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Original Article

Molecular epidemiology of *Pseudomonas aeruginosa* isolated from lower respiratory tract of ICU patients

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(With 3 figures)

Abstract

Lower respiratory tract infections (LRTIs) caused by *Pseudomonas aeruginosa* are the most common infection among hospitalized patients, associated with increased levels of morbidity, mortality and attributable health care costs. Increased resistant *Pseudomonas* worldwide has been quite meaningful to patients, especially in intensive care unit (ICUs). Different species of *Pseudomonas* exhibit different genetic profile and varied drug resistance. The present study determines the molecular epidemiology through DNA fingerprinting method and drug resistance of *P. aeruginosa* isolated from patients with LTRIs admitted in ICU. A total of 79 *P. aeruginosa* isolated from patients with LRTIs admitted in ICU were characterized by Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) and Repetitive Extrapalindromic PCR (REP-PCR). Antibiotic resistance was determined by minimum inhibitory concentration (MIC) assay while MDR genes, viz, blaTEM, blaOXA, blaVIM, blaCTX-M-15 were detected by polymerase chain reaction (PCR). Of the 137 *Pseudomonas* sp isolated from ICU patients, 57.7% of the isolates were reported to be *P. aeruginosa*. The overall prevalence of *P. aeruginosa* among the all included patients was 34.5%. The RAPD analysis yielded 45 different patterns with 72 clusters with 57% to 100% similarity level. The RFLP analysis yielded 8 different patterns with 14 clusters with 76% to 100% similarity level. The REP PCR analysis yielded 37 different patterns with 65 clusters with 56% to 100% similarity level. There was no correlation among the different DNA patterns observed between the three different methods. Predominant of the isolates (46.8%) were resistant to amikacin. Of the 79 isolates, 60.8% were positive for *blaTEM* gene and 39.2% were positive for *blaOXA* gene. *P. aeruginosa* was predominantly isolated from patients with LRTIs admitted in ICU. The difference in the similarity level observed between the three DNA fingerprinting methods indicates that there is high inter-strain variability. The high genetic variability and resistance patterns indicates that we should continuously monitor the trend in the prevalence and antibiotic resistance of *P. aeruginosa* especially in patients with LRTIs admitted in ICU.

Keywords: Pseudomonas aeruginosa, lower respiratory tract infection, DNA typing, intensive care unit, RFLP, RAPD and REP-PCR markers.

Epidemiologia molecular de *Pseudomonas aeruginosa* isolada em pacientes com infecções do trato respiratório inferior admitidos em UTI

Resumo

Infecções do trato respiratório inferior (ITRIs) são as infecções mais comuns entre pacientes internados em unidade de terapia intensiva (UTI). *Pseudomonas aeruginosa* é a causa mais comum de ITRIs e está associada ao aumento da mortalidade. Diferentes espécies de *Pseudomonas* exibem diferentes perfis genéticos e resistência variada as drogas. O presente estudo determina a epidemiologia molecular através do método de *fingerprinting* de DNA e resistência as drogas de *P. aeruginosa* isoladas de pacientes com LTRIs internados em UTI. Um total de 79 *P. aeruginosa* isoladas de pacientes com ITRIs internados em UTI foram caracterizados por Polimorfismo de Comprimento de Fragmentos de Restrição (RFLP), DNA Polimórfico Amplificado ao Acaso (RAPD) e PCR Extrapalindrômico Repetitivo (REP-PCR). A resistência aos antibióticos foram determinadas pelos ensaios de concentrações inibitória mínima (MIC), enquanto os genes MDR, blaTEM, blaOXA, blaVIM, blaCTX-M-15 foram detectados pela reação em cadeia da polimerase (PCR). Das 137 *Pseudomonas* sp isoladas de pacientes de UTI, 57,7% dos isolados foram relatados como *P. aeruginosa*. A prevalência geral de *P. aeruginosa* entre os pacientes incluídos foram de 34,5%. A análise RAPD renderam 45 padrões diferentes com 72 clusters com nível de similaridade de 57% a 100%. A análise RFLP renderam 8 padrões diferentes com 14 clusters com 76% a 100% de similaridade. A análise de PCR do REP produziram 37 padrões

diferentes com 65 clusters com nível de similaridade de 56% a 100%. Não houveram correlações entre os diferentes padrões de DNA observados entre os três diferentes métodos. Predominantes dos isolados (46,8%) eram resistentes à amicacina. Dos 79 isolados, 60,8% foram positivos para o gene blaTEM e 39,2% foram positivos para o gene blaOXA. *P. aeruginosa* foi predominantemente isolado de pacientes com ITRIs internados em UTI. A diferença no nível de similaridade observado entre os três métodos de fingerprinting do DNA indica que há alta variabilidade inter-strain. A alta variabilidade genética e os padrões de resistência indicam que devemos monitorar continuamente a tendência na prevalência e resistência a antibióticos de *P. aeruginosa*, especialmente em pacientes com ITRIs internados em UTI.

Palavras-chave: Pseudomonas aeruginosa, infecção do trato respiratório inferior, DNA, unidade de terapia intensiva, marcadores RFLP, RAPD, REP-PCR.

1. Introduction

Lower respiratory tract infections (LRTIs) are the most common infection among patients admitted in intensive care unit (ICU) (Bhatta et al., 2019). Patients admitted in ICUs are highly susceptible to nosocomial infections due to prolonged hospitalization, decreased mobility and exposure to various medications (Sydnor and Perl, 2011). The incidence of nosocomial infections are higher among developing countries when compared to developed countries and it ranges from 2.3% to 49.2% across different clinical settings (Alberti et al., 2002). *Pseudomonas* sP. is the common cause of nosocomial infections and are highly associated with the LRTIs with increased mortality. The ICU is considered as established endemic settings favoring the growth of *Pseudomonas* sP. Its ability to colonize in-patients made it as highly critical organisms within ICUs settings (Erbay et al., 2003). Among *Pseudomonas* sp, *P. aeuroginosa* is considered as the most predominant species associated with severe LRTIs among patients admitted in ICUs and the second most common pathogen associated with ventilator-associated pneumonia (Weinstein et al., 2005; Sievert et al., 2013). According to the National Nosocomial Infection Surveillance System (NNISS) from China, *P. aeruginosa* was the predominant pathogen isolated from the lower respiratory tract, accounting for 12.8%, 12.3% and 13.4% during 2002-2004, 1999-2001 and 2005-2007, respectively (Ding et al., 2016; Wen et al., 2011). A multicenter cross sectional analysis from the United States reported *P. aeuroginosa* (36.2%) as the most common Gram negative organisms isolated from patients with LRTIs acquired during ICU stay (Claeys et al., 2018).

P. aeruginosa with a high genetic variation is related to several infectious diseases. Due to its genetic diversity, the existing differences in the pathogenicity, and certain genotypes of *P. aeruginosa* were isolated from certain clinical settings. Understanding the relatedness and distribution of the pathogen is highly important to determine the epidemiology of *P. aeruginosa* infections and designing appropriate pathogen control measures (Nazik et al., 2007). Phenotypic methods including biotyping, serotyping, hemagglutination, cytotoxin activity assay, bacteriocin typing and immunoblotting were available. However, they lack reproducibility and the fact that *P. aeruginosa* is phenotypically unstable makes it difficult to rely on these methods for epidemiological studies. Genotypic methods are including pulse field gel electrophoresis, multiple-locus variable number tandem repeat analysis and multilocus sequence typing have high discriminatory power (Maatallah et al., 2013). However, they are require technical expertise, expensive, laborious and time-consuming. The PCR based molecular techniques gained importance in elucidating the epidemiology of *P. aeruginosa*. The restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) and Repetitive Extrapalindromic PCR (REP-PCR) analyses have been developed for *P. aeruginosa* genotyping (Nanvazadeh et al., 2013; Joseph et al., 2010; Ratkai et al., 2010). These techniques are reported to be less laborious, rapid with high discriminatory power and could be employed in different clinical laboratory settings.

Anti-pseudomonal quinolone or an aminoglycoside along with β-lactams is considered as the primary treatment option for *P. aeruginosa* infections (Chinese Thoracic Society, 1999; Chinese Society of Critical Care, 2013). However, the treatment of *P. aeruginosa* infection is challenging due to increasing drug resistance. The increasing prevalence of multidrug resistant (MDR) strains is recognized as a global problem during the treatment of *P. aeruginosa* infections (Rossi Goncalves et al., 2017). The MDR *P. aeruginosa* strains are associated with increased morbidity and mortality, prolonging hospital stay and higher costs. The pan-drug resistance, the lack of effective antibiotics against MDR *P. aeruginosa* strains and continues the dissemination of such strains pose serious challenges in the infection control management (Murugan et al., 2018). Hence, understanding the epidemiology of pathogens and antibiotic resistance patterns of *P. aeruginosa* is highly warranted. The present study determined the prevalence, genetic heterogeneity and antibiotic resistance patterns of *P. aeruginosa* isolated from sputum samples of patients admitted with LRTIs in ICU by RFLP, RAPD, REP-PCR, drug resistance by MIC and resistance gene detection by PCR.

2. Material and Methods

2.1. Sample collection

A total of 229 samples (sputum-167, broncho alveolar lavage-62) were collected from patients with LRTIs adimitted in the ICU of Ninth Hospital of XI'an, Xi'an, Shaanxi, China between April 2017 and December 2018. From all patients or their legal representatives a written informed consent was obtained before collecting the samples. The hospital review board approval was obtained for the study. Samples were aseptically transported and processed immediately for microbiology analysis.

2.2. Isolation and identification of P. aeruginosa

The samples were sub-cultured on to the MacConkey agar (HiMedia, India), incubated at 37 °C for 18-24 hours. Non-fermenting, irregular, green to brown color, catalase and oxidase positive colonies showing typical colony morphology of *Pseudomonas sP.* were selected. The API® 20 NE microbial identification system for the non-fastidious, non-enteric gram negative bacilli (Biomerieux, USA) was used to identify the organisms. After identification, the isolates were sub-cultured on nutrient agar (HiMedia, India) and stored at -40 °C in 20% glycerol containing nutrient broth until used for further analysis.

2.3. DNA extraction

DNA extraction was performed by boiling lysis method. Briefly, a loop full of overnight culture was suspended in 200 µL of sterile water and boiled for 10 minutes at 95 °C. Then the suspension was spin for 10 minutes at 14,000 g and supernatant was collected. The DNA present in the supernatant was quantified by spectrophotometry (NanoDrop ND-1000, USA) and stored at -20 °C until used.

2.4. Randomly amplified polymorphic DNA

RAPD was performed as described by Nanvazadeh et al. (2013). The PCR reaction mixture contains 12.5 µL of ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, USA), 5 µL of the template, 10 pm of primer 3'-AGCGGGCCAA-5' and molecular grade PCR water to made up to 25 µL. The PCR was performed with the following cycling conditions: initial denaturation for 2 minutes at 96 °C, followed by 3 cycles for 1 minute at 94 °C, 2 minutes at 36 °C, 2 minutes at 72 °C. Then 29 cycles for 1 minute at 94 °C, 1 minute at 58 °C, 1 minute at 72 °C and a final extension for 5 minutes at 72 °C. After PCR, DNA patterns resolved in 1.5% (w/v) agarose gel were analyzed against 1 Kb DNA marker.

2.5. Restriction fragment length polymorphism

RFLP was performed as described by Joseph et al. (2010). A 50 µL reaction mixture contains 5 µL of the template, 10 pm of each primer (forward: 5'- GACGGGTGAGTAATGCCTA -3' and reverse: 5'- CACTGGTGTTCCTTCCTATA -3') and 25 µL of ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, USA). The PCR was performed with the following cycling conditions: initial denaturation for 2 min at 95 °C, followed by 30 cycles for 20 sec at 94 °C, 20 sec at 54 °C, 40 sec at 72 °C and a final extension for 1 minute at 72 °C. Restriction digestions was performed with 10 µL of the amplicons (expected size of 618 bp) by 2 U of *HinfI* (ThermoFisher Scientific, USA) restriction enzymes as per manufacturer's instruction for 16 h at 37 °C. DNA patterns resolved in 1.8% (w/v) agarose gel were analyzed against 100 bp DNA marker.

2.6. Repetitive extrapalindromic PCR

REP-PCR was performed as described by Sorkh et al. (2017). A 50 µL reaction mixture contains 5 µL of the template, 20 pm of each primer (forward: 5'- ICGICTTATCIGGCCTAC -3' and reverse: 5' - IIIICGICGICATCIGGC-3') and 25 µL of ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, USA). The PCR was performed with the following cycling conditions: initial denaturation for 2 minutes at 95 °C, followed by 45 cycles for 30 sec at 95 °C, 1 minute at 38 °C, 2 minutes at 72 °C and a final extension for 16 minutes at 72 °C. DNA patterns resolved in 1.8% (w/v) agarose gel were analyzed against 100 bp DNA marker.

2.7. Phylogenetic tree analysis

DNA fingerprints obtained for RAPD, RFLP and REP-PCR, respectively, were analyzed separately for phylogenetic tree construction. They were coded to 1 or 0 binary forms, respectively. Using the NTSYSpc 2.11p software (Exeter Software, Setauket, USA) clustering (NJ-UPGMA method) was performed and phylogenetic tree was constructed.

2.8. Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of various antibiotics including gentamicin, amikacin, piperacillin-tazobactam, ciprofloxacin, cefepime, ceftazidime, imipenem and meropenem (Sigma-Aldrich, USA) against *P. aeruginosa* was determined. Innoculum was prepared by adjusting the overnight culture to 0.5 McFarland's standard. The MIC assay was performed at a concentration of each antibiotics ranging from 0.03 µg/mL to 256 µg/mL by micro broth dilution method; using Muller-Hinton Broth (MHB, HiMedia, India) as described in Clinical Laboratory Standard Institute (CLSI, 2015) guidelines. Briefly, 10 µL of culture was inoculated into MHB containing different concentration of antibiotics and incubated at 37 °C for 24 h. The MIC was determined visually by the highest concentration showing the absence of growth.

