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Identification of Antibiotics Susceptibility Patterns at Sub-inhibitory Concentrations in *Pseudomonas aeruginosa*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Pseudomonas aeruginosa is a Gram negative bacterium that has been recognized as an opportunistic pathogen. It is the most common bacterium associated with nosocomial infections and ventilator-associated pneumonia. It exhibits high innate resistance to various ranges of antibiotics thereby causing high morbidity and mortality rate. This research was aimed to identify the antibiotics susceptibility patterns at sub-inhibitory concentration in *Pseudomonas aeruginosa*. One hundred and fifty (150) clinical swab specimens were collected from urinary catheters; wound and ear infections of patients and the swabs inoculated using standard microbiology method. The isolates were characterized based on the bacteriological methods such as morphology and biochemical tests. The isolates were further confirmed by species specific by PCR amplification of 16S rRNA

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and the amplicons were analyzed by gel electrophoresis; and further genomic sequencing was done and blast with NCBI database mining. The antimicrobial susceptibility of the isolates was done by disc diffusion methods. The result of the isolation showed 22(59.46%) from wound infections, 12(32.43%) from ear infections and 3(8.11%) from urinary catheter. The isolates were Gram-negative, produced β-hemolysis on blood agar and the morphology is small pigmented circular. The isolates showed positive results to catalase, oxidase, citrate, nitrate and indole tests. The amplification of the 16S rRNA gene region resulted in the band size of 1500bp PCR product and the BLAST analysis gave 99% similarity. The results of susceptibility analysis showed that the isolates from the urinary catheter, wound and ear infection were 82%, 68% and 47% respectively, resistance to Piperacillin tazobactem, Cefoperazon, Ofloxacin, Tetracycline, Amikacin, Gentamycin, Bacitracin, Clarithromycin, Cefalotin, Levofloxacin and Cefpiroma. Antimicrobial susceptibility tests in *P. aeruginosa* isolates revealedshowed that they were multi-drug resistant. At sub-inhibitory concentrations of antibiotics within the microbial environment, *Pseudomonas aeruginosa* becomes more resistant. Perhaps, these antimicrobials could have other signaling activities within the environment. Therefore, there is a need for more research work to develop therapeutics combination to combat the recalcitrant nature of *Pseudomonas aeruginosa.*

Keywords: Pseudomonas aeruginosa; antibiotics; susceptibility; bacteria.

1. INTRODUCTION

Overuse of antibiotics has resulted in pathogenic bacteria developing a broad resistance. This resistance, along with the slow development of new medicines, has caused a crisis in the management of numerous diseases that were once easily treatable. Innovative medications that successfully target microbial life have been created, but for the vast majority of these substances, only the effects on growth inhibition at the maximum inhibitory concentration (MIC) were thoroughly investigated. Unanswered concerns include how small antibiotic concentrations influence bacterial existence, but more importantly, is it possible for the drugs to perform additional functions at sub-inhibitory concentrations (SIC)? According to the top studies on the reactions of different significant human infections, antibiotics have an effect on bacterial gene expression at sub-inhibitory concentrations and they also interrupt bacterial cell-cell communication. These genes are involved in virulence determinant factors, biofilm formation, stress response, colonization, survival response and as well as motility. This was supported by Diego et al. [1] research work which has given rise to the hypothesis that these small amounts of antibiotics exist in the natural environments.

P. aeruginosa, a Gram-negative bacterium, lives in both biotic and abiotic environments. They are a significant human pathogen that is resistant to a number of drugs that could possibly kill other bacteria. *P. aeruginosa* is a significant source of bacteremia in burn patients, UTIs in patients using catheter and pneumonia contracted from the hospital especially patients using respirators. *P. aeruginosa* can colonize the lungs for an extended period of time in cystic fibrosis patients because of damaged epithelia, which results in morbidity and mortality. Due in part to the bacterium's built-in antibiotic resistance, these infections are nearly tough to treat and ultimately result in lung failure and death [2].

Pseudomonas aeruginosa was identified in 2014 as the third-highest cause of healthcare infections in hospitals by the National Healthcare Safety Network (NHSN). Additionally, *Pseudomonas aeruginosa* was said to be responsible for 51, 000 infections annually in the United States, according to Weiner et al. [3]. *Pseudomonas aeruginosa* has been isolated from urine, the female reproductive organ, and wound swabs in Nigeria, according to a South-west research. In another study from the north, *Pseudomonas aeruginosa* was discovered in urine samples. In a separate south-west study, *Pseudomonas aeruginosa* was isolated from 41.9% of ear swabs and 39.3% of wound swabs. *Pseudomonas aeruginosa* from the south-south was isolated from 41% of people who had ears that discharged [4].

Pseudomonas aeruginosa treatment is extremely difficult due to the bacteria's multi-drug resistance. According to the World Health Organization, it is crucial to discover novel treatments for *P. aeruginosa* infections in order to effectively treat the infections [5]. Hence, in order to treat this infection, there is need to identify the antibiotics susceptibility patterns at

sub-inhibitory concentration in *Pseudomonas aeruginosa* and this present study tends to address it.

2. MATERIALS AND METHODS

2.1 Study Area

The research was conducted at the University of Abuja Teaching Hospital in the Gwagwalada Area Council of the Federal Capital Territory of Abuja (N 8º 57' 1.4976", E 7º 3' 45.4212), Nigeria.

2.2 Sample Collection

The casualty and surgical wards of the Teaching Hospital were the source of 150 clinical samples from various sexes. The samples came from the urinary catheter, burns on the skin, and ear infections. According to Perry [6] adopted method for the recovery live bacterial pathogen at 4ºC and nutrient agar inoculation and incubations at 37ºC for 48hours; all swab samples were kept at 4ºC, which is better for recovering live bacterial pathogens. The clinical specimens were swiftly injected in nutrient agar and incubated overnight at 37°C for 48 hours aerobically.

2.3 Isolation, Identification and Biochemical Characterization of *Pseudomonas aeruginosa*

The method described by Amutha & Kokila [7] was used to evaluate and classify the growth mediums, three isolates, one from each clinical sample source, were randomly chosen for characterization based on morphological and biochemical tests like Gram stain characteristics, methyl and nitrate test, oxidative-fermentation test, catalase, citrate and oxidase tests, and indole production (Table 3).

2.4 Extraction of Genomic DNA

The phenol-chloroform kit was used to perform the DNA extraction technique. The steps were completed in accordance with the kit's instructions. 200µl of the sample was added into a 1.5ml tube. 400 µl of lysis buffer was added to the sample contained in the 1.5ml tube and 10µl of proteinase K was added to the tube, and incubate at 55°C for 10-60 minutes. 400µl 0f equilibrated phenol (pH 7.8) was added, vortexed, and centrifuged at 12000rpm for 5minute. Supernatant was taken and 700µl of Chloroform was added: Isoamyl alcohol (24:1) (672µl Chloroform: 28µl Isoamyl alcohol) mixed well and centrifuged at 12000rpm for 5 minute and the layer was transferred to a fresh tube. 40µl of 3M sodium acetate (PH 5.2) was added. 400µl of 100% ethanol was added and incubate for a minimum of 1hour. It was centrifuged for 15 minutes at 4°C at 14000rpm to pellet the DNA. Supernatant was carefully removed without disturbing the DNA pellet.150ul of 70% cold ethanol was added and sample was centrifuged at 4°C for 2 minutes at 14000rpm and the supernatant was discarded. The DNA pellet was dried at room temperature for 5-10 minutes. The DNA pellet was resuspended in 100 µl of TE buffer or molecular grade water by pipetting up and down. It was centrifuged briefly to collect the sample and the tube was placed on ice (Plate 1)

2.5 PCR Assay

For molecular confirmation of the isolates validated by conventional test, PCR Assay was carried out. All of the isolates that were presumed to be *P. aeruginosa* by morphological and chemical testing had their species identity further confirmed by PCR. The 16S rRNA gene was amplified. The primers 27F-AGAGTTTGATCCTGGCTCAG and 1492R 5'-GGTTACCTTACGACTT-3' were used. According to the kit instructions, in a final volume of 20µl, the PCR was conducted using the following master mix parameters: Master Mix (10µl), MGW (3µl), Primers (F/R) (2µl), and DNA Template (5µl). The denaturation of the template occurred over 40 cycles of 30 seconds each at 94°C during the PCR process, 40 seconds at 50°C for annealing, and 1 minute at 72°C extension prior to 10 minutes of final extension at 72°C for 40 cycles (Plate 1).

