Prevalence of some virulence genes and antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolated from different clinical specimens

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Abstract

Background: *Pseudomonas aeruginosa* is an opportunistic human pathogen and are reported to cause acute and chronic infectious diseases. Due to its high ability to acquire resistance to many antibiotics, it has become a global public health threat. It consists of some virulence genes that may lead to its pathogenicity. The main objective of this cross-sectional study was to detect the virulence genes and antibiotic susceptibility pattern of *P. aeruginosa* isolated from clinical specimens collected from governmental hospital of Nepal.

Methods: A total of 7898 clinical specimens were analyzed for the period of six months from November 2018 to April 2019. The specimens were cultured on Nutrient agar, Blood agar, MacConkey agar, Chocolate agar, Cysteine-Lactose, Electrolyte Deficient agar plates and were incubated at 37°C for 24 hours. All the isolates were identified by standard biochemical tests and further confirmed by growth on Cetrimide agar plate. The antibiotic susceptibility testing was performed by modified Kirby-Bauer disc diffusion method following CLSI guideline. Multiplex-PCR was done to detect the virulence genes *oprL* and *toxA.* Statistical analysis was carried out using IBM SPSS Statistic ver. 25 and the p-value was calculated at significance level (0.05%) by using Chi square.

Results: Out of these specimens investigated, 87 isolates were tentatively identified to be *P. aeruginosa* in which 20 (22.98 %) were found to be multidrug resistant. Comparatively, most of the *P. aeruginosa* were isolated from outpatients 63 (72.41 %) than inpatients 24 (27.58 %), from male 56 (64.36 %) than female 31 (35.63 %) and in age group 60-79 years (41.37 %). AST result showed the highest resistance of 100% with cefixime whereas susceptibilities of 83.9% and 81.6% with polymixin B and tobramycin were noticed respectively. The PCR results showed that all *P. aeruginosa* isolates carried *oprL* 87 (100%) and 83 (95.4 %) isolates showed *toxA genes*.

Conclusion: The studies revealed that almost all *P. aeruginosa* harbors both *oprL* and *toxA* genes.

Background

Pseudomonas aeruginosa is known as one of the most-widely spread opportunistic human pathogen causing 18 to 63% infection worldwide (1, 2). It has the ability to grow at temperature 42 °C, this unique character helps to differentiate from many other *Pseudomonas* species (3). Most strains produce water soluble pigments, such as pyocyanin, pyoverdin, pyorubin and pyomelanin (4–5). The ability of *P. aeruginosa* to grow in minimum nutritional requirements and to withstand various physical condition has assigned these organism to persist in both hospital and community settings (6–7).

P. aeruginosa having great diversity and capable of causing life threatening contagion infections in a multifariousness of patients population (8). There are several extracellular and cell associated virulence factors that may lead to its pathogenicity. The colonization of these factors can cause blood stream invasive, extensive tissue damage and dissemination. Some genes, normally encode and participate in the virulence factors are, *toxA, exoS, exoY, exoU, oprL, oprI, lasA, lasB, oprD, plcH, plcN* and *nan1* etc. (9).

Virulence genes such as *oprL, oprI* and *oprD* are the major outer membrane lipoproteins, specific to *P. aeruginosa* only and true factors for identification of *P. aeruginosa* infections (10). Similarly, *toxA* gene is one of the virulence genes that encode exotoxin A produced by *P. aeruginosa* and inhibits protein biosynthesis by stop elongation of polypeptide chains (11–13).

Among all the investigating tools, DNA typing techniques have been often used to investigate the divergence of *P. aeruginosa* (14). Proteins can also be identify by matrix-assisted leser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (15).

This cross-sectional study is designed to assist the current resistant against different classes of antibiotics and to characterize *P. aeruginosa* by identifying virulence genes *oprL* and *toxA* using polymerase chain reaction (PCR).

In Nepal, the increasing trend of antimicrobial resistant property of *P. aeruginosa* is becoming an alarming health issues. There are no exact observable scheme for marking resistant pattern and its use in Nepal. Additionally, few researches and some available secondary data are not sufficient to study current scenario and it is really hard to describe the true positive trends of antibiotic resistant of *P. aeruginosa* in Nepal. Therefore this cross-sectional study is designed to assist the current resistant pattern of *P. aeruginosa* against different classes of antibiotics and to identify the involvement of several virulence genes in resistant mechanisms by using Polymerase Chain Reaction (PCR) which ultimately helps to select appropriate antibiotics useful for the treatment of infectious disease caused by *P. aeruginosa*.

