# ANTIBIOTIC SUSCEPTIBILITY PATTERN OF PSEUDOMONAS AERUGINOSA AND DETECTION OF VIRULENCE GENES IN A TERTIARY CARE HOSPITAL, KATHMANDU, NEPAL

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## ABSTRACT

*Pseudomonas aeruginosa* is one of the most common causes of hospital-acquired infections. It threatens worldwide public health because of its intrinsic antibiotic resistance and virulence. In this context, this research aimed to determine the occurrence of *P. aeruginosa* in a clinical setting, examine its susceptibility to antibiotics, and detect the presence of virulence genes, viz. *exoY*, *oprL* and *toxA* in all isolates. It was a cross-sectional study carried out from June 2022 to December 2022. A total of 1356 clinical specimens were collected and processed in the laboratory for Gram staining, culture techniques, and biochemical tests. The Kirby-Bauer disk diffusion method was applied to examine the antibiotic susceptibility pattern and conventional PCR was used to detect the virulence genes in all confirmed *P. aeruginosa* isolates. 40.5% of specimens showed bacterial growth, of which only 3.02% were identified as *P. aeruginosa*. Among them, 61.0% were multidrug resistant and 29.2% were β-lactamase producers. Aztreonam (70.7%) was the most effective antibiotic. Among all *P. aeruginosa* isolates, 68.3% isolates were *exoY* positive, 61.0% were *oprL* positive and 56.1% were *toxA* positive. Strategic interventions are required to stop the emergence and spread of antimicrobial resistance as a result of the isolation of multidrug resistant (MDR) isolates carrying these virulence genes. This study can benefit the medical staff and the entire community in building a surveillance system and enhancing infection control procedures.

## KEYWORDS

Pseudomonas aeruginosa, antimicrobial resistance, MDR, β-lactamase, virulence genes

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## INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic pathogen that causes the most severe infections, particularly in people whose immune system is compromised and associated with high death and morbidity rate.[1,2] It is associated with variety of illnesses, such as wound infection, urinary tract infection, pneumonia, soft tissue infection, skin infection and endocarditis.[1,3,4] The ability of *P. aeruginosa* to cause disease is due to the emergence of an antibiotic resistance, biofilm formation and the production of virulence factors. [5]

*P. aeruginosa* exhibits antibiotic resistance due to its outer membrane's lipopolysaccharides, serving as a permeability barrier.[6] Various resistance mechanisms, including multi-drug resistance, efflux pumps, biofilm formation, aminoglycoside modifying enzymes and β-lactamase production contribute to its ability to persist and cause infections.[7] The presence of virulence factors further enhances its pathogenicity, leading to invasive infections and extensive tissue damage. [8,9]

Multidrug resistant *P. aeruginosa*, poses a global health concern, with cephalosporins, carbapenems, aminoglycosides, monobactams, fluoroquinolones, penicillins with β-lactamase inhibitors, fosfomycin and polymyxins used for treatment.[1,10] Despite the effectiveness of β-lactam antibiotics, the bacterial defense mechanisms called β-lactamase enzymes break down β-lactam ring in the structure of β-lactam antibiotics, causing a complete loss of their antimicrobial properties.[11–13] Among the identified β-lactamase, extended-spectrum-β-lactamases (ESBL) and metallo-β-lactamases (MBL) are most clinically significant.[14] ESBLs are the bacterial enzymes carried by plasmid, which provide resistance against penicillins, aztreonam and cephalosporins, but their effectiveness can be neutralized with the help of β-lactamses inhibitors like clavulanic acid.[15] Meanwhile, MBLs are associated with carbapenem resistance and globally prevalent.[16] Their activity can be inhibited with the help of metal-chelating agents like ethylenediaminetetraacetic acid.[17]

