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In silico Assessment of the Genotypic Distribution of Virulence and Antibiotic Resistance Genes in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa harbours virulence and antibiotic resistance genes that contribute to life-threatening infections. *In silico* PCR amplification detected both virulence and antibiotic resistance genes of eighteen *Pseudomonas aeruginosa* isolates. L lipoprotein (*oprL)* gene was found in 72.22% of the isolates and is used usually to rapidly identify *Pseudomonas aeruginosa* species. Alkaline protease (*apr*) was detected in 66.67% of the isolates. Protein biosynthesis inhibited by exotoxin A (*exoA*), was found in 55.56% of the isolates. Fifty percent (n=9) of the isolates had the exoenzyme S (*exoS*) and elastase (*lasB*) genes. Thirteen isolates (72.22%) which harboured the phospholipid C (*plcH*) genes, were also found to be positive for rhamnolipid AB (*rhlAB*) genes. Thirteen isolates (72.22%) were found to possess alginate (*algD*) and neuraminidae (*nan2*) genes, respectively. *Pseudomonas aeruginosa* PA7 harboured both *aph(3")* and *aph(6)*-1d genes. Only sulfonamide resistance gene, *sul1* was present in 11.11% (n=2) of the isolates. Beta-lactamase gene, bla_{TEM} was present in only one isolate and no tetracycline resistance gene was found. Fluoroquinolone resistance-determining region of the *gyrA* and *parC* genes were present in fourteen (77.78%) and thirteen (72.22%) isolates, respectively. *Pseudomonas aeruginosa* NCGM2.S1 is a multidrug resistant bacterium since it harboured class 1 integrase gene. *In silico* pulsed-field gel electrophoresis (PFGE) analysis was able to group eighteen isolates into three genotypes. Gene distribution pattern within genotypes was almost similar and not dependent on genotypes. The data generated here helps to predict virulence and antibiotic resistance profile of *Pseudomonas aeruginosa*species based on genotype.

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that infects virtually all tissues. Fegan *et al.* (1990) reported that *P. aeruginosa* is colonized on respiratory tract and contributed to cystic fibrosis infection. Yetkin *et al.* (2006) reported that *P. aeruginosa* is responsible for nosocomial infection and affects patients in intensive care units. Pollack (2000) stated that immunocompromised cancer patients, burn patients and catheterized patients also suffer from serious infections that are caused by *P. aeruginosa*. Trautner and Darouiche (2004) reported that biofilms developed by *P. aeruginosa* are attached to the site of infection and caused chronic and recurrent infections. *Pseudomonas* is responsible for waterborne outbreaks of dermatitis, conjunctivitis and otitis media (Adesoji *et al.*, 2015). Van Delden and Iglewski (1998) stated that virulence properties of *P. aeruginosa* are tightly regulated by cell to cell signaling systems. Virulence factors of *P. aeruginosa* are divided into specific groups such as adhesins or other secreted exotoxins. Bradbury *et al.* (2010) reported that type I (TISS), type II (T2SS) or type III (T3SS) secretion system actively secreted exotoxins. Bradbury *et al.* (2010) also stated that previous studies have found the association of virulence factors with different diseases.

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Protein synthesis inhibiting exotoxin A was reported earlier by Thamir and Al-Jubori (2014). Khattab *et al.* (2015) demonstrated that PCR based amplification of L lipoprotein (*oprL*) is used for identification of the species of *Pseudomonas*. Pollack (2000) demonstrated that multidrug-resistant *P. aeruginosa* is medically important and caused several infections. Antibiotic resistance genes can be transferred between different microorganisms with the help of integrase gene, *int1* (Adesoji *et al.*, 2015). Yah *et al.* (2006), Bonomo and Szabo (2006), Chen *et al.* (2009) reported that plasmids or transposons also contributed to developing multidrug-resistant *Pseudomonas*.

Strateva and Yordanov (2009) concluded that risk factors identification for multidrug-resistant *Pseudomonas* is important to minimize this problem. Several clinical studies are needed to minimize severe infections caused by *Pseudomonas* and maximize the outcomes in the treatment of infectious diseases. The aim of the present study is to characterize the virulence and antibiotic resistance property of 18 *Pseudomonas aeruginosa* isolates by PCR and to find the distribution in genotypes based on pulsed field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Strains used in the study

Strains used in the study are summarized in Table 1.

