Molecular Characterization and Antibiogram of *Staphylococcus* and *Pseudomonas* species in the Ear and Throat among Patients Attending Rivers State Teaching Hospital

ABSTRACT

Disease of ear, nose and throat (ENT) affect the functioning of adults as well as children, often with significant impairment of the daily life of affected patients. *Staphylococcus* and *Pseudomonas* species are the most prevalent etiologic agent of ENT infections. Therefore the aim of this study was to determine the Antibiogram molecularly Characterized the *Staphylococcus* and *Pseudomonas* species in the Ear and Throat among Patients Attending Rivers State Teaching Hospital. Total number of Eighty samples of throat swab, and aural (ear) swab were collected aseptically using sterile Evepon swab sticks and the sample collected were properly labelled with patients' number, date and the side. The swabs were immediately transported with a transportation medium in sterile cotton plugged test tube to the microbiology laboratory for further analysis. Qquestionnaires were administered for degraphic data collections. Each throat and aural swab sample was inoculated onto Mannitol salt agar and Cetrimide agar plates for isolation of *Staphylococcus* and *Pseudomonas* species respectively. The plates were incubated at 37°C and examined for growth after 24–48 hours. Pure culture of the isolates were primarily identified biochemically and confirmed using molecular approaches. Antibiogram of the isolates was performed by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute guideline. Results shown that out of the 50 ear swabs and 30 throat swab samples, 17 (34%) and 8 (26.7%) of the throat swabs ear swabs were positive of *Pseudomonas* spp. While for the *Staphylococcus* spp. 8 (16%) out of the 50 ear swabs and 4 (13.7%) out of 30 throat swabs were positive. The molecular results of the isolates show that *Pseudomonas fluorescens* NR_115715.1. and *Pseudomonas aeruginosa* NR_113599.1. *Staphylococcus aureus* NR_113956.1. were the predominates species isolated from both ear and throat swabs. The results of antibiogram of the *Staphylococcus* and *Pseudomonas* species isolated revealed that commonly uses antibiotics especially Ceftazidime, Cefuroxime, Cloxacillin Gentamicin and Augmentin were 100% resistant to the isolates only Ofloxacin, Ceftriaxone and Nitrofurantoin were susceptible to the isolates with a percentage of 80.2%, 85.7% and 90%. The findings of this study points to the high level of frequent and indescrimate uses of antibiotics among patient suffering from symptoms of ENT resulting to difficulty in the treatment of infections using common antibiotics. It is strongly recommended that ccontinuous monitoring of patients is required for preparing antibiograms. These susceptibility results will help the physician for deciding the empirical regime for the patients

Introduction

Ear (Anural), Nose (Nasal), and Throat (ENT) infection are among the most widespread and serious infections that compel an individual to seek medical attention [1]. Disease of ear, nose and throat (ENT) affect the functioning of adults as well as children, often with significant impairment of the daily life of affected patients according to [2]. It has been envisaged that with increase in global population, infection remain the most important causes of disease with upper respiratory infections causing hearing loss and learning disability particularly in children [3] *Staphylococcus aureus* is a ubiquitous, and highly adaptive pathogen that colonizes the skin and mucous membrane of the anterior nares, gastro- intestinal tracts, perineum, the genitourinary tracts and pharynx [4]. It is the causative agent of a wide range of infections including Chronic Suppurative Otitis Media (CSOM) which is defined as a prolonged inflammation of the middle ear cleft characterized by a persistent otorhoea through a perforated tympanic membrane, lasting more than 6-12 weeks in humans and animals with a significant impact on public health [5]. Clinically, *S. aureus* is the most pathogenic member of the genus *staphylococci* and the etiologic agent of a wide variety of diseases that

ranges from superficial skin abscess, food poisoning and life threatening diseases such bacteremia, necrotic pneumonia in children and endocarditis [4]. The anterior nares of nose are the primary reservoir for replication and spread to other body sites.

Ear infection such as chronic otitis (tinnitus), have serious consequences in developing countries such as retarded language development and progress in school among children [5] