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Evaluation of the roles of Mex genes in Antibiotic Resistance of some Strains of *Pseudomonas aeruginosa* and the use of Antibiotic Combinations to boost their Susceptibility pattern

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Abstract

Pseudomonas aeruginosa *is a prevalent pathogen responsible for various infections, and exhibits significant resistance to a broad spectrum of antibiotics, leading to increased mortality rates. This study aimed to assess the role of mex genes in antibiotic resistance among indigenous strains of Pseudomonas aeruginosa and enhance their susceptibility through antibiotic combinations. Ten isolates were selected based on their appearance on selective media, microscopy and biochemical characterization. Antibiotic resistance profiles for fluoroquinolones (ofloxacin, pefloxacin, sparfloxacin, ciprofloxacin), aminoglycosides (gentamicin, streptomycin), beta-lactams (amoxicillin) Sulfonamide (septrin) and Macrolide (septrin) were determined using the Bauer-Kirby disk diffusion method. Minimum inhibitory concentrations (MICs) for 13 individual antibiotics and 19 combined antibiotics were determined using two-fold serial dilution techniques. DNA was extracted from* P. aeruginosa *isolates and analyzed by polymerase chain reaction (PCR) to detect resistance genes- MexA, MexB, and MexZ. The* MexA *and* MexZ *resistance genes were detected in all isolates, while* MexB *was present in all except 3 of the isolates.* P. aeruginosa *exhibited the highest susceptibility to gentamicin (40%) and complete resistance to sparfloxacin (100%). The multiple antibiotic resistance (MAR) index ranged from 0.22 to 1. The MIC values for cefexime and ciprofloxacin was 128 µg/mL, while ciprofloxacin + amoxicillin was 32 µg/mL showing increased susceptibility. This study highlights the presence of highly resistant* P. aeruginosa *strains, it showed a relationship between the resistant genes and antibiotic resistance. Drug combination of ciprofloxacin and amoxicillin proved to be most susceptible against the pathogen. This brings the need to study the mechanism of resistance in the pathogen and target silencing these resistance genes. There should be sensitization, increased awareness among patients and healthcare providers regarding the rising antibiotic resistance of the strain.*

Keywords*:* Antibiotics, Antibiotic Resistance, Mex genes, *Pseudomonas aeruginosa*,

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INTRODUCTION

Pseudomonas aeruginosa, a Gram-negative bacterium, is a causative agent of nosocomial infections affecting various anatomical sites, including the respiratory, urinary, integumentary and circulatory systems among others. *P. aeruginosa* exhibits characteristics of an opportunistic microorganism that thrives in diverse terrestrial ecosystems, encompassing aquatic environments, abiotic surfaces, and the biotic milieu of both human and animal hosts. Morphologically, it presents as a curved rod with dimensions spanning approximately 0.5 to 1.0 micrometers in width and 1 to 3 micrometers in length (Pier and Ramphal, 2010). The bacterium is motile and lacks sporulation capability, displaying parallel termini and typically existing as singular entities or forming abbreviated chains. The fimbriae found on *Pseudomonas aeruginosa* distinguish them from those of other Gram-negative rods due to their unique capacity to induce hemagglutination (Poole, 2011). Hematogenous dissemination of *Pseudomonas aeruginosa* can result in bacteremia, a grave condition characterized by the presence of the pathogen in the bloodstream. Bacteremia represents a severe manifestation of pseudomonas infection and can precipitate a state of pronounced hypotension termed hemodynamic shock, culminating in multi-organ dysfunction, including cardiac, renal, and hepatic impairment. Additionally, *Pseudomonas aeruginosa* can infect the pulmonary, auditory, and cutaneous systems. Cutaneous infections often target hair follicles, giving rise to a phenomenon referred to as folliculitis (Oliver *et al*., 2015).

