DRUG RESISTANCE AND BIOFILM PRODUCTION AMONG PSEUDOMONAS AERUGINOSA CLINICAL ISOLATES IN A TERTIARY CARE HOSPITAL OF NEPAL

Shrestha R, Nayak N, Bhatta DR, Hamal D, Subramanya SH, Gokhale S

Department of Microbiology, Manipal College of Medical Sciences, Pokhara, Nepal

ABSTRACT

Clinical isolates of *Pseudomonas aeruginosa* often exhibit multidrug resistance due to their inherent ability to form biofilms. Drug resistance in *Ps. aeruginosa* is a major clinical problem, especially in the management of patients with nosocomial infections and those admitted to ICUs with indwelling medical devices. To evaluate the biofilm forming abilities of the clinical isolates of *Ps. aeruginosa* and to correlate biofilm formation with antibiotic resistance. A total of 90 consecutive isolates of *Ps. aeruginosa* obtained from various specimens collected from patients visiting the Manipal Teaching Hospital, Pokhara, Nepal between January 2018 - October 2018 were studied. Isolates were identified by standard microbiological methods. Antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion method. All the isolates were tested for their biofilm forming abilities by employing the tissue culture plate assay. Of the 90 *Ps. aeruginosa* isolates, maximum i.e 42 (46.6%) were from patients in the age group of > 50 years. Majority (30; 33.3%) of the isolates were obtained from sputum samples. However, percentage isolation from other specimens like urine, endotracheal tube (ETT), pus, eye specimens and blood were 18.9%, 16.7%, 16.7%, 7.8% and 6.7% respectively. All the isolates were sensitive to polymixin B and colistin, 91.1% of the organisms were sensitive to imipenem, and more than 80% to aminoglycosides (80% to gentamicin, 83.3% to amikacin). A total of 29 (32.2%) organisms were biofilm producers. Maximum numbers of biofilm producing strains were obtained from ETT (8 of 15; 53.3%), pus (8 of 15; 53.3%) and blood (2 of 6; 33.3%) i.e from all invasive sites. None of the isolates from noninvasive specimens such as conjunctival swabs were biofilm positive. Significantly higher numbers of biofilm producers (23 of 29; 79.3%) were found to be multidrug resistant as compared to non-biofilm (6 of 61; 9.8%) producers (p=0.000). *Ps. aeruginosa* colonization leading to biofilm formation in deep seated tissues and on indwelling devices is a therapeutic challenge as majority of the isolates would be recalcitrant to commonly used antipseudomonal drugs. Effective monitoring of drug resistance patterns in all *Pseudomonas* clinical isolates should be a prerequisite for successful patient management.

KEYWORDS

Pseudomonas aeruginosa, antibiogram, biofilm, multi-drug-resistance

CORRESPONDING AUTHOR

Dr. Rajani Shrestha
Lecturer,
Department of Microbiology
Manipal College of Medical Sciences
Pokhara, Nepal
Email: rajani_sth7@hotmail.com

INTRODUCTION

The gram-negative bacterium, *Pseudomonas aeruginosa* belongs to a vast genus of obligate aerobic, non-fermenting, saprophytes, which are present in water, soil and on plants.[1] Moreover, *Ps. aeruginosa* can be frequently isolated from tap water. In its natural habitat, this organism is endowed with weak pathogenic potential. However, its profound ability to survive on inert materials, its minimal nutritional requirement, tolerance to a wide variety of physical conditions and relative resistance to several antimicrobial agents and antiseptics, contribute enormously to its ecological success and its role as an effective opportunistic pathogen.[2] *Ps. aeruginosa* is a notoriously difficult organism to control with antibiotics or disinfectants and has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance.

