

Antibiotic resistance profiles of clinical and environmental isolates of *Pseudomonas aeruginosa* in Calabar, Nigeria

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Abstract: Healthcare-associated infections due to multidrug-resistant Gram-negative bacteria, including *Pseudomonas aeruginosa* have become increasingly difficult to treat. The hospital environments, particularly in ICUs, are common habitats for *Pseudomonas aeruginosa*. Spread of pathogen may occur through direct patient contact with contaminated fomites and from patient-to-patient on the hands of healthcare workers. The present study was conceived to evaluate the antibiotic resistance profiles of clinical and environmental isolates of *Pseudomonas aeruginosa* in Calabar Metropolis. A total of 1000 different clinical specimens were non-repetitively obtained from patients in four health care facilities in Calabar while 75 samples were also collected from the hospitals environment. From these specimens, 197 clinical isolates were recovered, whereas 26 environmental isolates were identified. Pus/wound swab produced the highest number of clinical *P. aeruginosa* isolates 83(42.1%) followed by ear swab 53(26.9%); surgical wound swab 24(12.2%); eye swab 14(7.1%); respiratory secretions 12(6.1%); mid-stream urine 7(3.6%) and blood specimens 4(2.0%) in that order. The highest number of environmental isolates 12(46.2%), were recovered from the hospital floors whereas only one (3.8%) isolate was recovered from drug dispensing tray. Antibiotic susceptibility testing was done using 12 agents from six classes of antibiotics and included antipseudomonal penicillin - carbenicillin; aminoglycosides - amikacin, gentamicin and tobramycin; fluoroquinolone - ciprofloxacin; cephalosporin - ceftazidime, ceftriaxone and cefepime; monobactam - aztreonam; carbapenem - imipenem, meropenem and doripenem. The isolates exhibited highest resistance (>70%) to the cephalosporins while 30(15.2%) clinical isolates were carbapenem-resistant. None of the environmental isolates was resistant to the carbapenems and the monobactam, aztreonam. The overall prevalence of *Pseudomonas* infection in Calabar from the study was 19.7%. On the other hand, prevalence of carbapenem-resistant *P. aeruginosa* (CR-PA) was 15.2%. Although the clinical use of carbapenems is quite low in Calabar, resistance to this very important class of antibiotics already exists. This calls for surveillance and the judicious use of carbapenems for only infections that defy all other treatment options.

Keywords: Antibiotic resistance, *Pseudomonas aeruginosa*, Healthcare-associated infections

I. Introduction

Healthcare-associated infections (HAIs) are a leading cause of morbidity and mortality worldwide. Treatment of HAIs has become very difficult due to the increasing antibiotic resistance among pathogens. According to a recent review, over the last decade, multidrug-resistant Gram-negative bacteria (MDR-GNB), including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and Enterobacteriaceae producing extended-spectrum- β -lactamases (ESBLs) and carbapenemases, have been increasingly recovered in severe HAIs [1]. *P. aeruginosa* readily colonizes hospitalized and immunocompromised individuals, although it rarely causes infection in immunocompetent persons [2], the resultant infections comprise about 10% of HAIs in the United States of America [3,4,5].

P. aeruginosa is the most common Gram-negative bacterium found in HAIs especially in patients who have been hospitalized longer than one week [6,7] and is reported in outbreaks of HAIs, like pneumonia, bacteremia, urinary tract infection (UTI) and endocarditis, in different parts of the world, which are often complicated and potentially life-threatening [5]. Wound infection due to *P. aeruginosa* is the primary cause of limb amputation in children [8] and outbreaks among burn patients is associated with death rates as high as 60% [9]. General risk factors for acquiring infections include being seriously ill, being hospitalized, having undergone invasive procedures, having a compromised immunity and therapy with broad spectrum antibiotics [4].

