ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 10 (1): 70-77, 2011
ISSN 1682-296X / DOI: 10.3923/biotech.2011.70.77

Genetic Fingerprinting of *Pseudomonas aeruginosa* Involved in Nosocomial Infection as Revealed by RAPD-PCR Markers

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Abstract: Genetic fingerprinting of 30 *Pseudomonas aeruginosa* (*Pa*) isolates from three types of nosocomial infection cases from two Osun State Teaching Hospitals was compared using RAPD-PCR markers. Ten out of 50 operon primers tested showed polymorphism with reproducible results among the isolates and produced 131 bands of which 74 were polymorphic with sizes ranging between 200 and 3,000 bp. Cluster analysis using the 74 polymorphic markers classified the 30 *Pseudomonas aeruginosa* isolates into two (*PgA* and *PgB*) genetic groups. Comparing isolates proportion in each genotype based on their site of infection, antibiotics resistance pattern and geographical location, it was revealed that the proportion of urinary tract infection isolates in *PgA* genotype was significantly less than those in *PgB* genotype ($z = -1.195$, $p<0.05$) while the proportion of septicaemia isolates in *PgA* genotype was significantly higher than its proportion in *PgB* genotype ($z = 1.348$, $p<0.05$). However the proportion of wound infection isolates of *PgA* and *PgB* genotypes were significantly the same ($z = -0.278$, $p>0.05$). The *PgA* genotype contained few isolates with increased virulence and resistance to new antimicrobial modules and could possibly be new emerging *P. aeruginosa* strains from *PgB* genotype population. The study has critically revealed the genetic diversity and distribution among *P. aeruginosa* isolates in Osun State.

Key words: *Pseudomonas aeruginosa*, isolates, operon primers, polymorphism, genetic group, genotype, virulence, antibiotic resistance

INTRODUCTION

Pseudomonas aeruginosa (Pa) is a non sporulating, Gram negative motile bacterium. Its motility is ensured through a polar flagellum it possesses. It is an ubiquitous organism widely distributed in soil, water and on living hosts (Kiewitz and Tummler, 2000). Despite its ubiquitous nature, it is still regarded as an opportunistic pathogen because it does not cause colonization and infection except in immuno-compromised individuals (Kiewitz and Tummler, 2000). These immuno-compromised states range from simple or complex skin abrasion, abrasion of any other epithelial surface, to much more complex serological states of reduction in humoral immunity (Ruimy *et al*., 2001). Once colonization and infection are established, *P. aeruginosa* becomes one of the worst pathogens of humans. It is highly versatile in virulence and it is known to possess intrinsic multi-drug resistance capabilities (Agarwal *et al*., 2005). The various types of infection it causes include wound infections, pulmonary infections, urinary tract infections and infections in immuno-compromised patients with Acquired Immunodefficiency Syndrome (AIDS) and cancer (Kiewitz and Tummler, 2000). *Pseudomonas aeruginosa* is the second most common bacteria cause of nosocomial infections, accounting for 21% of cases. Incidences reported 16% of nosocomial pneumonia, 12% of urinary tract infections, 17-26% of wound infections and 10% of septicaemia are due to *P. aeruginosa* (Micek *et al*., 2005; Savafi *et al*., 2005). The reported attendant mortality rates are 30% in nosocomial pneumonia, 20% in septicaemia, 60% in burn unit, 50% in bacteria caused deaths in AIDS and it is also the leading cause of death in cystic fibrotic patients (Ruimy *et al*., 2001; Micek *et al*., 2005; Savafi *et al*., 2005; Van Delden and Iglewski, 1998). In its large genome of 6.3 million base pairs (bp) houses 8 virulence genes were identified. Moreover, the large genome size increases the probability of possible mutation sites and thus gives reasons for its virulence versatility, its growing multi drug resistance and the high mortality rate associated with its infection (Stover *et al*., 2000; Agarwal *et al*., 2005). *Pseudomonas aeruginosa* ever growing multi-drug resistance has also been widely reported

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(Agarwal *et al*., 2005). This cuts across the third and fourth generation cephalosporins, the generic flouroquinolones, the aminoglycosides and the advanced beta-lactam antibiotics (Micek *et al*., 2005). A high rate of spread of resistant gene has been suspected as the cause of increased antibiotic resistance cases in it. Plasmid carried genes could be spread by conjugation and transduction while the genome-based resistant genes are also spread by replication (Ramisse *et al*., 2000).