Several different epidemiological studies tracked its emergence as multi-drug-resistant *Ps. aeruginosa* (MDRPA) strains among the clinical isolates,[3] and this organism was accounted for a significant proportion of nosocomial infections.[4] MDRPA are often isolated from patients suffering from nosocomial infections, particularly those in the intensive care unit (ICU).[5] Thus, infections caused by *Ps. aeruginosa* especially in ICU patients are problematic because the organism apart from being inherently resistant to many drug classes, is able to acquire resistance to many effective antimicrobial drugs [2] and therefore infections caused by *Ps. aeruginosa* are frequently life threatening and difficult to treat.[6,7] Such multidrug resistance could be due to the slowly growing state of *P. aeruginosa* in the deeper layers of thick biofilms, which the organism has a tendency to form in many *in vivo* situations.[8] Biofilm cells have less access to antibiotics due to the impaired diffusion of antibiotic molecules through the biofilm matrix. Moreover, there is increased horizontal gene transfer among biofilm organisms , with high plasmid transfer rates, which aggravates the problem of drug resistance further.[8] As organisms growing in a biofilm are more resistant to antimicrobial agents than free floating planktonic cells, high antimicrobial concentrations (1000 fold higher) are often required to inactivate cells growing in the interior of a biofilm.[9] Thus, the aim of this study was to evaluate the biofilm forming abilities of the clinical isolates of *Ps. aeruginosa* and to determine the correlation of biofilm formation with multidrug resistance.

MATERIALS AND METHODS

A total of 90 consecutive isolates of *Ps. aeruginosa* obtained from various clinical specimens such as pus/wound swab, blood, sputum, urine, endotracheal tube (ETT) and conjunctival swabs collected from patients visiting the Manipal Teaching Hospital, Pokhara, Nepal between January 2018 and October 2018 were studied. Organisms were identified by standard microbiological techniques.[10] Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available antibiotic discs (Hi-media, Mumbai, India) using Kirby Bauer disc diffusion technique[11] and interpreted as per the guidelines of CLSI. The antibiotic discs (conc.) used were piperacillin/tazobactum (100/10mcg), ceftazidime (30mcg), ciprofloxacin (5mcg), amikacin (30mcg), imipenem (10mcg), cefepime (30mcg), polymyxin B (300units), gentamicin (10mcg), and colistin (10mcg). *Ps. aeruginosa* strain ATCC 15442 was used as the control. Isolates showing resistance to at least one agent in three or more antimicrobial categories based on the guidelines recommended by joint initiative of the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC),[12] were leveled as MDRPA.

All these isolates were tested for their biofilm forming abilities by employing the tissue culture plate assay, earlier described by Christensen *et al.*[13] Briefly, a single colony of *Ps. aeruginosa* was grown overnight at 37°C in 2 mL of trypticase soy broth (TSB). The bacterial culture was then diluted (1:100) with sterile fresh TSB. Each well of a 96-well flat-bottomed polystyrene tissue culture plate was filled with 200 µL of the diluted culture. The plate was incubated for 24 h at 37°C. The content of each well was aspirated carefully and then plate was gently washed with phosphate buffer saline (PBS), pH 7.2 in order to remove free-floating bacteria. Adherent bacteria were fixed with 99% methanol for 10–15 min. The content of the plate was decanted, allowed to dry and stained for 10 min with 1% crystal violet (CV). Excess stain was removed by rinsing the plate with tap water. Optical density (OD) of stained adherent biofilm was measured by using a micro-ELISA reader (Human) at a wavelength of 570 nm. *Ps. aeruginosa* ATCC strain 15442 was used as the positive control. Ten wells stained exactly in the same manner, but without any organism (sterile broth only) served as the negative control (blanks). The interpretation of biofilm production was made as per the criteria of Stepanovic *et al*,[14] and the cut off was calculated as 3XSD above the mean of the ten blanks.

RESULTS

A total of 90 *Ps. aeruginosa* isolates were identified during the study period. Distribution of *Ps. aeruginosa* isolates with respect to age and gender showed that 54 isolates were from male patients and 36 were from females. Irrespective of differences in gender, maximum strains were isolated in the age group of > 50 years.

Rates of isolation of *Ps. aeruginosa* from various clinical specimens have been shown in table 1. Maximum numbers of isolates (30; 33.3%) were obtained from sputum. However, percentage isolation from other specimens like urine, endotracheal tube (ETT), pus, eye specimens and blood were 18.9%, 16.7%, 16.7%, 7.8% and 6.7% respectively.

Table 1: *Pseudomonas aeruginosa* isolates from various clinical specimens.

Sources of specimen	n	%
Eye specimens (conjunctival swab)	7	7.8
Blood	6	6.7
Urine	17	18.9
Sputum	30	33.3
Pus/wound	15	16.7
ETT	15	16.7
Total	**90**	**100.0**

Overall antibiotic sensitivity pattern of the isolates are shown in Fig. 1. Whereas 100% of the organisms were sensitive to both polymixin B and colistin. Ninety one percent were sensitive to imipenem, and more than 80% to aminoglycosides (80% to gentamicin, 83.3% to amikacin). Sensitivity patterns were less promising against ciprofloxacin, piperacillin tazobactam, cefepime and ceftazidime; the rates of sensitivities being 65.5%, 58.9%, 42.2% and 26.7% respectively.