Primer used in the study

Primer used for virulence and antibiotic resistance gene detection in the study are summarized in Table 2 and 3.

PCR amplification

In silico PCR amplification was done in the website http://insilico.ehu.eus/PCR/ (San Millán *et al.*, 2013; Bikandi *et al.*, 2004).

PFGE digestion

Pulsed field gel electrophoresis (PFGE) digestion was done in the website http://insilico.ehu.es/digest/ (San Millán *et al.*, 2013; Bikandi *et al.*, 2004). The enzyme used for the digestion was *Pac*I. Banding patterns were scored in a binary matrix and a dendrogram was constructed using Complete Linkage method by SPSS 16 (IBM, USA).

Table 1: Name of the isolates.

Number	Isolate
1	NC_002516 *Pseudomonas aeruginosa* PAO1
2	NC_020912 *Pseudomonas aeruginosa* B136-33
3	NC_018080 *Pseudomonas aeruginosa* DK2
4	NC_023066 *Pseudomonas aeruginosa* LES431
5	NC_011770 *Pseudomonas aeruginosa* LESB58
6	NC_017548 *Pseudomonas aeruginosa* M18
7	NC_023019 *Pseudomonas aeruginosa* MTB-1
8	NC_017549 *Pseudomonas aeruginosa* NCGM2.S1
9	NC_022808 *Pseudomonas aeruginosa* PA1
10	NC_022806 *Pseudomonas aeruginosa* PA1R
11	NC_009656 *Pseudomonas aeruginosa* PA7
12	NC_022594 *Pseudomonas aeruginosa* PAO1-VE13 genome
13	NC_022591 *Pseudomonas aeruginosa* PAO1-VE2 genome
14	NC_022361 *Pseudomonas aeruginosa* PAO581 genome
15	NC_021577 *Pseudomonas aeruginosa* RP73
16	NC_023149 *Pseudomonas aeruginosa* SCV20265
17	NC_008463 *Pseudomonas aeruginosa* UCBPP-PA14
18	NC_022360 *Pseudomonas aeruginosa* c7447m genome

Table 2: Primer for virulence gene detection.

Target	Gene	Primer	Amplicon Size bp	Reference
L lipoprotein	*oprL*	ATG GAAATGCTGAAATTCGGC CTTCTTCAGCTCGACGCGACG	504	De Vos *et al.*, 1997
Alkaline protease	*apr*	TGTCCAGCAATTCTCTTGC CGTTTTCCACGGTGACC	1017	Fazeli and Momtaz, 2014
Exotoxin A	*toxA*	GGAGCGCAACTATCCCACT TGGTAGCCGACGAACACATA	250	Sabharwal *et al*., 2014
Exoenzyme S	*exoS*	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	504	Stover *et al.*, 2000
Elastase	*lasB*	GGAATGAACGAAGCGTTCTCCGAC TGGCGTCGACGAACACCTCG	283	Fazeli and Momtaz, 2014
Rhamnolipid AB	*rhlAB*	TCATGGAATTGTCACAACCGC ATACGGCAAAATCATGGCAAC	151	Sabharwal *et al*., 2014
Phospholipase C	*plcH*	GAAGCCATGGGCTACTTCAA AGAGTGACGAGGAGCGGTAG	307	Sabharwal *et al*., 2014
Alginate	*algD*	ATGCGAATCAGCATCTTTGGT CTACCAGCAGATGCCCTCGGC	1311	Stover *et al.*, 2000
Putative neuraminidase	*nan2*	ACAACAACGGGGACGGTAT GTTTTGCTGATGCTGGTTCA	1161	Stover *et al.*, 2000

Table 3: Primer for antibiotic resistance gene detection.