Several molecular studies have been carried out to investigate diversity amongst *P. aeruginosa* strains, polymorphism of certain of its genes and also genetic comparison of *P. aeruginosa* isolates from different hosts and environments (Kiewitz and Tummler, 2000; Martin *et al*., 1999; Ruimy *et al*., 2001; Onasanya *et al*., 2010). These were done to generate knowledge that would be useful in improving on the general management of infections due to *P. aeruginosa* (Onasanya *et al*., 2003). The molecular techniques widely used in these studies include random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), restriction fragment length polymorphism (RFLP) and multi-locus enzyme electrophoresis (MLEE) among others (Onasanya *et al*., 2003; Kiewitz and Tummler, 2000; Martin *et al*., 1999; Ruimy *et al*., 2001; Onasanya *et al*., 2010; Onasanya *et al*., 2007). Most of these studies reported high rate of polymorphism and genetic diversity among *P. aeruginosa* strains.

However, no report was found on the genetic study of *P. aeruginosa* in Nigeria. Besides, reports on genetic comparison of isolates from different infection types in man was scarce except for comparison of isolates from pneumonia with that from cancer patients and environmental water (Ruimy *et al*., 2001). Population studies of antibiotics resistance in *P. aeruginosa* isolates relative to their molecular diversity was also scarce internationally with no report of such from Nigeria. The objective of the study was to carryout *Pseudomonas aeruginosa* isolates genetic fingerprinting using RAPD-PCR markers as basis to differentiate *Pseudomonas aeruginosa* isolates from nosocomial septicaemia, urinary tract infections and wound infections from patients on admission at the two teaching hospitals (Obafemi Awolowo University Teaching Hospital Ile-Ife and Ladoke Akintola University of Technology Teaching Hospital Osogbo) in Osun State, Nigeria.

MATERIALS AND METHODS

Research location: Bacterial isolate propagation and RAPD-PCR analysis were carried out at Central Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. This study was conducted between February and July 2007.

***P. aeruginosa* isolate:** The *Pseudomonas aeruginosa* Pseudomonas aeruginosa isolates (Table 1) used in this study were obtained from the Obafemi Awolowo University Teaching Hospital Ile-Ife and Ladoke Akintola University of Technology Teaching Hospital Osogbo, Osun State, Nigeria where their identity had been confirmed by Gram staining technique and oxidase biochemical test as well as confirmation of antibiotic susceptibility test (Sivakumari and Shanthi, 2009; Masaadeh and Jaran, 2009). Isolates preservation and storage were performed in accordance with Onasanya *et al*. (2003).

Isolate propagation: *Pseudomonas aeruginosa* isolates, stored frozen in 50% glycerine, were removed, thawed and propagated using a modified procedure developed by Onasanya *et al*. (2003). Two hundred microliter of the *P. aeruginosa* isolate was transferred into 75 mL of Nutrient broth (pH 7.5) in a 250 mL conical flask and this was kept under constant shaking at 37°C for 24 h. The bacteria cells were removed by centrifugation, washed with 0.1 mM Tris EDTA pH 8.0, and kept at -20°C until DNA extraction.

Genomic DNA extraction: DNA extraction was done according to the methods of Onasanya *et al*. (2003) where 0.3 g of washed bacterial cell were suspended in 200 μL of CTAB buffer (50 mM Tris, pH 8.0; 0.7 mM NaCl; 10 mM EDTA; 2% hexadecyltrimethylammonium bromide; 0.1% 2-mercaptoethanol), followed by the addition of 100 μL of 20% sodium dodecyl sulfate and incubated at 65°C for 20 min. DNA was purified by two extractions with phenol:chloroform:isoamyl alcohol (24:25:1) and precipitated with -20°C absolute ethanol. After washing with 70% ethanol, the DNA was dried and resuspended in 200 μL of sterile distilled water. DNA concentration was measured using DU-65UV spectrophotometer (Beckman Instruments Inc., Fullerto CA, USA) at 260 nm. DNA degradation was checked by electrophoresis on a 1% agarose gel in TAE buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0).

