RANDOM AMPLIFIED POLYMORPHIC DNA TYPING OF MULTIDRUG-RESISTANT CLINICAL AND ENVIRONMENTAL Pseudomonas aeruginosa STRAINS FROM ABEOKUTA, NIGERIA.

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ABSTRACT

Pseudomonas aeruginosa, a multidrug-resistant organism is responsible for most opportunistic infections. Genetic relatedness between clinical and environmental strains has always been limited to hospital settings. This study utilized RAPD-PCR typing method to evaluate genetic relatedness between multidrug-resistant P. aeruginosa strains from diverse water samples (bottled water, tap water, sachet water, well water, hospital storage tank, and swimming pool water) and clinical strains (wound, blood, urine, eye and ear swab) collected from different locations in Abeokuta, Nigeria. Polymorphic DNA bands with sizes ranging between 250 and 3000bp were generated from both clinical and environmental strains. Within each population, both clinical and environmental strains were divided phylogenetically into two groups of Pc1 and Pc2 at 55% and Ps1 and Ps2 at 70% respectively. Genetic similarities between clinical and environmental strains yielded a total of 7 unique fingerprints. Cluster 2 (51.2%) had the largest number of strains in which strains from wound, blood and ear, clustered with strains from hospital storage tank, tap water, swimming pool water, sachet water and well water. These fingerprints proof genetic relatedness between clinical and environmental strains in Abeokuta, southwest Nigeria which is of public health significance, particularly, for immunocompromised individuals.

KEY WORDS: Pseudomonas aeruginosa; multidrug resistance; genetic relatedness; water quality; RAPD-PCR.

INTRODUCTION

Pseudomonas aeruginosa, a gram-negative rod, ubiquitous in nature (Finnan et al., 2004), accounts for significant proportion of nosocomial infections (Micek et al., 2005; Taheri et al., 2008). It causes different infections such as, urinary tract, respiratory system, soft tissue, bone and joint, gastrointestinal and a variety of systemic infections, dermatitis and bacteremia particularly in patients with severe burns, cancer and AIDS patients (Oni et al., 2002). Pseudomonas aeruginosa infection is significant particularly in immuno-compromised patients (Fichtenbaum et al., 1997; Asboe et al., 1998; Kiewitz and Tummler, 2000; Lang et al., 2004; Andualem, 2012) such as HIV/AIDS and cancer. It is an opportunistic bacterium responsible for chronic lung infection in cystic fibrosis patients, as well as the leading cause and death
Pseudomonas aeruginosa is found in moist environment including surface water, drinking water, distilled water, swimming pool and spas. Its ability to thrive in a broad range of environment is in parts caused by the fact that it possesses a large and diverse genome (Stover et al., 2000). The pathogenicity of P. aeruginosa is enhanced by production of both extracellular (cyanide, proteases) and cell-associated (pili, flagella, lipopolysachharide, Type III system effector proteins, type III secretion system and alginate) virulence factors (Finnan et al., 2004).

Environmental strains of P. aeruginosa can serve as the source for human infection (Wolfang et al., 2003). The role of tap water outlets and patients in hospital environment has been a subject of investigation by various research such as Cholley et al., 2008; Trautmann et al., 2009; Jiun-Ling et al., 2009; Salama et al., 2012 with serious implications of water contributing to nosocomial infections.

Studies on the origin and clonal diversity of P. aeruginosa have become very important because of increasing incidence of infections caused by this organism worldwide. Genetic relatedness between clinical and environmental Pseudomonas with the use of Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) has been proven to be highly discriminatory (Bogiel and Gospodarek, 2010; Deligianni, 2010; Mansefelid et al., 2010; Kidd et al., 2011; Maatallah et al., 2011; Fazeli et al., 2012; Gawish et al., 2013).

This study was therefore, designed to examine isolates of multidrug resistant P. aeruginosa from diverse clinical and environmental sources for genetic relatedness using RAPD-PCR and to determine if water is a significant source of clinical infections in Abeokuta, Nigeria.

MATERIALS AND METHODS

Source of bacterial culture
A total of 43 clinical P. aeruginosa strains from wound (16), ear swab (9), urine (9), blood (5), and eye swab (4) were obtained from two hospitals (Sacred Heart Hospital and Federal Medical Centre). A total of 41 environmental strains from bottled water (1), sachet water (5), tap water (6), well water (12), swimming pool (9) and hospital storage tanks (8) were isolated from diverse locations in Abeokuta, Nigeria. The isolation procedures have been described previously.

