Molecular Identification of Biofilm-forming *Pseudomonas aeruginosa* Isolates from Noncritical Surfaces of a Tertiary Healthcare Center in Abia State, Southeast Nigeria

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**INTRODUCTION**

*Pseudomonas aeruginosa*, albeit widespread in almost all habitats, is occasionally discovered in specialized reservoirs within the hospital environment, such as sinks. This organism's mode of transmission has not been fully explored, but it is evident that direct contact with contaminated surfaces and indirect contact routes, such as insufficient hand hygiene on the part of healthcare professionals, are potential routes of infection. *Pseudomonas aeruginosa* can survive in the environment for...
many days to six months on surfaces, which may contribute to cross-contamination in a healthcare setting [1–3]. Although airborne transmission of this organism has been proposed, scientific proof on the subject is lacking. Different biofilm morphologies can be generated depending on Pseudomonas aeruginosa strains and/or nutritional circumstances. For example, in glucose basal medium, the biofilm lifecycle cycle of Pseudomonas aeruginosa, PAO1, a derivative of the original Australian PAO isolate that is a standard isolate for research distributed worldwide to laboratories and strain collections, can be subdivided into five major phenotypic steps depicting the typical formation stages of microbial biofilms. These stages can be divided into five major phenotypic steps portraying the pretty standard formation phases of microbial biofilm. The process begins with the reversible adherence of planktonic bacteria to a suitable surface for growth, proceeded by the irreversible attachment of bacteria, which forms micro colonies in the EPS matrix. Bacterial micro-colonies increase gradually, and their confinements result in a more organized phenotype. Non-colonized gaps are then colonized by bacteria, which eventually cover the entire surface. Finally, bacteria are disseminated from the sessile structure and re-enter the planktonic stage, where they can spread and colonize new surfaces [4,5].

Pseudomonas aeruginosa produces at least three polysaccharides (alginate, Pel, and PsI) that are important for the biofilm structural stability [6]. Pseudomonas aeruginosa mucoid and non-mucoid strains differ in the qualitative composition of their polysaccharides in the biofilm matrix, which is primarily alginate or PsI/Pel, respectively [7]. Alginate, a linear unbranched polymer consisting of D-mannuronic acid and L-glucuronic acid, contributes to biofilm structural stability and protection, as well as water and nutrient retention [8]. Pel polysaccharide is mostly a glucose-rich matrix material with an unknown composition [9], whereas PsI is a repeating pentasaccharide composed of D-mannose, L-rhamnose, and D-glucose [10].

Pel and PsI are engaged in the early phase of biofilm formation and can act as a major structural scaffold for biofilm development [7]. Extracellular DNA (eDNA) is a critical functional component of the Pseudomonas aeruginosa biofilm matrix. Its involvement in Biofilm formation is very significant, for instance;

(i) DNase inhibits Pseudomonas aeruginosa biofilm formation [11];
(ii) eDNA-deficient biofilms are more sensitive to some detergents [12];
(iii) eDNA facilitates twitching motility-mediated biofilm widening by preserving coherent cell alignments [13];
(iv) eDNA has been proposed to play an important role in the initial and early development of Pseudomonas aeruginosa biofilm.
(v) Finally, during hunger, eDNA serves as a nutrition source for bacteria [15].

Pseudomonas aeruginosa extracellular appendages such as flagella, type IV pili, and cup fimbriae, which are also called matrix components, have sticky functions in cell-to-surface interactions (irreversible attachment) as well as microcolony development in biofilms. When compared to wild-type strains, mutants faulty in flagella-mediated movement and mutants defective in the synthesis of the polar-localized type IV pili do not produce microcolonies [16]. The primary goal of this research is to identify the molecular characteristics of Biofilm forming Pseudomonas aeruginosa identified from non-critical hospital surfaces.

MATERIALS AND METHODS

The Tertiary Health facility where the study was carried out is 800 beds capacity Federal Medical Center, Umuahia, Abia State, Southeast, Nigeria after obtaining approval from the Research and Ethics committee of the Hospital. Samples were collected using the swab technique from different noncritical surfaces surrounding hospitalized patients. Sampling was carried out in six busy wards of the hospital. Sample collection was repeated three (3) times per critical surface. One hundred and thirty-nine (139) isolates of Pseudomonas species on noncritical surfaces in hospital wards were isolated using the swab technique from 1314 samples. These non-critical surfaces include tap heads, sinks, bed rails, tabletop and medical equipment like sphygmomanometer and Thermometer. Bacterial analysis was performed by using conventional microbiological techniques, routine biochemical tests and the Microbact 24E assay. Developed biofilms were visualized with crystal violet staining method and then estimated by measuring the optical density through spectrophotometer [17].