RAPD-PCR analysis: RAPD-PCR analysis was carried out according to Onasanya *et al*. (2003). DNA primers tested were purchased from Operon Technologies (Alameda, California, USA) and each was 10 nucleotides long. Two concentrations of each DNA (24 and 96 ng per reaction) were used to test reproducibility and eliminate sporadic amplification products from the analysis. Thirty primers (OPA, OPJ,

Table 1: *Pseudomonas aeruginosa* isolates, site of infection, collection locality and sensitivity to antibiotics

			Antimicrobial Activity		
Isolate Code	Site of Infection	Locality	Ofloxacin	Imipenem	Gentamicin
Pa1	B	Ife	S	S	S
Pa2	B	Osogbo	S	S	S
Pa3	U	Ife	S	S	R
Pa4	U	Ife	S	S	S
Pa5	U	Ife	S	S	S
Pa6	W	Ife	R	S	R
Pa7	U	Ife	S	R	S
Pa8	U	Ife	S	S	S
Pa9	U	Osogbo	S	S	S
Pa10	B	Osogbo	S	S	S
Pa11	B	Osogbo	S	R	R
Pa12	B	Ife	S	S	S
Pa13	B	Osogbo	R	S	R
Pa14	W	Osogbo	S	R	R
Pa15	W	Ife	R	S	R
Pa16	W	Ife	S	S	S
Pa17	W	Ife	S	S	R
Pa18	U	Osogbo	S	S	S
Pa19	B	Osogbo	S	S	S
Pa20	B	Osogbo	S	S	S
Pa21	B	Ife	S	R	S
Pa22	U	Ife	S	S	S
Pa23	W	Osogbo	S	S	S
Pa24	B	Osogbo	S	S	S
Pa25	W	Osogbo	S	S	R
Pa26	W	Ife	S	S	S
Pa27	U	Ife	S	S	S
Pa28	W	Ife	S	S	R
Pa29	W	Ife	S	S	R
Pa30	U	Ife	S	S	S

Pa = *Pseudomonas aeruginosa*; B = Blood (for cases of septicaemia); W = Wound (wound infection cases), U = Urine (urinary tract infection); S = Sensitive and R = Resistant

Table 2: Oligonucleotide primers that showed genetic diversity among the *P. aeruginosa* isolate population using RAPD-PCR analysis

Operon primer code	Nucleotide sequence 5' - 3'	No. of fragment amplified	No. of polymorphic bands
OPJ-13	CCACACTACC	12	5
OPJ-18	TGGTCGCAGA	10	6
OPX-01	CTGGGCACGA	14	7
OPX-03	TGGCGCAGTG	13	8
OPX-07	GAGCGAGGCT	17	11
OPX-11	GGAGCCTCAG	12	6
OPX-13	ACGGGAGCAA	14	7
OPY-04	GGCTGCAATG	16	9
OPY-06	AAGGCTCACC	11	8
OPY-10	CAAACGTGGG	12	7
Total		131	74

OPX, OPY and OPW series) were screened with two isolates (Pa1 and Pa2) for their ability to amplify the *P. aerugionsa* isolate DNA. Ten of these primers (Table 2) were found useful since they gave polymorphism. These were used in amplifying the DNA from all *P. aeruginosa* isolates. Amplifications were performed in 25 μL reaction mixture consisting of genomic DNA, 1X reaction buffer (Promega), 100 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM Operon random primer, 2.5 μM $MgCl_2$ and 1U of Taq polymerase (Boehringer, Germany). A single primer was used in each reaction. The reaction mixture was overlaid with 50 μL of mineral oil to prevent evaporation. Amplification was performed in a thermowell microtiter plate (Costa Corporation) using a Perkin Elmer programmable Thermal Controller model 9600. The cycling program was (I) 1 cycle of 94°C for 3 min; (ii) 45 cycles of 94°C for 1 min for denaturation, 40°C for 1 min for annealing of primer and 72°C for 2 min for extension; and (iii) a final extension at 72°C for 7 min. The amplification products were resolved by electrophoresis in a 1.4% agarose gel using TAE buffer (45 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 2 h. A 1 kb ladder (Life Technologies, Gaithersburg, MD, USA) was included as molecular size marker. Gels were visualized by staining with ethidium bromide solution (0.5 μg mL^{-1}) and banding patterns were photographed over UV light using a red filter.

Phylogenetic analysis: Positions of unequivocally scorable RAPD bands were transformed into a binary character matrix ("1" for the presence and "0" for the absence of a band at a particular position). Pairwise distance matrices were compiled by the Numerical Taxonomy System (NTSYS) 2.0 software (Rohlf, 2000) using the Jaccard coefficient of similarity (Ivchenko and Honov, 1998). Phylogenetic tree was created by the unweighted pair-group method arithmetic (UPGMA) average cluster analysis (Sneath and Sokal, 1973; Jakó *et al*., 2009).