Methods

This cross-sectional study was conducted between November 2018 to April 2019 at Bir Hospital (a tertiary care hospital), National Academy of Medical Sciences (NAMS), Kathmandu, Nepal and Department of Microbiology at National College (NIST), Kathmandu Nepal. In this study, clinical and socio-demographic study of patients was also performed. In total, 7898 different clinical specimens were collected and cultured semi-quantitatively on Nutrient agar, blood agar, MacConkey agar. It was confirmed by using cetrimide agar medium and standard biochemical tests. The antibiotic susceptibility testing was performed on Muller Hinton agar by modified Kirby-Bauer disc diffusion method. Strains were considered as multi drug resistant (MDR) if they were resistant to at least 4 of following antimicrobial agents belonging to different classes of antibiotics: Imipenem, Ceftazidime, Tobramycin or Gentamicin and Ciprofloxacin (16). Diameter of the zone of inhibition for each antibiotic was measured and interpreted as recommended by Clinical and Laboratory Standard Institute (CLSI) guideline (2018) (17–18).

DNA Extraction

For the identification of virulence genes (*oprL* and *toxA*), chromosomal DNA was extracted from each *P. aeruginosa* isolate by phenol-chloroform assay (19).

Detection of Virulence Genes by using PCR

The PCR amplification was carried out by using temperature gradient thermal cycler (PCR tube 96 wells, Takara/Japan) with specific forward and reverse primer for the detection of *oprL*, and *toxA* genes respectively. The primer was then diluted to working concentration of 10 Pm by using nuclease free water (Table 1).

The PCR was carried out in total 20 µl volume of reaction mixtures containing 2 µl of template DNA, 1 µl of each primer, 4 µl of master mixtures, 12 µl of nuclease free water and Taq-polymease enzyme with 35 cycles. The annealing temperature was 61.8 °C for *oprL*, and 58.2 °C for *toxA*. The PCR condition is depicted in Table 1. The PCR products were separated by gel electrophoresis on 1% agarose gel containing 5 µl of ethidium bromide. The band of size about 500 bp and 352 bp of *oprL* and *toxA* were produced respectively along with 100 bp DNA marker and positive control (20).

Table 1
Nucleotide sequence of primers and condition used to amplify species specific virulence genes in *P. aeruginosa* by PCR

Virulence factors	Target genes	Primer name	Sequence (5 to 3)	Annealing temperature, °C	Amplicon size (bp)
Exotoxin A	*toxA*	toxA-f	GGT AAC CAG CTC AGC CAC AT	58.2	352
		toxA-r	TAG TGT CCA GGT CAT GCT TC		
Sialidase enzyme	*oprL*	oprL-f	ATG GGA ATG CTG AAA TTC GGC	61.8 °C	500
		oprL-r	CTT CTT CAG CTC GAC GCG ACG		

Statistical analysis

Data was analyzed using IBM SPSS ver. 25. Pearson's correlation test were used to detect significance of the result. The p-value was calculate at significance level (0.05%) (20).

Results

Among 7898 clinical specimens, a total of 2026 (26%) showed significant growth (i.e., 10^5cfu/ml) out of which 87 (4.29%) isolates were *P. aeruginosa*.

Clinical and socio-demographic study

The highest number of *P. aeruginosa* was isolated from male patients 56 (64.36%) and community acquired infections was found to be greater which accounts 63 (72.41%). In addition, the substantial number 36 (41.37%) of *P. aeruginosa* were found in age group 60–75 (Table 2).

Table 2
Clinical and socio-demographic study

S. No.	Status of patients	Number (%)
1	Male	56 (64.36)
2	Female	31 (35.63)
S.No.	**Gender**	**Number (%)**
1	In-patients	24 (27.58)
2	Out-patients	63 (72.41)
S.No.	**Age distribution (years)**	**Number (%)**
1	1–19	6 (6.89)
2	20–39	16 (18.39)
3	40–59	24 (27.58)
4	60–79	36 (41.37)
5	> 79	5 (5.47)

Antibiotic Susceptibility Pattern of *P. aeruginosa*

Table 3 shows the antibiotic susceptibility pattern of *P. aeruginosa* isolates. Polymyxin, Tobramycin, Gentamicin, Imipenem and Ceftazidime were the most effective antibiotics *in-vitro* with sensitivitities of 73 (83%), 71 (81.60%), 69 (79.31%), 63 (72.41%), and 61 (70.11%) respectively. On the other hand, all the *P. aeruginosa* isolates were resistant (100%) to cefexime.