Genomic DNA Extraction
Genomic DNA was extracted as described by Abd-El-Haleem et al. (2003). Total bacterial DNA was prepared using the boiling approach. Bacterial cells were pelleted by centrifugation, resuspended in 50µL of TE buffer and then lysed by boiling for 10 min. The lysate was centrifuged and the supernatant was transferred to a new tube. The crude cell lysate was used directly for PCR.

RAPD-PCR Amplification
The genomic DNA was amplified with a RAPD-2 arbitrarily primer sequence forward 5’CCGTCAGCA 3’ and backward 3’GGCAGTCGT 5’. PCR was performed in 20µl of a reaction mixture containing DNA (200ng), a master mix containing (1 X PCR Buffer, 12.5Mm MgCl2, 1mM dNTP, 2.5 U of DNA Taq polymerase), 25µMol (each) primer, and sterile distilled water. Thermal cycling was conducted in Eppendorf vaporet (Germany thermal cycler). After an initial denaturation for 5min at 95°C, 40 amplification cycles were performed (30 s at...
95°C, 1 min at 28°C and 2 min at 72°C). The final cycle was extended at 72°C for 10 min. A reaction mixture containing sterile water was included as a negative control and a purified DNA mixture of the targeted bacteria was included as a positive control. The amplified PCR products were analysed by gel electrophoresis in 2% agarose gels stained with ethidium bromide, and then visualized and photographed using a photo documentation system under a short wave ultraviolet light trans illuminator.

**Phylogenetic Analysis of RAPD Fingerprints**
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 Dice coefficient of similarity. Phylogenetic relationship was created by the unweighted pair-group method arithmetic (UPGMA) average cluster analysis (Jako et al., 2009).

**RESULTS**

**Banding patterns of RAPD**
DNA banding pattern of various *P. aeruginosa* obtained from clinical and environmental sources shows molecular weight range between 250 and 3000 bp using RAPD-2 primer. Plate 1 shows the representative electrophoresis gel banding pattern.

**Phylogenetic analysis of multidrug resistant clinical *P. aeruginosa* strains**
Table 1 shows the phylogenetic analysis of clinical isolates of *P. aeruginosa* obtained at 55% similarity dividing strains into Pc1 and Pc2. Pc1 constitutes 9.3% while the remaining belonged to Pc2. Pc2 was subdivided into Pc2a (9.3%), Pc2b (11.6%), Pc2c (18.4%) and Pc2d (51.2%) at 70% similarity.

**Phylogenetic analysis of multidrug resistant environmental *P. aeruginosa* strains**
Figure 2 shows the dendogram while Table 2 shows the phylogenetic analysis of multidrug resistant environmental *P. aeruginosa*. Isolates were classified into two groups at 70% phylogenetic similarity, which consist of Ps1 (19.5%) and Ps2 (80.5%). Ps1 comprises of three sub-groups: Ps2a (53.7%), Ps2b (16.3%) and Ps2c (9.3%) at 75% genetic similarities.

Figure 3 and Table 3 show genetic polymorphism and sub-clonal diversity of *P. aeruginosa* obtained from clinical and water samples. All the isolates were grouped into seven clusters: cluster 1 (9.5%), cluster 2 (51.2%), cluster 3 (17.8%), cluster 4 (1.2%), cluster 5 (11.9%), cluster 6 (3.6%) and cluster 7 (4.8%).
Lane M: molecular marker: lane 34 to 43: environmental DNA; lane 44 to 65: clinical DNA
Plate 1: Banding patterns determined by RAPD showing the genetic relatedness of some multidrug-resistant clinical and environmental *Pseudomonas aeruginosa* strains.

Figure 1. Dendogram determined by RAPD showing the genetic relatedness of multidrug-resistant clinical *Pseudomonas aeruginosa* strains.
Table 1. Genetic relatedness of multidrug resistant clinical *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Clonal group</th>
<th>Clonal sub-groups percentage (%)</th>
<th>Wound</th>
<th>Blood</th>
<th>Ear swab</th>
<th>Urine</th>
<th>Eye swab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of isolates</td>
<td>Percentage (%)</td>
<td>No of isolates</td>
<td>Percentage (%)</td>
<td>No of isolates</td>
<td>Percentage (%)</td>
</tr>
<tr>
<td>Pc1</td>
<td>Pc1</td>
<td>9.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Pc2a</td>
<td>9.3</td>
<td>2</td>
<td>4.7</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Pc2b</td>
<td>11.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pc2c</td>
<td>18.4</td>
<td>4</td>
<td>9.3</td>
<td>2</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Pc2d</td>
<td>51.2</td>
<td>10</td>
<td>23.3</td>
<td>2</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>37.2</td>
<td>5</td>
<td>11.6</td>
<td>9</td>
</tr>
</tbody>
</table>