Hospital environments, particularly in ICUs, are common habitats for *Pseudomonas aeruginosa* [10]. The mode of transmission may include person-to-person, reservoir-to-person and colonization with subsequent auto-infection [2]. Spread may occur from patient-to-patient on the hands of healthcare workers and through direct patient contact with contaminated fomites [4]. The pathogenic success of *P. aeruginosa* is as a result of its array of virulence factors and its tendency to colonize surfaces in an intractable biofilm form, makes the

cells impervious to therapeutic concentrations of antibiotics [3,11]. It is innately tolerant to many antimicrobics and disinfectants because of its outer membrane permeability barrier [12]. In addition, it maintains resistance plasmids and is able to exchange same with other bacteria with which it lives as normal flora, through the mechanism of Horizontal Gene Transfer (HGT), particularly conjugation and transduction [3].

The introduction of carbapenems, example imipenem and meropenem, into clinical practice represented a great advance for the treatment of serious bacterial infections caused by beta-lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most beta lactamases, the carbapenems have been the drug of choice for treatment of infections caused by penicillin- or cephalosporin-resistant Gram-negative bacteria [13]. Recently in Calabar, there have been increased therapeutic failures to common antibiotics as well as mortality among patients with *Pseudomonas* infections. This research was conceived to investigate the antibiotic susceptibility and multi-drug resistance profiles of clinical isolates and hospital environment isolates of *Pseudomonas aeruginosa* with commonly used antibiotics as well as the carbapenems, which constitute last line therapeutic options in Calabar.

II. Materials And Methods

Collection of specimen

A total of 1000 clinical specimens were obtained from patients in four health facilities in Calabar Metropolis; University of Calabar Teaching Hospital (THC), General Hospital Calabar (GHC) and two privately managed clinics, one in each of the two Local Government Councils that make up Calabar Metropolis, Private Clinic 1 (PC1) and Private Clinic 2 (PC2) respectively. These specimens which included wound swabs from various sites such as ulcers, burns, sores and surgical wounds; respiratory secretions, eye and ear swabs, urine and blood were collected over a period of 10 months from February to November 2013. Ethical approval was obtained from Cross River State Ministry of Health. Another 75 samples were also collected from the hospital environment. These included liquid and semi-solid environmental samples which were collected directly, and solid environmental samples which were sampled using swab sticks from floors, sinks, drains, bed coverings, dispensing trays, bed pans and other hospital equipment handled by hospital staff and patients alike.

Processing of specimen

Swab samples and clean-catch, mid-stream urine samples were inoculated unto Blood agar (Oxoid), Cysteine Lactose Electrolyte Deficient agar (CLED) and *Pseudomonas* Isolation Agar (PIA) (Oxoid) using standard microbiological methods as recommended by American Society for Microbiology [14]. The plates were incubated at 37°C for 24 – 48 hours. The presence of $\geq 10^5$ colony forming units (CFU) per millilitre was considered clinically significant for diagnosis of UTI as recommended by World Health Organization [15]. About two milliliters of blood sample was dispensed into 10ml of tryptic soy broth (TSB) (BBL) in blood culture bottles and thoroughly mixed to prevent clotting. Aseptic technique was employed to prevent contamination. This was then incubated at 37°C for 48 hours. Positive growth was subcultured unto Blood Agar, CLED and PIA and incubated at 37°C for 48 hours.

Preliminary identification of isolates

The morphology of the colonies on pure culture was used in the preliminary identification of *P. aeruginosa* isolates. Colony characteristics such as size, colour, shape and consistency were observed. Other characteristics included the outline of the edges, pigment production on PIA, haemolysis on blood agar, no fermentation on CLED, positive oxidase reaction and production of a distinctive fruity odour. *P. aeruginosa* ATCC 27853 obtained from the Nigerian Institute of Medical Research (NIMR), Lagos, was used as control strain for all identification purposes.

