Antimicrobial Resistance and Molecular Typing of *Pseudomonas aeruginosa* Isolated from Surgical Wounds in Lagos, Nigeria

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Abstract- The aim of the study was to determine the resistance patterns of *Pseudomonas aeruginosa* isolates recovered from patients with surgical wounds in hospitals and also to investigate their epidemiological relatedness using molecular typing techniques. Twenty *Pseudomonas sp.* isolated from surgical wounds were subjected to antibiotic susceptibility testing by disk diffusion, plasmid profile, SDS-PAGE and PCR using the *par*C, *gyr* A gene and RAPD using the 1254 primer. The isolates showed resistance to 12 different antibiotics with six being 100% resistant. Plasmids were detected in 16 (80%) of the isolates. The RAPD-PCR using the primer 1254, SDS-PAGE classified the 20 *Pseudomonas spp*. into 5 and 6 types respectively. *Pseudomona aeruginosa* strains isolated from surgical wounds were generally resistant to a broad range of antibiotics and this is rather worrisome. The typing techniques classified the 20 isolates into 5 and 6 groups.

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Introduction

Surgical site infections are one of the most common nosocomial infections apart from the blood stream infections, pneumonia and urinary tract infections (1). *Pseudomonas aeruginosa* is ubiquitous in nature with minimal requirements for survival and its ability to adapt to a variety of environmental conditions. Emori and Gaynes, (2) reported that *Pseudomonas aeruginosa* accounted for about 10% of common hospital acquired infections . However another report by Betrand et al. [3] showed that in intensive care units (ICUs), *Pseudomonas aeruginosa* accounted for about 18% of nosocomial infections in such units with 6% of cases being from surgical ad non surgical sites. *Pseudomonas spp.* also accounted for 10.8% of infections from surgical sites in another report by Maksimovic *et al.* (1) although the colonization in nosocomial infections has been attributed most often to patients with neutropaenia and otherwise immunocompromised patients in intensive care units (4). It has also been implicated as an important opportunistic pathogen which causes high morbidity in cystic fibrosis patients (5). Its importance as an opportunistic pathogen is due to its ability to activate useful phenotypes under environmental stress and to persist in adverse conditions such as antibiotic or antiseptic substances.

In Nigeria, a study from the South West showed that *Pseudomonas aeruginosa* had been isolated from urine (4.6%), Reproductive tract (2.1%) and wound infections (16.3%) (6). Another study from the north reported the occurence of *Pseudomonas aeruginosa* in urine samples to be 4.6% (7). Another report from the south west isolated *Pseudomonas aeruginosa* from 39.3% in wound swabs, 41.9% in ear swabs. From the south south, *Pseudomonas aeruginosa* was isolated from 41% of cases with discharging ears (8).

Despite improvements in antibiotic therapy *Pseudomonas aeruginosa* is intrinsically resistant to a number of antimicrobial agents frequently including multiple classes of antimicrobial agents (9). Subsequently outbreaks due to multi resistant *Pseudomonas aeruginosa* have been reported in various nosocomial settings, such as ICUs (10,11). Typing techniques useful for establishing clonal relationships between individual isolates in hospital settings are therefore important to recognize nosocomial transmission and guide infection control practice.

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Typing techniques such as PFGE, SDS-PAGE and PCR-RAPD have been found to be useful for epidemiological study of *Pseudomonas aeruginosa* (9,12).

The aim of the study was to determine the resistance patterns of *Pseudomonas aeruginosa* isolates recovered from patients with surgical wounds in hospitals and also to investigate their epidemiological relatedness using molecular typing techniques.

Materials and Methods

Twenty *Pseudomonas aeruginosa* isolated from surgical wounds of patients from two hospitals in Nigeria were obtained from the Molecular Biology and Biotechnology Division stock and checked for purity before being subjected to the following tests:

Method of *Pseudomonas* identification: Isolation of *Pseudomonas aeruginosa* from clinical specimens is by the use of Columbia agar plates supplemented with 5% sheep blood and MacConkey agar with production of the blue pigment with grapelike odour. Growth on these media after 24 to 48h and the suspected colonies were then purified and subjected to biochemical characteristics for identification by growth at 42^0C, nitrate reduction and gas production. Others are arginine dihydrolase positive, acid production from glucose and xylose and positive in simmons citrate agar.