The expression of pigmentation stands as a pivotal morphological characteristic within laboratory culture. Among strains of *Pseudomonas aeruginosa*, four distinct pigment types are developed pyorubin, pyocyanin, pyoverdin, and pyomelanin. Pyocyanin, a prevailing blue-green phenazine pigment, holds prominence as it is actively excreted into the surrounding milieu (Pier and Ramphal, 2010). Differentiating it from other species within the genus, *Pseudomonas aeruginosa* is uniquely endowed with the capacity to synthesize pyocyanin, serving as a diagnostic trait. Certain strains exhibit variations in pigment coloration, yielding yellow-green to yellow-brown hues through the synthesis of pigments known as pyoverdin. In contrast, pigments named pyorubin and pyomelanin manifest as red and black colonies respectively. Cetrimide agar, a widely employed selective medium for pseudomonas isolation, incorporates a detergent to deter the growth of numerous organisms, though with exceptions. It also contains $MgCL_2$ and K_2SO_4 to promote the characteristic green pigmentation associated with Pseudomonas (Ezeador *et al.,* 2020). The genome of *P. aeruginosa*, spanning 5.5 to 7 megabase pairs (Mbp), boasts significant size compared to other sequenced bacteria like *Bacillus subtilis* (4.2 Mbp), *Escherichia coli* (4.6 Mbp), and *Mycobacterium tuberculosis* (4.4 Mbp). It encompasses a substantial portion of regulatory enzymes essential for metabolic processes, organic compound transport, and efflux. This extensive coding capacity within the *P. aeruginosa* genome confers remarkable metabolic adaptability and heightened resilience to environmental fluctuations (Klockgether *et al.,* 2011). Information regarding systemic exposure and risk factors associated with *Pseudomonas aeruginosa* community-acquired pneumonia (*P. aeruginosa*-CAP) is notably scarce. Existing data on the prevalence of *P. aeruginosa* and its resistance to multiple drugs primarily stems from single-center investigations and studies characterized by limited methodological rigor (Shugang *et al.,* 2022).

Pseudomonas aeruginosa isolates have been sourced from numerous healthcare facilities and diagnostic settings across various countries, including Nigeria. Yusuf *et al*. (2014) conducted a study that identified *P. aeruginosa* as one of the multi-drug resistant pathogens recovered from diverse clinical specimens. In 2020 Adejobi *et al.,* reported the prevalence of *P. aeruginosa* in clinical specimen in Obafemi Awolowo University Teaching Hospital, were they recorded

high resistance of the isolates to gentamicin. Furthermore, *P. aeruginosa* has been isolated from both clinical and environment specimens in Anambara State, Nigeria (Ezeador *et al.,* 2020). The Centers for Disease Control reported in 2019 that an estimated 32,600 infections attributed to *Pseudomonas aeruginosa* occurred among immunocompromised hospital patients, resulting in approximately 2,700 estimated fatalities. The continental prevalence of *P. aeruginosa* was estimated at 5.5% in Africa, 5.2% in Asia, 3.8% in Europe, 4.3% in North America, 3.1% in Oceania, and 4.3% in the Northern Hemisphere (Marcos *et al.,* 2018). The World Health Organization (WHO) documented around 15 million annual cases of *P. aeruginosa* infections in children under the age of 5, with a mortality rate of 59%. In Nigeria, UNICEF reported an estimated 162,000 deaths in 2018 attributable to *P. aeruginosa* infections in this age group.

The primary strategies employed by *Pseudomonas aeruginosa* to counteract the impact of antibiotic assault can be categorized as intrinsic, acquired, and adaptive resistance mechanisms. Intrinsic resistance in *P. aeruginosa* encompasses factors such as limited permeability of the outer membrane, the presence of efflux pump systems responsible for expelling antibiotics from within the bacterial cell, and the synthesis of enzymes that render antibiotics inactive. Acquired resistance in *P. aeruginosa* can arise through either the horizontal transfer of resistance genes or mutational alterations (Breidenstein *et al.,* 2011). Adaptive resistance in *P. aeruginosa* involves the development of biofilms within the pulmonary tissues of infected patients. These biofilms function as diffusion barriers, constraining antibiotic penetration to reach bacterial cells effectively. The antibiotic resistance exhibited by *Pseudomonas aeruginosa* has been closely linked to both its efflux system for antibiotics and the low permeability of its outer membrane. The up-regulation of MexXY-OprM, for instance, has a notable impact on aminoglycoside resistance. In various instances, efforts have been made to disrupt the cell's outer membrane permeability barrier using agents such as polycations (Pang *et al.,* 2019). However, it is important to note that low outer membrane permeability alone is insufficient to confer resistance; it must be coupled with a secondary resistance mechanism, typically involving the efflux system. In laboratory experiments, three mutational modifications have been identified, namely nalB, nfxB, and nfxC mutants. These mutations impact the regulatory genes responsible for triggering the overexpression of efflux pumps and β-lactamase production. However, the emergence of resistance mechanisms in *Pseudomonas aeruginosa* involving acquired extended-spectrum β-lactamase (ESBL) and metallo β-lactamase (MBL) is of considerable significance. When it comes to addressing suspected cases of *P. aeruginosa* infections, empirical antibiotic therapy has been a conventional approach (Rafiee *et al.,* 2014). Nonetheless, managing *P. aeruginosa* infections has become increasingly challenging due to the bacterium's resistance to numerous presently available antibiotics (Park *et al*., 2012).