*P. aeruginosa* has a numbers of virulence genes namely *exoY, exoS, toxA, oprL, oprI, oprD, lasA, lasB, alg, plcH, plcN* and *nan1*.[8,18] These genes encodes different virulence factors that contributes to its pathogenicity.[19] The outer membrane lipoprotein of *P. aeruginosa* is encoded by *oprL*, *oprI* and *oprD* genes, and is also used as marker for the identification of *Pseudomonas* infections. [8,20] The *oprL* gene protects bacteria from oxidative stress and helps maintain cellular integrity.[19] The *toxA* gene encodes exotoxin A which is a cytotoxic agent that inhibits the protein synthesis by stopping the elongation of polypeptide chains, leading to host tissue damage.[21] Similarly, the *exoY* gene encodes exotoxin Y, which is responsible for producing an enzyme called adenylate cyclase that increases the level of cyclic nucleotides, especially cyclic AMP (cAMP), inside the cell.[22,23]

The knowledge of bacterial antibiotic susceptibility profile is crucial for selecting the most effective empirical therapy, as it helps to determine the proper choice of antibiotics against bacterial infections.[24] The combination of virulence gene and antibiotic resistance genes makes the bacterial isolates more difficult to treat and control.[25] In Nepal, multidrug resistance challenges the effectiveness of treatments especially for the infections caused by *P. aeruginosa* producing ESBL and MBL, which exhibit higher rates of death and present a growing global concern.[26] Hence, understanding β-lactamase-mediated resistance rates, knowing the involvement of some virulence genes, and identifying alternative antibiotic strategies are crucial for effective *P. aeruginosa* treatment and infection control in hospital setting.

## MATERIALS AND METHODS

It was a hospital-based cross sectional study conducted among both outpatients and inpatients of all age groups including both male and female visiting in Annapurna Neurological Institute and Allied Sciences (ANIAS), Maitighar, Kathmandu, Nepal from June 2022 to December 2022. Ethical approval for carrying out the study was received from the Institutional Review Committee of Central Department of Microbiology, Tribhuvan University, Nepal with the registration number IRCIOST-22-0041.

All the samples collected during the study periods for culture and AST was taken for the study. The specimens included in the study were urine, sputum, blood, CSF, pus, swab, biopsy, catheter tip, stool and other body fluids. Samples collected on sterile leak proof container and well labelled samples were included in the study whereas improperly labelled and leaked samples were excluded from the study.

**Sample Processing and Identification of Isolates:** The specimens were cultured on MacConkey agar and blood agar. The non-lactose fermenting colony on MacConkey agar was subjected for Gram staining and the gram

negative bacteria were further characterized using a set of standard biochemical tests such as catalase, oxidase, oxidative/fermentative, indole, methyl red, Voges-Proskauer, citrate utilization, urease and triple sugar iron for the identification of *P. aeruginosa*. Furthermore, growth on cetrimide agar, pigment production and growth at 42°C confirmed *P. aeruginosa*.[27,28] The media used in this study were procured from HiMedia Laboratories, India and all points related to quality control were considered.

**Antibiotic Susceptibility Testing:** The Kirby-Bauer disc diffusion method was used to test the antibiotic susceptibility pattern of all collected isolates on Muller Hinton Agar (MHA) following the criteria set by the Clinical and Laboratory Standards Institute (CLSI, 2021). The antibiotics discs used were piperacillin (PI, 100 µg), piperacillin-tazobactam (PIT, 100/10 µg), ceftazidime (CAZ, 30 µg), cefepime (CPM, 30 µg), aztreonam (AT, 30 µg), imipenem (IPM, 10 µg), gentamicin (GEN, 10 µg), ciprofloxacin (CIP, 5 µg), amikacin (AK, 30 µg) and levofloxacin (LE, 5 µg) (products of HiMedia Laboratories, India). Broth culture of test organism adjusted to 0.5 McFarland standards were swabbed using sterile swab on the surface of MHA (4 mm thickness and pH 7.2) (HiMedia, India) and the antibiotics discs were placed on the top of agar plate. The plates were then incubated at 37º for 24 hours and the diameter of zone of inhibition was measured and results were analyzed based on CLSI standards, classifying them as either resistant (R), intermediate (I) or sensitive (S).[29] Multidrug resistance (MDR) was defined by resistance to more than three antibiotic classes.[30] To standardize the Kirby-Bauer test, a control strain of *P. aeruginosa* ATCC 27853 was employed.