Target	Gene	Primer sequence (5'-3')	Amplicon size bp	Reference
Class 1 integrase gene	*int1*	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	270	Houang *et al.*, 2003
Class 2 integrase gene	*int2*	TTATTGCTGGGATTAGGC ACGGCTACCCTCTGTTATC	233	Roe *et al.*, 2003
Variable region of class 1 integron	*vt1*	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Variable	Hall and Collis, 1995
Variable region of class 1 integron	*vt2*	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	Variable	White *et al.*, 2001
Sulfonamide resistance gene	*sul1*	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	433	Vinue *et al.*, 2010
Sulfonamide resistance gene	*sul2*	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	293	Vinue *et al.*, 2010
Sulfonamide resistance gene	*sul3*	TCAAAGCAAAATGATATGAGC TTTCAAGGCATCTGATAAAGAC	787	Vinue *et al.*, 2010
Streptomycine resistance gene	*aph (3")*	GCTCAAAGGTCGAGGTGTGG CCAGTTCTCTTCGGCGTTAG	515	van Overbeek *et al.*, 2001
Streptomycine resistance gene	*aph (6)-1d*	GACTCCTGCAATCGTCAAGG GCAATGCGTCTAGGATCGAG	560	van Overbeek *et al.*, 2001
Streptomycine resistance gene	*ant (3")*	CAGCGCAATGACATTCTTGC GTCGGCAGCGACATCCTTCG	294	van Overbeek *et al.*, 2001
Tetracycline resistance gene	*tet(A)*	TTGGCATTCTGCATTCACTC GTATAGCTTGCCGGAAGTCG	494	Call *et al.*, 2003
Tetracycline resistance gene	*tet(B)*	CAGTGCTGTTGTTGTCATTAA GCTTGGAATACTGAGTGTAA	571	Call *et al.*, 2003
Tetracycline resistance gene	*tet(M)*	ATTTCCGCAAAGTTCAGACG CCGTCATGCAATTTGTGTTC	536	Call *et al.*, 2003
Beta lactamase gene	bla_{TEM}	AAAGATGCTGAAGATCA TTTGGTATGGCTTCATTC	425	Henriques *et al.*, 2006
Beta lactamase gene	bla_{SHV}	GCGAAAGCCAGCTGTCGGGC GATTGGCGGCGCTGTTATCGC	538	Henriques *et al.*, 2006
DNA gyrase	*gyrA*	GTGTGCTTTATGCCATGAG GGTTTCCTTTTCCAGGTC	287	Gorgani *et al.*, 2009
Topoisomarase IV	*parC*	CATCGTCTACGCCATGAG AGCAGCACCTCGGAATAG	267	Gorgani *et al.*, 2009

RESULTS AND DISCUSSION

Eighteen isolates were subjected to pulsed-field gel electrophoresis (PFGE) analysis with *Pac*I restriction digestion. The recognition sequence of *Pac*I restriction digestion was TTA_AT'TAA. Twenty-five distinct band sizes were observed upon gel electrophoresis. The banding patterns were converted to binary data and this data can be interpreted by SPSS software to form a dendrogram (Fig. 1).

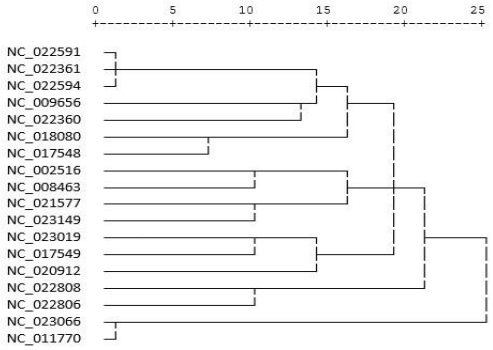

Fig. 1: Phylogenetic diversity of *Pseudomonas aeruginosa* identified by PFGE.

It was able to group the 18 isolates into 3 genotypes at 20% dissimilarity cutoff value. Genotype 1 was more prevalent (77.7%) (Fig. 2). Genotype 2 and genotype 3 were found to contain 11.11% of the isolates.

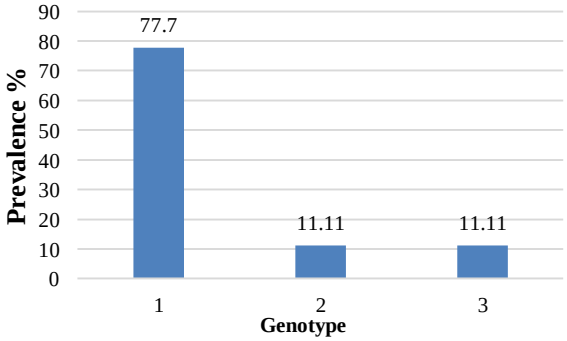

Fig. 2: Prevalence of genotypes.