Table 3
Antibiotic Susceptibility Pattern of *P. aeruginosa*

Antibiotics used	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Aztreonam	58 (66.66)	21 (24.13)	8 (9.19)
Ceftazidime	61 (70.11)	0 (0)	26 (29.88)
Carbenicillin	30 (34.48)	16 (18.39)	41 (47.12)
Cirpofloxacin	59 (67.81)	1 (1.14)	27 (31.03)
Gentamicin	69 (79.31)	0 (0)	18 (20.68)
Imipenem	63 (72.41)	0 (0)	24 (27.58)
Norfloxacin	56 (64.36)	0 (0)	31 (35.36)
Nitrofurantoin	0 (0)	0(0)	40 (100)
Piperacillin	54 (62.06)	10 (11.49)	23 (26.43)
Piperacillin-tazobactam	37 (78.72)	1 (2.12)	9 (19.14)
Polymixin	73 (83.90)	14 (16.09)	0 (0)
Cefexime	0 (0)	0 (0)	87 (100)
Tobramyycin	71 (81.60)	0 (0)	16 (18.39)
As shown in Table 4, out of 87 *P. aeruginosa* isolates, the maximum number of MDR *P. aeruginosa* (MDRPA) was isolated from pus (30.76%) which followed by urine (30%) and sputum (16.66%).			

Table 4
Distribution of MDR *P. aeruginosa* isolates among different clinical specimens

Specimens	Number of *P. aeruginosa* isolates N (%)	Number of MDRPA isolates N (%)
Urine	40 (3.09)	12(30)
Sputum	24 (12.06)	4(16.66)
Pus	13 (4.48)	4(30.76)
Blood	2 (1.81)	0(0)
Body fluid	8(6.01)	0(0)
Total	**87**	**20**

Out of 87 total isolates, 83 (95.4%) *P. aeruginosa* isolates showed the presence of *toxA* virulence genes whereas *oprL* gene was detected in all of the collected *P. aeruginosa* isolates 87 (100%) (Table 5).

Table 5
Prevalence of virulence genes (*oprL* and *toxA*) in *P. aeruginosa*

Virulence genes	Specimen type					Total N (%)
	Urine n(%)	Sputum n(%)	Pus n(%)	Blood n(%)	Body fluid n(%)	
oprL	40 (100)	24 (100)	13 (100)	2 (100)	8 (100)	87 (100)
toxA	40 (100)	21 (87.5)	12 (92.30)	8 (100)	2 (100)	83 (95.40)

Discussion

P. aeruginosa is associated as a versatile opportunistic human pathogen and its ultimate infection is reported to complete by attachment, colonization, local invasion and dissemination as a systemic disease (21–22).

In this study, the prevalence of *P. aeruginosa* isolates was 4.29%, in which the distribution in male patients 56 (64.36%) was higher than in female patients 31 (35.63%). The possible reasons might be types of studied populations, different geographical locations, type of hospitals. In addition, other reasons may be the male have a routine outdoor work and they are frequently in the risk of infection from the infected environments (16). The prevalence rate of infections was higher in outpatients 63 (72.41%) compared to the hospital admitted patients 24 (27.58%) which may be due to frequent exposure to infected environment. In addition, the occurrence of *P. aeruginosa* isolates to be greater in the age group 60–79 (41.37%), infection caused by *P. aeruginosa* is more common in patients of old age group. This could be described as because of decrease immune system, prolonged duration of hospitalization (23–25).

In our study, the difference between gender of patients and *P. aeruginosa* isolation from different clinical specimens was found to be statistically significant ($p = 0.016$). Likewise, the difference between type of patients and *P. aeruginosa* isolated from different clinical specimens was found to be statistically significant ($p = 0.009$). There was no statistical significance between the different age groups and occurrence of *P. aeruginosa* in male and female patients ($p = 0.071$) at 5% level of significance.