The World Health Organization (WHO) has recently identified carbapenem-resistant *P. aeruginosa* as one of the top three bacterial species necessitating urgent development of novel antibiotics for effective treatment (Tacconelli *et al.,* 2017). There is an urgent requirement for the development of new antibiotics or alternative therapeutic strategies to tackle *P. aeruginosa* infections that exhibit resistance to conventional antibiotics. Recent years have witnessed exploration into new antibiotics with unique mechanisms of action, alternative routes of administration, and resistance to modification by bacterial enzymes. Some of these novel antibiotics have demonstrated excellent *in vitro* antibacterial efficacy against *P. aeruginosa*, coupled with lower minimum inhibitory concentrations (MIC) when compared to conventional antibiotics (Cigana *et al.,* 2016).

There is a rapid and global spread of multi-drug resistant *Pseudomonas aeruginosa*. The roles of mexA, mexB, mex Z in antibiotic resistance among local strains of *Pseudomonas aeruginosa* have not been established. Moreover, *Pseudomonas aeruginosa* significantly contributes to the disease burden of Nigeria. Infection by multidrug resistant strains of *Pseudomonas aeruginosa* leads to high mortality rate in patients. The research was aimed at correlating the presence of the resistance genes in the local strains of pseudomonas aeruginosa to antibiotic resistance.

MATERIALS AND METHODS

Study area

The selected hospitals are located within Kaduna metropolis which provide healthcare facilities and services to the residents of the state.

Collection of Samples

Clinical samples were collected from the Department of Microbiology National Ear and Throat center and Saint Gerald Hospital Kakuri, Kaduna within a period of Four months (November 2022 to February 2023). Cotton swabs and plates collected from the laboratory were transferred into sterile ziplock bags, into an ice box and transported immediately to the laboratory at the department of Biochemistry Kaduna State University.

Isolation and Characterization of Bacteria

Cotton swab samples were streaked evenly on MacConkey agar plates for distinct colonies and identified through the pale colored colonies observed (Umar *et al.,* 2019). A colony was picked using sterile wire loop and transferred into Nutrient broth medium and was incubated at 37^0C for about 24hours. The overnight culture was transferred on Cetrimide agar plate a selective agar, evenly streaked and incubated for another 24hours at 37^0C to give distinct colonies. The overnight cultured plate was then carefully observed for fluorescent green pigmentation, with smooth round colonies. A single colony was picked using sterile wire loop and transferred into nutrient broth. Identified samples were stored in glycerol by pipetting 0.5ml glycerol and 0.5ml overnight broth culture into Eppendorf tubes and kept at 4^0C for further studies (Ezeador *et al.,* 2020).

Samples were further tested for, using several tests. morphological characterization encompassing Grams reaction: Biochemical tests: indole, spore, motility, Triple Sugar Iron (TSI) Test coagulase test (Tube Coagulase Test Procedure), urea hydrolysis, citrate, and oxidase tests, and microscopy were conducted (Adekunle *et al.,* 2022). The acquired results were subsequently compared with the criteria outlined in the 21st edition of Bergey's Manual of Systematic Bacteriology. The Isolates selected for further studies were isolate 12(1), 33(2), 6(3), 3(4), 5(5), 11(6), 27(7), 8(8), 4(9), and 9(10). These were selected because the isolates produced the best result for the parameters used during identification, biochemical test, and microscopy.

DNA Extraction

5ml of *P. aeruginosa* culture was grown overnight in nutrient broth. Bacterial cells were harvested by centrifugation at 5000rpm for 5 minutes for pellet. The supernatant was discarded and the pellet re-suspend in 1ml sterile buffer. The cultured cells were re-suspended in an isotonic buffer- PBS in 100 µl, 200 µl of sample was added to a microcentrifuge tube. BioFluid of 200 µl and 20 µl of Proteinase K was added. Mixture was vortex for 10-15sec and then incubated at 55^0C at 10mins. 0.5ml of Genomic Binding Buffer was added to digest the mixture and was vortexed for 10secs. The mixture was transferred into a collection tube and centrifuged at 10,000rpm for 1minute. 400 µl of DNA Pre-Wash Buffer was added and

transferred into a new Collection Tube and centrifuged at 10,000xg for 1 minute, the collection tube was then emptied. g-DNA (700 µl) wash buffer was added to the spin column and centrifuged at 10,000rpm for 1 minute, after which the collection tube was emptied. Another 200 µl of g-DNA wash buffer was added to the spin column and centrifuged at 10,000rpm for 1 minute, the collection tube was then discarded with the flow through. The spin column was transferred to a micro-centrifuge tube, 50 µl of DNA Elution Buffer was added to the matrix and incubated at room temperature for 5 minutes, then centrifuged at maximum speed for 1 minute to elute the DNA.