**Phenotypic Detection of Extended Spectrum β-Lactamase (ESBL) Positive Isolates:** Screening for ESBL production was done using ceftazidime and/or cefepime disc. *P. aeruginosa* isolates exhibiting ≤22 mm zone of inhibition for ceftazidime (30 µg) were considered potential ESBL producers. The phenotypic confirmation of ESBL production on suspected isolates was done by combination disk test (CDT). In this method, a lawn culture of test strain was made on MHA. Then, ceftazidime and ceftazidime plus clavulanic acid discs (30 µg/10 µg) were placed over the culture. The plates were incubated at 37º for 18-24 hours. An increase in zone diameter of ≥5 mm in disc containing clavulanic acid in comparison with the inhibition zone of antibiotic tested alone confirmed ESBL production.[31,32]

**Phenotypic Detection of Metallo-β-lactamase (MBL) Positive Isolates:** Isolates exhibiting resistance to imipenem in Kirby-Bauer disc diffusion method were considered potential MBL producer. Imipenem - EDTA disc method was employed for the phenotypic confirmation of MBLs in imipenem resistant *P. aeruginosa*. A test inoculum equivalent to 0.5 McFarland was inoculated onto plates of MHA. Two 10 µg imipenem discs were placed on the plates, and 10 µl of 0.5 M EDTA of pH 8 was added to one of the discs to obtain 750 µg concentration. The distance between the center of imipenem and imipenem-EDTA discs was maintained to be 20 mm. after 16-18 hours of incubation in aerobic condition at 37º, the inhibition zones of imipenem and imipenem-EDTA discs were measured and compared with each other.

**Table 1: Primers used in this study**

| Primers | Sequences (5' to 3') | Cycles | Initial denaturation | Cycling | Final Extension | Length (bp) | Ref |
|---|---|---|---|---|---|---|---|
| *toxA* | GACAACGCCCTCAGCATCACCAGC (Forward)<br>GACAACGCCCTCAGCATCACCAGC (Reverse) | 35 | 5 min, 94º | 1 min, 94º<br>1 min, 58º<br>1 min, 72º | 10 min, 72º | 352 bp | 8 |
| *exoY* | CGGATTCTATGGCAGGGAGG (Forward)<br>GCCCTTGATGCACTCGACCA (Reverse) | 35 | 10 min, 94º | 40 sec, 94º<br>50 sec, 64º<br>55 sec, 72º | 10 min, 72º | 289 bp | 36 |
| *oprL* | ATGGGAATGCTGAAATTCGGC (Forward)<br>CTTCTTCAGCTCGACGCGACG (Reverse) | 35 | 5 min, 94º | 1 min, 94º<br>1 min, 60º<br>1 min, 72º | 10 min, 72º | 500 bp | 8 |

If the difference in inhibition zone between imipenem disk and imipenem-EDTA disk was ≥7 mm, the isolate was considered as MBL-producer.[33]

**Extraction of chromosomal DNA:** For the identification of *exoY, oprL* and *toxA* genes, the extraction of bacterial DNA was done from each isolates of *P. aeruginosa* by phenol-chloroform method.[34] The extracted DNA was preserved in Tris-EDTA (10 mM Tris-HCl, 0.10 mM EDTA, pH 8) buffer (HiMedia, India) at 4º for further analysis. Later, it was used for polymerase chain reaction (PCR).

**Detection of Virulence Genes Using PCR:** The PCR was done using a conventional PCR machine (ProFlex, Thermo Fisher, USA). The PCR mixture, with a final volume of 25 µl, was prepare. It comprised 12.5 µl of master mix (Roche, Germany), 0.5 µl of the forward primer (10 pM), 0.5 µl of the reverse primer (10 pM), 4 µl of DNA template, and 7.5 µl of distilled water.[35] PCR was performed as per the conditions described in Table 1. The final hold temperature is 4º during PCR. A reagent blank was included in every PCR reaction which contained all the components of reaction mixture except the bacterial DNA. *P. aeruginosa* ATCC 27853 carrying *exoY*, *oprL* and *toxA* genes was used as positive control. The agarose gel electrophoresis (1.5%) in 1X TAE buffer, stained with 0.5 µg/ml ethidium bromide (HiMedia, India) was utilized for achieving resolution of the amplified PCR products. Following this, under UV light using a gel documentation system, the DNA bands were visualized and photographed.[35]