Biofilm forming ability determined by tissue culture plate method revealed that 29 (32.2%) of the 90 isolates were biofilm producers. Distribution of biofilm producing organisms according to their sources of isolation has been depicted vide table 2. It was interesting to note that maximum numbers of biofilm producing organisms were obtained from ETT (8 of 15; 53.3%), pus (8 of 15; 53.3%) and blood (2 of 6; 33.3%) i.e all from invasive sites. Contrary to this none of the isolates from noninvasive specimens such as conjunctival swabs were shown to be biofilm positive.

Table 2: Biofilm producer and non-biofilm producer organisms obtained from various clinical specimens.

Specimens	Biofilm		Total
	Non producer (%)	Producer (%)	
Eye samples	7 (100%)	0 (0%)	7
Blood	4 (66.7%)	2 (33.3%)	6
Urine	11 (74.85%)	6 (25.2%)	17
Sputum	25 (83.4%)	5 (16.6%)	30
Pus	7 (46.7%)	8 (53.3%)	15
ETT	7 (46.7%)	8 (53.3%)	15
Total	61 (67.7%)	29 (32.3%)	90

P value=0.025

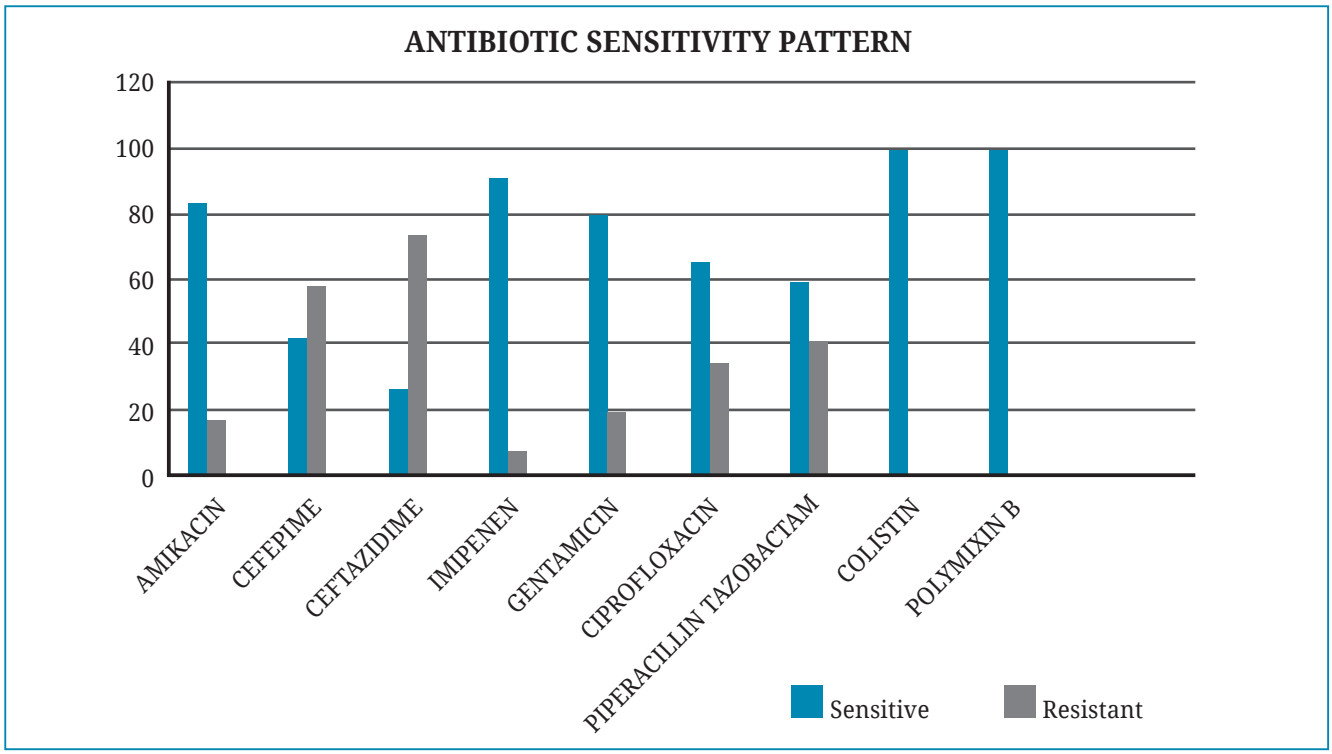

Fig. 1: Antibiotic sensitivity pattern of the isolates.