Antimicrobial susceptibility testing, according to NCCLS (13) using 12 antimicrobials, DNA extraction as described by Birnboim and Doly (14). PCR using the *gyr*A and *par*C genes and the following primers: for fluoroquinolone resistant gene *gyr A* (5'TTA AAA TTT GTC ACG AAT ATG CC 3'; 5' AAC GAT ACG CTC ACG ACC AGT 3'), *parC* (5' AAA AAC TAC TCT ACA TTC TTT GAA AGG AG3'; 5' CAG TTG GGT GGT CAA TCA TGT ACC 3'). The PCR was performed in a 25 µl reaction mixture. After an initial denaturation of 95°C for 3 minutes, the PCR conditions consisted of 35 cycles for 45 sec at 95°C, 1 min at *gyr*A 58, *par*C 60°C, and 1 min. 30 sec at 72°C, in a Techne, US thermal cycler. 100bp ladder was used as DNA molecular weight standards.

RAPD-PCR with primer 1254

Using primer 1254 (5'-CCG CAG CCA A-3'), the PCR conditions consisted of an initial 5 cycles of consecutive denaturation, annealing, and DNA extension (30 sec at 94°C, 2 min at 20°C, and 2 min at 72°C), followed by 35 cycles of 30 sec at 94°C, 1 min at 32°C, and 2 min at 72°C in Techne thermal cycler (Techne, US). DNA fragments were separated in 1.5% agarose gels and visualized by ethidium bromide staining. HIND III digest of λ-DNA marker was used as DNA molecular weight standards.

Protein induction and extraction/SDS-PAGE

Induction and outer membrane protein of *Pseudomonas* was according to Murakami *et al.* (15) Briefly, the cells were grown in TSB at 32°C to log phase (4-6 h). After 6h, 5 ul of ofloxacin was added to induce the cell and incubated for 90 mins at 32°C. The incubated cells were dispended into eppendorf tubes and centrifuges at 13,000 × rpm for 5 mins. The supernatant was discarded and 200 ul was added to the pellet and incubated for 30 min, after which it was centrifuged at 13,000 rpm for 5 min and the supernatant was discarded and the pellet was resuspended in 100 ul 1 × SDS-PAGE lysing buffer and boiled for 5 min at 100 0C. This was then centrifuged at 5000 rpm for 10 min. Ten percent freshly prepared APS was used for the SDS-PAGE. The supernatant (10 ul) was loaded onto 10% PAGE gel and allowed to run for 4 h. The separating gel was placed in small plastic plate for staining with coomasie blue for at least 2h followed by another 2h of destaining.The gels were then viewed and photographs taken of the protein bands.

Results

The 20 *Pseudomonas aeruginosa* isolates showed resistance to 12 different antibiotics; cotrimoxazole (100%), amoxycillin (100%), tetracycline (95%), augmentin (95%), ofloxacin (80%), gentamicin (80%), nalidixic Acid (100%), nitrofurantoin (100%), ciprofloxacin (70%), imipenem (60%), cefuroxime (100%) and cefoxitin (100%) (Table 1). Plasmids were detected in 16 (80%) of the isolates and mainly one band (27.1 kb) in 12 (75%) (Figure 1).

The RAPD-PCR using the primer 1254, classified the 20 *Pseudomonas spp*. into 5 types Type A-E. Only 3 (15%) of the isolates possessed the *gyr*A gene for fluoroquinolone, while none of the isolates possessed the *par*C gene. SDS-PAGE analysis following induction with ofloxacin grouped the isolates into 6 types (Figure 2).

Discussion

The high rate of resistance of our isolates of *Pseudomonas aeruginosa* to 12 antibiotics is worrisome. The least resistant antibiotic was imipenem with only 40% of the isolates being sensitive. From various studies on antibiotic resistance of *Pseudomonas aeruginosa* , resistance to imipenem was 16.3% in Brazil (9).