RESULTS

Genetic fingerprinting of 30 *Pseudomonas aeruginosa* isolates from three types of nosocomial infection cases from two Osun State Teaching Hospitals was compared using RAPD-PCR markers. Ten primers showed good level of polymorphism and reproducibility among the 30 arbitrary primers tested (Table 2). The amplification reaction with the 10 primers generated 131 bands and 74 of which were polymorphic (Table 2) with sizes ranging between 200 and 3,000 bp (Fig. 1). The 74 polymorphic RAPD markers were used to construct the phylogenetic relationship dendrogram among 30 *P. aeruginosa* isolates that classified the isolates into two major group genotypes (*PgA* and *PgB*) at 50% similarity (Fig. 2). The *PgA* genotype was further differentiated into two subgroups (*PgA1* and *PgA2*) at approximately 63% similarity while the *PgB* group was further differentiated into two subgroups (*PgB1* and *PgB2*) at 55% similarity. The *PgA* group comprised of 10 (33%) of the *P. aeruginosa* isolates analyzed while the remaining 20 (67%) formed the *PgB* group (Table 3).

Isolates from wound infections were well distributed across the two genotypes with 30% in *PgA* and 35% in *PgB* (Table 4). Although the *PgA* seemed to have a lower percentage of wound infection isolates, statistically with a Z value of -0.278 calculated by the comparison of difference in proportion the percentage of wound isolates in *PgA* group was not significantly different from those in *PgB* ($p>0.05$) (Table 5). Isolates from cases of septicaemia made up 50% in *PgA* group while 25% were in *PgB* group (Table 4). On testing the difference between the two proportions, the Z value of 1.348 obtained proved that the proportion of blood infection isolates in *PgA* was significantly higher than the proportion in *PgB* group ($p<0.05$) (Table 5). Isolates from urinary tract infection made up 20% in *PgA* and 40% in *PgB* (Table 4). The Z value of -1.195 from the statistical testing of difference in proportion further revealed that urinary tract infection isolates in *PgA* genotype were significantly less in proportion than those in *PgB* ($p<0.05$) (Table 5).

On geographical distribution of isolates in the groups, 4 of the 10 isolates in *PgA* group were from Osogbo based hospital while 8 out of 20 isolates in *PgB* were likewise from the Osogbo based hospital (Table 6). On the other hand, 6 out of 10 isolates in *PgA* group were from the Ile-Ife hospital while 12 of the 20 in *PgB* group were likewise from the Ile-Ife Hospital (Table 6). The *PgA* and *PgB* genotypes constitute the *P. aeruginosa* isolates population structure in both the Osogbo and Ile-Ife hospitals.

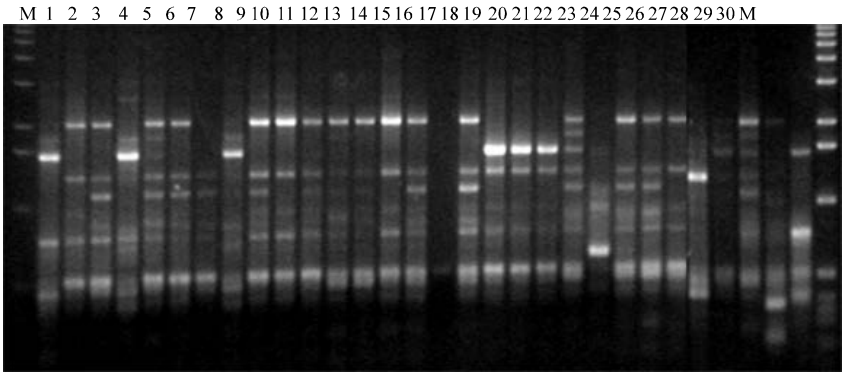

Fig. 1: DNA fingerprinting patterns of 30 *P. aeruginosa* isolates using OPY-04 RAPD primer. M=molecular size marker, 1-30 = *P. aeruginosa* isolates number

Table 3: *P. aeruginosa* isolate group genotype and percentage distribution

Groups	No. of Isolates	Percentage	Sub-group	No. of Isolates	Percentage
PgA	10	33.33	*PgA1*	7	23.33
			PgA2	3	9.99
PgB	20	66.67	*PgB1*	10	33.33
			PgB2	10	33.33

Table 4: Number of isolates and their percentage in each of the 3 types of infections studied categorized by phylogenetic group

		Blood		Wound		Urinary tract	
Group	No. of isolates	No. of isolates	Percentage	No. of isolates	Percentage	No. of isolates	Percentage
PgA	10	5	50	3	30	2	20
PgB	20	5	25	7	35	8	40