2.9. Resistant gene detection

Antibiotic resistant genes including bla_{TEM}, bla_{OXA}, $bla_{CTX\text{-}M\text{-}15}$, bla_{VIM} were detected through PCR as described earlier (Murugan et al., 2018). PCR was performed using ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, USA). Briefly, 25 µL of ReadyMix™, 5 µL of template DNA, 5pmol of each primer described in Table 1, and molecular grade PCR water to make up to 50 µL was used. Mastercycler® X50s (Eppendorf, Germany) was used for all PCR assays (RAPD, RFLP, REP-PCR). The PCR cycling conditions for resistant gene detection included: initial denaturation for 5 min at 94 °C followed by 35 cycles for 30 sec at 94 °C, 30 sec at 56 °C, 1.5 min at 72 °C and a final extension for 7 min at 72 °C. After PCR, amplicons were resolved in 1.2% agarose gel electrophoresis.

2.10. Statistical analysis

Mean and ranges were used to represent the continuous variables; number and percentages were used to represent categorical variable. Statistical significance ($P<.05$ was considered statistically significant) was determined through Chi-Square and ANOVA test using MINITAB statistical software (MINITAB version 13.1; MINITAB Inc, PA, USA).

Table 1. Primer sequence of ESBL-MBL resistant genes.

Genes	Primer sequence (5'-3")	Amplicon size (bp)
bla_{TEM}	Forward: TCAACATTTTCGTGTCGCCC Reverse: AACTACGATACGGGAGGGCT	766
bla_{OXA}	Forward: AGATCCTTGACCCGCAGTTG Reverse: CGCCGTCCCATCGAAAAATC	928
bla_{CTX-M}	Forward: AGACTGGGTGTGGCATTGAT Reverse: TTAGGTTGAGGCTGGGTGAAGT	676
bla_{VIM}	Forward: TGTCCGTGATGGTGATGAGT Reverse: GTGCTTCCGGGTAGTGTTGT	456

Table 2. Characteristics of patient population.

Demographic characteristics	No of patients (%)	*P*
Mean age ± SD (years))	43.3±5.3 years	
Male	145 (63.3%)*	<.05
Female	84 (36.7%)	
Smoker	157 (68.6%)*	<.05
Non-smoker	72 (31.4%)	
Bronchitis	118 (51.5%)*	<.05
Pneumonia	64 (27.9%)	
Lung abscess	47 (20.5%)	
Mechanical ventilator support	82 (35.8%)	NA
Male (n=145)		
Smoker	112 (77.2%)*	<.05
Non-smoker	33 (22.8%)	
Bronchitis	78 (53.8%)*	<.05
Pneumonia	41 (28.3%)	
Lung abscess	26 (17.9%)	
Mechanical ventilator support	49 (33.8%)	NA
Female (n=84)		
Smoker	55 (65.5%)*	<.05
Non-smoker	29 (34.5%)	
Bronchitis	40 (47.6%)	>.05
Pneumonia	23 (27.4%)	
Lung abscess	21 (25.0%)	
Mechanical ventilator support	33 (39.2%)	NA

*Significantly higher (Chi-Square test); SD= Standard Deviation, P= P value of < .05 is considered as statistically significant, NA = Not applicable.

3. Results

3.1. Patients and isolates

Of the 229 patients, 145 (63.3%) were male and 84 (36.7%) were female (mean age 43.3±5.3 years). Among these 157 (68.6%) patients had the history of smoking and 82 (35.8%) patients were under mechanical ventilator support. A detailed patient's characteristics are presented in Table 2. Of the 229 sputum samples, a total of 137 (59.8%) samples isolated *Pseudomonas sp.*by culture method. All *Pseudomonas sP.* isolates were characterized using API® 20 NE microbial identification system (Biomerieux, USA). Among the *Pseudomonas sp.,* 79 (57.7%) isolates were identified as *P. aeruginosa*. Of these, 47 (34.3%) isolates were from male patients and 32 (23.4%) were from female patients. The overall prevalence of *P. aeruginosa* among the all included patients was 34.5% (male 20.5%; female, 14.0%). The presence of *P. aeruginosa* was significantly higher than other *Pseudomonas sp* (*P* = .04). Among the 82 patients who were under mechanical ventilator support, 23 (28%) patients isolated *P. aeruginosa*.

3.2. Randomly amplified polymorphic DNA

The RAPD-PCR revealed 45 different patterns with 1-10 different sized amplicons per strain with sizes ranging from 200->2700 bP. The phylogenetic analysis revealed 72 clusters with a similarity level ranging from 57-100%. Seventeen (20.3%) strains (strain numbers 1, 4, 11, 14, 17, 27, 38, 44, 48, 51, 54, 60, 62, 64, 72, 75) showed 100% intra-strain similarity with 8 different clusters. Other strains revealed high variability (Figure 1).