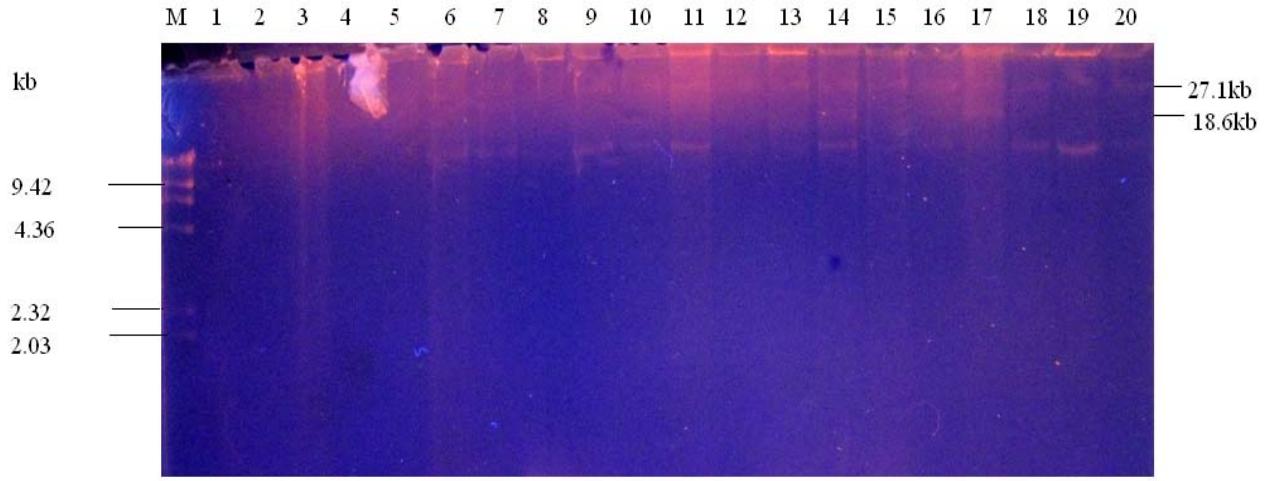

Figure 1. Plasmid profile analysis of 20 *Pseudomonas aeruginosa* isolates

Lanes 2, 6, 7, 8, 9, 10, 11, 12, 15, 16, 18 and 20 all had one plasmid each of molecular weight 27.1Kbp, while Lanes 13, 14 and 17 all had two plasmids of molecular weight 18.6Kb and 27.1Kb. M is the molecular weight marker.

Table 1. Table showing antimicrobial susceptibility patterns of the 20 *Pseudomonas aeruginosa* isolates.

Sample No.	COT	AMX	TET	AUG	OFL	GEN	NAL	NIT	CIP	FOX	IPM	CXM
PS1	R	R	R	R	R	R	R	R	R	R	R	R
PS2	R	R	R	R	R	R	R	R	R	R	S	R
PS3	R	R	R	R	R	R	R	R	R	R	R	R
PS4	R	R	R	R	R	R	R	R	R	R	S	R
PS5	R	R	R	R	R	R	R	R	R	R	R	R
PS6	R	R	R	R	R	R	R	R	R	R	R	R
PS7	R	R	R	R	R	R	R	R	R	R	R	R
PS8	R	R	R	R	R	R	R	R	R	R	R	R
PS9	R	R	R	R	R	R	R	R	R	R	R	R
PS10	R	R	R	R	R	S	R	R	R	R	S	R
PS11	R	R	R	R	R	S	R	R	R	R	S	R
PS12	R	R	R	R	R	R	R	R	S	R	R	R
PS13	R	R	R	R	R	R	R	R	S	R	R	R
PS14	R	R	R	S	R	R	R	R	R	R	R	R
PS15	R	R	R	R	S	S	R	R	S	R	S	R
PS16	R	R	R	R	R	R	R	R	R	R	R	R
PS17	R	R	R	R	S	S	R	R	S	R	S	R
PS18	R	R	R	R	R	R	R	R	R	R	S	R
PS19	R	R	R	R	S	R	R	R	S	R	S	R
PS20	R	R	S	R	S	R	R	R	S	R	R	R

Cot: cotrimoxazole, amx: amoxicillin, tet: tetracycline, aug: amoxy/clav, ofl: ofloxacin, gen: gentamicin, nal: nalidixic acid, nit: nitrofurantoin, cip: ciprofloxacin, fox: cefuroxime, ipm: imipenen, cxm: cefoxitin