Motility and Preliminary biochemical tests techniques

Motility is the main physiological character of interest here and was determined by the hanging drop method, while preliminary biochemical tests like Sugar reaction, mainly oxidation of glucose, were determined by phenol red broth base (10g/L peptone, 1g/L yeast extract, 5g/L sodium chloride, 0.018g/L phenol red, with the addition of filter-sterilized sugar solution at a final concentration of 0.5%). The reaction time was within 24 hours at an optimum temperature of 37°C. Other biochemical tests like the methyl-red test were performed by the addition of an indicator (0.1g methyl red test per 300ml 95% ethanol) to cultures grown for 48h in 4ml of MR-VP broth. Voges Proskauer test was performed by the addition of 40% potassium hydroxide in water and 5% l-naphthol in 95% ethanol to cultures grown for 24h in MR-VP broth. Indole production test was determined by Kovacs reagents (5g p-dimethylamino benzaldehyde, 25ml HCL and 75ml sulphanilamide pentanol-1-ol). The nitrate reduction test was carried out by the addition of 1% of 1M HCL and 0.02% N-1 naphthlene diamine HCL in water to nitrate test broths [18].

Microbact 24E biochemical identification assay for Pseudomonas spp.

All Pseudomonas isolates identified by conventional biochemical tests were again subjected to the Microbact test [19] in line with the manufacturer’s instruction for confirmation. The Microbact 24E system (Fig. 1) is a miniaturize pH dependent and substrate utilization identification test system for Enterobacteriaceae and other miscellaneous Gram-negative bacilli.

Crystal Violet Assay

Biofilm forming Pseudomonas aeruginosa was confirmed using the Crystal Violet Assay technique [19]. Crystal violet Assay is a staining method (Fig. 2) which helps in the quantitation of biofilm mass though, does not give information on biofilm viability as it stains both the bacteria cells and the extracellular matrix [20].

Molecular Identification

Conventional primed Polymerase Chain Reaction (PCR) was used for the molecular identification of biofilm-forming Pseudomonas aeruginosa isolates for confirmation of their true identity according to Spike et al. [21]. Steps involved in molecular characterization of biofilm-forming Pseudomonas aeruginosa by Polymerase Chain Reaction include DNA extraction, PCR amplification, Gel Electrophoresis and sequencing.
DNA extraction
Biofilm-forming *Pseudomonas aeruginosa* isolates were sub-cultured and grown in Mueller Hinton Broth (MHB) at 37°C for 24h. Then 1ml of the liquid culture was transferred into a 1.5 ml volume microfuge tube. Bacteria cells were harvested by centrifugation at 12,000 rpm for 5 min. The supernatant was discarded, and the pellet was washed twice with ultrapure water and re-suspended in 1 ml ultrapure water. DNA extraction was carried out (ethanol precipitation method) by adding 0.1 vols. 3M Sodium acetate 2.5-vols. ice cold 100% ethanol, this is vortexed to mix thoroughly, and precipitated at -20°C for 1 hour. After centrifugation, the clear supernatant containing the DNA was transferred to a new microfuge tube and used directly in a specific Polymerase Chain Reaction amplification step for the determination of the Genus and Specie of the isolate.

Amplifications and cycling.
PCR amplifications were performed on a thermocycler (A&Ep Laboratories, UK Model Cyl-005). The reaction volume was 25µl and consisted of 10X PCR buffer, 25 mM MgCl2, 10 mM dNTP’s mixture, 5 U/µl of Taq DNA polymerase with 0.5 µg/ml of ethidium-bromide for 45 min and de stained EDTA, pH 8.3) performed at 70 V for 1 hour. Gels were stained according to manufacturer's instruction.

Amplified products (10 µl) were separated using 1.5 % agarose Gel Electrophoresis. Amplifications were performed for 35 cycles of 96°C for 1 min, 10 s annealing, 72°C for 1 min. Amplified products were separated and detected on an ABI 3730xL genetic analyzer (Clinitx, Japan). In the study, a major band matching to the expected band size in comparison to the DNA mass ladder was taken into account.