The resultant purified DNA was quantitatively assessed for both yield and purity, determined through the ratio of absorbance readings at 260/280nm, using a Nano Drop spectrophotometer device. Subsequently, a qualitative assessment of total bacterial DNA was conducted via 1% agarose gel electrophoresis at 80V for 25 minutes, with visualization facilitated by a Desktop Gel Imager (Suhad and Marrib 2018).

Polymerase Chain Reaction

For PCR amplification, a total reaction volume of 25 µl was prepared, comprising 12.5 µl of 2X Master Mix with Standard buffer, 0.5 µl of the forward primer, 0.5 µl of the reverse primer, 10 µl of nuclease-free water, and 2 µl of DNA lysate. The polymerase chain reaction setup involved the addition of the master mix, forward and reverse primers, and the extracted DNA into a PCR vial. Amplification commenced with an initial denaturation at 94°C for 30sec, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at temperatures of 55°C, 68°C, and 68°C for 1 minute each, corresponding to Mex A, Mex B, and Mex Z, respectively. extension at 68°C for 5minute and final ° extension procedure was done for 4 minutes at 10°C (Adekunle *et al.,* 2022).

Preparation of Agarose Gel for Electrophoresis

30ml of 1X Tris Borate EDTA buffer was measured using the measuring cylinder and transferred into a beaker, 1% Agarose concentration, (ie 0.3g dissolved into the buffer was used to make the gel). Microwave was used to heat the mixture to form a clear colourless solution and allow to cool slightly, 1 µL of ethidium bromide was added and was shaken gently until it mixed properly. The gel block was covered at both ends with masking tape. The solution was poured into the block and allow for some few minutes to solidify. The tapes were removed and the gel block transferred into a tank filled with 1X TAE buffer and the comb removed. Prepared Genomic DNA samples or PCR product were loaded into the wells and DNA ladder loaded into the first well to serve as the control. Electrophoresis was carried out at 80volt 400mA for 25 minutes using the agarose gel electrophoretic machine and viewed under the blue light gel trans illuminator. L represent 1000bp marker ladder used to estimate the Genomic DNA bands sizes.

Primer Sequence Design.

The three primers utilized in this study were designed using the Primer-BLAST tool available on the NCBI website. Before synthesis, the quality of the primers was verified using the Oligo-analyzer and Primer-Stat as seen in table 2.

Antimicrobial Susceptibility Test.

The antimicrobial sensitivity test of 10 isolated *Pseudomonas aeruginosa* strains were conducted employing the disk-diffusion methodology, commonly referred to as the Kirby-Bauer method developed in 1950s standardized in 1961. This approach involved the utilization of Mueller-Hinton agar plates. An overnight broth single colony suspension technique was employed to create a microbial suspension in normal saline (0.9%) and achieving a density equivalent to

the McFarland 0.5 turbidity standard using light spectrophotometer. The entire process adhered to the guidelines established by the National Committee for Clinical and Laboratory Standards (CLSI) in 2020. Antibiotics tested were commercially gotten, they include Streptomycin (30µg), OFloxacin(10µg), Pefloxacin(30µg), Septrin (30µg), Chloramphenicol (30µg), Sparfloxacin (10µg), Amoxicillin (30µg), Gentamycin (10µg), Ciprofloxacin(10µg). (Ezeador *et al.,* 2020).

Multi-Antibiotic Resistance Index

The Multiple Antibiotic Resistance (MAR) of the ten isolates were calculated using the formula

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

The Multiple Antibiotic Resistance (MAR) index is a tool used to evaluate the level of resistance of bacterial strains to multiple antibiotics (Afunwa *et al.,* 2020)

Minimum Inhibition Concentration

An Isolate was selected for Minimum inhibition concentration (MIC) test because it was observed that it has high MAR and does not contain MexB gene. The MIC was done using two-fold serial dilution methods according to the CLSI (2020) procedure(s). A stock solution of 1ml was created for each antibiotic used including: gentamicin (80mg/2ml), amoxicillin (500mg), erythromycin (500mg), metronidazole (400mg), ampiclox (500mg), tetracycline (250mg), chloramphenicol (250mg), penicillin (312.5mg), ampicillin (250mg), deoxycycline (100mg), cefixime (400mg) and cefuroxime (500mg). A pure overnight culture was prepared and its density was adjusted to 0.5 McFarland standard. Sterile solid Mueller Hinton agar plates were inoculated using sterile inoculating loop. Cork borer was used to bore holes into the plates and the antibiotics were introduced using a micro pipette. The plates were incubated at 35^0C for about 20hrs in an incubator (Babak *et al.,* 2016).