Characterization of *P. aeruginosa* isolates

This was carried out using the Microgen GN A+B-ID system (Microgen Bioproducts, UK) which employs 24 standardized biochemical substrates in microwells to identify the family Enterobacteriaceae and other non-fastidious gram negative bacilli (oxidase positive and negative).

Principle of the test

The Microgen GN-ID system comprised two separate microwell test strips (GN A and GN B). Each test strip contained 12 standardised biochemical substrates which had been selected on the basis of extensive computer analysis of published databases for the identification of the family Enterobacteriaceae and commonly encountered non-fastidious oxidase positive and negative gram negative bacilli [14]. The dehydrated substrates in each well were reconstituted with a saline suspension of the organism to be identified. If the individual substrates were metabolized by the organism, a colour change occurred during incubation or after addition of specific reagents. The permutation of metabolized substrates were interpreted using the Microgen Identification

System Software (MID-60) which provided identification based on percentage probability and likelihood with an analysis of the quality of differentiation (Microgen Bioproducts, UK). The Microgen GN A+B-ID kit contained additional requirements which included; mineral oil, Kovac's reagent, Voges-Proskauer I and VP II reagents, Nitrate A and B reagents, TDA reagent, Colour chart for reading results and result forms. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as positive and negative controls.

Test procedure

Oxidase test was carried out on the isolate to be identified after which a single colony from an 18 – 24 hour culture was emulsified in 3 – 5 ml sterile 0.85% saline and mixed thoroughly. The adhesive tape sealing the microwell test strip was carefully peeled back, but not discarded as they were required later. Using a sterile Pasteur pipette, 3 – 4 drops of the bacterial suspension were added to each well of the microwell test strips. After inoculation, wells 1,2,3 and 9 (GN A) and well 24 (GN B) were overlaid with 3 – 4 drops of mineral oil. These wells were highlighted with a black circle to assist in decision making in respect of oil overlays. The top of the microwell test strips were sealed with the adhesive tape removed earlier and incubated at 35 – 37°C. They were read after 18 – 24 hours incubation for Enterobacteriaceae and after 48 hours for oxidase positive isolates. The adhesive tape was removed and all positive reactions were recorded on the result forms provided with the aid of the colour chart. Appropriate reagents were added to specified wells according to the manufacturer's instructions and results read after 60 seconds.

Identification of isolates

Identification of *P. aeruginosa* isolates was based on their observed characteristics compared with the characteristics of reference *P. aeruginosa* as published [14,16]. On the Microgen GN A+B-ID report form, the substrates were organized into triplets (sets of 3 reactions) with each substrate assigned a numerical value (1, 2 or 4). The sum of the positive reactions for each triplet formed a single digit of the Octal code that was used to determine the identity of the isolate. The Octal code was entered into the Microgen Identification System Software (MID-60 Ver 1.2.5.26), which generated a report of the five most likely organisms in the selected database.

Antimicrobial susceptibility testing of *P. aeruginosa* isolates

The modified disc diffusion method of Bauer and Kirby was used to determine susceptibility of *P. aeruginosa* isolates to the following antibiotics: imipenem (10µg), meropenem (10µg), doripenem (10µg), ciprofloxacin (5µg), amikacin (30µg), ceftazidime (30µg), ceftriaxone (30µg), cefepime (30µg), gentamicin (10µg), tobramycin (10µg), carbenicillin (100µg), aztreonam (10µg), (Hardy Diagnostics, Santa Maria, USA). Mueller-Hinton Agar (Oxoid) medium was used for the test as recommended by CLSI [17].

Determination of multiple antibiotic resistance (MAR) index

The Multiple Antibiotic Resistance (MAR) index was determined for *P. aeruginosa* isolates in each hospital by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested [18-19].

$$\text{MAR index} = \frac{\text{Number of antibiotics isolate is resistant to}}{\text{Total number of antibiotics tested}}$$

III. Statistical Analysis

Data generated in the course of this study was analyzed using statistical package software (SPSS version 15.0) for test of significance. Results were presented as percentages unless otherwise indicated. Relationships at a P-value of less than 0.05 (P<0.05) was considered statistically significant.