Table 3: Correlation between biofilm formation and multidrug resistance (MDR)

Biofilm production	MDR n (%)	No MDR n (%)	Total
Producer	23 (79.3)	6 (20.7)	29
Non-producers	6 (9.8)	55 (90.2)	61
Total	**29 (32.2)**	**61 (67.8)**	**90**

P=0.000

Table 3 shows the correlation between biofilm formation and multi-drug resistance among the isolates. Higher number of biofilm producers (23 of 29; 79.3%) were found to be multidrug resistant as compared to only 6 of the 61; (9.8%) non-bioflim producers. This difference was found to be statistically significant (p<0.001)

Table 4: Resistance patterns in relation to biofilm production

Resistance to antibiotics	Biofilm		p value
	Non producer (%) (n-61)	Producer (%) (n-29)	
Amikacin	5 (8.2)	10 (34.5)	**0.002**
Cefepime	27 (44.3)	25 (86.2)	**0.000**
Ceftazidime	41 (67.2)	25 (86.2)	0.057
Imipenem	2 (3.3)	6 (20.7)	0.007
Gentamicin	6 (9.8)	13 (44.9)	**0.000**
Ciprofloxacin	10 (16.4)	21 (72.4)	**0.000**
Piperacillintazobactam	15 (24.6)	22 (75.4)	**0.000**
Polymixin B	0 (0)	0 (0)	-
Colistin	0 (0)	0 (0)	-

Table 4 depicts the resistance patterns of biofilm producers vs. biofilm non-producers against individual antibiotics tested. Significantly higher numbers of biofilm producers were resistant to all the antibiotics except ceftazidime for which no significant difference in the resistance rates were detected among both the groups. However, all the organisms, irrespective of biofilm production, were found to be sensitive to both polymixin B and colistin.

DISCUSSION

Ps. aeruginosa is a leading cause of nosocomial infections, ranking only second among the gram-negative pathogens.[2] Resistance to antimicrobials among *Ps. aeruginosa is* an increasing clinical problem and is a recognized public health threat.15

In cases of polymicrobial bacteremia, *Ps. aeruginosa* is the main cause of mortality and second most common bacterium causing sepsis in the intensive care unit.[16,17] In addition, *Ps. aeruginosa* is one of the infuriating pathogens implicated in urinary tract infections (UTIs), burn and post-operative wound infections, lower respiratory tract infections (LRTIs), cystic fibrosis (CF), and ventilator associated pneumonia (VAP).[16] According to CDC, the overall incidence of *Ps. aeruginosa* infection in the US hospitals averages about 0.4% (4 per 1000 discharges), and the bacterium is the fourth most commonly isolated nosocomial pathogen accounting for 10.1% of all hospital acquired infections.[3]

There are limited number of antimicrobial agents with reliable activity against *Ps. aeruginosa* including anti-pseudomonal penicillins and cephalosporins, carbepenems, aminoglycosides and fluoroquinolones. However, *Ps. aeruginosa* clinical isolates show remarkable properties for the development of resistance, which is the reason for increased rate of mortality and morbidity due to infection by this organism.

We found multidrug resistance among 32.2% (29/90) of our isolates. Other workers from Nepal reported 18.6% and 20.5% of the *Pseudomonas* isolates to be multidrug resistant (MDR).[18,19] Yet lower rates of MDR were noted by studies outside Nepal.[20] Contrarily, higher resistance rates noted in our study as compared to others may be because the organisms in our investigation were isolated not only from blood but from other specimens like urine, sputum, pus as well. Obritsh *et al*,[3] however, were of the view that rates of isolation of MDRPA varied between 0.6-32%, and this variation was dependent upon the geographical location and the type of study.[3]

Nevertheless, multidrug resistance among *Ps. aeruginosa* is a major clinical problem especially among ICU patients, and in those with CF and on various indwelling devices, who provide enough opportunity for biofilm production by this organism in the above clinical situations.[21] Over and above, the pathogenesis of the majority of chronic *Ps. aeruginosa* infections is mainly due to the capacity of this organism to form biofilm, that imparts resistance to many antibiotics. Such biofilms embedded bacteria are difficult to eradicate by

conventional antibiotic therapy because bacterial cells living inside biofilms are more tolerant towards antibiotics than their planktonic counterparts.[22]