Table 5: "Z" value and level of significance calculated from the statistical comparison of the proportion of isolate of a particular site of infection belonging to each of the two different group genotypes

	Infection site		
Groups	Blood	Wound	Urinary tract
Percentage in *PgA*	50.0	30.0	20.0
Percentage in *PgB*	25.0	35.0	40.0
Z-Value (calculated)	01.348*	-0.278ns	-1.195*

*=significance at $p<0.05$; ns=not significant

Table 6: Number and percentage of isolates from each of the 2 locations of isolate collection belonging to each of the phylogenetic group

	Ile-Ife		Osogbo	
Groups	No. of isolates	Percentage	No. of isolates	Percentage
PgA	6	60	4	40
PgB	12	60	8	40
Total	18		12	

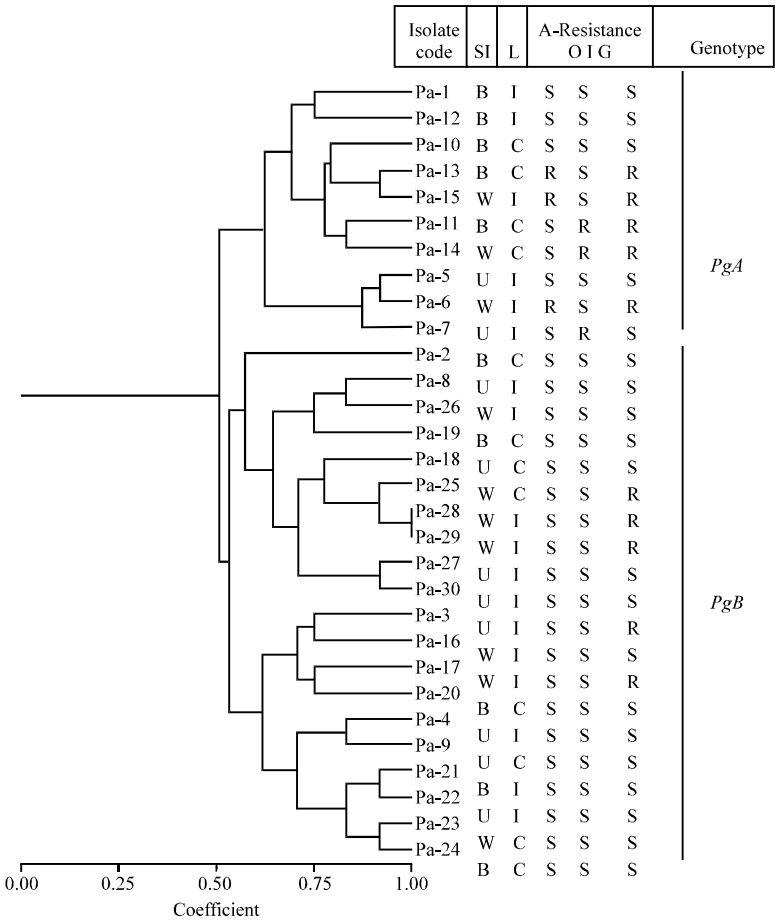

Fig. 2: Dendrogram showing genetic diversity among 30 *P. aeruginosa* isolates. SI = site of infection (B = blood; W = wound; U = urinary tract); L = locality (O = Osogbo; I = Ile-Ife); S = sensitive; R = resistant; A = antibiotics (O = ofloxacin; G = gentamicin; I = imipenem)

Table 7: Number and percentage of isolates resistant to a particular antibiotic in each of the group genotypes

	Ofloxacin		Imipenem		Gentamicin	
Groups	No. of isolates	Percentage	No. of isolates	Percentage	No. of isolates	Percentage
PgA	3	100	3	75	5	50
PgB	0	0	1	25	5	50
Total	3		4		10	

The status of the *PgA* and *PgB* genotypes relative to antibiotic sensitivity revealed that 3 *P. aeruginosa* isolates resistant to imipenem was in *PgB* group ofloxacin and imipenem were in *PgA* group while only one *P. aeruginosa* isolate resistant to(Table 7). However, resistant isolates to gentamicin were the same within the *PgA* and *PgB* groups (Table 7).