2.6 Analysis of PCR Products (amplicons)

The results of the PCR reaction were analyzed using trisborate-EDTA buffer (pH = 8.0), and the amplicons were then subjected to 1.5% agarose gel electrophoresis. The agarose gel was next stained with ethidium bromide and examined using a UV transilluminator device running at 120V for 30 minutes. The estimated size of the amplicons was established by comparing the size of the amplifications (PCR products) to a pertinent DNA ladder of 25/100bp [8].

2.7 Genomic Sequencing

The genomic sequencing of *Pseudomonas aeruginosa* was carried out and NCBI database mining was used to identify the strain [9] Fig. 1.

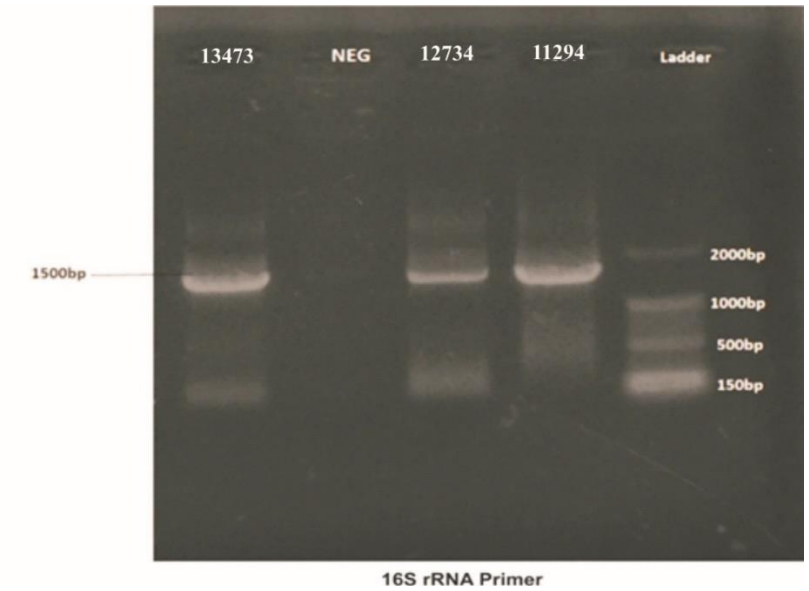

Plate 1. An electrophoregram of 16S rRNA of *Pseudomonas aeruginosa* samples separated on agarose gel electrophoresis stained by ethidium bromide. The amplification of DNA appears as a lane-like pattern. Lane (ladder) DNA Marker (150bp), lane (13473, 12734, 11294) represent positive Isolates and lane (N) the negative control

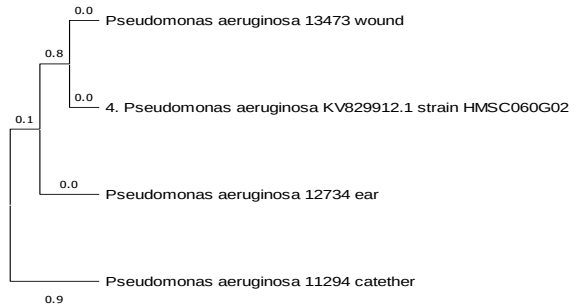

Fig. 1. Phylogenetic tree of Pseudomonas aeruginosa from different sources using the Neighbor-Joining method (Mega 11 software)