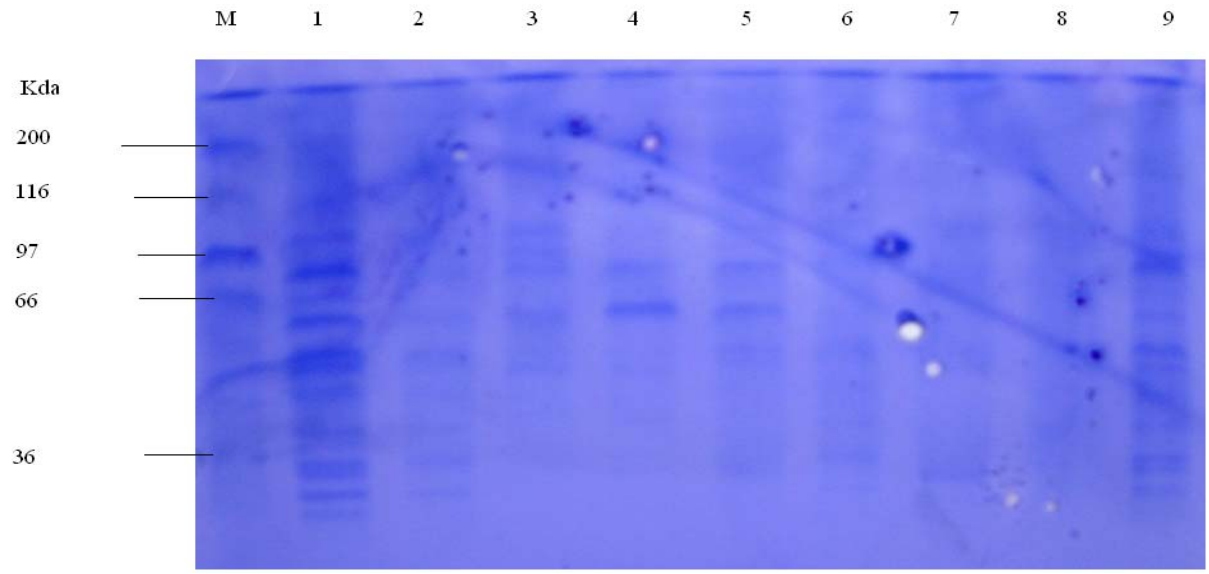

Figure 2. SDS-PAGE analysis of the *Pseudomonas aeruginosa* isolates
Lanes M: Molecular weight marker (36 MDa-200 MDa); Lanes 2-10: isolates 1-9.

In other countries also e.g. from a study from Iran, (16) 70% of their isolates obtained from cystic fibrosis patients were resistant, in an earlier study in Iran resistance to imipenem was 2.9% (17), Greece 21% (18), Spain 13% (19). These studies all had low resistances to imipenem which is the drug of choice recommended for treatment of *Pseudomonas* infections. In this study, although it is the least resistant drug for the isolates, majority were resistant to it (60%). Only the fluoroquinolones were next in terms of susceptibility. The cephalosporins tested were all resistant (cefuroxime, cefoxitin). Other reports from Iran showed 15.4% resistance to the ciprofloxacin (17); Greece 31% (18).

From this study, 60% of the isolates were resistant to the 12 antibiotics used in this study; this is quite worrisome due to the fact that if the majority were resistant to 12 antibiotics constant antibiotic screening must be done before drugs are prescribed for *Pseudomonas aeruginosa* infections in our environment. This result is similar to that of Tassios *et al*. (18) with 52% of their isolates being multidrug resistant and contrary to Freitas *et al*. (9) with only 3.2% resistant to all antimicrobial agents. In Nigeria, several studies have reported on the sensitivity of *Pseudomonas* isolates to fluoroquinolones (>70%) (20-22), while there was varying sensitivies to the cephalosporins (cefuroxime 76.6%, ceftazidime (50.7%) (21). The report by Odusanya (6) showed varying degrees of resistance in which 30 isolates showed 43.3% sensitive to perfloxacin, cefuroxime. In another study by Yoon *et al*. (23) 56% of Korean *Pseudomonas aeruginosa* isolates were multidrug resistant (MDR) out of which 44% showed resistance to five or more antibiotics.

The typing of the 20 isolates by plasmid profiles showed the technique had low typeability as the same patterns was also distributed in the two hospitals. This is similar to a previous report from Iran by Nikbin *et al.* (17) where the isolates by plasmids profile showed low typeability and discriminatory power.

RAPD-PCR yielded five types and the technique produce better discrimination as the isolates that were grouped into one from the plasmid profile were subdivided into four patterns. However from other reports RAPD-PCR using primer sets 272 and 208 were found to be highly discriminatory for study in Iran as majority had unique fingerprints (16) while that of Sazakli *et al.* (12) using two different primer opa-13 showed that one of the primer had a low discriminatory power similar to that from this study. Yoon *et al*. (23) also highlighted the importance of RAPD-PCR for DNA genotype analysis of their Korean strains. The *gyrA* gene for quinolone resistance only showed 15% of the isolates to be resistant to fluoroquinolones and so was not useful for typing our isolates, while the SDS-PAGE typing after induction of fluoroquinolone could type the isolates into 6 types. This method seems to be the best

for typing our isolates in our environment albeit after induction. Another report by Sazakli *et al*. (12) found SDS-PAGE useful for typing of *Pseudomonas spp.* from the aquatic environment.

There was generally no relationship between the genetic profiles and antibiotic susceptibility of the isolates. A combination of SDS-PAGE and RAPD-PCR typed the isolates into nine patterns.

In conclusion, *Pseudomonas aeruginosa* isolated from surgical wound infections in this study have multi drug resistance to a variety of drugs. The best typing system is that of the SDS-PAGE following induction of the fluoroquinolone, however, there seems to be no relationship between the genetic profiles and antibiotic susceptibility of the isolates. This is the first report to our knowledge looking at molecular typing of *Pseudomonas aeruginosa* in Nigeria.

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