

Antibiotic resistance pattern of *Pseudomonas* spp. from patients in a tertiary hospital in South-West Nigeria

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Abstract

Introduction Pseudomonads constitute critical agents of opportunistic infections in hospital settings particularly in immunocompromised patients and *Pseudomonas aeruginosa* is a major flagship member of these infectious agents. This study assessed the distribution of *Pseudomonas* spp. associated with infections in patients and their antibiotic resistance patterns as part of an antibiotic stewardship intervention program and resistance surveillance.

Methods One hundred and fifty *Pseudomonas* spp. from different clinical specimens were obtained from the Obafemi Awolowo University Teaching Hospitals Complex Ile-Ife. Culture was carried out on MacConkey and blood agar while phenotypic characterization was done by Gram staining, oxidase, and catalase test. Species identification was done using MICROBACT™ 24E bacterial identification kit and confirmed by 16S rDNA polymerase chain reaction (PCR) assay. Antibiotic susceptibility testing to eight antibiotics in four classes was done.

Results *Pseudomonas aeruginosa* was the most frequently occurring species (96.0%); *P. putida* (2.67%) and *P. fluorescens* (0.67%) were also identified as well as an isolate of *Burkholderia pseudomallei* (0.67%). The highest resistance rate among isolates was observed towards gentamicin (35.4%); piperacillin/tazobactam was the most active antibiotic. Multidrug-resistant (MDR) strains constituted 12.8% of the isolates and most MDR strains also displayed a high multiple antibiotic resistance index (MAR).

Conclusions *Pseudomonas aeruginosa* is emerging as a highly MDR pathogen in our hospital setting. This calls for the establishment of a surveillance system and antimicrobial stewardship programme in place. Furthermore, we propose a review of the current antibiotics prescription policy, and infection control programmes (ICPs) if we must control the spread of MDR-*P. aeruginosa* in this environment.

Keywords *Pseudomonas* spp., multiple antibiotic resistance index (MAR), multidrug resistance (MDR), antibiotics stewardship.

Introduction

Pseudomonas species represent a metabolically versatile and ubiquitous class of organisms naturally found in soil, water, and vegetation. They are not considered as part of the normal flora of humans and are often implicated in

opportunistic infections.¹ *Pseudomonas aeruginosa*, a flagship member of *Pseudomonas* spp. is one of the main opportunistic pathogens involved in nosocomial infections globally, particularly in immunocompromised patients due to a combination of intrinsic and acquired

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mechanisms used to develop resistance to all effective antibiotics.²

Pseudomonas spp. exhibits an ever-growing multidrug-resistance that cuts across fluoroquinolones, aminoglycosides, third and fourth generation cephalosporins and advanced beta-lactams and was assigned to a serious level of threat as a result of this observation.^{3,4} Emergence of multidrug resistant (MDR) *Pseudomonas aeruginosa* is a serious healthcare challenge with significant morbidity and mortality worldwide.⁵ Infections caused by MDR pathogens require timely and appropriate therapy to improve patients' survival.⁶

Pseudomonas aeruginosa infection is usually severe, with high mortality rates in hospitalized patients with cancer, cystic fibrosis, and burns.⁷ It is accountable for about 10% of all nosocomial infections and considered as one of the most critical agents of Gram-negative bacterial infections with reports of increasing antibiotic resistance worldwide.⁸

There is a dearth of data on the magnitude of this problem for developing countries; hence, this study was done to assess the distribution of *Pseudomonas* spp. associated with infections in patients and their antibiotic resistance surveillance patterns with a view on antibiotic stewardship intervention to control the spread of MDR pseudomonads in our hospital setting.

Methods

Study area, sample size and ethical approval

This cross-sectional study was undertaken at Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC) Ile-Ife and Department of Medical Microbiology and Parasitology, Obafemi Awolowo University (OAU), Ile-Ife, Osun State, Nigeria. Ethical approval was sought and obtained from the Ethics and Research Committee of the OAUTHC (IRB/IEC/0004553). A total of 150 non-repetitive *Pseudomonas* spp. isolated from different clinical specimens from patients at the hospitals between March-September 2018 were employed as a non-patient contact for this study. Relevant demographic and clinical data of the patients were collected using a predesigned questionnaire.