IV. Results

The summary of the distribution of *P. aeruginosa* isolates according to the source of clinical specimen and hospital is shown on Table 1. A total of 197 isolates were recovered. Overall, pus/wound swab produced the highest number of *P. aeruginosa* isolates 83(42.1%) followed by ear swab 53(26.9%); surgical wound swab 24(12.2%); eye swab 14(7.1%); respiratory secretions 12(6.1%); mid-stream urine 7(3.6%) and blood specimens 4(2.0%) in that order. Table 2 presents the rate of isolation of *P. aeruginosa* according to the source of environmental and inanimate specimens from the four hospitals. Twelve (46.2%) isolates were recovered from the floors, sinks and drains yielded 8(30.8%) isolates while disinfecting liquids/handwash yielded 2(7.7%) isolates. Three (11.5%) isolates were recovered from water storage containers in two hospitals, THC and GHC whereas only one (3.8%) isolate was recovered from drug dispensing tray in THC. Beddings did not yield any isolate.

The results of in vitro activity of the twelve antibiotics tested against 197 clinical *P. aeruginosa* isolates are displayed on Table 3. Analysis of the results showed varying degrees of resistance among isolates. The isolates exhibited highest resistance to the cephalosporins while the carbapenems were >80% effective (Fig. 1). Thirty (15.2%) isolates were carbapenem-resistant. The antibiotic susceptibilities of the environmental *P. aeruginosa* isolates were also tested using the same antibiotics as for the clinical isolates. Table 4 shows the resistance profiles of the isolates, 26 of them from the four health care facilities. None of the isolates was resistant to the carbapenems and the monobactam, aztreonam. Of the cephalosporins, cefepime was most active against the isolates. The isolates showed varying degrees of resistance to other antibiotics. However, the resistance rates were low. Data on Table 5 shows that *P. aeruginosa* isolates from GHC had MAR index of 0.5 (6/12). The result was similar to what was obtained for both PC1 and PC2 while that of THC was slightly higher at 0.6 (7/12) which was not significant ($P>0.05$).

V. Discussion

This study carried out in Calabar Metropolis, Cross River State in Nigeria has demonstrated the occurrence of significant levels of infections due to *Pseudomonas aeruginosa* in the health care facilities surveyed. The overall prevalence of 19.7% was recorded for *P. aeruginosa* infections in this study (Table 1). This figure is higher than those reported in other studies conducted in parts of Nigeria and other climes [20-23]. The variations in *P. aeruginosa* prevalence may be due to differences in geographical location, differences in study population, number of specimens, exposure to broad spectrum antibiotics and contact with hospital setting [24]. Rice and coworkers [25] suggested that such factors may contribute to acquisition and establishment of *P. aeruginosa* infection in hospitals. The clinical specimens with the highest frequency of *P. aeruginosa* isolation were pus/wound swabs (83 isolates) ear swabs (53 isolates) and surgical wound swabs (24 isolates) (Table 1). These results agree with the findings of Basak et al. [26] where pus and wound swab yielded the highest number of *P. aeruginosa* strains in that study. To begin an infection, the organism often requires a substantial break in first-line barriers. Such a break may be as a result of bypass or breach of normal cutaneous or mucosal barriers example trauma, surgery, serious burns or indwelling devices [3]. In this study, a significant number of patients with wound infections had burns or skin lesions discharging pus. These conditions may have contributed to the significant level of *P. aeruginosa* isolated from this group of patients.