2.8 Antimicrobial Susceptibility

Disk diffusion technique was used to determine antibiotic susceptibility, and it was carried out in accordance with Clinical Laboratory Standard Institute (CLSI) standards. According to SIC interpretation Standard, a commercially available antimicrobial disc was used to test against *Pseudomonas aeruginosa* isolates. This disc contained known antibiotics such as Piperacillin tazobactam, Cefoperazon, Ofloxacin, Tetracycline, Amikacin, Gentamycin, Bacitracin, Clarithromycin, Cefalotin, Levofloxacin, and Cefpiroma. They were kept in an environment (candle jar) that was enhanced with 5-10% CO_2 for 48 hours at 37°C. It was measured how wide the zone of inhibition was. According to the CLSI recommendations on the measurement of zone of inhibition [10] the results were recorded by classifying the zone of inhibitions as sensitive (S) or resistant (R) (Tables 4, 5 and 6).

3. RESULTS

3.1 The Clinical Specimens

A total of 150 clinical specimens were received from casualty and the surgical wards from

Table 1. Demography of the clinical specimens and the number of *P. aeruginosa* obtained from the different age group and catheter from University of Abuja Teaching Hospital, Nigeria

Age	No of clinical samples (%) N= 150		No of *Pseudomonoas a*. spp N=37	
	Sample No	(%)	Sample No	(%)
<10	6	4.00	6	16.22
11-20	20	13.33	4	10.81
21-30	50	33.33	2	5.41
31-40	32	21.33	3	8.11
>40	27	18.00	19	51.35
Catheter	15	10.00	3	8.11

Table 2. Distribution of clinical isolates from the sample's sources and the percentage gender distribution in UATH, Nigeria

Sources	No of the isolates (%) N=37	Gender	No of the isolate by gender (%) N=34
Wound swabs	22 (59.46)	Male	22(64.71)
Ear swabs	12(32.43)	Female	12(35.29)
Urinary Catheter	3(8.11)		

Table 3. Morphological and biochemical identification of the isolates

S/N	Source of Isolate	Morphology	Gram Reaction	Oxidase	Catalase	Indole	Citrate Test	Methyl Red	Nitrate
1.	Wounds	Small Pigmented circular	Gram -ve Rods	+	+	+	+	-	+
2.	Ear	Small Pigmented circular	Gram -ve Rods	+	+	+	+	-	+
3.	Catheter	Small Pigmented Circular	Gram -ve Rods	+	+	+	+	-	+

Key: + (Positive) – (Negative)

Table 4. Antibiotics susceptibility Test of *Pseudomonas aeruginosa* (n=3) isolates from hospital urinary catheter from UATH Abuja, Nigeria

Antibiotics	% Sensitivity	% Resistance	No of Isolates
Piperacillin tazobactem	1 (11.1)	2 (88.9)	3
Cefoperazone	1(19.1)	2 (80.9)	3
Ofloxacin	1 (8.7)	2 (91.3)	3
Tetracycline	1 (33.3)	2 (66.7)	3
Amikacin	1 (10.5)	2 (89.5)	3
Gentamycin	1 (5.3)	2 (94.7)	3
Bacitracin	0 (0.0)	3 (100.0)	3
Clarithromycin	1 (30.0)	2 (70.0)	3
Cefalotin	1 (38.0)	2 (62.0)	3
Levofloxacin	1 (28.0)	2 (72.0)	3
Cefpirome	1 (14.3)	2 (85.7)	3

Table 5. Antibiotics Sensitivity Test of *Pseudomonas aeruginosa* (n=22) isolate from patient wound UATH Abuja, Nigeria

Antibiotics	%Sensitivity	%Resistance	No of isolates
Piperacillin tazobactem	6 (33.3)	16 (66.7)	22
Cefoperazone	10 (41.0)	12 (59.0)	22
Ofloxacin	7 (39.1)	13 (60.9)	22
Tetracycline	4 (33.3)	18 (66.7)	22
Amikacin	8 (36.8)	14 (63.2)	22
Gentamycin	8 (36.8)	14 (63.2)	22
Bacitracin	14 (60.0)	8 (40.0)	22
Clarithromycin	0 (0.0)	22 (100.0)	22
Cefalotin	0 (0.0)	22 (100.0)	22
Levofloxacin	10 (44.0)	12 (56.0)	22
Cefpirome	4 (28.6)	18 (71.4)	22