## RESULTS

**Distribution of *P. aeruginosa* according to age, sex, specimen type and patient type:** Of the total 1,356 clinical specimens processed, the bacterial growth was observed in 549 (40.5%) samples. Among them, 41 (3.02%) isolates were identified as *P. aeruginosa*. Out of them, the highest growth was seen in the age group 60+ years (16; 39.0%) and the lowest number of isolates was observed in the age group below 20 years (5; 12.2%). Of the total *P. aeruginosa* isolates, (26; 63.4%) were from male patients while (15; 36.6%) were from female. Sample wise distribution study revealed that *P. aeruginosa* was predominant in sputum samples (13, 31.7%), followed by urine (9, 22.0%) and the least from swab and biopsy, and most strains (63.4%) were isolated from the inpatient department (Table 2).

**Antibiotic Susceptibility Pattern of *P. aeruginosa:*** The antibiotic susceptibility pattern of all *P. aeruginosa* isolates were shown

**Table 2: Distribution of *P. aeruginosa* according to demographic information of patients**

| Characters | No. of cases | No. of *P. aeruginosa* isolated | Percentage of total *P. aeruginosa* isolated | Percentage of total specimens |
|---|---|---|---|---|
| **Age group (in years)** | | | | |
| <20 | 122 | 5 | 12.2 | 0.37 |
| 20-39 | 427 | 9 | 22 | 0.66 |
| 40-59 | 371 | 11 | 26.8 | 0.81 |
| 60 and above | 436 | 16 | 39 | 1.18 |
| Gender | | | | |
| Male | 737 | 26 | 63.4 | 1.91 |
| Female | 619 | 15 | 36.6 | 1.11 |
| **Specimen types** | | | | |
| Sputum | 214 | 13 | 31.7 | 0.96 |
| Urine | 498 | 9 | 21.9 | 0.66 |
| Pus | 68 | 5 | 12.2 | 0.37 |
| Blood | 172 | 4 | 9.8 | 0.29 |
| CSF | 243 | 3 | 7.3 | 0.22 |
| Swab | 84 | 2 | 4.9 | 0.15 |
| Biopsy | 22 | 2 | 4.9 | 0.15 |
| Catheter tips | 22 | 3 | 7.3 | 0.22 |
| Others | 33 | - | - | - |
| **Patient type** | | | | |
| Inpatient | 1063 | 26 | 63.4 | 1.9 |
| Outpatient | 293 | 15 | 36.6 | 1.12 |
| **Total** | **1356** | **41** | **100** | **3.02** |

*Others: Body fluid and stool

**Table 3: AST pattern of *P. aeruginosa***

| Antibiotic discs | Resistant | Intermediate | Sensitive |
|---|---|---|---|
| Piperacillin (100 µg) | 13 (31.7%) | 10 (24.4%) | 18 (43.9%) |
| Piperacillin-tazobactam (100/10 µg) | 8 (19.5%) | 9 (22%) | 24 (58.5%) |
| Ceftazidime (30 µg) | 24 (58.5%) | 4 (9.8%) | 13 (31.7%) |
| Cefepime (30 µg) | 15 (36.6%) | 1 (2.4%) | 25 (61%) |
| Aztreonam (30 µg) | 12 (29.3%) | - | 29 (70.7%) |
| Imipenem (10 µg) | 17 (41.5%) | 1 (2.4%) | 23 (56.1%) |
| Gentamicin (10 µg) | 15 (36.6%) | 4 (9.8%) | 22 (53.7%) |
| Ciprofloxacin (5 µg) | 20 (48.8%) | 7 (17%) | 14 (34.2%) |
| Amikacin (30 µg) | 9 (22%) | 7 (17%) | 25 (61%) |
| Levofloxacin (5 µg) | 20 (48.8%) | 4 (9.8%) | 17 (41.5%) |

