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DETERMINATION OF RESISTANCE CONFERRING GENES (SHV, CTX-M) IN PSEUDOMONAS AERUGINOSA ISOLATED FROM CLINICAL SAMPLES OBTAINED FROM SOME COMMUNITY HOSPITALS IN AMASSOMA, NIGER DELTA AREA, NIGERIA

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ABSTRACT

Antimicrobial resistance is a global public health challenge in the clinical setting and the huge indiscriminate use of antimicrobial agents has been a major contributing factor; this promotes the increasing emergence of antibiotic resistant organisms. This work was carried out between January and October 2019 to determine the presence of resistance conferring genes (SHV and CTX-M) in *Pseudomonas aeruginosa* isolated from clinical samples. Two hundred (200) samples wound swab (65), ear swab (75) and mid-stream urine (65)] collected were analyzed, characterized and identified using microbiological standard techniques. Pure *Pseudomonas aeruginosa* isolates were subjected to antibiotic susceptibility testing using disc diffusion method by Kirby-Bauer and molecular analysis using Polymerase Chain Reaction (PCR) technique to reveal the Extended Spectrum Beta-Lactamases (ESBLs) (SHV and CTX-M). The total number of *Pseudomonas aeruginosa* isolated were eighty 80 (40%) from all the two hundred samples, 20(25%) mid-stream urine, 36(45%) wound swab and 24(30%) from ear swab. Gentamicin (100%) was the most potent antibiotic followed by Streptomycin (95%), Ofloxacin (86.25%), Pefloxacin (82.5%), Ciprofloxacin (75%), Ceftazidime (61.25%), and the least was Nalidixic acid (12.5%), Co-trimoxazole (2.5%) but resistant to Augmentin (100%) and Ampicillin (100%). A total of twelve (12) Multi Resistant *Pseudomonas aeruginosa* Isolates subjected to molecular analysis revealed the presence of ESBLs resistant genes; SHV in 11(91.6%) and CTX-M 4(33.3%). Observation showed that *Pseudomonas aeruginosa* was more prevalent in the wound (45%), followed by ear (30%) and the least was in the urine (25%) samples. Based on these findings, Gentamicin and Streptomycin are recommended for *Pseudomonas aeruginosa* infections in hospitals.

Keywords: Antimicrobial Susceptibility Testing; *Pseudomonas aeruginosa*; Resistance Conferring Genes SHV; CTX-M.

INTRODUCTION

Antibiotics are one of the frequently prescribed drugs among hospitalized patients and their continuous excessive indiscriminate use has been reported as a major factor that promote the increasing emergence of antibiotic resistant organisms (Breidenstein, 2012) Antimicrobial resistance is a global public health in the clinical setting and the huge use of antimicrobial agents in both community and hospital setting has been described as a major contributing factor. The major consequence of this resistance is the increasing rate of treatment failures in the management of common human infections caused by microorganisms such as *Pseudomonas aeruginosa* with the available agents in an environment where the discovery and manufacturing of newer antimicrobial agents is very slow and scarce (Keen *et al*., 2010) and the resultant could be mortality according to Daniel *et al.* (2016). Extended-spectrum Beta-lactamases (ESBLs) are a major cause of resistance to Beta-lactam antibiotics such as Penicillins, cephalosporins and monobactams. They (ESBLs) are derived from narrow-spectrum beta-lactamases as a result of plasmid possession (SHV-1, CTX-M, TEM-1 and TEM-2), but molecular analysis using Polymerase Chain Reaction technique can be used to detect these genes Kanji and Sexton, 2016; Kerr*et al*., 2009. *Pseudomonas aeruginosa* is rarely a healthy human micro flora; its ubiquity, stubborn resistance and virulence factors such as flagella and type 4 and 3 pilli have led this organism to cause several infections which include soft tissue, urinary tract, bacteremia, diabetic foot, respiratory/ pneumonia, otitis externa, keratitis (corneal infection), Otitis media folliculitis (Alvarez *et al*., 2011). This study was carried out to determine the resistance genes of Extended Spectrum beta-lactamases (SHV and CTX-M) of *Pseudomonas aeruginosa* isolatesfrom the clinical samples. The objectives were the isolation, identification of *Pseudomonas aeruginosa* strains and subjected them to antimicrobial susceptibility testing and molecular analysis to determine the presence of resistance genes using Polymerase Chain Reaction technique.