Data Analysis

Descriptive data analysis including measures of central tendency (mean) and measures of Variability (standard deviation) were used. Visual tools such as bar and pie chart were used to analyze the data set for this study

RESULTS

Properties of Purified DNA: The isolates extracted DNA, showed corresponding concentration in micrograms per milliliter (µg/mL) and the absorbance ratios measured at 260/280 nm, which is typically used to assess the purity of nucleic acids (DNA/RNA), this data is presented in Tabl.1

Table 1: Properties of Purified DNA

Isolate	Concentration(µg/mL)	Absorbance at 260/280nm
1	51.7	1.80
2	31.5	2.10
3	28.4	2.34
4	26.1	2.5
5	33.7	2.31
6	29.5	3.15
7	24.8	2.92
8	31.0	2.68
9	39.9	2.98
10	27.2	2.90

The three primers used for the amplification of MexA, MexB, and MexZ genes in PCR (Polymerase Chain Reaction) is seen in Table 4.2. Each gene has a forward primer and a reverse primer nucleotide sequence, which are short sequences of DNA used initiate the replication process during PCR.

Table 2: Primers Designed for the Study

Gene	Nucleotide Sequence	Size (bp)
Mex A	Forward Primer: ACAAGTGGCTGGTTACCGAA	175-182
	Reverse Primer: AGCCCTTGCTGTCGGTTTT	
Mex B	Forward Primer: CCAACATCCAGGACCCACTC	276-289
	Reverse Primer: GTCGGGATTGACCTTGAGCA	
MexZ	Forward Primer: ACAAGATCGAGGTCTGCCT	460-465
	Reverse Primer: CCAGCAACAGGTAGGGAGAA	

The gel picture showing *Pseudomonas aeruginosa* samples amplified using Mex Z (Fig 1), Mex B (Fig 2) and MexA (Fig 3) primer sequences. The primer sequences were used in polymerase chain reaction to amplify the resistance genes. L represent 1Kbp marker ladder used to estimate the PCR product sizes. Mex Z and Mex A shows band in all Lane, while in Mex B Lane 5, 6 and 9 forms no band.

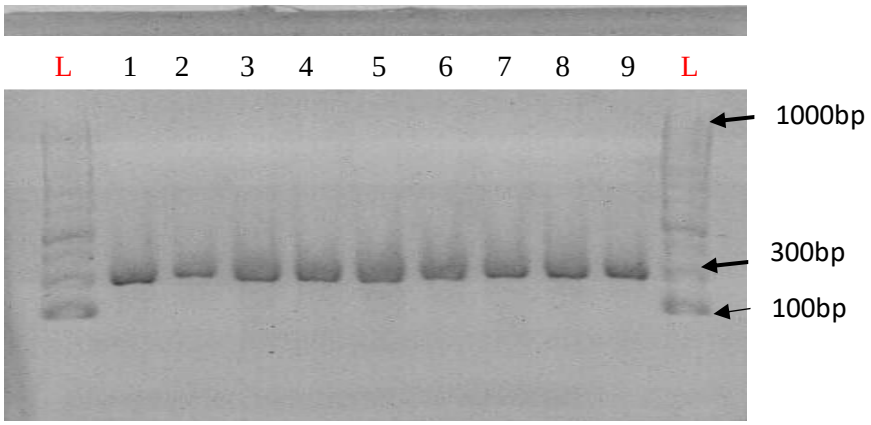

Fig 1: Stained Agarose Gel Electrophoresis of Mex Z Gene in some strains of *Pseudomonas aeruginosa.* L: DNA Ladder, Lanes 1-9: various strains of *Psedomonas aeruginosa*

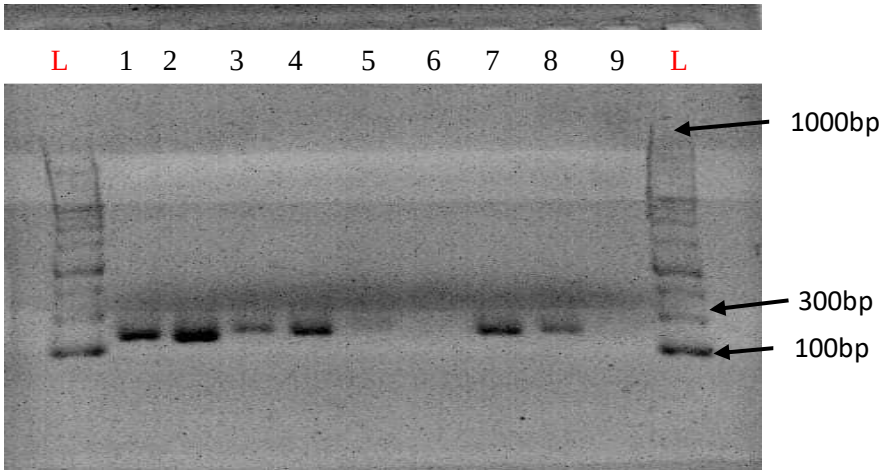

Fig 2: Stained Agarose Gel Electrophoresis of Mex B Gene in some strains of *Pseudomonas aeruginosa.* L: DNA Ladder, Lanes 1-9: various strains of *Psedomonas aeruginosa*