Isolates collection and characterization

The isolates were collected on nutrient agar slants and transported to the Research Laboratory of the Department of Medical Microbiology and Parasitology of OAU, Ile-Ife for further analysis. Pure colonies of isolates were obtained by culture on MacConkey agar, blood agar and Trypticase soy agar and incubating at 35°C for 24 h. Morphological and biochemical characteristics of the isolates were determined by Gram staining, catalase test, oxidase test and growth at 42°C in line with standard procedures.⁹ Definitive species of isolates was confirmed using MICROBACT™ 24E identification kit and Polymerase Chain Reaction (PCR).

Antibiotic susceptibility testing

Commercially available antibiotic discs (Mast, United Kingdom) were used to determine the susceptibility profile of the isolates. The Modified Kirby Bauer technique was employed and inhibition zones were interpreted according to the guidelines of Clinical and Laboratory Standard Institute (CLSI).¹⁰ The antibiotics tested include piperacillin (75 µg), piperacillin/tazobactam (100/10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), amikacin (30 µg), gentamicin (30 µg), imipenem (10 µg), and meropenem (10 µg). *P. aeruginosa* ATCC 27853 was used as a reference strain for the test. Multidrug-resistance was defined as resistance to at least one antibiotic in three or more classes of antibiotics and the multiple antibiotic resistance (MAR) index of the isolates was recorded as defined by Krumperman.^{4,11}

DNA extraction

DNA was extracted from isolates using the boiling method. Three colonies of each isolate were emulsified in 100 µL of sterile distilled water in an Eppendorf tube, boiled for 15 minutes and centrifuged at 10,000 rpm for five minutes in a microcentrifuge. The supernatant was transferred to a new Eppendorf tube after centrifugation and was used as template DNA for polymerase chain reaction (PCR).

Identification of *Pseudomonas* spp. and *Pseudomonas aeruginosa* by PCR

PCR was used to confirm the identities of the isolates. A 25 µL PCR mixture (12.5 µL one Taq Quick-Load 2X master mix with standard buffer, 0.5 µL of 10 µM each of forward primer and reverse primer, 3 µL template DNA and 8.5 µL of nuclease-free water) was set up to amplify the genes of *Pseudomonas* spp. and *Pseudomonas aeruginosa* using the primers PAGES 618 bp (F: GGGGGATCTTCGGACCTCA, R: TCCTTAGAGTGCCACCCG) and PASS 956 bp (F: GGGGGATCTTCGGACCTCA, R: TCCTTAGAGTGCCACCCG) respectively.¹² PCR conditions were observed according to Ghosh et al.¹³ Each amplicon (10 µL) was electrophoresed on a 1.5% agarose gel pre-stained with 0.5 µg/mL ethidium bromide in 1X Tris-Acetate-EDTA buffer and viewed with a transilluminator (Avebury, UK). The positions of the PCR products were determined by the positions of the 100 bp molecular weight marker (Biolabs, UK).

Data analysis

Data analysis was done using the IBM Statistical Product and Service Solutions (SPSS) version 20 (IBM Corp, USA). Descriptive statistics (frequencies, percentages, etc.) of data were presented.

Results

During the study period from March to September 2018, 150 isolates of *Pseudomonas* spp. from diverse clinical specimens were obtained from our hospital and employed as a non-patient contact for this study. The majority of the isolates (n=90; 60%) were recovered from patients with no recent (>4 weeks) hospital admission and the rest (n=60; 40%) were from those with recent (<4 weeks) hospital admission. There were slightly more isolates recovered from female patients n=76 (51%) compared to males n=74 (49%). The highest frequency of the isolates (n=43; 28.7%) was recovered from patients in the age range of 30-39 years, while the least (n=11; 7.3%) was from the age group less than 10 years old (Table 1).