Biofilms have an enormous impact on health care. According to a recent study, biofilm related infective conditions are estimated to be responsible for almost 65% of all nosocomial infections.[23] Till date, there are scanty reports in the literature on the discovery and evaluation of any antibiofilm agent which could attenuate the pathogenicity of biofilm forming *Ps. aeruginosa*.[22]

In the above context, we extended our study further in order to find if there were any biofilm producers among the isolates in our series. Our results showed that 32.2% of the isolates were biofilm producers. Heydari *et al*[24] reported 43.5% of the *Ps. aeruginosa* isolates from patients in the burn care units to be biofilm producers. Worlitzh and colleagues[25] demonstrated that *Ps. aeruginosa* formed robust biofilms in CF patients, which reflected persistence of the organism in the CF lesions. In yet another recent study, Gilperotin *et al*[21] noted that biofilm formation on the surfaces of ETT was a frequent occurrence and was related to the pathogenesis of VAP allowing persistence of the organism in ETT biofilms, affecting the prognosis adversely.

We detected biofilm-producing *Ps. aeruginosa* isolates from 53.3% of the ETT, 53.3% of pus specimen, 33.3% of blood, 25.3% of urine and 16.6% of sputum samples. Interestingly, we observed that a significantly higher number of biofilm forming *Ps. aeruginosa* showed MDR (79.3%), as compared to non-biofilm producers. Abidi *et al*[26] studied 22 *Ps. aeruginosa* isolates and concluded that biofilm production was significantly higher among MDRPA isolates. Further, Nithyalakshmi *et al*[27] showed a statistically significant association of MDR in bacteria with their biofilm activity and thus showed that 12(25%) of their 48 biofilm producing *Ps. aeruginosa* were multidrug resistant as compared to only 5(7.8%) of the 64 non-biofilm producers. Gurung *et al*[28] also observed that 57% of biofilm producing *Ps. aeruginosa* were of the multidrug resistant phenotype.

Overall antibiotic sensitivity pattern of the isolates revealed that 91.1% of the isolates were sensitive to imipenem, >80% to aminoglycosides, 65.6% to ciprofloxacin, 58.9% to pipearacillin tazobactam. Alarmingly, 73.3% of the organism exhibited resistance against ceftazidime, which is supposed to be the drug of choice against *Ps. aeruginosa,* often preferred by many clinicians because of its optimum antipseudomonal activity. Various other studies in the past documented that 69 to 89% of pseudomonas nosocomial isolates were resistant to ceftazidime, according to the views of the researchers, could be the outcome of frequent use of this drug in clinical practice.[2,29,30]

Much in agreement with the present study, several reports in the past evidenced that multidrug resistant strains of *Ps. aeruginosa* were uniformly susceptible to polymixin B.[31,32] All the isolates in the present study were sensitive to polymixin B and colistin. Similar observations were noted by Kalaivoni *et al*[33] from India. However, high incidence of nephrotoxicity and neurotoxicity associated with the administration of these drugs could be a limitation for their use in clinical practice.[34] In recent years there had been dramatic increase in the resistance rates shown by *Ps. aeruginosa* towards imipenem. Fortunately, our study documented only 8.9% of the isolates to be resistant to this drug, which was much lower compared to the resistance rates presented by a study conducted in Nepal.[35,18] Kateete *et al* from Uganda reported 40% carbapenemase and 66% metallo-betalactamase (MBL) producing carbapenem resistant *Ps. aeruginosa*.[36]

In our study, irrespective of differences in gender, maximum number of *Pseudomonas* isolates (46.6%) were obtained from the patient in the age group of >50 years. Similar observations were made by Chandel *et al*[37] who reported that 31% of all *Pseudomonas* isolates from different samples (pus, sputum, urine tracheal aspirate, BAL fluid, bile, catheter, high vaginal swab) were from patients who were more than 60 years of age. These findings are of clinical relevance, as patients in these age groups may have decreased immunity, invariably have other co morbidity conditions, and are more likely to undergo prolonged hospitalization with indwelling medical devices, making them vulnerable to develop biofilm related infections.