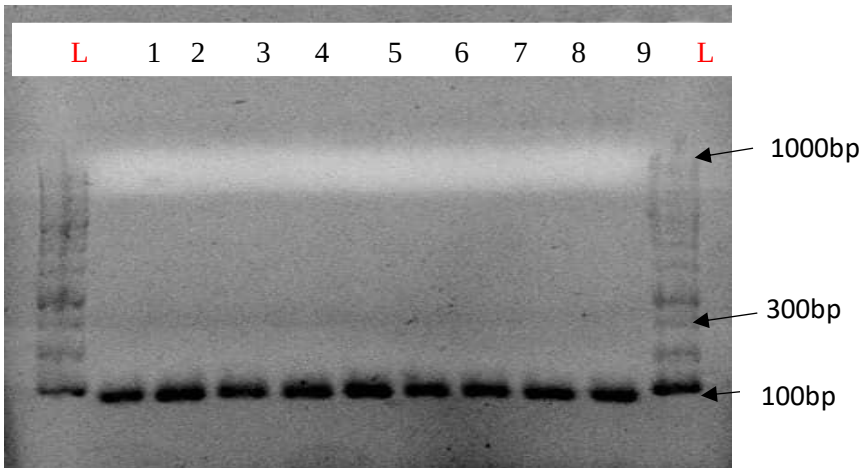

Fig 3: Stained Agarose Gel Electrophoresis of Mex A Gene in some strains of *Pseudomonas aeruginosa.* L: DNA Ladder, Lanes 1-9: various strains of *Psedomonas aeruginosa*

Antibiotic susceptibility profile: The antibiotic sensitivity profile was determined by calculating the zone of inhibition for the drug. The chart in fig 4 showed the range of inhibition from 3mm-26.7mm.

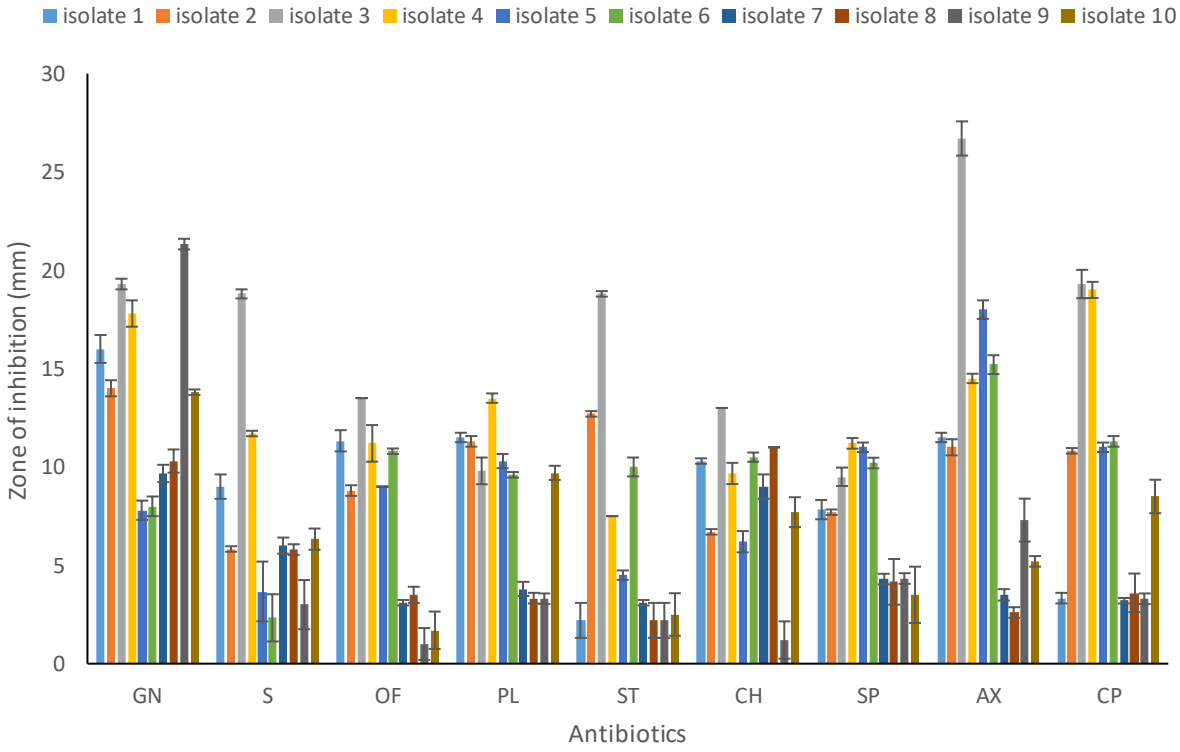

Fig **4**: Antibiotic sensitivity profile of isolates showing the zone of inhibition in mm.