**Sequencing**
The amplified product was then purified using MinElute UF plates (Qiagen) following the manufacturer's protocol before being used in a sequencing reaction. Sequencing was carried out on each DNA strand with Big-Dye Terminator Ready Reaction Mix v3.1 (PE Bio-systems, Foster City, US) under the following conditions: initial denaturation at 96°C for 1 min, 30 cycles of 10 sec at 96°C, 5 sec at 51°C and 2 min at 60°C. Unincorporated dye terminators were removed by precipitation with 95% alcohol. The reaction products were separated and detected on an ABI PRISM genetic analyzer 3100 (PE Bio-systems) using a standard sequencing module with a Performance Optimized Polymer and 5 cm array. The universal Primer pair used for sequence analysis was 27F-1492R primers [22]. The sequencing protocol optimized using this universal primer helps reduce the reaction turn-around time (TAT) and very cost-effective.

**Molecular Identification of biofilm-forming Pseudomonas aeruginosa**
Molecular identification of biofilm-forming *Pseudomonas aeruginosa* isolates was carried out using conventional primed polymerase chain reaction (PCR). Bands that were clearly in alignment with the molecular mass ladder were subsequently chosen as positive for either genus or species of isolates, having separated during gel electrophoresis to the same position of the ladder. PCR products were digested before sequencing using protease. Of the eighty-three (83) samples of Biofilm forming *Pseudomonas aeruginosa*, isolates identified from Microbatch 24E biochemical and crystal violet assay, Forty (40) samples proceeded to Sequencing after conventional PCR Analysis. Sequence analysis identified two groups of biofilms forming *Pseudomonas aeruginosa* isolates with the following nucleotide sequence (Fig. 3) compared to the control strain obtained from gene library.

**RESULTS**
The majority of *Pseudomonas* species isolated in this study exhibited characteristic biochemical characters as confirmed by Microbact biochemical identification assay (Table 1). Out of 139 isolates of *Pseudomonas* spp. 115(82.73%) were identified as biofilm formers among biofilm forming bacteria (Tables 2 and 3). Of this number of biofilm formers, *Pseudomonas* spp. discovered from noncritical surfaces classified further as 83 (72.17%) belonged to *Pseudomonas aeruginosa*, 30 (22.09%) *Pseudomonas fluorescens*, 20 (17.39%) belong to *Pseudomonas putida and* 6 (5.22%) belong to *Pseudomonas chlororaphis* (Table 4). The crystal violet assay technique was suitable for quantitation of *Pseudomonas aeruginosa* biofilm formation as it is suitable for both motile and non-motile bacterial isolates [23]. The former adheres to both the bottom and walls of the reaction wells while the latter can only be found at the bottom of the well thus making it easy for biofilm identification and quantitation (Fig. 2).

**Table 1. Motility and biochemical identification reactions of diverse bacterial species in Hospital environment.**

| Sample Code | Colonial characteristics | Gram reaction | Oxidase | Urease | Coagulase | Neutral sugar utilization | Acid formation | Outdoor motility | Indoor motility | Probable organism |
|-------------|--------------------------|---------------|---------|--------|-----------|--------------------------|----------------|-----------------|-----------------|-----------------|
| SE, IE, MU   | Gram-positive cocci in chains | + + + + + + + + + + + | G G G | + + + + + + + + + + + | + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | S. pneumoniae |
| OE, SE      | Slightly raised edge colony, 1-2 mm in diameter | Gram-positive cocci in clusters | + + + + + + + + + + + | G G G | + + + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | S. aureus |
| WFE, SE     | Convex, smooth and white colony with entire edge | Gram-positive cocci in Clusters | + + + + + + + + + + + | G G G | + + + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | S. aureus |
| EE, IE       | Smooth, high colony with irregular edge | Gram-negative rods | A A A | + + + + + + + + + + + | + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | Pseudomonas spp. |
| IE, WFE      | Swarming, flat colony with fishing odour | Gram-negative rods | G G G | + + + + + + + + + + + | + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | Ag Proteus spp. |
| SE, QE      | Mucoid, colony, 1-4 mm in diameter | Gram-negative rod | A A A | + + + + + + + + + + + | + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | E. coli |
| PW, RT      | Moderate sized smooth and gummy colony | Gram-negative rod | A A A | + + + + + + + + + + + | + + + + + | + + + + + + + + + | + + + + + + + + + | + + + + + + + + + | K. pneumoniae |