3.3. Restriction fragment length polymorphism

The RFLP analysis revealed 8 different patterns (designated as genotype I to VIII) with 1-3 different sized amplicons per strain with sizes ranging from 180-618 bP. The phylogenetic revealed 14 clusters with a similarity level ranging from 49-100%. Thirteen different clusters emerged with 100% intra-strain similarity of. Among these, 3 major restriction patterns were identified; 17 (21.5%) strains (strain numbers 2, 8, 12, 14, 16, 18, 25, 28, 32, 44, 47, 50, 56, 59, 74, 75, 77) (genotype I) followed by 17 (21.5%) strains (strain numbers 3, 6, 20, 23, 29, 30, 33, 42, 48, 51, 54, 63, 66, 68, 69, 72, 78) with genotype II and 10 (12.7%) strains (strain numbers 7, 13, 21, 31, 34, 35, 38, 62, 73, 76) with genotype III. Other strains exhibited high heterogeneity (Figure 2).

3.4. Repetitive extrapalindromic PCR

The REP-PCR analysis revealed 37 different patterns with 1-17 different sized amplicons per strain with sizes ranging from 100-1,000 bP. The phylogenetic analysis of RAPD yielded 65 clusters at a similarity level ranging from 56-100%. Eighteen (22.8%) strains (strain numbers 3, 6, 18, 19, 21, 25, 37, 42, 44, 45, 49, 51, 53, 64, 66, 68, 69, 79) showed 100% intra-strain similarity with 9 different clusters. Other strains revealed high variability, overall, the REP-PCR analysis revealed high heterogeneity among our isolates (Figure 3).

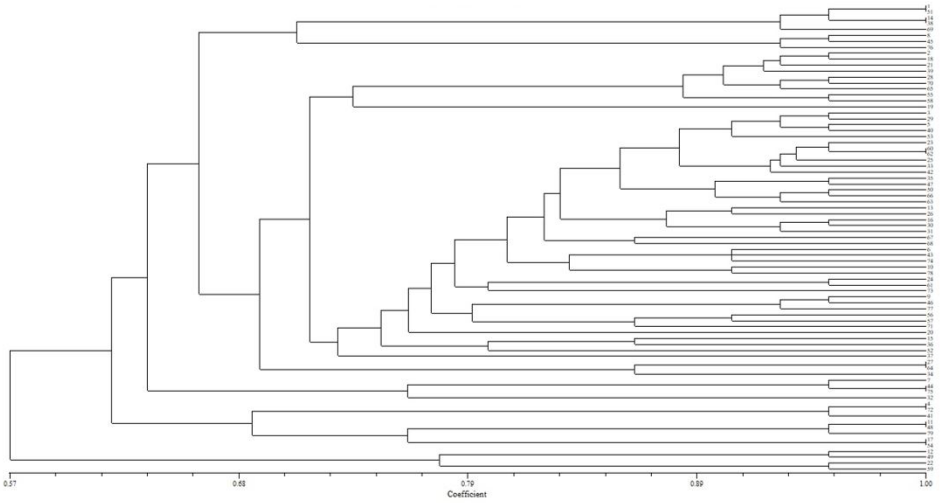

Figure 1. Phylogenetic tree analysis of RAPD.

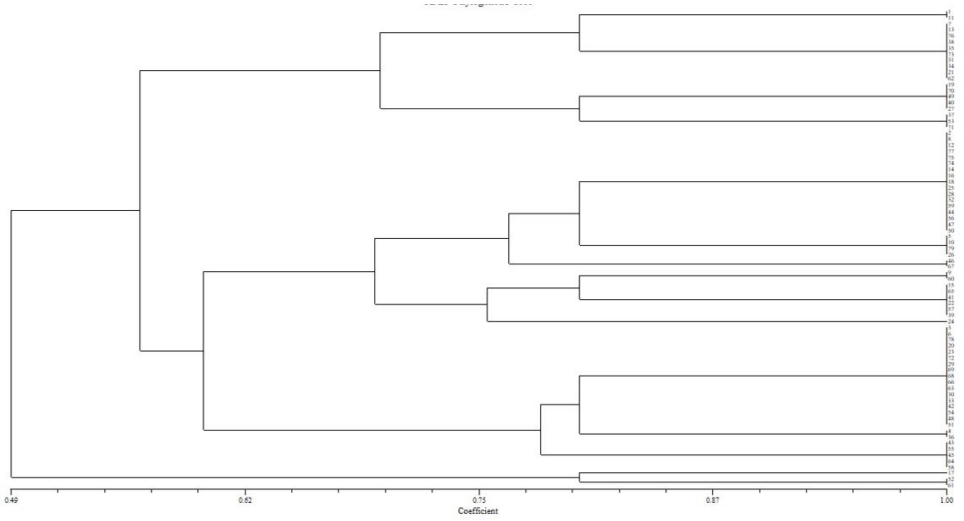

Figure 2. Phylogenetic tree analysis of RFLP.