As is evident from the data provided vide table 4, higher number of biofilm producing organisms exhibited resistance to all antibiotics tested except ceftazidime. This signifies that patients, especially those belonging to the older age groups, who are more likely to have indwelling medical devices, and are prone to develop biofilm on these devices, would be recalcitrant to majority of the routinely used antimicrobial agents.[21]

In conclusion, our study emphasizes on the dynamic relationship between *Ps. aeruginosa* colonization, biofilm formation and development of serious life threatening deep-seated infections. Biofilm once formed are responsible for bacterial persistence and their impaired response to antimicrobial therapy.

REFERENCES

1. Valles J, Mariscal D, Cortes P *et al*. Patterns of colonization by *Pseudomonas aeruginosa* in intubated patients: a 3-year prospective study of 1,607 isolates using pulsed-field gel electrophoresis with implications for prevention of ventilator-associated pneumonia. *Intensive Care Med* 2004; 30: 1768–75.
2. Gales AC, Jones RN, Turnidge J, Rennie R, Ramphal R. Characterisation of *Pseudomonas aeruginosa* isolates: occurrence rate, antimicrobial susceptibility pattern and molecular typing in the Global SENTRY antimicrobial surveillance pro- gram 1997–1999. *Clin Infect Dis* 2001; 32: S146–155.
3. Obritsch MD, Fish DN, MacLaren R, Jung R. Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. *Pharmacotherapy* 2005; 25: 1353-64.
4. Blanc DS, Petignat C, Janin B *et al*. Frequency and molecular diversity of *Pseudomonas aeruginosa* upon admission and during hospitalization: a prospective epidemiologic study. *Clin Microbiol Infect* 1998; 4: 242–7.
5. Tassios PT, Gennimata V, Spaliara-Kalogeropoulou L *et al*. Multiresistant *Pseudomonas aeruginosa* serogroup O:11 outbreak in an intensive care unit. *Clin Microbiol Infect* 1997; 3: 621–8.
6. Poole K. *Pseudomonas aeruginosa:* Resistance to the max. *Front Microbiol* 2011; 2: 1-13.
7. Kerr KG, Snelling AM. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *J Hosp Infect* 2009; 73: 338-44.
8. Diaz MA, Hernandez JR, Martinez-Martinez L, Rodrifuez-Bano J, Pascual A. Extended-spectrumbeta-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in Spanish hospitals: 2nd multicenter study (GEIHBLEE project, 2006). *Enferm Infect Microbiol Clin* 2009; 27: 503-10.
9. Stewart PS, Costerton J. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001; 358: 135.
10. Forbes BA, Sham DF, Weissfeld AS. Bailey and Scott's diagnostic Microbiology, 10th ed. New York: Mosby; 1998. p. 167-87
11. Bauer KBR, Kerby WMM, Sherris JC, Turck. M. Antibiotic Susceptility testing by a standardized single disc method. *Am J Clin Pathol* 1966; 45: 493-6.
12. Magiorakos AP, Srinivasan A, Carey RB *et al*. Multidrug-resistant, extensively drug-resistant and pandrug- resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; 18: 268-81.
13. Christensen GD, Simpson WA, Younger JJ *et al*. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* 1985; 22: 996- 1006.
14. Stepanovic S, Vukovic D, Hola V *et al.* Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Acta Pathol Microbiol Immunol Scandinavia* 2007; 115: 891-9.
15. Arruda EA, Marinho IS, Boulos M *et al*. Nosocomial infections caused by multiresistant *Pseudomonas aeruginosa. Infect Control Hosp Epidemiol* 1999; 20: 620–3.
16. Marra AR, Bar K, Bearman GM *et al*. Systemic inflammatory response syndrome in nosocomial bloodstream infections with *Pseudomonas aeruginosa* and Enterococcus species: comparison of elderly and nonelderly patients. *J Am Geriatr Soc* 2006; 54: 804–8.
17. Vincent JL, Sakr Y, Sprung CL *et al*. Sepsis in European inten- sive care units: results of the SOAP study. *Crit Care Med* 2006; 34: 344 – 53.
18. Mishra SK, Acharya J, Kattel HP, Koirala J, Rijal BP, Pokhrel BM. Metallo-beta-lactamase producing gram negative bacterial isolates. *J Nepal Health Res Counc* 2012; 10: 208-13.
19. Shrestha S, Amatya R, Adhikari RP. Prevalence and antibiogram of Pseudomonas aeruginosa isolated from clinical specimens in a Teaching Hospital, Kathmandu: *Nepal Med Coll J* 2015; 17: 132-5.
20. Traub WH, Scheidhauer R, Leonhard B, Bauer D. Surveillance of *Pseudomonas aeruginosa* in intensive care units: clusters of nosocomial cross-infection and encounter of a multiple-antibiotic resistant strain. *Chemotherapy* 1998; 44: 243–59.
21. Gil-Perotin S, Ramirez P, Marti V *et al*. Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept. *Crit Care* 2012, 16: R93
22. Furiga A, Lajoie B, El Hage S, Baziard G, Roques C. Impairment of *Pseudomonas aeruginosa* Biofilm Resistance to Antibiotics by Combining the Drugs with a New Quorum-Sensing Inhibitor. *Antimicrobial Agents Chemother* 2016; 60: 1677-86.
23. Potera C. Forging a link between biofilms and disease. *Science* 1999; 283: 1837–9.
24. Heydari S, Eftekhar F. Biofilm formation and β-lactamase production in burn isolates of *Pseudomonas aeruginosa*. *Jundishapur J Microbiol*. 2015 Mar 21; 8: e15514.
25. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC *et al.* Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 2002; 109: 317–25.
26. Abidi SH, Sherwani SK, Siddiqui TR, Bashir A, Kazmi SU. Drug resistance profile and biofilm forming potential of *Pseudomonas aeruginosa* isolated from contact lenses in karachi Pakistan. *BMC Opthalmol* 2013; 13: 57.doi:10.1186/1471-2415-13-57.
27. Nithyalakshmi. J, Akila. K, Mohanakrishnan. K and Smanthi G. Evaluation of resistance profile of *Pseudomonas aeruginosa* with reference to biofilm production-An emerging challenge. *IOSR-J Dental Med Sci 2015;* 14: 13-8.
28. Gurung J, Khyriem AB, Banik A, Lyngdoh WV, Choudhury B and Bhattacharya P. Association of biofilm production with multidrug resistance among clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from intensive care unit. *Indian J Crit Care Med* 2013; 17: 214-8.