Analysis of antibiotic susceptibility test results revealed high level resistance to a significant number of antibiotics by *P. aeruginosa* isolates. Overall, more than 70% of isolates tested were resistant to ceftriaxone, ceftazidime and carbenicillin while over 50% were resistant to cefepime, ciprofloxacin, tobramycin and gentamicin (Table 3 & Table 4). The most effective agents in this study were the carbapenems (percentage mean resistance <7.0) followed by aztreonam (percentage mean resistance, 23.6) (Fig. 1). The multidrug resistance pattern observed is consistent with reports of Olayinka et al. [19] who suggested the importance of instituting a system for antimicrobial resistance surveillance. MDR criteria in this study was defined as non-susceptibility to at least three of five drug groups including the antipseudomonal penicillins, cephalosporins, aminoglycosides, fluoroquinolones and carbapenems [27-28]. Numerous studies have demonstrated that previous exposure to antimicrobial drug is a serious risk factor for colonization and infection by drug resistant bacteria [29-33]. Fluoroquinolones and third-generation cephalosporins have been implicated often in promoting the spread of MDR-bacteria [1]. The high level resistance of *P. aeruginosa* isolates to the cephalosporins observed in this study is alarming. Over 70% of isolates were resistant to this antibiotics class. This high resistance level may be explained by the indiscriminate empirical use of broad-spectrum antibiotics (cephalosporins) and the non-availability of data to inform medical personnel about bacterial resistance patterns within their institution in the study area, resulting in continuous use of these drugs. Pinheiro et al. [31] linked inappropriate initial treatment directly to bacteria resistance. Other studies also reinforced the idea that the empirical treatment adopted in hospital routine induced selective pressure of multidrug resistant strains resulting in treatment failure [29-30]. In contrast, the *P. aeruginosa* isolates in this study were significantly susceptible ($P<0.05$) to carbapenems (imipenem, meropenem and doripenem) and aztreonam. All environmental and >80% of clinical isolates were susceptible to these antibiotics. These findings agree with those of other researchers [20,26,33] and may be due to the fact that these group of antibiotics are relatively new to Nigeria, costly and are not widely available for abuse and misuse in this part of the world. Thirty carbapenem resistant isolates were identified. Carbapenem resistance, in this study, was defined as non-susceptibility to at least one of the three carbapenems tested [34]. The highest resistance rate to carbapenem was recorded for meropenem (mean 6.7%) followed by imipenem (mean 5.9%) and doripenem (mean 5.4%). This trend is consistent with the observations of other studies [34-35] which also observed greater meropenem resistance in Thailand.

Analysis of the MAR index of the *P. aeruginosa* strains showed that all of them had MAR index above 0.2 (Table 5). MAR index higher than 0.2 has been said to be an indication of isolates originating from an environment where antibiotics were often used [18]. The practical significance of the index may be lost in Nigeria, where antibiotic abuse and misuse is common place, since the cut off point was determined in countries

with tight antibiotic control protocols [20]. The MAR values may however be viewed as an indication of the extent of microbial exposure to antibiotics in our hospitals.

In conclusion, the prevalence of *P. aeruginosa* infections in the various hospitals ranged from 16.6 % to 29.0% with overall prevalence of 19.7%. On the other hand, prevalence of carbapenem-resistant *P. aeruginosa* (CR-PA) was 15.2%. Although the clinical use of carbapenems is quite low in Calabar, resistance to this very important class of antibiotics already exists. This calls for surveillance and the judicious use of carbapenems for only infections that defy all other treatment options. The occurrence of significant multidrug-resistant *P. aeruginosa* (MDR-PA) and carbapenem-resistant *P. aeruginosa* (CR-PA) in Calabar, as observed in this study, will have serious implications on chemotherapy and clinical prognosis of *P. aeruginosa* infections if appropriate control measures are not implemented.