**Table 4: Prevalence of *P. aeruginosa* and distribution of MDR isolates among types of specimen and patient type**

| Character | No. of cases | MDR | Non- MDR | Total isolates |
|---|---|---|---|---|
| **Specimen type** | | | | |
| Sputum | 214 | 11 (26.8%) | 2 (4.9%) | 13 (31.7%) |
| Urine | 498 | 2 (4.9%) | 7 (17.1%) | 9 (22%) |
| Pus | 68 | 2 (4.9%) | 3 (7.3%) | 5 (12.2%) |
| Blood | 172 | 4 (9.8%) | - | 4 (9.7%) |
| CSF | 243 | 2 (4.9%) | 1 (2.4%) | 3 (7.3%) |
| Swab | 84 | 1 (2.4%) | 1 (2.4%) | 2 (4.9%) |
| Biopsy | 22 | - | 2 (4.9%) | 2 (4.9%) |
| Catheter tips | 22 | 3 (7.3%) | - | 3 (7.3%) |
| Others | 33 | - | - | - |
| **Patient type** | | | | |
| Inpatient | 1063 | 20 (48.8%) | 6 (14.6%) | 26 (63.4%) |
| Outpatient | 293 | 5 (12.2%) | 10 (24.4%) | 15 (36.6%) |
| **Total** | **1356** | **25 (61%)** | **16 (39%)** | **41 (100%)** |

**Table 5: Presence of virulence genes in *P. aeruginosa* isolated from clinical specimens**

| Character | *toxA* gene | *exoY* genes | *oprL* genes |
|---|---|---|---|
| Sputum | 9 (22%) | 8 (19.5%) | 10 (24.4%) |
| Urine | 5 (12.2%) | 7 (12.2 %) | 4 (9.8%) |
| Pus | 2 (4.9%) | 4 (9.8%) | 3 (7.3%) |
| Blood | 3 (7.3%) | 2 (4.9%) | 1 (2.4%) |
| CSF | 1 (2.4%) | 3 (7.3 %) | 3 (7.3%) |
| Swab | 1 (2.4%) | 2 (4.9%) | - |
| Biopsy | 1 (2.4%) | 2 (4.9%) | 2 (4.9%) |
| Catheter tip | 1 (2.4%) | 1 (2.4%) | 2 (4.9%) |
| Others | - | - | - |
| **Total** | **23 (56.1%)** | **28 (68.3%)** | **25 (61%)** |

**Table 6: Virulence genes among multidrug resistant and β-lactamase producing *P. aeruginosa* isolates**

| Drug spectrum | *toxA* gene | *exoY* gene | *oprL* gene |
|---|---|---|---|
| MDR | 15 (36.6%) | 15 (36.6%) | 17 (41.5%) |
| Non-MDR | 8 (19.5%) | 13 (31.7%) | 8 (19.5%) |
| ESBL | 7 (17.1%) | 6 (14.6%) | 3 (7.3%) |
| Non-ESBL | 16 (39%) | 22 (53.7%) | 22 (53.7%) |
| MBL | 7 (17.1%) | 7 (17.1%) | 5 (22.2%) |
| Non-MBL | 21 (51.2%) | 21 (51.2%) | 20 (48.8%) |
| **Total** | **23 (56.1%)** | **28 (68.3%)** | **25 (61%)** |

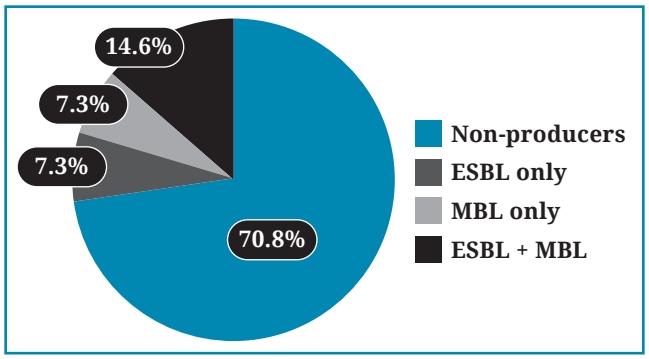


**Fig.1:** Beta lactamase production in *P. aeruginosa* isolates

in the Table 2. Aztreonam was found most effective antibiotics in vitro with sensitivity of 70.7%. Similarly, the least effective drug was ceftazidime which sensitive against 31.7% of the *P. aeruginosa* isolates (Table 3).