Table 6. Antibiotics Sensitivity Test of *Pseudomonas aeruginosa* (n=12) isolates from patient ear infections UATH Abuja, Nigeria

Antibiotics	%Sensitivity	%Resistance	No of isolates
Piperacillin tazobactem	7 (55.6)	5 (44.4)	12
Cefoperazone	6 (50.0)	6 (50.0)	12
Ofloxacin	7 (52.2)	5 (47.8)	12
Tetracycline	4 (33.3)	8 (66.7)	12
Amikacin	7 (52.6)	5 (47.4)	12
Gentamycin	7 (57.9)	5 (43.1)	12
Bacitracin	4 (40.0)	8 (60.0)	12
Clarithromycin	9 (70.0)	3 (30.0)	12
Cefalotin	8 (63.0)	4 (38.0)	12
Levofloxacin	5 (48.0)	7 (52.0)	12
Cefpirome	7 (57.1)	5 (42.9)	12

different age groups and urinary catheter from University of Abuja Teaching Hospital between June, 2021 and August, 2022 and a total of 37 isolates of *Pseudomonas aeruginosa* were identified from the specimens (Table1).

The three sources of the clinical specimens such as urinary catheter tip swabs, wound swabs and ear infection swabs showed 3(8.11%), 22(59.46%) and 12(32.43%) respectively and the percentage gender distribution of the isolates showed 22(64.71%) from male patients and 12(35.29%) from the female patients (Table 2).

3.2 Morphological and Biochemical Identification of the Isolate

Table 3 shows the morphological and biochemical characters which identify the isolates. The isolates area small pigmented circular, which shows positive, results to catalase, oxidase, indole, citrate and nitrate testes; negative test to methyl red test.

3.3 PCR Amplification of 16s rRNA Gene

At the molecular level, *Pseudomonas aerugi*nosa was recognized and validated. The band size of the amplification of the 16S rRNA gene region is 1500bp (Plate 1). According to the BLAST analysis, 98 sequences from the NCBI data exhibited 99% similarity. The phylogenetic tree produced by the NCBI program shows that this organism is related to other species genetically.

3.4 Antimicrobial Susceptibility Patterns

Antimicrobial susceptibility pattern of isolate from catheter swabs: The isolates from urinary catheter showed 82% resistance to Piperacillin tazobactem, Cefoperazon, Ofloxacin, Tetracycline, Amikacin, Gentamycin, Bacitracin, Clarithromycin, Cefalotin, Levofloxacin and Cefpiroma and 18% sensitive to the same antibiotics (Table 4).

Antimicrobial susceptibility pattern of isolate from wound swabs: The isolates from wound

infections showed 68% resistance to Piperacillin tazobactem, Cefoperazon, Ofloxacin, Tetracycline, Amikacin, Gentamycin, Bacitracin, Clarithromycin, Cefalotin, Levofloxacin and Cefpiroma and 32% sensitive to the same group of antibiotics (Table 5).

Antimicrobial susceptibility pattern of isolate from ear swabs: The isolates from ear infections showed 47% resistance to Piperacillin tazobactem, Cefoperazon, Ofloxacin, Tetracycline, Amikacin, Gentamycin, Bacitracin, Clarithromycin, Cefalotin, Levofloxacin and Cefpiroma and 52.6% sensitive to the same group of antibiotics (Table 6).

3.5 Evolutionary Relationships of Taxa

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. (Next to the branches). The evolutionary distances were computed using the Poisson correction method and are in the units of the number of nucleotide substitutions per site. This analysis involved 4 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 282 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [9].

4. DISCUSION

The results of the isolates obtained from the clinical specimens showed that wound specimens have greater number of solates in comparison with other specimens. Garba et al. [11], stated that this is not unexpected given that wounds, particularly those caused by thermal burns, have vast exposed areas of dead tissues devoid of any defenses, making them prime locations for infection by bacteria from the environment or natural microbiota.