Figure 2. Dendogram estimated by RAPD-RAPD showing the genetic relatedness of multidrug-resistant environmental *Pseudomonas aeruginosa* strains.
### Table 2. Genetic relatedness of multidrug resistant environmental Pseudomonas aeruginosa

<table>
<thead>
<tr>
<th>Clonal</th>
<th>Sub-clonal Percentage (%)</th>
<th>Hospital storage tank No of isolates</th>
<th>Percentage</th>
<th>Tap water No of isolates</th>
<th>Percentage</th>
<th>Swimming pool No of isolates</th>
<th>Percentage</th>
<th>Bottled water No of isolates</th>
<th>Percentage</th>
<th>Well water No of isolates</th>
<th>Percentage</th>
<th>Sachet water No of isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps1</td>
<td>Ps1</td>
<td>19.5</td>
<td>2</td>
<td>4.9</td>
<td>2</td>
<td>4.9</td>
<td>1</td>
<td>2.4</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>Ps2</td>
<td>Ps2a</td>
<td>53.7</td>
<td>2</td>
<td>4.9</td>
<td>4</td>
<td>9.8</td>
<td>8</td>
<td>19.5</td>
<td>1</td>
<td>2.4</td>
<td>7</td>
<td>17.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ps2b</td>
<td>17.1</td>
<td>1</td>
<td>2.4</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>4</td>
<td>9.8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Ps2c</td>
<td>9.7</td>
<td>3</td>
<td>7.3</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>1</td>
<td>2.4</td>
<td>0</td>
</tr>
</tbody>
</table>

![Dendogram](image)

**Fig. 3.** Dendogram estimated by RAPD showing the genetic relatedness of multidrug-resistant clinical and environmental Pseudomonas aeruginosa strains.  
Key: C = Cluster group
Table 3. Genetic relatedness of multidrug resistant clinical and environmental *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Cluster groups</th>
<th>Wound</th>
<th>Blood</th>
<th>Ear swab</th>
<th>Urine</th>
<th>Eye swab</th>
<th>Hospital tank</th>
<th>Tap water</th>
<th>Swimming pool</th>
<th>Bottled Water</th>
<th>Well Water</th>
<th>Sachet Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1</td>
<td>8</td>
<td>0</td>
<td>0.0</td>
<td>1.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>2.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>C 2</td>
<td>43</td>
<td>11</td>
<td>13.1</td>
<td>4.8</td>
<td>3.6</td>
<td>2.4</td>
<td>4.8</td>
<td>4.8</td>
<td>0.0</td>
<td>0.0</td>
<td>8.0</td>
</tr>
<tr>
<td>C 3</td>
<td>15</td>
<td>3</td>
<td>3.6</td>
<td>0.0</td>
<td>2.4</td>
<td>2.4</td>
<td>0.0</td>
<td>2.4</td>
<td>0.0</td>
<td>4.8</td>
<td>1.2</td>
</tr>
<tr>
<td>C 4</td>
<td>1</td>
<td>1</td>
<td>1.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>C 5</td>
<td>10</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.2</td>
<td>3.6</td>
<td>1.2</td>
<td>2.4</td>
<td>2.4</td>
<td>1.2</td>
<td>0.0</td>
</tr>
<tr>
<td>C 6</td>
<td>3</td>
<td>1</td>
<td>1.2</td>
<td>1.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>C 7</td>
<td>4</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>4.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total Isolate</td>
<td>84</td>
<td>16</td>
<td>19.1</td>
<td>5.9</td>
<td>9.10</td>
<td>4.8</td>
<td>8.9</td>
<td>6.7</td>
<td>9.0</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Note: Percentage values are calculated based on the total number of isolates.*
DISCUSSION

Multidrug resistant *Pseudomonas aeruginosa* has been previously reported from both hospital and water environment from across the nation, Nigeria. This study investigated genetic relatedness between clinical and diverse environmental *P. aeruginosa* strains using RAPD-PCR typing method which has been proven to be highly discriminatory. A very high polymorphism with banding sizes ranging from 250-3000bp generated was used to construct the dendogram and phylogenetic relationship in order to infer clonality among strains. The polymorphism observed in this study is supported by the fact that *Pseudomonas aeruginosa* is said to exhibit an epidemic population structure with horizontal transfer of DNA resulting in high frequency of genomic islands (Morales-Espinosa et al., 2012).