Note: + Acid, G-Gal, (Positive), - (Negative) CoNStaph = Coagulase negative Staphylococcus
Table 2. Classification of Biofilm forming bacterial isolates.

| S/N | Bacterial Isolates     | Total No of Isolates | Total No of Isolated Formed Biofilm | % Biofilm occurrence in each isolate |
|-----|------------------------|----------------------|------------------------------------|-------------------------------------|
| 1   | Staphylococcus aureus  | 79                   | 13                                 | 16.46                               |
| 2   | Pseudomonas spp.       | 139                  | 115                                | 82.73                               |
| 3   | Escherichia Coli       | 117                  | 21                                 | 17.94                               |
| 4   | Klebsiella pneumonia   | 27                   | 6                                  | 22.22                               |
| 5   | Streptococcus pneumonia| 42                   | 4                                  | 9.52                                |
| 6   | Proteus spp.           | 20                   | 3                                  | 15.00                               |
| 7   | Coagulase negative     | 26                   | 1                                  | 3.70                                |

Table 3. Microbact 24E biochemical identification assay of Pseudomonas spp. isolates.

| Test       | Total Sample collected | P aeruginosa | P fluorescens | P putida | P chlorophis | No of probable organism |
|------------|------------------------|-------------|---------------|----------|--------------|-------------------------|
| Oxidase    |                        |             |               |          |              | 83                      |
| Motility   |                        | +           | +             | +        |              | 30                      |
| Nitrate    |                        | -           | -             | -        |              | 21                      |
| Lys        |                        | -           | -             | -        |              | 27                      |
| Orn        |                        | -           | -             | -        |              | 24                      |
| H2S        |                        | -           | -             | -        |              | 32                      |
| Glu        |                        | +           | -             | -        |              | 20                      |
| Mann       |                        | -           | -             | -        |              | 33                      |
| Xyl        |                        | -           | -             | -        |              | 23                      |
| ONPG       |                        | -           | -             | -        |              | 21                      |
| Ind        |                        | -           | -             | -        |              | 18                      |
| Ure        |                        | -           | -             | -        |              | 17                      |
| V-P        |                        | -           | +             | -        |              | 16                      |
| Cit        |                        | -           | +             | -        |              | 15                      |
| TDA        |                        | -           | +             | -        |              | 15                      |
| Gel        |                        | -           | -             | -        |              | 14                      |
| Mal        |                        | -           | -             | -        |              | 13                      |
| Ino        |                        | -           | +             | -        |              | 12                      |
| Soerb      |                        | -           | -             | -        |              | 12                      |
| Succ       |                        | -           | -             | -        |              | 11                      |
| Lac        |                        | -           | -             | -        |              | 10                      |
| Ara        |                        | -           | -             | -        |              | 9                       |
| Raff       |                        | -           | -             | -        |              | 8                       |
| Sal        |                        | -           | -             | -        |              | 7                       |
| Arg        |                        | +           | -             | -        |              | 6                       |

Table 4. Distribution of Pseudomonas spp. Isolates in Different Hospital Wards.

| Wards | Sample collected | P aeruginosa | P fluorescens | P putida | P chlorophis | No of isolates |
|-------|------------------|-------------|---------------|----------|--------------|----------------|
| Accident & Emergency Paediatrics | 314 | 21(68.00%) | 7(22.00%) | 4(1.34%) | 3(0.97%) | 35(11.75%) |
| Intensive Care | 193 | 11(68.73%) | 4(21.37%) | 1(0.53%) | 0(0.00%) | 23(11.75%) |
| Obst & Gynaec | 207 | 16(69.57%) | 5(17.37%) | 4(1.55%) | 2(0.87%) | 23(11.75%) |
| Surgical Ward | 213 | 11(52.04%) | 6(28.75%) | 6(2.81%) | 1(0.47%) | 13(6.10%) |
| Internal Medicine | 207 | 15(62.50%) | 5(20.00%) | 5(2.45%) | 2(0.97%) | 14(6.71%) |
| Total | 1314 | 83 | 30 | 20 | 6 | 139 |

Fig. 1. Reaction well for Microbact assay (Oxoid, England).