Table 1. Demographic profile of patients with *Pseudomonas* spp. infections

| Parameter | No of <i>Pseudomonas</i> spp. isolated (n=150), n (%) |
|----------------------------------|---|
| Age groups | |
| <10 years | 11 (7.3) |
| 10-19 years | 13 (8.7) |
| 20-29 years | 24 (16) |
| 30-39 years | 43 (28.7) |
| 40-49 years | 18 (12) |
| 50-59 years | 11 (7.3) |
| ≥60 years | 30 (20) |
| Gender | |
| Male | 74 (49.3) |
| Female | 76 (50.7) |
| Hospital stay | |
| Recent admission (<4 weeks) | 60 (40) |
| No recent admission (>4 weeks) | 90 (60) |
| Invasive medical device | |
| IV/catheter use | 54 (36) |
| No IV/catheter use | 96 (64) |
| Drug use | |
| No antibiotic use | 123 (82) |
| Antibiotic use | 27 (18) |
| Comorbidity | |
| No comorbidity | 102 (68) |
| Comorbidity | 48 (32) |
| Type of clinical specimen | |
| Wound swab | 66 (44.0) |
| Urine | 45 (30.0) |
| Blood | 10 (6.7) |
| Ear swab | 12 (8.0) |
| Pus | 3 (2.0) |
| Eye swab | 2 (1.3) |
| Aspirate | 3 (2.0) |
| Urethral swab | 3 (2.0) |
| Pleural fluid | 2 (1.3) |
| Catheter tip | 2 (1.3) |
| Sputum | 1 (0.67) |
| HVS | 1 (0.67) |

As shown in Table 1, wound swab was the predominant specimen source (n=66; 44%) followed by urine. Forty-five (30%) isolates were recovered from urine samples out of which 40% were catheter-stream and 60% mid-stream urine. Ear swabs ranked next in frequency giving 8% of

the isolates recovered predominantly from chronic otitis media in adult patients, while blood was 6.7%. Sputum and high vaginal swab yielded the lowest frequency of isolates (n=1; 0.67%). A uniplex PCR was used to identify *Pseudomonas* spp. and *Pseudomonas aeruginosa*. PA-GS, a genus-specific primer pair used to amplify the 16Sr RNA gene of *Pseudomonas* spp. yielded 618 bp while PA-SS a species-specific primer pair used to amplify the 16SrRNA gene of *Pseudomonas aeruginosa* yielded 956 bp (Figures 1 and 2). Hence, all the isolates identified as *Pseudomonas* spp. and *Pseudomonas aeruginosa* by the phenotypic method were confirmed by PCR.

Of the isolates studied, 144 (96%) *P. aeruginosa* strains were recovered representing the highest frequency. Most of the isolates of *P. aeruginosa* were recovered from wound swabs (44%), *P. fluorescens* n=4 (2.67%) were from blood, ranking next in frequency, while an isolate each (0.67%) of *P. putida* (from catheter tip) and *Burkholderia (Pseudomonas) pseudomallei* (from sputum) were also recovered.

As shown in Table 2, the isolates exhibited the highest resistance to gentamicin (35%); imipenem and piperacillin-tazobactam were the most effective antibiotics with resistance rates of 7% and 6% respectively.

MDR strains constituted 12.7% of the isolates, out of which 26.3% were from outpatients and 73.7% from inpatients. All the MDR strains (n=19; 12.7%) had a high multiple antibiotic resistance (MAR) index (Table 3).