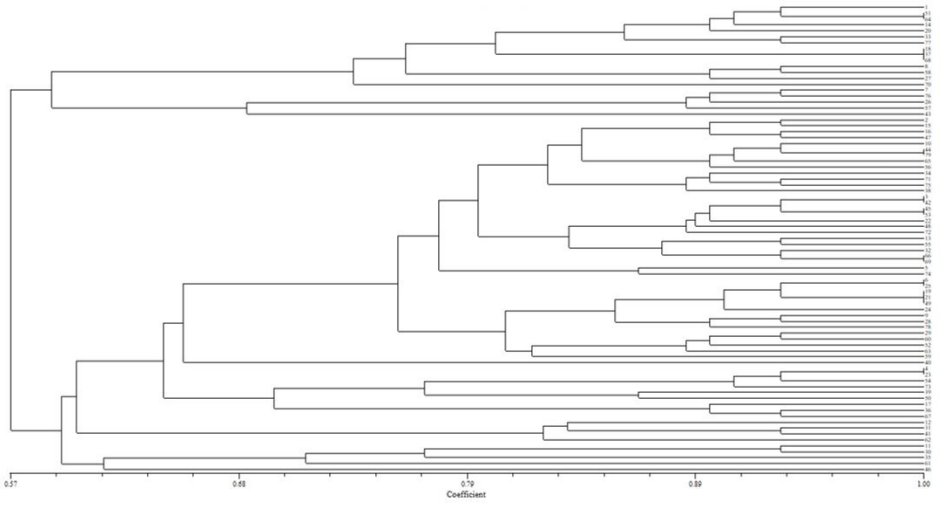

Figure 3. Phylogenetic tree analysis of REP-PCR.

Table 3. Antibiotic resistance and MIC of *P. aeruginosa* isolates.

Antibiotics	No. of isolates (n=79)			MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Range (µg/mL)
	S	I	R			
AM	36 (45.6%)	6 (7.6%)	37 (46.8%)	2	64	0.06-128
GN	33 (41.8%)	12 (15.2%)	34 (43%)	4	32	0.5-64
CIP	44 (55.7%)	8 (10.1%)	27 (34.2%)	4	16	0.06-32
CAZ	53 (67.1%)	2 (2.5%)	24 (30.4%)	8	128	2 - ≥256
MEM	52 (65.8%)	4 (5.1%)	23 (29.1%)	4	32	0.5-64
IPM	42 (53.2%)	16 (20.3%)	21 (26.5%)	4	64	0.25-64
CPM	53 (67.1%)	8 (10.1%)	18 (22.8%)	16	64	≤ 0.03 - ≥356
PTZ	62 (78.5%)	3 (3.8%)	14 (17.7%)	1	32	8-128

MIC= Minimum Inhibitory Concentration; S= Sensitive; I= Intermediate Resistant; R= Resistant;Gentamicin (GN); amikacin (AM); piperacillin-tazobactam (PTZ); ciprofloxacin (CIP); cefepime (CPM); ceftazidime (CAZ); imipenem (IPM); and meropenem (MEM).

3.5. Minimum inhibitory concentration

Of the 79 *P. aeruginosa* isolates tested for antibiotic susceptibility, 37 (46.8%) isolates were found to be resistant to amikacin, 34 (43%) were resistant to gentamicin, 27 (34.2%) were resistant to ciprofloxacin, 24 (30.4%) were resistant to ceftazidime, 23 (29.1%) were resistant to meropenem, 21 (26.6%) were resistant to imipenem, 18 (22.8%) were resistant to cefepime and 14 (17.7%) isolates were resistant to piperacillin-tazobactam (Table 3). The antibiotic resistance pattern did not differ significantly among the isolates (ANOVA, F = 0.00; $P = 1.00$).

3.6. Resistant gene detection

Of the 79 isolates, 48 (60.8%) isolates amplified bla_{TEM} gene, 31 (39.2%) isolates amplified bla_{OXA} gene, 21 (26.6%) isolates amplified $bla_{CTX\text{-}M}$ gene and 12 (15.2%) isolates amplified bla_{VIM} gene. Significantly higher number of isolates amplified *blaTEM* gene when tested by PCR ($P<.05$). All the resistant isolates amplified any of the four tested genes. Nine isolates which showed intermediate resistance towards the tested antibiotics, were positive for bla_{TEM} (5), bla_{OXA} (2) and $bla_{CTX\text{-}M}$ (2) genes.