In this study, the most effective drug for *P. aeruginosa* isolates was found to be polymyxin B 73 (83.90%), also called last resort antibiotic for Pseudomonadecae family in the hospitals and less effective antibiotic was cefixime 87 (100%). Out of 87 *P. aeruginosa* isolates, 20 (22.98%) were identified to be MDRPA. The development of antibiotic resistant towards *P. aeruginosa* might be due random use of antibiotics, production of different types of enzyme like carbapenamase, AmpC-lactamases, quorum sensing modification of different target side etc (26, 13). Furthermore, one of the major cause of the

emergence of *P. aeruginosa* is prescribing the antibiotics without performing susceptibility test due to lack of laboratory facilities in most of the healthcare centers in Nepal (16, 24, 27–28).

The PCR results showed that 87 (100%) of 87 *P. aerugiosa* isolates were positive for *oprL* genes. Similarly in this study 83 (95.40%) of 87 *P. aeruginosa* were positive for *toxA* gene. Almost comparable study was carried out by Ibraheem at 2018 which showed 100% of *toxA* and 98.8% *oprL* genes. According to the Khattab et al in (2015), 100% of *oprL* gene, and 63.33% *toxA* gene were reported among the *P. aeruginosa*. The divergences in the distribution of virulence factor genes in the different populations might be due to the probability that some *P. aeruginosa* strains are better adopted to the particular conditions found in infectious sites that may returned to the diverse geographical and environmental sources. The prevalence of *P. aeruginosa* and its virulence genes depends on various causes consisting nature of places, degree of contamination and type, immune status of individual patients and virulence of strains (29).

Exotoxins A are either actively secreted through the type 1 secretion system (T1SS), the type 2 secretion system (T2SS), and the type 3 secretion system (T3SS) or passively secreted via the cell (27). The exotoxin A is encoded by gene called *exoA* which is involved in the tissue necrosis and resistant to antibiotics (28). The L, and I are two outer membrane lipoproteins of *P. aeuginosa* are found only in this organism, so that they could be a suitable factors for rapid identification of *P. aeruginosa* in clinical specimens. This bacterium is also answerable for inherent resistance to antiseptics and antibiotics (29).

In this study, detection of *P. aeruginosa* by multiplex PCR for amplification of *oprL* gene, present study showed that of 87 tested *P. aruginosa* isolates 87 (100%) contained the *oprL* gene (sensitivity = 100%), where as other species of bacteria did not produce any positive result (specificity = 100%). While amplification of the *toxA* gene showed that of 87 tested *P. aeruginosa* isolates 83 (95.40%) contained the *toxA* gene (sensitivity = 95%), where as other species of bacteria did not yield any positive result (specificity = 100%). This study revealed that the multiplex PCR may be one of the rapid diagnostic tool for the identification of *P. aeruginosa* infections.

Conclusion

In this research, polymyxin and amynogycocides were found to be functional antibiotics for treatment and the studies revealed that almost all *P. aeruginosa* harbors both *oprL* and *toxA* genes. Additional studies might be required to confirm the pathogenicity and increasing trends of antibiotic resistance pattern of *P. aeruginosa*.

Limitations of study

Study has limitations as minimum inhibitory concentration and 16 s rRNA sequencing for *P. aeruginosa* were not performed because of cost and unavailability of laboratories respectively. Despite of all, only *tox A* and *opr L* genes were taken as an identification keys because of expensiveness.

Abbreviations

CLSI: Clinical and laboratory Standards Institute, **MDRPA**: Multidrug resistant *Pseudomonas aeruginosa*

Declarations

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Authors' contributions

YC designed the research proposal, conducted laboratory work, data analysis and drafted the manuscript under the guidance of PP and DKK. OPP, SK and DS were responsible for data analysis and reviewing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data sharing is not applicable to this study as the datasets created needed to be confidential

Ethical approval and consent to participate

The ethical clearance and consent to participate was approved by institutional review board (IRB), National Academy of Medical Sciences (NAMS), Bir Hospital, Kathmandu, Nepal.