Pseudomonas aeruginosa isolates are more prevalent in adults than in children, according to the demographic profile results from the clinical isolates. The result obtained was in line with the study of Iregbu & Eze [12], who stated that *Pseudomonas aeruginosa* isolates were 185(70%) in adults in comparison with the children. Due to their weakened immune system and higher frequency of comorbid conditions like diabetes and cardiovascular disease, older persons are typically at a higher risk of contracting nosocomial infections, especially MDR infections [13].

Additionally, more isolates *Pseudomonas aeruginosa* were found in males than in females. This is in line with findings from international research that demonstrated that males are more likely than females to be infected with Gram-negative bacteria and to develop antibiotic resistance to those infections, including *P. aeruginosa* [14]. Due to physiological reasons relating to sex chromosomes and hormones, men are typically more prone to bacterial infections. Additionally, behavioral variations between men and women, such as smoking rates or treatment compliance, may contribute to the discrepancy between men and women [15].

Pseudomonas aeruginosa was identified using both morphological and biochemical traits; however biochemical traits are shared by morphologically related species. The 16S rRNA sequence provided data that was helpful in matching the correct taxonomy and 99% similarity was found in the NCBI BLAST study, which confirms *Pseudomonas aeruginosa* as a molecular level organism. This is supported by the international study which stated that Species with comparable morphologies share biochemical characteristics and the 16S rRNA gene sequence, which is extremely conserved, aids in our ability to anticipate the correct classification [7].

The isolates from urinary catheters, wounds, and ear samples had antibiotic resistance patterns that demonstrated multidrug resistance to bacitracin (polypeptide), gentamicin (aminoglycosides), ofloxacin (fluoroquinolones), amikacin (aminoglycosides), Piperacillin tazobactem (Beta-lactem), cefpirome (Beta-lactem), cefoperazone (Beta-lactem), levofloxacin (quinolone), clarithromycin (macrolides), tetracycline (tetracyclines), and cefalotin (cephalosporin).When compared to wound and ear infections, urinary catheter isolates had a higher percentage of (MDR) multidrug resistant, which may be the result of indiscriminately using subpar disinfectants that build up at sub-inhibitory concentrations and cause biofilm formation. However, it has been demonstrated in other trials that using indwelling catheters increases the risk of infection [16]. This is also supported by the local and international studies which stated that due to the few available treatments, increased *Pseudomonas aeruginosa* resistance to third-generation antibiotics posed a serious threat to patient care. *P seudomonas aeruginosa* isolated from diverse clinical samples has become increasingly resistant to antibiotics,

including aminoglycosides, fluoroquinolones, quinolones, macrolides, tetracyclines, and polypeptide antibiotics, which are β-lactamase inhibitors [11] and the synthesis of metallo-lactamases (MBL), which can be chromosomally encoded or facilitated by plasmids, may also be the cause of the resistance [17].

5. CONCLUSION

According to the research methodologies, *Pseudomonas aeruginosa* was isolated from clinical samples taken from urinary catheters, wounds, and ear infections. The most precise approach for detecting *Pseudomonas aeruginosa* is by PCR (16S rRNA gene amplification) and the agarose gel of the band size of the amplicons is 1500bp. *P. aeruginosa* isolates antimicrobial susceptibility tests showed that these isolates were multi-drug resistant. Therefore, at sub-inhibitory concentration of antibiotics within the microbial environment, *Pseudomonas aeruginosa* becomes more resistant. Perhaps, these antimicrobials could have other signaling activities within the environment. Therefore, there is a need for more research works to develop therapeutics combination to combat the recalcitrant nature of *Pseudomonas aeruginosa.*

6. RECOMMENDATION

There should be further research to identify the signaling molecules of *Pseudomonas aeruginosa* in response to antibiotics at sub-inhibitory concentrations and there should be more research works to developed therapeutics combination that could possible combat the recalcitrant nature of *Pseudomonas aeruginosa*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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