Discussion

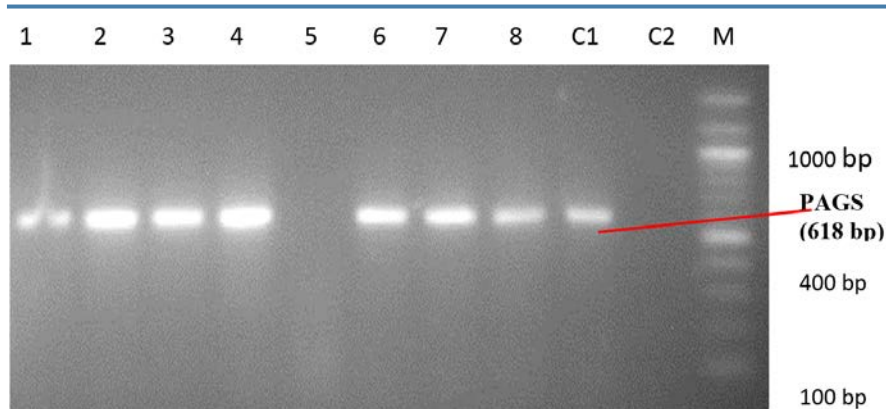
Although *Pseudomonas aeruginosa* was the predominant species isolated in this setting, other species of pseudomonads were infrequently encountered or isolated. In certain chronic health facilities, *Stenotrophomonas maltophilia* accounted for 80 percent of opportunistic infections by pseudomonads in immunocompromised clinical situations underscoring the need to identify pseudomonads from various clinical specimens to species level for epidemiological and surveillance purposes.¹⁴

We observed also that *S. maltophilia* was not represented in this study, however, the

predominance of *P. aeruginosa* reported by us aligns with the findings of Gad et al.¹⁵ that reported *P. aeruginosa* (75.7%) as the predominant species in a series of 107 *Pseudomonas* strains recovered from 445 clinical specimens. In our study, the majority of the *P. aeruginosa* isolates were recovered from wound swab (44%) while in the study reported by Gad et al.,¹⁵ urine (22.5%) gave the highest yield of *Pseudomonas aeruginosa*, but in another series of 102 *Pseudomonas aeruginosa* reported by Shrestha et al.,¹⁶ both urine and sputum specimens accounted for the highest number of isolates (n=37; 36.3%) each, which highlights the major regular specimen source for the recovery of pseudomonads, particularly in immunocompromised situations. However, the differences can be explained due to the different types of clinical specimens in the different studies and the inclusion of environmental pseudomonas isolates in the study reported by Gad et al.¹⁵

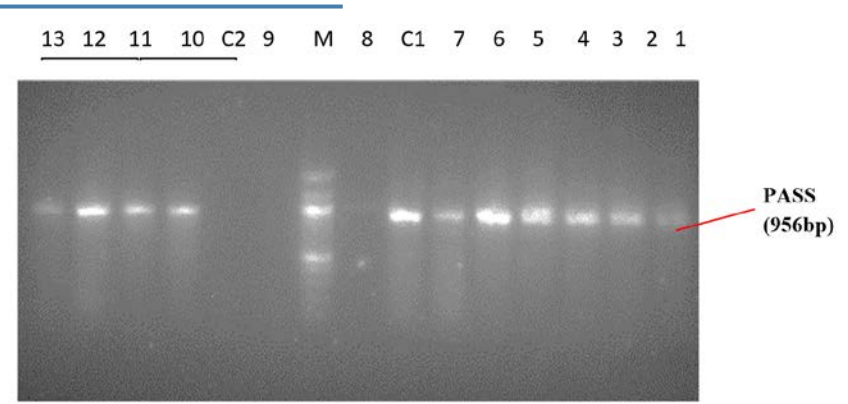
The highest frequency of the isolates (28.7%) was recovered from patients in the age range of 30-39 years, contrary to the observation of Okon et al.¹⁷ who reported the highest occurrence in the 20-29 years age range. Although there were slightly more isolates of pseudomonads recovered from female patients (51%) compared to males (49%) in this study, the anatomical structure of the female reproductive system makes the invasiveness of *Pseudomonas* spp. easier when immunity is compromised. Ear swabs ranked next in frequency giving 8% of the isolates recovered predominantly from chronic otitis media in adult patients. This finding is in tandem with reports by studies in and outside Nigeria.^{19,20} As an opportunistic pathogen, *P. aeruginosa* often requires a breach in the first-line defense of the skin to initiate infection, this breach in immunity was found to be of significant influence in the high frequencies of the patients' cases in trauma, burn wounds and surgery wounds from which the isolates were recovered.