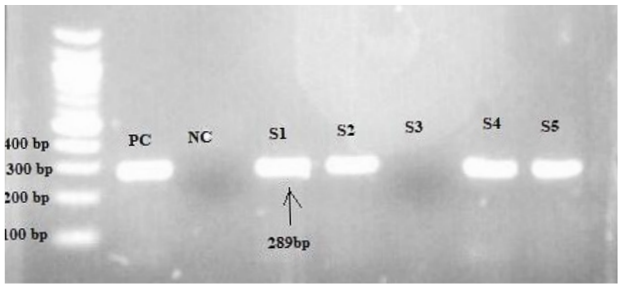


**Fig.2:** PCR amplification of *exoY* gene in *P. aeruginosa* isolate. Lane L1: Molecular marker (ladder 100bp), PC: Positive control for *exoY* gene, NC: Negative control for *exoY* gene, Lane L1-L5 represents samples S1-S5 in which S1, S2, S4 and S5 are *exoY*-positive

**Distribution of multidrug resistance (MDR) pattern in *P. aeruginosa*:** Out of 41 *P. aeruginosa* isolates, 25 (61.0%) isolates were identified to be MDR. The highest percentage of MDR isolates were from sputum samples (26.8%) followed by blood samples (9.8%). Similarly, the maximum MDR isolates were from inpatients (48.8%) (Table 4).

**β-lactamases production in *P. aeruginosa* isolates:** For the detection of β-lactamase production, ESBL and MBL were tested among the *P. aeruginosa* isolates. Out of 41 *P. aeruginosa*, 12 (29.2%) were found to produce at least one type of β-lactamase. Among them, 3 (7.3%) were ESBL producer only and 3 (7.3%) were MBL producer only. Co-production of ESBL and MBL was observed in 6 (14.6%) isolates. In addition to this, 7 (17.1%) MDR isolates were ESBL producer while 9 (19.5%) MDR isolates were MBL producer (Fig. 1).

**Prevalence of virulence genes (*exoY*, *oprL* and *toxA*) in *Pseudomonas aeruginosa* isolates:** The polymerase chain reaction (PCR) result showed the presence of virulence genes

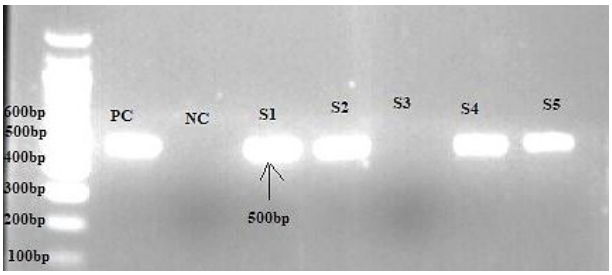


**Fig. 3:** PCR amplification of *oprL* gene in *P. aeruginosa* isolate. Lane L1: Molecular marker (ladder 100bp), PC: Positive control for *oprL* gene, NC: Negative control for *oprL* gene, Lane L1-L5 represents samples S1-S5 in which S1, S2, S4 and S5 are *oprL*-positive

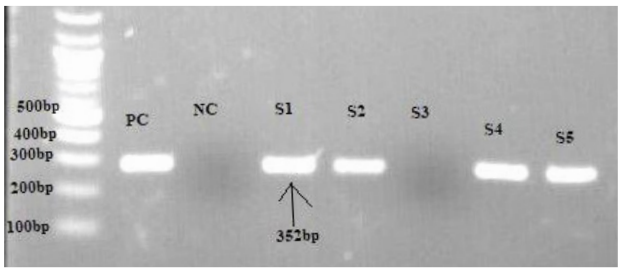


**Fig. 4.** PCR amplification of *toxA* gene in *P. aeruginosa* isolate. Lane L1: Molecular marker (ladder 100bp), PC: Positive control for *toxA* gene, NC: Negative control for *toxA* gene, Lane L1-L5 represents samples S1-S5 in which S1, S2, S4 and S5 are *toxA*-positive

in 41 isolates of *P. aeruginosa* in which 28 (68.3%) isolates were positive for *exoY* gene, 25 (61.0%) isolates were positive for *oprL* gene and 23 (56.0%) isolates were positive for *toxA* gene. The maximum number of virulence genes was found in sputum followed by urine sample (Table 5).