Virulence factors of *Pseudomonas* spp. tightly regulated by cell to cell signaling systems was reported by Van Delden and Iglewski (1998). Total eighteen isolates were studied for virulence and antibiotic resistance genes. Out of eighteen isolates, 13 isolates had the L lipoprotein (*oprL*) with 504 bp gene product. de Vos *et al.* (1999), Masuda *et al.* (1995) and Nikaido (1994) previously described that *P. aeruginosa* contained outer membrane protein L (*oprL*) that is responsible for inherent resistance to antibiotics and antiseptics. This *oprL* gene is used as a marker for

rapid identification of *P. aeruginosa* species. According to previous studies (Pitt, 1998; Engel, 2003) collagen, C1q and C3 of the complement pathway, serum protease inhibitors, fibrin, fibrinogen, laminen and elastin are the substrates of alkaline protease, *apr*. Twelve isolates were found to possess *apr* gene and produce 1017 bp gene product. Hence the prevalence was 55.56%. Genotypic distributions were similar in genotype 2 and 3 (Fig. 3). All the isolates present in genotype 2 and 3 harboured *oprL* and *apr* genes (100%). Gene distribution patterns were different in genotype 1 and 64.29% and 57.14% present in genotype 1 carried *oprL* and *apr* genes, respectively.

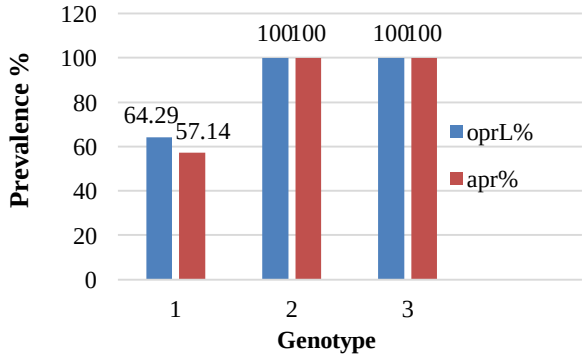

Fig. 3: Genotypic distribution of *oprL* and *apr* genes.

Sabharwal *et al.* (2014) identified that only *P. aeruginosa* contained exotoxin A (*toxA*) gene, whereas other species of this genus did not yield *toxA* gene. Out of the 18 isolates, 10 (55.56%) isolates had the exotoxin A genes (*toxA*) with an approximate length of 250 bp. Hamood (2004) reported that protein biosynthesis is inhibited by the action of exotoxin A (*toxA*). Nikbin *et al.* (2012) described that *toxA* gene was found in 90.7% *P. aeruginosa* isolated from burn, wound and pulmonary tract infections sample. According to Riese *et al.* (2002) and Anthonv *et al.* (2007), type-III secretion system directly secrete exoenzyme S (*exoS*) into the cytosol of human epithelial cell and other studies (Olson *et al.*, 1999; Yahr *et al.*, 1996) found that this inactivated cellular function, inhibited DNA synthesis and microvillus effacement. Nine isolates were found to possess exoenzyme S (*exoS*) and gave 504 bp gene product. So, the percentage of prevalence was 50. Recently, Thamir and Jubori (2014) described that *exoS* gene was present in higher number in samples collected from cystic fibrosis patients, wound, urine and blood than from sputum and bronchial washer. The 283 bp gene product of *lasB* gene was found in nine isolates. Hence the prevalence was 50%. Sabharwal *et al.* (2014) reported that elastin, collagen, fibronectin and laminin are substrates of T2SS secreted proteolytic enzyme, *lasB* gene. Elastase, *lasB* gene showed elastolytic activity on human lung tissue and was reported by Lomholt *et al.* (2001). Phospholipase C (*plcH*) hydrolyzing phospholipid was reported by Sabharwal *et al.* (2014). The *plcH* gene produces 307 bp gene product and 13 isolates (72.22%) were found to express *plcH* genes. Proinflammatory activities and pulmonary inflammation caused by phospholipase C (*plcH*) was reported by Wieland *et al.* (2002). Terada *et al.* (1999) examined that oxidative burst of neutrophils is also inhibited by phospholipase C (*plcH*). Genotypic distribution of exotoxin A (*toxA*), exoenzyme S (*exoS*), elastase (*lasB*) and phospholipase C (*plcH*) gene was similar in genotype 2 and 3 (Fig. 4). All the isolates present in genotype 2 and 3 harboured these four genes (100%). Low prevalence of *exoS* and *lasB* gene were encountered in genotype 1 (35.71%) while 42.86% and 64.29% isolates present in genotype 1 contained *toxA* and *plcH* genes, respectively.