Increased resistance of *P. aeruginosa* to antibiotics continues to pose a major threat to patient care due to limited treatment options.¹⁶ In this study, we observed resistant strains of



PAGES positive isolates- 1:205IK, 2:729, 3:720, 4:721, 6:725WG, 7:912, 8: 609.
 PAGES negative isolate - 5:101IK. C2: negative control *E. coli* ATCC 25922. C1:
 positive control *P. aeruginosa* ATCC 27853. M: 100 bp ladder N0551S (New
 England Biolabs Inc.).

Figure 1. PCR amplicons of *Pseudomonas* spp.



PASS positive isolates: 1-803, 2-S16, 3-720, 4-721, 5-S84K, 6-725WG, 7-912,
 10-609, 11-718, 12- 604, 13-144K. PASS negative isolates- 8:101IK, 9:416WG
 C2- negative control *E. coli* ATCC 25922. C1- positive control *P. aeruginosa*
 ATCC27853. M - 100bp ladder N0551S (New England Biolabs Inc.).

Figure 2. PCR amplicons of *Pseudomonas aeruginosa*

Table 2. Antibiotic susceptibility patterns of *Pseudomonas* spp. (n=150)

| Antibiotic class | Antibiotics | Susceptible isolates, n (%) | Resistant isolates, n (%) | | | | Total |
|------------------|-------------------------|-----------------------------|-------------------------------|---------------------------|--------------------------------|----------------------------------|---------|
| | | | <i>Pseudomonas aeruginosa</i> | <i>Pseudomonas putida</i> | <i>Pseudomonas fluorescens</i> | <i>Burkholderia pseudomallei</i> | |
| Aminoglycosides | Gentamicin | 98 (65) | 47 (31.3) | 1 (0.7) | 3 (2) | 1 (0.7) | 52 (35) |
| | Amikacin | 130 (87) | 17 (11.3) | 1 (0.7) | 1 (0.7) | 1 (0.7) | 20 (13) |
| Quinolone | Ciprofloxacin | 106 (71) | 40 (26.7) | 1 (0.7) | 2 (1.3) | 1 (0.7) | 44 (29) |
| | Levofloxacin | 108 (72) | 39 (26) | 1 (0.7) | 1 (0.7) | 1 (0.7) | 42 (28) |
| Beta-lactam | Piperacillin | 124 (83) | 25 (16.7) | 1 (0.7) | 1 (0.7) | 1 (0.7) | 28 (17) |
| | Piperacillin-tazobactam | 141 (94) | 8 (5.3) | 1 (0.7) | 0 (0) | 0 (0) | 9 (6) |
| Carbapenem | Imipenem | 139 (93) | 11 (7.3) | 0 (0) | 0 (0) | 0 (0) | 11 (7) |
| | Meropenem | 134 (89) | 13 (8.6) | 1 (0.7) | 1 (0.7) | 1 (0.7) | 16 (11) |

Table 3. Distribution of multidrug resistant strains of *Pseudomonas* spp. isolated

| <i>Pseudomonas</i> spp. | No (%) of isolates | Multidrug resistant strains, n (%) | Multiple antibiotic resistance (MAR) index |
|----------------------------------|--------------------|------------------------------------|--|
| <i>Pseudomonas aeruginosa</i> | 144 (96.0) | 16 (84.2) | 0.5-1.0 |
| <i>Pseudomonas fluorescens</i> | 4 (2.7) | 1 (5.6) | 0.75 |
| <i>Pseudomonas putida</i> | 1 (0.67) | 1 (5.6) | 0.875 |
| <i>Burkholderia pseudomallei</i> | 1 (0.67) | 1 (5.6) | 0.75 |
| Total | 150 (100) | 19 (12.7) | 0.5-1.0 |