**Occurrence of virulence genes (*toxA*, *exoy* and *oprL*) among multidrug resistant and β-lactamase producing *P. aeruginosa* isolates**: Among 25 MDR *P. aeruginosa*, 36.6% were found to have *toxA* gene and *exoY* gene while 41.5% were found to have *oprL* gene. Among 9 ESBL producing *P. aeruginosa*, 17.1% had *toxA* gene, 14.6% had *exoY* gene and 7.3% had *oprL* gene. Among 9 MBL producing *P. aeruginosa*, 17.1% had *toxA* gene and *exoY* gene while 12.2% had *oprL* gene (Table 6).

## DISCUSSION

The prevalence of *P. aeruginosa* in this study was 3.02%. The lower prevalence found in this study is supported by Gyawali *et al*,[8] Chand *et al*,[37] and Bhandari *et al*.[38] which reported the frequency of 2.2%, 4.29% and 1.09%, respectively. On contrary, Adhikari *et al*,[6] Maharjan *et al*,[26] Mahaseth *et al*,[39] and Shrestha *et al*.[40] the prevalence was reported as 8.6%, 6.48%, 11.29% and 7.9%, respectively.

This variation might be due to difference in the type of clinical specimens received, study population, study duration and type of hospital.[8]

In this study, a significant proportion of the isolates (39.0%) were isolated from individuals aged 60+ years suggesting that the infections attributable to *P. aeruginosa* are more prevalent among this age group. Chand *et al*[8] and Shrestha *et al*[41] in Nepal and Gautam and Gopi[42] in India have also found that most of the cases of pseudomonal infection in this age group. This age group is highly vulnerable to infection and also longer duration of stay at the hospital due to decreased immunity and other associated co-morbidities.[8,42]

Gender-wise, majority of the isolates were from male patients (63.4%). This finding correlates with various previous reports from Nepal. Baral *et al*,[6] Shrestha *et al*,[26] Shrestha *et al*,[40] Sathyavathy and Madhusudhan,[43] Maharjan,[44] Mahaseth *et al*,[45] reported highest percentage of male patients which were 61.0%, 55.6%, 60.0%, 57.1%, 51.47% and 62.25%, respectively. However, Bhattarai *et al*[46] and Anil and Shahid[47] in Nepal and Mohammadzadeh *et al*[48] in Iran showed maximum infection in female patients by 80.08%, 55.17% and 68.4%, respectively. The variation in the prevalence of *P. aeruginosa* between male and female patients may be due to differences in various factors such as immune system, lifestyle choices and occupational performance among individuals who come into contact with this opportunistic pathogen.[48]

Sample-wise distribution study revealed sputum as the most common source of the isolates accounting for 31.7% of the total *P. aeruginosa* growth. Previous studies from Nepal have also reported higher proportion of *P. aeruginosa* (33.3% to 65.8%) from sputum samples.[6,43,44,49] On contrary, some other studies from Nepal have reported maximum isolation from pus sample.[44,47] The occurrence of *P. aeruginosa* in various specimens may differ from hospital to hospital, since each hospital has its own unique environment that can influence bacterial distribution.[50]

More than one-fourth of the *P. aeruginosa* isolate was isolated from inpatients (75.6%) compared to outpatients (24.4%). Similar findings have been reported by Ansari *et al*[6] Mahaseth *et al*[49] and Sujakhu *et al*[51]. The majority of the isolates were isolated from inpatients since the duration of hospital stay is directly proportional to the infection.[6] In contrast, another study carried out by Chand *et al*[8] have found majority isolates in outdoor patients which may be due to the frequent exposure of those patients with infected surrounding.

*P. aeruginosa* isolates in this study were tested for susceptibility to ten different antibiotics by modified Kirby-Bauer disc diffusion method. Aztreonam (AT/30 µg) was the most effective (70.7%) drug. Mahaseth *et al*[6] and Chand *et al*[8] have also reported similar results, for example, 62.2% and 66.7%, respectively. This study revealed that more than half (61.0%) of our isolates were MDR. However, the rate of MDR in the study was lower than previously reported by Maharjan *et al*[52] (83.0%). In contrast to this, a lower rate of MDR have also been noted by various researchers such as 32.2% by Shrestha *et al*[44] and 20.7% by Anil & Mohammad Shahid[47]. The high percentage of resistance may be due to indiscriminate use of antibiotics resulting in the failure of commonly used drugs for the management of Pseudomonal infection. Furthermore, mutation or acquisition of resistant genes through horizontal gene transfer can occur during antibiotic therapy resulting in the over expression of endogenous beta lactamases, efflux pump genes and expression of specific porins. This could contribute to high level of resistance.[53] Self-medication by patients and incomplete course of treatment are also probable contributing factors.[54] Highest frequency of MDR *P. aeruginosa* were identified in sputum sample (26.8%) and from inpatient department (48.8%).