Key

GN- Gentamicin
S- Streptomycin
O- ofloxacin
PL- Pefloxacin
ST- Septrin
CH- Chloramphenicol
AX- Amoxicillin
CP- Ciprofloxacin

SP- Sparfloxacin

The effectiveness of various antibiotics against the isolates, is presented in table 2, the results are categorized into three groups: Susceptible, Intermediate, and Resistance. The values in each column represent the number (and percentage) of cases where the isolates were classified into these categories based on its response to each antibiotic.

Table 3: Total Number of Isolates Susceptible, Intermediate and Resistance

Antibiotics	Susceptible n(%)	Intermediate n(%)	Resistance n(%)
Gentamicin	4(40)	2(20)	4(40)
Streptomycin	1 (10)	0	9(90)
Ofloxacin	0	2(20)	8(80)
Pefloxacin	0	2(20)	8(80)
Septrin	1(10)	1(10)	8(80)
Chloramphenicol	1(10)	1(10)	8(80)
Sparfloxacin	0	0	10(100)
Amoxicillin	1(10)	1(10)	8(80)
Ciprofloxacin	0	2(20)	8(80)
TOTAL	5(9)	11(12)	71(79)

n: number of isolates

The Multiple Antibiotic Resistance (MAR) Index for all ten isolates was determined after the antibiotic sensitivity test. The MAR index ranges from 0.22 to 1, where: A value closer to 1 indicates high resistance to multiple antibiotics. A value closer to 0 indicates low resistance or susceptibility to antibiotics, this data is presented in table 4.

Table 4: Multi-Antibiotic Resistance Index of Antibio-gram of Various Strains of *Pseudomonas aeruginosa*

ISOLATES	MAR INDEX
1	0.89
2	0.67
3	0.22
4	0.56
5	1
6	0.89
7	1
8	0.89
9	0.89
10	0.89

The minimum inhibition concentration for 13 antibiotics (table 5) and 19 combinations (Table 6) was determined for isolate 5 using two-fold serial dilution. The results revealing high resistance as the drugs were all beyond breakpoints.

Table 5: Minimum Inhibition Concentration (MIC) of Single Drug on an isolate

Antibiotics	MIC (µg/ml)
Amoxicillin 500mg	<1024
Ampiclox 500mg	<1024
Gentamicin 80mg/2ml	512
Ampicillin 250mg	<1024
Cefexime 400mg	128
Chloramphenicol 250mg	<1024
Erythromycin 500mg	<1024
Metrinidazole 400mg	<1024
Tetracycline 250mgs	1024
Penicillin 312.5mg	<1024
Deoxycline100mg	1024
Ciprofloxacin 500mg	128

Cefuroxime 500mg	<1024

Table 6: Minimum Inhibition Concentration (MIC) of Drug Combination on isolate 5

Antibiotics	MIC (µg/ml)
Amo + Metrinidazole	<1024
Amo + Gentamicin	128
Amo + Cefexime	512
Amo + Chloram	64
Amo + Erythromycin	<1024
Amo + Metrinidazole	<1024
Amo + Tetracycline	128
Amo + Penicillin	<1024
Amo + Deoxycline	128
Amo + Ciprofloxacin	32
Amo + Cefuroxime	<1024
Pen + Metrinidazole	<1024
Pen + tetracycline	<1024
Pen + ciprofloxacin	<1024
Pen + deoxycycline	1024
Pen + erythromycin	<1024
Pen + chloram	64
Pen + gentamicin	64
Pen + cefexime	64
Pen + cefuroxime	<1024

Key
Amo- Amoxicillin
Pen- Pencillin
Chloram- Chloramphenicol

DISCUSSION

Pseudomonas aeruginosa was isolated from cotton swab samples taken from both the left and right ears. The bacteria exhibited positive pigmentation on the agar plates used for culture. Microscopic examination revealed them to be rod-shaped. The catalase, citrate, and oxidase tests all yielded positive results, whereas the urease, indole, and coagulase tests were negative. Additionally, the fermentation test results were negative similar to the reports of Jidda *et al.* (2016).