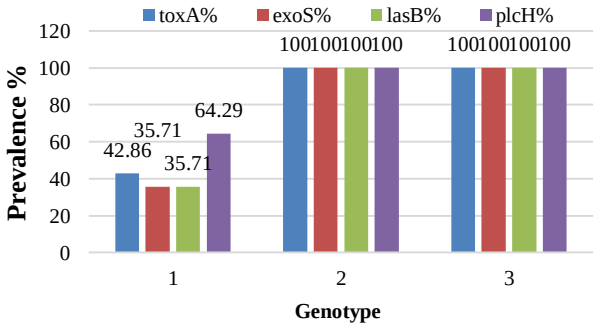

Fig. 4: Genotypic distribution of *toxA*, *exoS*, *lasB* and *plcH* genes.

Isolates were also screened for rhamnolipid AB (*rhlAB*), alginate (*algD*) and putative neuraminidase (*nan2*). Isolates those harboured *plcH* genes were also found to be positive for rhamnolipid AB (*rhlAB*) gene. The *rhlAB* gene produces 151 bp gene product. Van and Iglewski (1998) reported that phospholipids of lung surfactant are solubilized by rhamnolipid and this solubilized phospholipid is more easily cleaved by phospholipid C (*plcH*). Thirteen isolates were found to possess alginate (*algD*) genes and produced 1311 bp gene product. Hence the prevalence was 72.22%. Govan and Deretic (1996) described that chronic pulmonary inflammation is caused by alginate (*algD*) gene. It also helps to escape host immune surveillance and provide protection from antibiotics. Putative neuraminidase (*nan2*) was found in 72.22% of the isolates with 1161 bp gene product. Daniel *et al.* (2007) reported that spreading of *P. aeruginosa* within the host cells is mediated by neuraminidase gene. Neuraminidase also required for implantation of the bacterium was described by Cacalano *et al.* (1992) and Davies *et al.* (1999). Genotypic distribution of *rhlAB*, *algD* and *nan2* gene was similar in all genotypes (Fig. 5). All the isolates present in genotype 2 and 3 carried these three genes (100%) while 64.29% isolates present in genotype 1 expressed all these three genes.

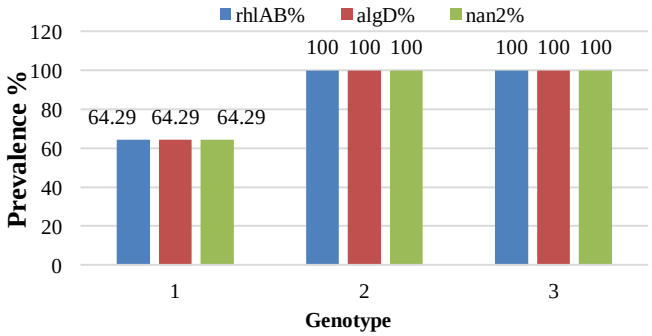

Fig. 5: Genotypic distribution of *rhlAB*, *algD* and *nan2* genes.

Antibiotic resistance is now a global concern. Isolates were tested for the streptomycin resistance genes. The *aph(6)-1d* gene produces 560 bp gene product and was present in *Pseudomonas aeruginosa* PA7. *P. aeruginosa* PA7 isolate was also found to express 515 bp gene product for *aph(3")* gene. Only *P. aeruginosa* NCGM2.S1 isolate had the *ant(3")* gene with 294 bp gene product. So, isolate *P. aeruginosa* PA7 hariboured both *aph (3")* and *aph (6)-1d* genes. Adesoji *et al.* (2015) also reported about a *Pseudomonas* bacterium that carried more than one streptomycin resistance genes. These isolates had the aminoglycoside modifying enzyme. Llano-Sotelo *et al.* (2002) reported that antibiotic molecule is modified by AMEs enzyme that linked antibiotic molecule with phosphate, adenyl or acetyl radical. Thus, modified antibiotics could not bind with its target. Aminoglycoside modifying enzyme (AME) genes which includes *aac(6')-I* and *ant(2")-I*, were found in 50 and 45% of *Pseudomonas* isolates, respectively as reported by Odumosu *et al.* (2003). Seveno *et al.* (2002) examined that streptomycin resistance is high in clinical and agricultural habitats. Genotype 2 and 3 carried no streptomycin resistance gene (Fig. 6). Gene distribution pattern was similar in genotype 1 and 14% isolates present in genotype 1 harboured these three genes.