4. Discussion

Lower respiratory tract infections are one of the important infectious diseases in developing countries and are often associated with high mortality (Agrawal and Kombade, 2014). Patients who are under ventilator support in the ICU settings are at high risk of having LRTIs with *P. aeruginosa* (Bobik and Siemiątkowski, 2014). The microbiological diagnosis of lower respiratory infection is often challenging due to possible contamination of the specimen by upper respiratory tract microorganisms (Ahmed et al., 2013). Thus, accurate diagnosis through the appropriate clinical specimen and appropriate techniques plays an important role. In this study, sputum and broncho alveolar lavage samples were aseptically collected and processed immediately. In our study, 57.7% of the *Pseudomonas* strains were identified as *P. aeruginosa* accounting up to 34.5% of the overall samples collected. Our study reported a higher (34.5%) prevalence rate of *P. aeruginosa* than that reported from Nepal (13.9%), Pakistan (24%) and India (26.8%) in patients with LRTIs (Bhatta et al., 2019; Fatima et al., 2012; Ahmed et al., 2013). The NNISS from China reported lower prevalence of *P. aeruginosa* ranging from 12.3-13.4% during 1999-2007 in patients with LRTIs (Ding et al., 2016; Wen et al., 2011). A multicenter cross sectional analysis from the United States reported that 36.2% of the Gram negative organisms isolated from patients with LRTIs admitted in ICU were identified as *P. aeruginosa*, which is comparable (34.5%) to that reported in our study (Claeys et al., 2018). While a study from Brazil, when compared to our results, reported a higher percentage (51.9%) of *P. aeruginosa* isolates from ICU patients (Matos et al., 2016). Although the prevalence of *P. aeruginosa* varies in a different region, our study reported a higher rate compared to other studies from Asian countries including China. Thus, there is a need to continuously monitor the prevalence of *P. aeruginosa* especially in patients admitted in ICU with LRTIs in this region. Among the 79 patients who were isolated with *P. aeruginosa*, predominant (52, 65.8%) of them were from male patients. Similarly, a study from Pakistan reported that predominant (70.8%) of the *P. aeruginosa* were isolated from male patients with LRTIs; the reported rate was slightly higher than our report (Fatima et al., 2012). In our study, *P. aeruginosa* infection was reported to be significantly higher in male patients ($P<.05$). Our result indicates that *P. aeruginosa* isolated from patients admitted in ICU with lower respiratory infection showed varied prevalence rates and have epidemiological diversity, compared to other studies from different geographies.

Genetic complexity and the presence of large genome allows *P. aeruginosa* to thrive in different ecological conditions. Genomic heterogeneity forms the basis of several molecular techniques and is considered a valuable tool in epidemiological studies. Although several molecular techniques are available, PCR based molecular techniques, which are less laborious and cost-effective has gained importance in elucidating the epidemiology of *P. aeruginosa*. The RAPD, RFLP and REP-PCR are widely used in several epidemiological studies of *P. aeruginosa* (Nanvazadeh et al., 2013; Joseph et al., 2010; Ratkai et al.,

2010). The RAPD analysis in our study yielded 45 different patterns, RFLP yielded 8 different patterns and REP-PCR yielded 37 different patterns. A study from Iran, which included 50 *P. aeruginosa* from burn patients for RAPD, reported only 9 different genotypes which are lower than our report (Nanvazadeh et al., 2013). A study from Czech Republic which included 47 *P. aeruginosa* isolated from various clinical samples of patients admitted in ICU reported 22 different genotypes when analyzed by RAPD (Vaněrková et al., 2017). A study from Switzerland which included 55 (54-clinical, 4- environmental) *P. aeruginosa* isolated from burnt unit reported 8, 12 and 12 different patterns when digested the amplicons with *BamHI, EcoRI and PstI*, respectively (Blanc et al., 1993). A study from Iran, which analyzes 75 *P. aeruginosa* isolated from burn patients by REP-PCR reported 40 different REP types, a comparable number of REP-PCR pattern was obtained from our study (Sorkh et al., 2017).Although we did not include different isolates from the same patients, we observed that 20.3% of our isolates showed 100% intra-strain similarity with 8 different clusters when analyzed by RAPD. The RFLP analysis revealed 13 different cluster with 100% intra-strain similarity. While in REP-PCR 22.8% of the isolates 100% intra-strain similarity with 9 different cluster. We found that 12 isolates had common patterns across three different techniques. Overall, all three methods revealed high heterogeneity among our isolates. However, RAPD and REP-PCR had good discriminatory power and yielded different patterns with high strain variability when compared to RFLP. Apart from the isolates, which showed 100% intra-strain similarity majority of our stains, yielded several different DNA fingerprints and showed high genetic variability. The genetic variation among our isolates suggests continues monitoring of genotypes with a geographical specific approach is required for effective identification of different types of *P. aeruginosa* strains.