The phylogenetic tree for clinical strains classified the strains into two major groups of Pc1 with 4 (9.3%) strains and Pc2 with 39 (81.7%) strains at 55% phylogenetic similarity expressing their high diversity. Pc1 comprised only strains from ear swab whereas Pc2 was further differentiated into four subgroups at approximately 65% similarity. Pc2a (9.3%), comprised strains from wound (2), blood (1), and ear (1) while Pc2b (11.6%), consists of strains from urine (3) and eye swab (2) only. Pc2c (18.4%), clustered strains from wound (4), blood (2), ear swab (1), and eye swab while Pc2d (51.2%) clustered strains from all clinical samples from wound (10), blood (2), ear swab (3), urine (5) and eye swab (2). Similar results of diversity by RAPD-PCR typing of clinical strains from Nigeria with unique fingerprints were observed by Akanji et al. (2011) on nosocomial infections and Smith et al. (2012) on wounds.

The dendogram for the environmental strains revealed two major clonal groups indicated as Ps1 and Ps2 at 70% genetic similarities. Out of 41 strains, only 8 (19.5%) belonged to Ps1 group while the remaining 33 (80.5%) belonged to Ps2, implying that all the environmental strains have relative genetic diversity. Though, Ps2 was further divided into sub-group (Ps2a, Ps2b and Ps2c) at 82% similarity, there is little diversity in their genetic polymorphism, showing that despite their different sources, they are more closely related than clinical strains. Strains from hospital storage tank spread across all the genotypes with 4.9% in Ps1 and 14.6% in Ps2 showing clonality.

In this study, the combined dendogram of both the clinical and environmental strains of *P. aeruginosa* classified them to seven different clusters. In cluster 1 (8 strains), strains from blood (1), clustered with hospital storage tank (2), well water (3) and sachet water (2). Cluster 2 with 43 (51.2%) strains had the largest number whereby wound (11), blood (3), and ear (3), clustered with strains from hospital storage tank (2), tap water (4), swimming pool (4), sachet water (3) and well water (8). It is notable that in cluster 3 with 15 (17.6%) strains, wound (3), ear (2), urine (2), clustered with strains from hospital storage (2), swimming pool (4), bottled water (1), and well water (1). From this result, it is observed that multidrug resistant *P. aeruginosa* from the environment is clonally related with clinical strains similar to Gad et al. (2007), Ndip et al. (2007), El-Bialy et al. (2008), Taheri et al. (2008), and Salimi et al. (2010).

Though, cluster 4 (1.2%) and cluster 7 (4.8%) had stand alone wound (1) and ear swab (4) strains respectively, while cluster 6 had 3 clinical strains only, each respectively from wound, blood and ear, the results re-
reflect the genetic uniqueness of these strains. This study revealed genetic relatedness between clinical and environmental multidrug-resistant *P. aeruginosa* by RAPD-PCR typing suggesting little variation in virulence, infection types and environmental survival. Gawish et al. (2013) observed that virulence genes in genome of *P. aeruginosa* strains regardless of their origin whether clinical or environmental were conserved, as detected by whole-genome DNA microarray, and this lead to the suggestion that the environmental strains possess the ability to cause human infections despite the low probability of encountering a human host. Similarly, Wolfang et al. (2003) studied clinical and environmental *P. aeruginosa* using a whole genome microarray, which revealed no correlation between genome content and infection type and concluded that the organism possesses a highly conserved genome that encodes genes important for survival in numerous environments which allows it to cause a variety of human infections. Finnan et al. (2004) observed that phylogenetically, isolates from cystic fibrosis patients and that of hospital environment, clustered together with one another on the *mdh* gene tree.

The finding from this study implies that environmental strains could contribute to clinical infections and that water could serve as a major route of dissemination of infections within hospitals and other community. This is significant for other types of *P. aeruginosa* infections that were not considered in this study such as diarrhea particularly for immunocompromised patients, because *P. aeruginosa* is also one of the opportunistic bacterium in HIV/AIDS patients with diarrhoea (Fichtenbaum et al., 1998; Osazuwa et al., 2011, Andualem, 2012). Also, in respiratory tract infections, once established, eradication of *P. aeruginosa* from the respiratory tract of HIV-seropositive individuals with advanced immunosuppression is problematic and a chronic infective state appears common (Asboe et al., 1998).

Prior to this study, there have been few studies on genetic relatedness of *Pseudomonas aeruginosa* from clinical infections in Nigeria. This is the first report on molecular typing of multidrug resistant clinical and environmental *P. aeruginosa* in Nigeria.

**CONCLUSION**

The fingerprints of *P. aeruginosa* provide genetic relatedness between clinical and environmental multidrug resistant strains which is of public health significance, particularly, in immunocompromised patients.

**RECOMMENDATIONS**

There is a need to evaluate virulence and resistance genes in environmental *P. aeruginosa* strains as well as evolutionary pathways between clinical and environmental strains. Also, in order to evaluate the full burden of multidrug resistant *P. aeruginosa* on immunocompromised individuals such as HIV in Nigeria, there is a need to investigate its co-infection in south western Nigeria.

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