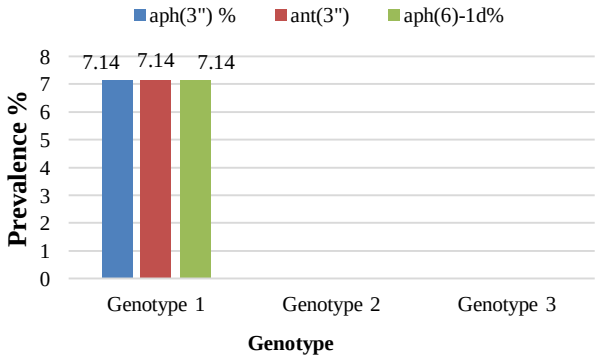

Fig. 6: Genotypic distribution of streptomycin resistance genes.

Sulfonamide resistance gene, *sul1* was present in 2 isolates (11.11%) (*P. aeruginosa* NCGM2.S1, *P. aeruginosa*PA7) with an amplicon of 433 bp. No isolate harboured the *sul2* and *sul3* gene. Adesoji *et al.* (2015) also found sulfonamide resistance gene *sul1* (31.8%) and no *sul3* genes. But their study found *sul2* gene (27.3%). Only *P. aeruginosa* NCGM2.S1 had the bla_{TEM} gene with 425 bp gene product. The bla_{SHV} gene was not present in any one of the isolates. Adesoji *et al.* (2015) found that among the beta lactamase gene, bla_{TEM} gene was more prevalent (40.9%) than bla_{SHV} (27.3%). Bradford (2001) reported that enterobacteriaceae mainly harboured the enzyme, bla_{TEM} and $bla_{SHV.}$ However, *Pseudomonas* harboured the bla_{TEM} and bla_{SHV} gene possibly due to the overuse of beta lactam antibiotics. Adesoji *et al.* (2015) examined that tetracycline resistance of *tet(A)*, *tet(B)*, and *tet(E)* gene is acquired by efflux pump system and for *tet(O)* and *tet(M)* gene, resistance mechanism is developed by ribosomal protection. No isolate was found that contained tetracycline resistance *tet(A)*, *tet(B)* or *tet(M)* gene. Zhang *et al.* (2009) stated that various environmental genera carried the broad host range tetracycline resistance gene *tet(A)*. Shababi *et al.* (2011) studies found that the prevalence of *tet(B)* gene was higher than *tet(A)*gene of *P aeruginosa* samples isolated from municipal sewage. Genotype 2 and 3 contained no sulfonamide, *sul1* or beta lactamase bla_{TEM} resistance genes (Fig. 7). The *sul1* and bla_{TEM}genes were present in 14.29% and 7.14% isolates in genotype 1.

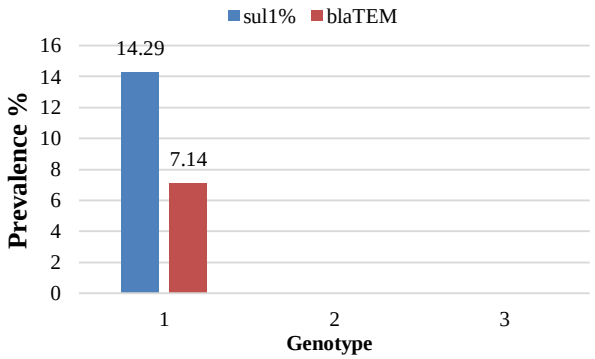

Fig. 7: Genotypic distribution of *sul1* and bla_{TEM} genes.