The treatment of *P. aeruginosa* infection is often challenging due to its inherent resistance to a broad range of antibiotics, its ability to develop MDR and acquired resistance through chromosomal mutations (Gales et al., 2001; Zahraa et al., 2014). In our study, 46.8% (MIC range 0.06-128 µg/mL) of our isolates were found to be resistance to amikacin while only 43% (MIC range 0.5-64 µg/mL) of isolates were found to be resistance to gentamicin. In contrast, a meta-analysis from China reported amikacin resistance in 22.5% of *P. aeruginosa* isolated from patients with VAP, which is lower than our result (Ding et al., 2016). While the study reported higher (51.1%) gentamicin resistance (Ding et al., 2016). A study from Nepal that included 19 *P. aeruginosa* isolated from patients admitted in ICU with LRTIs reported higher resistance to cefepime (68.4%), ciprofloxacin (68.4%), amikacin (52.6%), gentamicin (52.6%) and lower resistance to imipenem (10.5%) when compared to our results (Bhatta et al., 2019). Similarly, a study from Brazil reported higher rates of antibiotic resistance for meropenem (64.8%), imipenem (64.8%), ceftazidime (63%), cefepime (48.1%) and piperacillin/tazobactam (46.3%) (Matos et al., 2016). While a study from Pakistan when compared to our results, reported lower resistance to amikacin (35%), imipenem (24%) and higher resistance to cefepime (40%) among *P. aeruginosa* isolated from patients with LRTIs (Fatima et al., 2012). A study from India that included *P. aeruginosa* isolates from patients admitted in ICU with LRTIs reported varied results; the resistance rate of ceftazidime (30.4%) and ciprofloxacin (34.8%) corroborates with that reported in our study (Agrawal and Kombade, 2014). While resistance to imipenem (86.9%), cefepime (34.8%) was higher and gentamicin (8.7%) was lower than our result (Agrawal and Kombade, 2014). A study from China that included *P. aeruginosa* from patients with VAP in intensive care units reported that 50% of their isolates showed resistance to ceftazidime and all (100%) the isolates showed resistance to ciprofloxacin, which is much higher than our result (Xiao et al., 2013). In our study, the highest antibiotics resistance rate was observed for amikacin (46.8%) while the lowest was observed for piperacillin-tazobactam (3.8%).

Due to the stability against ESBLs, carbapenems (imipenem and meropenem) are considered as the most potent agents for MDR *P. aeruginosa* (Zavascki et al., 2010). In our study, meropenem and imipenem resistance were comparable, where 26.5% of our isolates were resistant to imipenem and 29.1% of isolates were resistant to meropenem. Our result on meropenem resistance (29.1%) was slightly higher (25%) than that reported from India (Murugan et al., 2018). A study from Brazil reported much higher rates of resistance (64.8%) for meropenem and imipenem among *P. aeruginosa* isolated from ICU patients (Matos et al., 2016). Majority of our isolates found to be resistant to amikacin and gentamicin, however, there was no significant difference in resistance rates was found among the isolates ($P = 1.00$). In our study, 24 (30.4%) of isolates were found to be MDR strains which were lower than that reported from Brazil (37%) and Asia (42.8%) (Chung et al., 2011; Matos et al., 2016). Of the 24 MDR isolates 9 (11.4%) were isolated from patients who were under mechanical ventilator support. All our MDR isolates were found to be resistance to gentamicin which is in agreement with the findings of a study from Qatar (AbdulWahab et al., 2017).

The family of genes, *TEM, SHV, CTX-M, PER, VEB, GES,* and *IBC* encoding the ESBL enzymes was prevalent among *P. aeruginosa* (Rawat and Nair, 2010). Among several ESBL genes present, bla_{TEM} gene was predominantly carried by *P. aeruginosa* that confers resistance towards penicillin group of antibiotics (Dumas et al., 2006). In our study bla_{TEM} was predominant gene (60.8%) present among our isolates. Our findings are in agreement with a study from India, which reported that bla_{TEM} was predominant gene present among the *P. aeruginosa* isolate, however, with higher rates (72.5%) to that our result (Murugan et al., 2018). The study also reported that the presence of bla_{OXA} (33.5%), bla_{VIM} (11.5%) and bla_{CTX-M} gene (5%) genes were lower than our result (Murugan et al., 2018). In our study 26.6% of isolates were positive for bla_{OXA} gene, in contrast a study from Iraq reported that 93% of their isolates were positive of bla_{OXA} gene which was much

higher than out report (Zahraa et al., 2014). The bla_{OXA} gene is considered as a naturally occurring gene, the high prevalence indicate a potential horizontal transfer in which Class D β-lactamases might get introduced by other co-inhabiting bacterial species (Girlich et al., 2004; Kong et al., 2005). We report that 15.2% of our isolates possessed bla_{VIM} gene lower (1.6%) or absence of bla_{VIM} gene among their *P. aeruginosa* isolates (Fallah et al., 2013; Moosavian and Rahimzadeh, 2015; Amini and Mobasseri, 2017). When compared to the antibiotic resistance and the presence of resistance genes with the 3 different molecular typing, we did not find any specific patterns or genotypes associated with antibiotic resistance or resistance genes.

5. Conclusion

P. aeruginosa was the predominant isolate with an overall prevalence of 34.5% isolated from patients (male 20.5%; female, 14.0%) with LRTIs admitted in ICU. Majority of the isolates were resistant to amikacin and bla_{TEM} was the predominant gene detected among our isolates. The difference in the similarity level observed between the three DNA fingerprinting methods indicates that there is high inter-strain variability. The differences in resistance patterns emphasize the need for personalized antibiotic treatment. The high genetic variability and resistance patterns indicate that we should continuously monitor the trend in the prevalence and antibiotic resistance of *P. aeruginosa* especially in patients with LRTIs admitted in ICU.

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