All the isolates in this study were tested for ESBL and MBL production. It was found that 29.2% isolates showed β-lactamases production. This finding was supported by Ansari *et al*[49] and Shrestha *et al*[26] who reported 33.1% and 36.0% ESBL producing *P. aeruginosa* respectively. However, in some studies such as Poudyal *et al*[15] and Bhandari *et al*[55] ESBL producing *P. aeruginosa* was not reported. Similarly, this study showed 29.2% MBL producing isolates which is supported by the study done by Acharya *et al*[56] who had reported 30.9% MBL producing *P. aeruginosa*. Shrestha *et al*[26] have reported that 8.0% *P. aeruginosa* as MBL producer which is much lower compared to our study. The high prevalence of β-lactamase production may be due to the long-term hospital stay, maximum use of β-lactam antibiotics and horizontal transfer of β-lactamases encoding genes. Detection of β-lactamase producing multidrug-resistant (MDR) bacteria is crucial as it poses challenges in therapeutic management and restricts available treatment choices. Among 25 MDR *P. aeruginosa*, 17.0% were ESBL producer and 21.9% were MBL producers.

In this present study, PCR showed that 56% *Pseudomonas aeruginosa* isolates were *toxA* gene positive, 68.3% isolates were *exoY* gene positive and 61.0% isolates were *oprL* gene positive. Mapipa *et al*[20], Chand *et al*[8] and Mohammadzadeh *et al*[48] revealed the occurrence of *toxA* gene as 100.0%, 95.4% and 100.0% respectively. Bogiel *et al*[57] and Rodrigues *et al*[58] showed the prevalence of *exoY* as 99.1% and 75.9% respectively. Chand *et al*[8] and Aslani *et al*[59] illustrated prevalence rate of *oprL* gene as 100.0% and 96.0% respectively. The possible reason for low prevalence rate of these three virulence genes (*toxA*, *exoY* and *oprL*) in this study might be because during infection the bacterium may undergo genomic reduction.[60] This process may lead to the loss of virulence factors that are no longer necessary for survival in the host. As a result, the bacterium may become less virulent but better adapted to persist in the host environment.[60,61] The prevalence of *P. aeruginosa* and its virulence genes depends upon nature of place, degree of contamination, immunity of patients and virulence of strains.[8]

Among 25 MDR, the prevalence of *toxA, exoY* and oprL gene are 36.6%, 36.6% and 41.5% respectively. Among 9 ESBL producing isolates, prevalence of these genes are 17.1%, 14.6% and 7.3% respectively while among 9 MBL producing isolates, the prevalence of these genes are 17.1%, 17.1% and 12.2% respectively.

This study could be helpful for health worker to improve infection control measures and to establish a surveillance system. This effort may contribute to control the emergence and transmission of MDR, MBL and ESBL producing bacteria in clinical setting. Additionally, this study could be a significant reference for further study on prevalence of virulence genes in *P. aeruginosa.*

For conclusion, Aztreonam was found to be most effective antibiotics for treatment of infection caused by *P. aeruginosa* followed by imipenem, gentamicin and amikacin. The study revealed that more than half of *P. aeruginosa* isolates harbor at least one of virulence genes, namely *toxA, exoY* and *oprL*. Out of total *P. aeruginosa* isolates, majority of them were MDR while nearly one third of the isolates showed β-lactamase production. Multidrug resistant and β-lactamase production in *P. aeruginosa* have been considered as one of the challenging nosocomial as well as community acquired pathogen. So, the special attention is required in regular surveillance of antibiotic susceptibility patterns. Similarly, the presence of intrinsic virulence and pathogenicity of bacteria is indicated by existence of virulence genes such as *toxA, oprL* and *exoY*. Therefore, the detection of these genes by PCR is highly recommended.

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