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MATERIALS AND METHOD

Study Area

This study was carried out at Niger Delta University, Amassoma Community's (with 500,000 people) General Hospital and Tantua Private Hospital. Clinical samples were collected from the two (Government and Private) hospitals with 60-beded space and 40-beded space respectively. Amassoma community (in Southern Izon Local Government area) is an island called Wilberforce Island in Bayelsa State, located in Southern Nigeria and share a common boundary with two other states: Rivers state to east and Delta state to the west. Bayelsa state has a population of 1.7 million people and eight (8) Local Government areas which include; Sagbama, Brass, Yenagoa, Ekeremor, Ogbia, Nembe, Southern Izon Local Government Areas.

Study Design

Two hundred (200) clinical samples were collected from symptomatic patients from Amassoma Community General Hospital and Tantua

private hospital; their demographic data was obtained through their informed consent. Ethical approval was obtained from the Hospital Medical Directors.

Laboratory Materials used

Sterile universal bottles, swab sticks, platinum wire loop, Bunsen burner, filter paper (What man),Mc Cartney bottles, Microscopy slides, cover slips, disposable petri dishes, test tubes, Volumetric flask (100, 250 and 500ml), and Pasteur pipette. Kovac's reagent, oxidase reagent, hydrogen peroxide, sodium chloride (normal saline), gram stain reagent, Mc Farland standard solution, master mix, primers, ethidium bromide, Tris-boris EDTA, DNA ladder. Autoclave, triple beam balance, analytical balance, binocular light microscope, hot air oven, incubator, microwave, El- tech Vortexing machine, Wealtecorp heating block, Eppendorf micro tubes (2ml, 1.5ml), pipette, centrifuge 10000xg, Bio-Rad Dyad PCR machine, Thermo Scientific Nanodrop 1000 spectrophotometer and Gel electrophoresis machine.

Sample Collection and Method of Processing

The two hundred (200) clinical samples collected from in- patients of all age group include Urine (60); Wound swab (65) and ear swab (75). The samples were collected at convenient into commercially available universal sterile bottles, sterile swab sticks and were immediately taken to the laboratory for analysis using Cetrimide agar, Nutrient agar, Muller Hinton agar, Luria Betani broth, DNase agar, Citrate agar, Agarose gel. The pure standardized microbial culture with 0.5 Mac Farland turbidity standard were characterized and identified on the basis of their colonial morphology, gram staining and biochemical tests (Catalase, Indole, Citrase utilization test, Oxidase test, DNase test). Antibiotic susceptibility testing was carried out using Kirby – Bauer modified agar disc diffusion method (Bauer *etal*., 1966) and Muller Hinton agar with Gram negative multidisc containing the following (Augumentin 30ug, Ofloxacin 5ug, Pefloxacin 5ug, Ceftazidine 30ug, Gentamycin 10ug, Streptomycin 10ug, Ciprofloxacin 5ug, Ampicillin 10ug, Septrin 2ug and Nalidixic acid 30ug). The antibiotic susceptibility testing results were interpreted accordingly based on National Committee for Clinical Laboratory Standard (CLSI, 2010). Multi- drug resistant isolates were subjected to molecular analysis using Polymerase Chain Reaction and Agarose Gel electrophoresis method to detect the presence of resistance conferring genes (SHV and CTX-M).

