infection comprising attachment, colonization, local invasion and spread as a systemic disease (27, 28). This study documented its growth in 21.4% of total clinical specimens. Different studies conducted in Nepal reported its growth rate of 13-58% in clinical specimens (29–31). The prevalence of *P. aeruginosa* in this study was 5.14% which is in tune with the earlier studies done in Nepal (32, 33); however, the prevalence rate is lower than the previous studies done in Nepal (34), India (35) and Egypt (36). Growth of bacteria in different clinical specimens differs with the hospitals, number of samples, type of patients, and time duration of the study.

In this study, *E. coli* was the most predominant Gram-negative bacterial isolate whereas *S. aureus* was the predominant Gram-positive bacterial isolate. This finding was in line with other studies reported from different hospitals/laboratory settings in Nepal (37–40). Observation of higher number of Gram-negative bacterial isolates in clinical specimens in different studies conducted in Nepal indicates Gram-negative bacteria are emerging as important health-care

TABLE 4 | Biofilm production and its association with antibiotic resistance in *Pseudomonas aeruginosa* (n=17).

Method	Strong	Moderate	Weak	p-value
Tube culture	14	1	2	0.34
Microtiter Plate	11	4	2	
From Fischer's Exact Test		Type of Biofilm Production p-values		
Resistant Isolates to	**Strong**	**Moderate**	**Weak**	
Piperacillin (PI)	0.005*	0.11	0.11	
Piperacillin-tazobactam (PIT)	1	1	1	
Cefepime (CPM)	0.02*	0.59	0.09	
Ceftazidime (CAZ)	0.64	1	1	
Aztreonam (AT)	0.02*	0.59	0.09	
Imipenem (IMP)	0.01*	0.24	0.24	
Meropenem (MRP)	0.04*	0.26	0.26	
Gentamicin (GEN)	1	0.59	0.28	
Tobramycin (TOB)	0.37	0.11	1	
Amikacin (AK)	0.37	1	0.11	
Ciprofloxacin (CIP)	1	1	0.61	
Levofloxacin (LEV)	0.65	1	0.58	
Ofloxacin (OF)	0.05*	0.53	0.53	

**Significant at 5% level of significance.*

associated problems (41, 42). The highest incidence of *E. coli* and *S. aureus* may be due to factors such as their ubiquity in hospital environments and propensity to form biofilm on numerous uninhabitable surfaces.

This study reported ofloxacin and meropenem as the choice of drug for *P. aeruginosa* infection whereas ceftazidime was the least effective drug. Similar to this finding, several studies conducted in Nepal reported ceftazidime as the least effective drug (29, 43, 44). The presence of naturally occurring inducible cephalosporinase in *P. aeruginosa* imparts a low-level resistance to cephalosporins and aminopenicillins (45). Prevalence of MDR among *P. aeruginosa* isolates was 55% which was higher than the prevalence rate reported in other studies (29, 42). The widespread use of antibiotics in humans and animals, as well as inadequate infection control and increased mobility of people might have all contributed to the fast spread of multidrug-resistant bacteria.

The pathogenesis of the majority of chronic *P. aeruginosa* infections is mainly due to the capacity of this organism to form biofilm that imparts resistance to many antibiotics. All MDR *P. aeruginosa* isolates showed strong to weak biofilm formation in the current study. This finding echoes with the study done in Nigeria (46); however, a significant lower incidence (33.7%) of biofilm forming *P. aeruginosa* was reported in the study done in India (47). Biofilms embedded bacteria are difficult to eradicate by conventional antibiotic therapy as bacterial cells living inside biofilms are more tolerant towards antibiotics than their planktonic counterparts (48). *P. aeruginosa* resistant to piperacillin, cefepime, aztreonam, imipenem and meropenem were reported as the strong biofilm formation. Biofilm producing *P. aeruginosa* exhibit increased resistance to several antibiotics including tetracycline, chloramphenicol, quinolones, β-lactams and these resistance patterns could be closely related to drug profiles that are actively effluxed by the MexAB-OprM pump (49).

In the current study, all seventeen MDR isolates were selected for PCR amplification, twelve isolates (70.58%) showed the presence of both *mexA* and *mexB* genes. A similar study done in Suez Canal University Hospital, Egypt reported both *mexA* and *mexB* genes in 58.8% *Pseudomonas* strains which is in commensurate with our finding (50). On the other hand, Abbas et al. reported *mexA* and *mexB* genes in 100% isolates (25). RND efflux pumps (MexAB–OprM) are transport proteins in *P. aeruginosa* that may play a role in the extrusion of all types of therapeutically relevant antimicrobial drugs from the cell into the external environment (51). This study reported an insignificant association between the presence of *mexA* and *mexB* genes with fluoroquinolone and beta-lactams. The result of this study is in contrast with that of the result of a work by Dupont et al. who concluded that MexAB-OprM is responsible for efflux of beta-lactams and quinolones (52). Apart from the MexAB-OprM efflux pump system, three additional efflux pump systems (MexCD-OprJ, MexEF-OprN, and MexXY-OprM) also contribute to antibiotic resistance. Biofilm and efflux pumps are not the only variables that contribute to an organism's antibiotic resistance, but many other factors also play a part in creating an organism drug resistant.