Fazeli and Momtaz (2014) demonstrated that multiplex PCR assays were used to detect fluoroquinolone resistance-determining region of the *gyrA* and *parC* gene. Fourteen isolates were found to express *gyrA* genes with an approximate length of 287 bp. So, the percentage of prevalence was 77.78. Strateva and Yordanov (2009) described that *gyrA* gene is modified within the enzyme's active site, quinolone-resistant-determinative region by a point mutation and changed the amino acid sequence of A and B subunits. This alteration created low affinity quinolone molecules. PCR amplification of *parC* gene produces 267 bp gene product. Thirteen isolates (72.22%) had the *parC* genes. All the isolates present in genotype 2 and 3 carried *gyrA* and *parC* genes (100%) (Fig. 8). Genotype 1 also harboured *gyrA* and *parC* genes and 71.43% and 64.3% isolates present in genotype 1 expressed *gyrA* and *parC* genes, respectively.

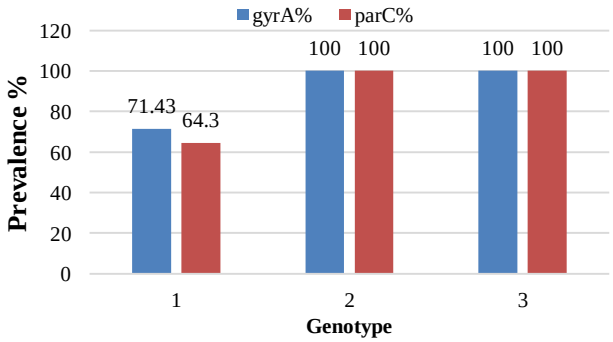

Fig. 8: Genotypic distribution of *gyrA* and *parC* genes.

Class 1 integrase, *int1* was found in only isolate *Pseudomonas aeruginosa* NCGM2.S1. No isolate was found to harbour class 2 integrase, *int2*. Mazel (2006), Rowe-Magnus and Mazek (2002) studied class 1 integrase and described that class 1 integrase facilitate the ability to shuttle antibiotic resistance genes

between different bacterial species. Isolates *P. aeruginosa* NCGM2.S1 contained class 1 integron gene and is considered as a multidrug resistant bacterium. Variable region of class 1 integron was found in one isolate (*P. aeruginosa* NCGM2.S1). Adesoji *et al.* (2015) concluded that the variable region of class 1 integrase helps to insert antibiotic resistant cassette in the integrase sequence. Class 1 integrase and variable region of class 1 integron genes were absent in genotype 2 and 3 (Fig. 9). Genotype 1 harboured similar number of class 1 integrase and variable region of class 1 integron gene (7.14%).

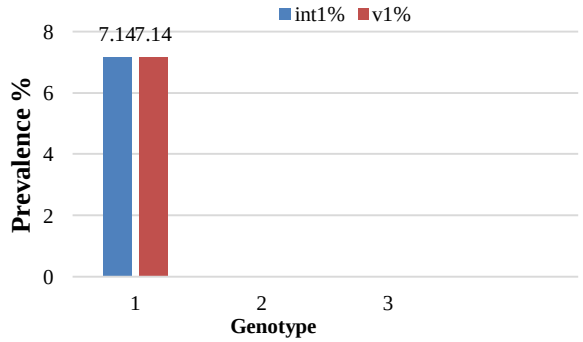

Fig. 9: Genotypic distribution of class 1 integrase and variable region of class 1 integron.

CONCLUSION

Identification of L lipoprotein, *oprL* precisely and rapidly identify *P. aeruginosa* species. Gene distribution pattern of virulence and antibiotic resistance genes within genotypes were almost similar. Different virulent genes are identified that may contribute to virulence and pathogenicity of the infections. The data has provided epidemiological information to study the characteristics of the *P. aeruginosa* and also the virulence factors associated with infections. *Pseudomonas* harbouring class 1 integron spread the antibiotic resistance gene in the environment which is now a global concern. This multidrug resistant *Pseudomonas* may contribute to many emergence and re-emergence infectious diseases. Finally clinical studies are needed to control multidrug-resistant *Pseudomonas* and maximize the clinical outcomes in the treatment of infectious diseases.

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Conflict of Interests: There are no conflicts of interest.

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