Sample Size Estimation

The sample size was calculated using the stanadard sampling technique.

$$n = \frac{z^2 \times P(1-P)}{E^2}$$

n = required sample size; z= confidence level of 95% (1.96);

p = estimated prevalence of the disease; E= % error (0.05);

$$P = \frac{200}{550} = 0.36 (36.36\%)$$

$$n = \frac{1.96^2 \times 0.36(1-0.36)}{0.05^2}$$

$$n = \frac{0.50}{0.05^2} = 200$$

Molecular analysis using Polymerase Chain Reaction Technique

Extraction of the bacteria DNA (Heating Block Method)

Multi drug resistant *Pseudomonas aeruginosa* pure colonies from the Nutrient agar plates were aseptically inoculated into the sterile Luria Betani broth (LB) which was prepared according to the manufacturer's instruction and incubated at 37℃ for 24h. The microbial broth from the LB was transferred into 2ml micro centrifuge tubes and centrifuged for 1 minute for cell condensation; the supernatant was discarded. 1.5ml of normal saline was added to the microbial cell and the samples were mixed by vortexing for five seconds and were allowed to stand at room temperature for ten minutes and the process of washing was repeated. The samples were then heated using the heating block at 95℃ for 20 minutes, and kept in a deep freezer for 5 minutes; it was removed and centrifuged to obtain a clean upper layer. A micropipette was then used to withdraw 300µl from the centrifuged samples into a 1.5 micro centrifuge pipette.

DNA Quantification

DNA quantification and purity testing were carried out on a Nano-drop 1000 spectrophotometer by loading 2ul of the extracted product on the lower pedestal. The concentration and purity of the DNA were determined using the Nanodrop 1000 software installed on a desktop computer.

Amplification of the SHV AND CTX-M genes

Polymerase Chain Reaction (PCR) technique was used to amplify SHV and CTX-M genes, amplification was done at final volume of 20ul, 1X master mix containing Magnesium Chloride (MgCl2), buffer, De oxy nucleoside triphosphate, dNTPs, and Tag polymerase with 5ul and 0.5ul of DNA template and 0.5C of both forward and reverse primers were used respectively for the PCR reaction. Primers used for amplification were SHV Forward: CGCCTGTGTATTATCTCCCT, SHV R: CGAGTAGTCCACCAGATCCT. CTX-M F:GGACTCTGCAACAAATATACGC, CTX-M Reverse: CGCTTTCCGATGTGCAG. The mixture was then placed in a thermo cycler (PCR machine), the machine is programmed to cycle within three different temperatures; denaturation at 92-95℃ (initial for 5 minutes, final for 30 seconds), annealing 45-65℃ for 30seconds to 1 minutes and extension at 70-72℃ (initial for 30 seconds and final for 1-10 minutes). The cycle was then programmed to be 30. Primer sizes of SHV is 300bp and 600bp for CTX-M).

Agarose Gel Electrophoresis

Agarose gel prepared with Ethidium bromide was used to run the amplified sample for visualization on UV trans-illuminator. Gel Electrophoresis was done with 1.5% agarose ran at 110v for 25mins and visualized under UV trans-illuminator. This was run alongside with the DNA molecular ladder and the control strains, different base pairs of the genes were compared with the DNA ladder.

Statistical Analysis

There is no significant difference in the efficacy of susceptible antibiotics at p-value of 0.05.

RESULT

Table1.0: Depicts the distribution of sample collected in relation to age and gender of the patients. Table 2.0: Depicts the distribution of *Pseudomonas aerugionsa* isolated from the test samples. Table 3.0: Depicts Antibiotics susceptibility profile of OXOID UK antibiotic disc

Table 1: Distribution of sample collected in relation to age and gender of the patients.