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TABLE 1: Isolation rate of *P. aeruginosa* according to hospital and source of clinical specimens

Specimen source	Hospital/No. of isolates				Total No. (%) ^a of <i>P. aeruginosa</i> isolates
	THC	PC1	GHC	PC2	
Eye swab	9	0	1	4	14(7.1)
Ear swab	27	7	13	6	53(26.9)
Respiratory secretions	5	2	2	3	12(6.1)
Blood	3	1	0	0	4(2.0)
Mid-stream urine	4	1	1	1	7(3.6)
Pus/wound swab	33	14	18	18	83(42.1)
Surgical wound swab	12	2	7	3	24(12.2)
Total	93	27	42	35	197(100)

^aPercentage is based on number of *P. aeruginosa* isolates

TABLE 2: Distribution of *P. aeruginosa* isolates from four hospitals in Calabar according to inanimate source of specimen

Specimen source	THC	PC1	GHC	PC2	Total (%)
Floor	5	1	3	3	12(46.2)
Beddings	0	0	0	0	0(0)
Drug dispensing tray	1	0	0	0	1(3.8)
Sinks/drains	3	1	2	2	8(30.8)
Disinfecting liquid/ handwash	1	0	1	0	2(7.7)
Water storage containers	2	0	1	0	3(11.5)
Total	12	2	7	5	26(100)

TABLE 3: Antibiotic resistance profiles of *P. aeruginosa* clinical isolates from four hospitals in Calabar

Hospital	No. of Isolates tested	Resistance rates to the indicated antibiotic (%)											
		AKN (30µg)	CB (100µg)	CIP (5µg)	NN (10µg)	CAZ (30µg)	CPM (30µg)	CRO (30µg)	GEN (100µg)	ATM (5µg)	IPM (10µg)	MEM (30µg)	DOR (30µg)
GHC	42	12(28.6)	32(76.2)	17(40.5)	25(59.5)	36(85.7)	21(50.0)	40(95.2)	23(54.8)	12(28.6)	2(4.8)	4(9.2)	3(7.1)
PC1	27	8(29.6)	18(66.7)	10(37.0)	15(55.6)	20(74.1)	15(55.6)	22(81.5)	14(51.9)	5(18.5)	2(7.4)	1(3.7)	1(3.7)
THC	93	27(29.0)	64(68.8)	47(50.5)	52(55.9)	68(74.1)	49(52.7)	77(82.8)	51(54.8)	26(28.0)	4(4.3)	9(9.7)	5(5.4)
PC2	35	15(42.9)	23(65.7)	25(71.4)	21(60.0)	28(80.0)	17(48.6)	21(60.0)	22(62.9)	8(22.9)	2(5.7)	3(8.6)	2(5.7)

AKN = Amikacin, CB = Carbenicillin, CIP = Ciprofloxacin, NN = Tobramycin, CAZ = Ceftazidime, CPM = Cefepime

CRO = Ceftriaxone, GEN = Gentamicin, ATM = Aztreonam, IPM = Imipenem, MEM = Meropenem, DOR = Doripenem

Susceptibility was determined in accordance with CLSI breakpoints.

TABLE 4: Antibiotic resistance profiles of *P. aeruginosa* environmental isolates from four hospitals in Calabar

Hospital	No. of Isolates tested	Resistance rates to the indicated antibiotic (%)											
		AKN (30µg)	CB (100µg)	CIP (5µg)	NN (10µg)	CAZ (30µg)	CPM (30µg)	CRO (30µg)	GEN (100µg)	ATM (5µg)	IPM (10µg)	MEM (30µg)	DOR (30µg)
GHC	7	1(14.3)	2(28.6)	3(42.9)	2(28.6)	2(28.6)	0	2(28.6)	2(28.6)	0	0	0	0
PC1	2	0	1(50.0)	1(50.0)	0	1(50.0)	0	1(50.0)	0	0	0	0	0
THC	12	3(25.0)	4(33.3)	5(41.7)	3(25.0)	4(33.3)	2(16.7)	3(25.0)	5(41.7)	0	0	0	0
PC2	5	2(40.0)	2(40.0)	0	1(20.0)	2(40.0)	1(20.0)	2(40.0)	2(40.0)	0	0	0	0