29. Srinivas B, Lalitha Devi D, Narasinga Rao B. A prospective study of *Pseudomonas aeruginosa* and its antibiogram in a Teaching Hospital of Rural setup. *J Pharm Biomed Sci* 2012; 22: 1-5.

30. Tahira M, Mohammed AM, Galnaz K , Mustafa K. Pseudomonas aeruginosa in chronic suppurative otitis media: sensitivity spectrum against various antibiotics in Karachi. *J Ayub Med Coll Abbottabad* 2009; 21: 120-3.

31. Koomanachai P, Tiengrim S, Kiratisin P, Thamliktikil V. Efficacy and safety of colistin (colistimethate sodium) for therapy of infections caused by multidrug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in Siriraj Hospital, Bangkok, Thailand. *Int'l J Infect Dis* 2007; 5: 402-26.

32. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases:the quiet before the storm? *Clin Microbiol Rev* 2005: 18; 306-25.

33. Kalaivani R, Shashikala P, Sheela Devi C, Prashanth K, Saranathan R. Phenotypic assays for detection of ESBL and MBL producers among the clinical isolates of multidrug resistant *Pseudomonas aeruginosa* from a tertiary care hospital. *Int'l J Cur Res Rev* 2013; Sep; 05: 28-35.

34. Falagas ME, Kaisiakov SK. Toxicity of polymixins: a systematic review of the evidence from old and recent studies. *Crit Care* 2006; 10: R27. doi: 10.1186/cc3995.

35. Shrestha RK, Dahal RK, Mishra SK *et al*. Ventilator Associated Pneumonia in Tertiary Care Hospital, Maharajgunj, Kathmandu, Nepal. *J Inst Med* 2013; 35: 21-8.

36. Kateete DP, Nakanjako R, Namugenyi J, Erume J, Joloba ML, Najjuka CF. Carbapenem resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* at Mulago Hospital in Kampala, Uganda (2007-2009). *Springerplus* 2016; 5: 1308. doi: 10.1186/s40064-016-2986-7. eCollection 2016.

37. Chandel A, Raza Mohammad S. Antimicrobial susceptibility patterns of Pseudomonas aeruginosa clinical isolates at a tertiary care hospital in Kathmandu, Nepal. *Asian J Pharm Clin Res* 2013; 6: 235-58.