DISCUSSION

The use of 10 RAPD primers for *P. aeruginosa* (Pa) generated a total of 131 amplified fragments of between 200 to 3000 bp and 74 polymorphic bands, this was more than the number of fragments generated from *Staphylococcus aureus* and *Escherichia coli* while using the same number of RAPD primers (Shehata, 2008; Onasanya *et al*., 2003; Salehi *et al*., 2008). This agrees with the expectation from an organism with a much larger genome (Nazik *et al*., 2007). Also, the generation of 74 polymorphic bands shows that there is a high rate of polymorphism in *P. aeruginosa* genome and this is consistent with the actual possibility for a bacterium genome of length with 5,570 predicted open reading frames (Stover *et al*., 2000). High levels of polymorphism or hypermutation in the genome of *P. aeruginosa* had earlier been reported (Wiegand *et al*., 2008; Jelsbak *et al*., 2007).

From the phylogenetic tree, the isolates were grouped into two main genotypes (*PgA* and *PgB*) as early as at 50% similarity, while the two main groups were further separated into two subgroups at 60% similarity. This put to rest the fact that there is definitely a high rate of genetic recombination leading to a high level of genetic diversity among the *P. aeruginosa* isolates population (Perron *et al*., 2008). High level of genetic diversity in *P. aeruginosa* isolates population had also been reported as the basis of *P. aeruginosa* multi-drug resistance and high tenacity in environmental adaptability (Perron *et al*., 2008; Nazik *et al*., 2007; Mena *et al*., 2008).

The relationship between the isolates site of infection in the body with their genetic diversity shows that isolates grouped as *PgA* were predominant in septicaemia infection cases and those grouped as *PgB* were urinary tract infection predominant while both *PgA* and *PgB* shared isolates from wound infection cases. This reveals the fact that genetic differences in *P. aeruginosa* strains could affect the site at which the organism is predisposed to be virulent, suggesting possible relationship between genetic diversity of *P. aeruginosa* isolates and the type of environment theypreferentially survive (Perron *et al*., 2008; Mena *et al*., 2008; Onasanya *et al*., 2010). Besides, *P. aeruginosa* isolates in the *PgA* group were predominant in surviving in the most adverse of the three environments in the blood stream. This suggests that *PgA* could be the more virulent group. This would not be unconnected with their genetic makeup which possibly would have enhanced the expression of virulence factors necessary to survive despite the blood immune responses (Warner and Moldawer, 2008; Oldak and Trafny, 2005; Choy *et al*., 2008). On the other hand, the *PgB* group has a significantly larger proportion of urinary tract infection *P. aeruginosa* isolates. The urinary tract is also a special environment with high osmotic pressure, pH fluctuations and movement of fluid. Bacteria known as pathogens and flora of this terrain have been known to possess good ATPase membrane pumps and good adhesive properties, and *P. aeruginosa* is generally known to be rich in such properties (Yang *et al*., 2008; Davies *et al*., 1999; Govan and Deretic, 1996). The genetic diversity captioned in the *PgB* group must involve a good number of genes with expressions that ensure survival in that terrain (Rajan *et al*., 2010).

The percentage of isolates from Osogbo in both *PgA* and *PgB* groups are the same as well as those from Ile-Ife. This shows that there is equal geographical distribution of nosocomial infection population of *P. aeruginosa* across Ile-Ife and Osogbo, thus revealing the similar genetic diversity witnessed possibly due to common factors in the two geographical locations (Kayabas *et al*., 2008).

The *PgA* genotype contained few isolates with increased virulence and resistance to new antimicrobial modules and could possibly be new emerging *P. aeruginosa* strains from *PgB* genotype population. This shows that the genetic diversity in *PgA* genotype involves genes with antibiotic resistance activity. It is an established fact that all antibiotics resistant traits are coded for by genes on either the generic DNA or the plasmids (Baer *et al*., 2009; Bennett, 2008). Only 50% of the isolates with resistance to gentamicin formed the *PgA* group. This shows that the

preponderance of antibiotic resistance activity of the *PgA* population were for the new generic module antibiotics, imipenem and ofloxacin and not the older generation gentamicin (Meyer *et al*., 2007). That strongly suggests that the genetic diversity reflected in the *PgA* group are more recently developed than those in *PgB*, since evolutionary trend in antibiotics resistance has been known to be towards increased survival (Wiegand *et al*., 2008).

CONCLUSION

This study has demonstrated that there is high DNA polymorphism in the *P. aeruginosa* (Pa) isolates population involved in nosocomial infection in Osun State hospitals which is apparently due to high rate of genetic recombination. The high genetic diversity of *P. aeruginosa* strains also affects the site at which they preferably cause colonization and infection. The preponderance of more virulent and multi-drug resistant population in *PgA* genotype show that it could possibly be the most recently developed genotype in the population.

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