AKN = Amikacin, CB = Carbenicillin, CIP = Ciprofloxacin, NN = Tobramycin, CAZ = Ceftazidime, CPM = Cefepime
 CRO = Ceftriaxone, GEN = Gentamicin, ATM = Aztreonam, IPM = Imipenem, MEM = Meropenem, DOR = Doripenem

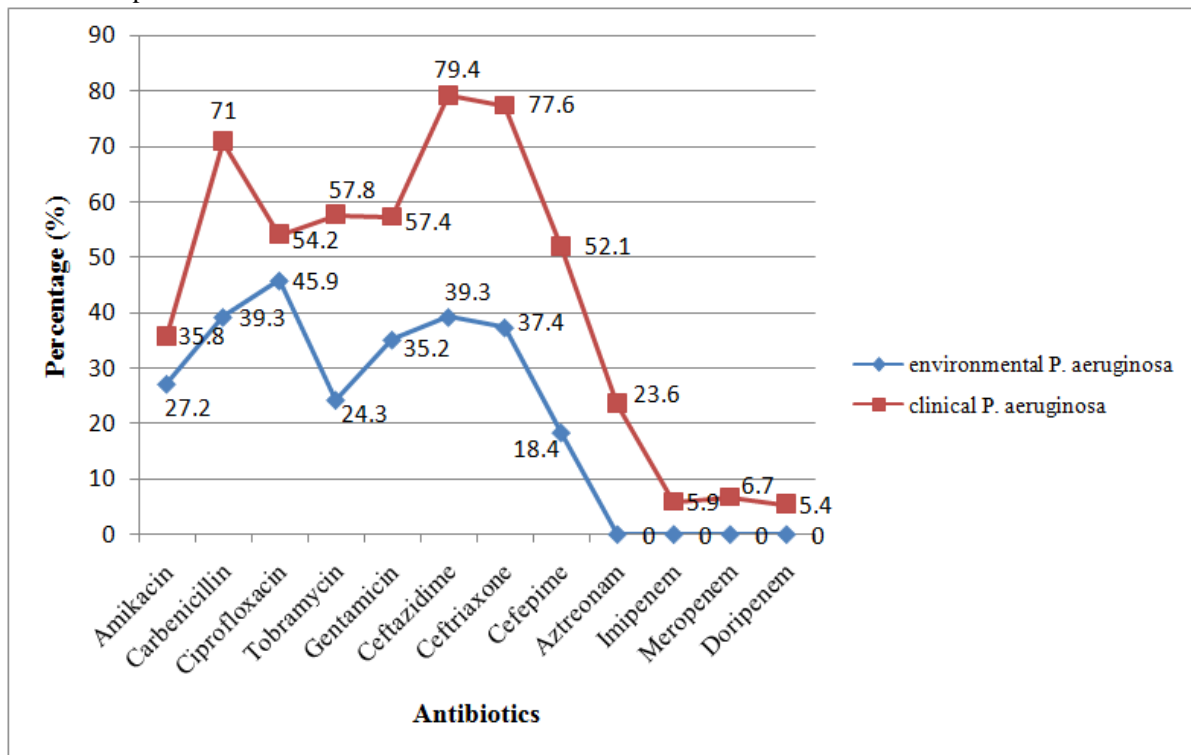


FIG. 1: Percentage mean resistance to antibiotics of clinical and environmental *P. aeruginosa* isolates from four hospitals in Calabar

TABLE 5: Multiple antibiotic resistance index of *P. aeruginosa* clinical isolates from hospitals in Calabar

Hospital	No. (%) ^a of isolates	MAR index ^b
GHC	42(21.3)	6/12 (0.5)
PC1	35(17.8)	6/12 (0.5)
THC	93(47.2)	7/12 (0.6)
PC2	27(13.7)	6/12 (0.5)
TOTAL	197	

^aPercentage is based on number of *P. aeruginosa* isolates

^bMAR index is based on number of antibiotics to which isolates are resistant divided by the number of antibiotics tested.