pseudomonads across the different classes of antibiotics tested. However, the highest resistance rates among isolates were observed towards gentamicin (35.4%) while piperacillin/tazobactam was the most active antibiotic with a low resistance rate (6%), and Peshattwar²⁰ reported similar findings in their study. The observed resistance rate in our study reflects the current antibiotic prescription pattern and the selective pressure that followed is that gentamicin and ciprofloxacin have been much longer in circulation which will explain their relatively higher rates of resistance compared to piperacillin/tazobactam and imipenem with lower resistance rate because they are relatively recent in hospital practice in this country. Our observation is slightly different from what Shrestha et al.¹⁶ reported from Kathmandu, Nepal. In their study, *P. aeruginosa* exhibited high rates of resistance to piperacillin (57.1%) and ciprofloxacin (36.7%) among others, while only 6.5% of the isolates were resistant to imipenem in agreement with our reported resistance rate of 7% for imipenem. Gad et al.¹⁵ from Egypt reported a higher rate for gentamicin (59%) and meropenem (22%) in an earlier study. Gentamicin was introduced to the market in the '60s and this suggests a higher chance of exposure within the study population in comparison to the carbapenems. The high probability of exposure to the drug is also a driver for resistance. The level of resistance to fluoroquinolones detected in this study is low when compared to a previous study.²¹ The isolation of an MDR *B. pseudomallei* from an end-stage renal disease patient in this study is of particular interest since a misdiagnosis of the disease is highly probable given the fact that it requires a high index of suspicion and the clinical

context of isolation for the clinical significance of the pathogen to be determined and not discarded as a contaminant or colonizer.

Previous studies from Nigeria have indicated high rates of multidrug resistance in *P. aeruginosa* especially to fluoroquinolones, aminoglycosides and third-generation cephalosporins. In a study by Adejuyigbe et al.²² on septicemia in high-risk newborns at a teaching hospital in Ile-Ife, Nigeria, *Pseudomonas aeruginosa* accounted for 18.8% of the causative organisms with a high degree of in-vitro antimicrobial resistance. Oluranti et al.²³ reported an incidence of 19.6% MDR *P. aeruginosa*. Igbalajobi et al.²⁴ reported 18-31% resistance to penicillins, aminoglycosides and fluoroquinolones. *P. aeruginosa* has also been implicated as a prominent cause of post-operative wound infection in Nigeria.²⁵ *Pseudomonas aeruginosa* is one of the most important opportunistic pathogens responsible for 10-15% of nosocomial infections worldwide.⁸

From the foregoing, it can be unequivocally stated that *P. aeruginosa* has now emerged as a highly multidrug-resistant pathogen with concomitant high multiple antibiotic resistance index in this environment. This is also in addition to the intrinsic nature of *Pseudomonas* being inertly impervious to most antibiotics due to the cell wall structure as well as its ease of spread in nosocomial settings. All the MDR strains (19; 100%) had a high MAR index suggesting a high-risk source where antibiotics are regularly and inappropriately used leading to high selective pressure. We can, therefore, safely speculate that the widespread, easy access and unrestrained antibiotic use have accelerated the incidence of antibiotic resistance and MDR

strains in this environment. MDR strains constituted 12.8% of the isolates out of which 26.3% were from outpatients and 73.7% from inpatients suggesting the hospital as an important reservoir of multidrug-resistant strains. MDR isolates of 12.8% were observed with a high MAR index that may just be a tip of the iceberg phenomenon since this study was hospital-based and not widespread community-based research.

Piperacillin/tazobactam and imipenem were the most active antibiotics observed in this study. Our study underscores the importance of antibiotic susceptibility testing of clinical isolates in this environment.

The study was conceptualized more as a laboratory based study with less patient contact and not being a funded research the scale was limited. Larger studies are needed to further investigate the magnitude of antimicrobial resistance in this environment.

Conclusions

Pseudomonas aeruginosa has now emerged as a highly multidrug-resistant MDR pathogen with concomitant high multiple antibiotic resistance index in our hospital setting. This calls for the establishment of a surveillance system and antimicrobial stewardship programme in place. We propose a review of the current antibiotics prescription policy, and infection control programmes if we must control the spread of MDR-*P. aeruginosa* in this environment.

Authors' contributions statement: AO and OO designed and supervised the study. AA and OA collected and analyzed the data and performed the background literature review for the manuscript. AA and BO carried out the laboratory work and conducted the statistical analysis. AO, OO and BO drafted the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest: All authors – none to declare.

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