Age (years)	Total number	Sample type					
		Wound		Ear		Urine	
		Male	Female	Male	Female	Male	Female
11-20	5	3		11		9	2
3							
0 -10	2	3		5			
				8	-	1	19
35							
31-40	11	5		10		1	2
31							
21-30	21	11		15			
				7	7	25	86
60							
41-50	3	1		4		5	3
2							
18							
Total	42	23		45		30	15
45							
200							

Table 2: Distribution of *Pseudomonas aeruginosa* isolated from the test samples

Sample type	Number of sample	Total number of positive (isolated) sample
Urine	60(30%)	20 (25%)
Wound	65 (32.5%)	36(45%)
Ear	75 (37.5%)	24(30%)
Total	200(100%)	80(100%)

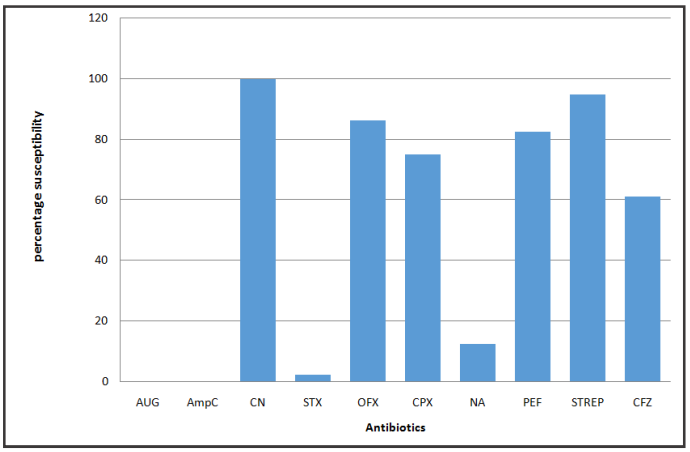

Fig 1: Bar chart showing the antibiotics percentage susceptibility profile to different antibiotics of *Pseudomonas aeruginosa* isolated from clinical sample.

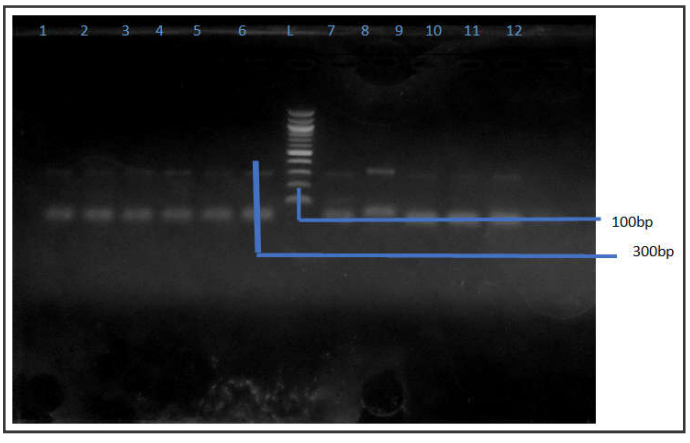

Fig 2: Agarose Gel Electrophoresis showing SHV resistance gene(300bp). Lane 1-12 represent Pseudomonas aeruginosa isolates while L represents the 100bp molecular ladder.

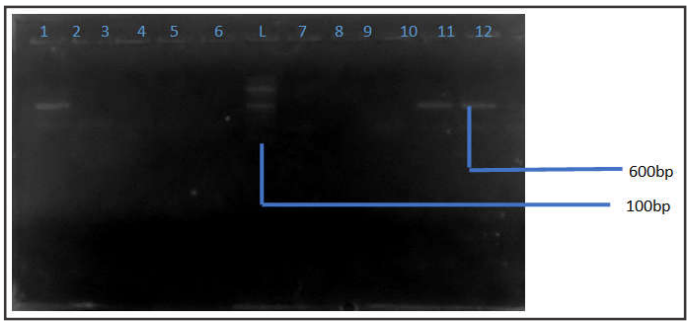

Fig 3: Agarose Gel Electrophoresis showing CTXM resistance gene(600bp). Lane 1-12 represents Pseudomonas aeruginosa isolates while L represents the 100bp molecular ladder