CONCLUSION

This study reported 21.4% bacterial growth in various clinical specimens at a tertiary-care hospital of which 5.1% were *Pseudomonas aeruginosa* and more than half of them were multi-drug resistant. All the isolates tested showed biofilm-forming capacity with majority being strong producers. The

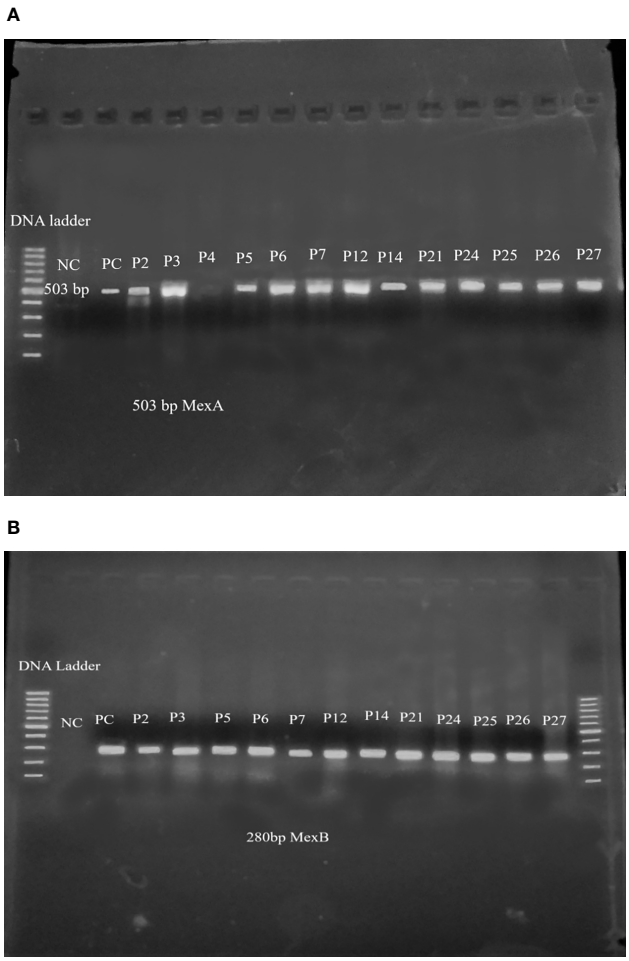

FIGURE 4 | **(A)** *mex*A gene amplified product after PCR in agarose gel electrophoresis. Lane 1-100bp DNA ladder, lane 2- negative control, lane 3- positive control, lane4-P2, lane5-P3, lane6-P4, lane7-P5, lane8-P6, lane9-P7, lane10- P12, lane11-P14, lane12- P21, lane13-P24, lane14- P25, lane15- P26, lane16- P27. **(B)** *mex*B gene amplified product after PCR in agarose gel electrophoresis. Lane 1-100bp DNA ladder, lane 2- negative control, lane 3- positive control, lane 4-P2, lane 5-P3, lane 6-P5, lane7-P6, lane8-P7, lane9-P12, lane10- P14, lane11-P21, lane12- P24, lane13-P25, lane14- P26, lane15- P27, lane16- 100bp DNA ladder.

TABLE 5 | Association of occurrence of *mex*A and *mex*B genes with few variables (N=17).

Clinical specimens	Gene Present N (%)	Gene Absent N (%)	Total N (%)	p-value
Sputum	7 (58.3)	1 (20)	8 (47.1)	
Body fluids	5 (41.7)	0 (0)	5 (29.4)	0.001*
Urine	0 (0)	4 (80)	4 (23.5)	
Relation between gene and fluoroquinolones antibiotic resistance				
Ciprofloxacin (CIP)	4 (57.1)	3 (42.9)	7	
Levofloxacin (LEV)	5 (55.6)	4 (44.4)	9	0.97
Ofloxacin (OF)	2 (50)	2 (50)	4	
Relation between gene (*mex*A and *mex*B) and development of MDR				
MDR	12 (70.6)	5 (29.4)	17	0.09
Relation between genes and beta-lactam antibiotic resistance				
Piperacillin (PI)	5 (71.4)	2 (28.6)	7	0.96
Piperacillin-tazobactam (PIT)	8 (61.5)	5 (38.5)	13	
Cefepime (CPM)	5 (71.4)	2 (28.6)	7	
Ceftazidime (CAZ)	6 (54.5)	5 (45.5)	11	
Aztreonam (AT)	5 (62.5)	3 (37.5)	8	
Imipenem (IMP)	7 (58.3)	5 (41.7)	12	
Meropenem (MRP)	4 (80)	1 (20)	5	

**means statistically significance.*

efflux-pump genes *mexA* and *mexB* were detected in 70.58% of the isolates tested. It's high time the concerned authorities employ serious and robust measures to prevent the dissemination of MDR isolates with biofilm-forming potential within and outside the hospital environments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Committee (IRC) of Institute of Science and Technology (IOST), Tribhuvan University, Nepal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceiving and study design: SB, KR, BL, and MR. Lab work and data collection: SB and DK. Supervision: BL, SA, AC, PJ, and KR. Data curation: SB, BD, MR, and KR. Data analysis: SS, BD, SA, and KR. Drafting the manuscript: SS, SA, BD, and KR. Review, editing and finalizing the manuscript: SA, BD, BL, and KR. All authors have read and approved to the published version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.