Fig. 2. Crystal violet assay indicating quantification of biofilms forming bacterial isolates. KEY: A= Strong Biofilms (OD=0.4± 0.03); B =Moderate Biofilms (0.2± 0.01); C= Weak Biofilms (0.1±0.001); D= No Biofilms (0.001 ± 0.004).

Fig. 3. Sequence lines of biofilm forming Pseudomonas aeruginosa isolates.
DISCUSSION

In this study, a high degree of reproducibility was achieved with conventional PCR for the confirmation of the identity of *Pseudomonas aeruginosa* isolates. The PCR showed a high degree of genomic similarity among the Biofilm forming *Pseudomonas aeruginosa* isolates in conformity with the research hypothesis that strain similarity exists among *Pseudomonas aeruginosa* isolates circulating within the hospital environment being studied. The similarity in the banding pattern of the isolates could be attributed to genetic convergence due to cross infections of the circulating biofilm forming *Pseudomonas aeruginosa* within this hospital Community.

The PCR results further showed that single oligonucleotide primers can be used to amplify genomic DNA segments and polymorphisms can be detected between the amplified products of isolates. This work clearly demonstrates the relevance of molecular typing techniques such as PCR in the analysis of relationships in an organism as well as providing reliable epidemiological data. The specific target sequence was determined by co-amplification of a highly similar sequence, an internal standard which was distinguished from the target sequence in the PCR product. A shortened version of the specific sequence was used as an internal standard [24]. It is possible to assert from this study that typing to evaluate the existence of genetically different strains of microorganisms like *Pseudomonas aeruginosa* is commonly advised in epidemiological investigations. However, the use of very high discriminating typing methods is recommended for differentiation between bacterial species [25].

The sequence of highly conserved gene region of Biofilm forming *Pseudomonas aeruginosa*, 16S rDNA helped in the final prediction of the correct identity of *Pseudomonas aeruginosa* isolates from this study. Complete knowledge of an organism’s genetic makeup through sequencing allows for exhaustive identification of antimicrobial targets [26]. The sequence of 16S rDNA was fully based on PCR amplification for identification and genetic level confirmation of biofilm-forming *Pseudomonas aeruginosa*.

Also observed in this study were common species of biofilm-forming *Pseudomonas aeruginosa* detected in the samples analyzed, thus indicating that there could be patient-to-patient spread in this Hospital or between the environments and the admitted patients. Complications due to biofilm forming *Pseudomonas aeruginosa* are usually associated with the opportunistic nature of the organism; therefore, rapid diagnosis of such infection in a reliable way becomes necessary. It is widely accepted that traditional phenotypic methods for typing *Pseudomonas aeruginosa* do not permit accurate investigation of infections because phenotypic markers are relatively unstable [24], thus molecular technique as applied in this study remains the gold standard.

CONCLUSION

Biofilm-forming bacteria especially *Pseudomonas spp.* seriously exist on noncritical surfaces of various hospital sections that could lead to nosocomial transmissions in the hospitals. In this study, the nucleotide sequence of the experimental isolates expressed genetic mutation which represents point of involvement to *Pel B* gene involved in biofilm formation. This was observed for (samples one and two) both of which represents the strong and weak biofilm forming *Pseudomonas aeruginosa* isolates. Previously published data showed that *Pel B* mutation resulted in a dramatic decrease in pellicle formation compared to the parent strain [27]. However, later biofilm formation stages are significantly influenced by *Pel B* gene. There may be other genes involved in biofilm formation in *Pseudomonas aeruginosa* which are mutants of the standard control isolate used in the study as most of the genes associated with biofilm formation are usually controlled by about 1% chromosomal genes. Although the gene responsible for biofilm formation is more or less mutant of the standard control isolates of *Pseudomonas aeruginosa*, there may be other factors like strain difference, culture conditions, rate of expression of these genes and presence of other genetic factors affecting biofilm formation of *Pseudomonas aeruginosa*. This study confirms the molecular character of biofilm-forming *Pseudomonas aeruginosa*. This calls for adequate healthcare infection control practices in hospitals [28] like Federal Medical Centre, Umuahia, Abia-Nigeria as nosocomial infection may pose serious health challenges and drug resistance due to mutation, among healthcare professionals, patients and patient relatives.

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CONTRIBUTION OF AUTHORS

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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