DISCUSSION

A total of eighty (80) *Pseudomonas aeruginosa* isolated from the study clinical samples were gotten from the following age groups: 21-30 years (30, 37.5%), followed by 31-40 years (22, 27.5%); this number was higher than what was found in a study done by Waheed *et al*. (2017) in Pakistan that revealed 40.7%virulence producing *Pseudomonas aeruginosa*out of the total number of 54 isolates among the study. This could be due to the decrease immunity of prolonged hospitalization and other associated co- morbidities. *Pseudomonas aeruginosa* had the highest prevalence in the wound (45%), followed by Ear (30%) and the least was in the urine (25%); this result is in concordance with the findings of Hossein *et al.*(2012) which reported a prevalence of 33.3% and 25% of *Pseudomonas aeruginosa* in the wound and urine samples respectively and stated that mucoid morphological types of *Pseudomonas aeruginosa* could be found in clinical samples such as urine. A high prevalence of Pseudomonas infection was found in the wound (55.5%) and ear (66%) specimen of the male patients while 80% of the organism was in the urine specimen of their female counterparts. This could be attributed to the fact that women are more prone to urinary tract infection because of intercourse, sexual development stages and puberty, their anatomical structure, pregnancy, childbirth, pre and post coital voiding, frequency of micturition, wiping pattern and douching according to Fuentefria *et al.* (2011). In this study, *Pseudomonas aeruginosa* had hundred percent (100%) susceptibility to Gentanycin; Ciprofloxacin (75%), Pefloxacin (82.5%) and Ceftazidine 61.25% a third generation Cephalosporin; this result is in correlation with the vales of Gentamycin (97%), Pefloxacin (90%),Ciprofloxacin (99%) and Ceftazidine 92% obtained by Lidia *et al*.,(2018), but in disagreement with the work done in Kasturba Medical School and hospital, Manipal, India by Syed *et al*.(2016) which stated that resistance rates were high for Fluoroquinolones 43.5%, Gentamycin 40.5% and Ceftazidine 32.5% compared with 26.25% got from this study. This is in concordance with study done by Chia-Jung *et al*. (2010) who reported that *Pseudomonas aeruginosa* was 34.9% resistant to Ceftazidine; but higher than the result of Ceftazidine(60.6%) obtained by Mai *et al*.(2014), this could be attributed to the presence of CTX-M gene present in this strain. The resistance rate of *Pseudomonas aeruginosa* to Co- trimoxazole (91.25%) Nalidixic acid (85%), Ampicillin (100%) and Augumentin (97.5%) recorded in this study is in agreement with the work of Daniel *et al*. (2016) with 99.2% resistance of Co- trimoxazole, followed by Nalidixic acid (89%), Ampicillin (99.6%) and Augumentin (97.6%) and attributed Nalidixic acid resistance in *Pseudomonas aeruginosa* to mutation. These *Pseudomonas aeruginosa* isolates produced

resistant genes ESBL (SHV 91.67%; CTM-X 33.3%), this was much higher than what was found in the study done by Gharib*et al*.(2009) in Egypt and Mai *et al*. (2014) in Cairo University Hospital, Egypt in which 24.5% and 7.4% were ESBL producers respectively.

Conclusion

In conclusion, Gentamycin had the highest potency (100%) against *Pseudomonas aeruginosa,* followed by Streptomycin (95%), Ofloxacin (86.25%), Pefloxacin (82.5%) and Ciprofloxacin (75%). Therefore, Gentamycin and Streptomycin are recommended for clinical use against *Pseudomonas aeruginosa* infections. Also resistance genes (SHV, CTX-M) Beta lactamases were found in the *Pseudomonas aeruginosa* which showed multidrug resistance to the test antibiotics, amounting to the difficulty this will pose on the treatment of infections caused by these strains which are also resistant to 3rd generation Cephalosporin's.

Recommendation

Laboratory diagnosis should be done before commencing the treatment and self -medication should be discouraged among the populace.

Acknowledgement

We acknowledge the staff and the management of Tantua and Amassoma Community Hospitals for permitting us to collect the samples.

Ethical Approval

The ethical committee of the study hospitals approved the collection of the samples, but the written document was waived because the already collected and worked on samples (duplicated) by the hospitals were used for our analysis. Only the Patients bio-data were collected from the Medical Lab Scientist –in-Charge.

Competing Interest
None

Source of Funding
None

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