Consent for application

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Figures

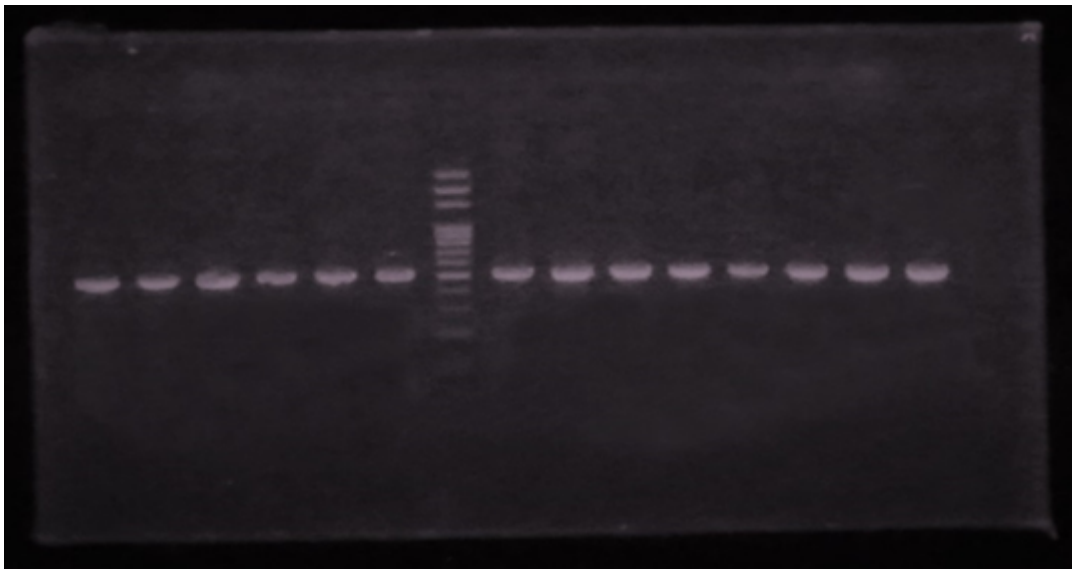

Figure 1

Agarose gel (1%) electrophoresis of PCR assay of oprL gene (500 bp). From left to right bands U8, U14, U5, U15, U7, U6, B1, BF1, BF2, BF3, S6, S7 and P8 indicates P. aeruginosa isolates with 100 bp marker, U12 represents positive control

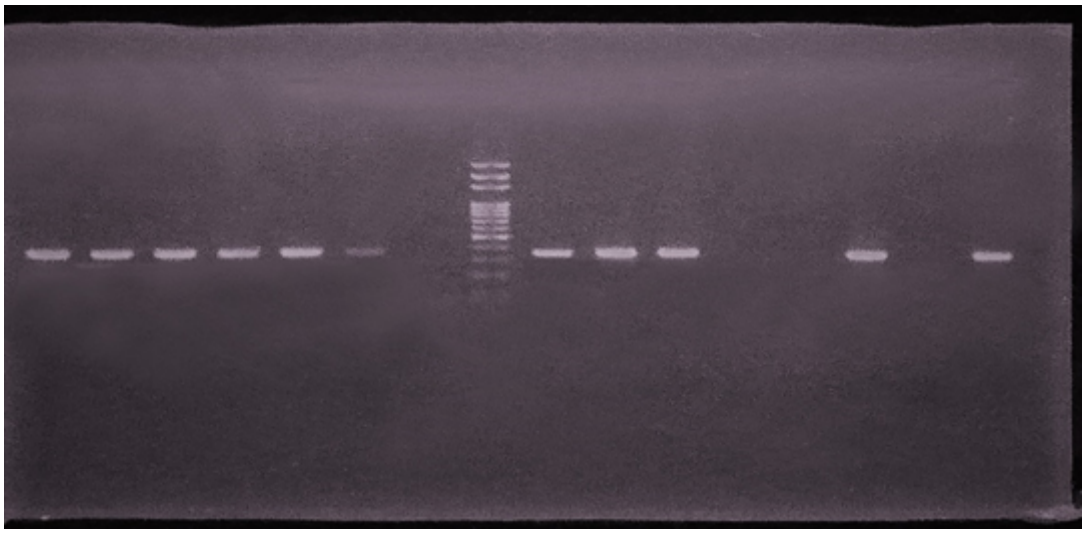

Figure 2

Agarose gel (1%) electrophoresis of PCR assay of toxA gene (352 bp). From left to right bands U7, U8, U18, U19, B1, BF1, P6, P10, S12 and S24 indicates P. aerogenosa isolates, bands P4, S4, S7 and S17 indicates negative isolates with 100 bp marker and band P5 represents positive control.