Polymerase Chain Reaction analysis produced products for Mex A (164 bp) and Mex B (217 bp), similar with the findings of Babak *et al.* (2016) and Mex Z (mutant gene) with a PCR product size of 462bp similar to the work of Thomas *et al*. (2013). Mex A and Mex B have been confirmed to induce resistance against beta-lactam and fluoroquinolones (ciprofloxacin, amoxicillin, sparfloxacin, plarfloxacin, and ofloxacin), as expounded by Pang *et al* 2019. Conversely, Mex Z functions by expelling aminoglycosides and beta-lactams (Gentamicin, amoxicillin, and streptomycin), as outlined by Guenard *et al*. (2014). The antibiotic susceptibility profiles were determined for nine distinct antibiotics belonging to the classes of aminoglycosides, fluoroquinolones, beta-lactams, sulfonamides, and macrolides. In the assessment of antibiotic susceptibility, resistance was observed across all tested antibiotics, with particularly high resistance noted for Amoxicillin, ciprofloxacin, and sparfloxacin. Compounds such as Septrin and chloramphenicol, although outside the specified antibiotic classes was affected by the resistance genes and exhibited intermediate resistance. These findings corroborate the assertions made by Juan *et al.* (2019), who highlighted the ability of *Pseudomonas aeruginosa* to mutate and acquire resistant genes, enables them to expand their resistance spectrum to a broad array of antibiotics, thereby manifesting a pan-drug resistant (PDR) phenotype. In the study 80% *Pseudomonas aeruginosa*, exhibited high resistance to

ciprofloxacin, accompanied by 20% intermediate resistance. This observation aligns with the findings of Iregbu and Eze (2015) conducted at the National Hospital in Abuja, Nigeria. Conversely, Adekunle *et al.* (2022) reported contrasting results, noting low resistance to ciprofloxacin and high resistance to gentamicin. Gentamicin, exhibiting approximately 40% susceptibility and 60% resistance, emerged as the most efficacious among all tested drugs, consistent with the study conducted by Ajiya *et al.* (2021) on Northwestern Nigerians with chronic suppurative otitis media infections. Moreover, a 100% resistance was documented for sparfloxacin, and 90% resistance to streptomycin among the ten *P. aeruginosa* strains assessed, reminiscent of the findings reported by Umar *et al*. (2019). A total percentage for Resistance Susceptible and Intermediate was recorded as (79%), (9%) (12%), respectively.

The MIC values for the antibiotics tested showed high resistance as none fell below the breakpoint standard of CLSI (2020). The breakpoint MIC signifies the maximum safe concentration achievable in blood with recommended dosages. Microorganisms are deemed susceptible to a drug if their MIC falls below the breakpoint MIC. Those with intermediate susceptibility are inhibited at concentrations nearing the breakpoint. Conversely, a resistant organism exhibits an MIC surpassing the drug's breakpoint MIC, where the risk of toxicity outweighs therapeutic benefits. In the single antibiotic tests, high resistance was observed, with most drugs registering above 1024 µg, except for ciprofloxacin and cefexime with MIC at 128 µg. However, the combined antibiotic therapy demonstrated a notable enhancement in efficacy against the organism, evidenced by considerably elevated MIC values, with most combinations ranging from 32 µg (ciprofloxacin + amoxicillin) to above 1024 µg. *Pseudomonas aeruginosa* exhibited high resistance to all other antibiotics tested which agrees to the work of Adekunle *et al*., 2022.

The Microbial Antibiotic Resistance (MAR) index is defined as the ratio of the count of antibiotics against which an organism exhibits resistance to the total count of antibiotics it encounters. A MAR index exceeding 0.2 indicates an elevated risk of contamination, signifying the usage of multiple antibiotics. In this investigation, all ten isolates demonstrated a substantial MAR index ranging from 0.22 to 1. This finding aligns with prior studies, such as Afunwa *et al*. (2020), which reported a *Pseudomonas aeruginosa* MAR ratio of 0.8, and Adejobi *et al*. (2021), who observed ratios ranging from 0.5 to 0.875.

CONCLUSION

The ten isolates selected for molecular studies showed gel image for all three genes except isolates 5, 6 and 9 that showed no band for Mex B. The investigation unveiled a notable prevalence of antibiotic resistance, particularly toward ciprofloxacin and amoxicillin, in *Pseudomonas aeruginosa* infections within selected hospitals in Kaduna state. This resistance phenomenon can be attributed to the extensive usage of these antibiotics for self-medication, leading to the development of resistance as a consequential side effect. Gentamicin emerged as the most efficacious antimicrobial agent against *P. aeruginosa*, displaying a susceptibility rate of 40%, with isolate 3 exhibiting the lowest MAR index at 0.22. This observation suggested a conscientious approach to medication by the patient in contrast to others. The antibiotic drug combinations of AMO + Cipro and AMO + Chloram were more effective than as single drugs, as they showed an increased MIC after the combination.

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