Genetic Fingerprinting of Pseudomonas aeruginosa Involved in Noscomial 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 Immune Deficiency 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 fluoroquinolones, 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 Tummier, 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 Tummier, 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 Ille-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 Ille-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% hexadecltrimethylammonium 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., Fullert 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, OPI,
OPX, OPY and OPW series) were screened with two isolates (Pa1 and Pa2) for their ability to amplify the *P. aeruginosa* 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 MgCl2, 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>
<thead>
<tr>
<th>Groups</th>
<th>No. of Isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgA</td>
<td>10</td>
<td>33.33</td>
</tr>
<tr>
<td>PgB</td>
<td>20</td>
<td>66.67</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sub-group</th>
<th>No. of Isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgA1</td>
<td>7</td>
<td>23.33</td>
</tr>
<tr>
<td>PgA2</td>
<td>3</td>
<td>9.99</td>
</tr>
<tr>
<td>PgB1</td>
<td>10</td>
<td>33.33</td>
</tr>
<tr>
<td>PgB2</td>
<td>10</td>
<td>33.33</td>
</tr>
</tbody>
</table>
Table 4: Number of isolates and their percentage in each of the 3 types of infections studied categorized by phylogenetic group

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood</th>
<th>Wound</th>
<th>Urinary tract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of isolates</td>
<td>No. of isolates</td>
<td>Percentage</td>
</tr>
<tr>
<td>PgA</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>PgB</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Infection site</th>
<th>Blood</th>
<th>Wound</th>
<th>Urinary tract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>Percentage in PgA</td>
<td>Percentage in PgB</td>
<td>Z-Value (calculated)</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>25.0</td>
<td>01.348*</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>35.0</td>
<td>-0.278ns</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td></td>
<td>-1.195*</td>
</tr>
</tbody>
</table>

*=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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ile-Ife</th>
<th>Osogbo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of isolates</td>
<td>Percentage</td>
</tr>
<tr>
<td>PgA</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>PgB</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

![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)](image-url)

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ofloxacin No. of isolates</th>
<th>Percentage</th>
<th>Imipenem No. of isolates</th>
<th>Percentage</th>
<th>Gentamicin No. of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgA</td>
<td>3</td>
<td>100</td>
<td>3</td>
<td>75</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>PgB</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>25</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>25</td>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

The status of the PgA and PgB genotypes relative to antibiotic sensitivity revealed that 3 P. aeruginosa isolates resistant to imipenem were 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 they preferentially 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 Trauft, 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 captured 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
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.

REFERENCES


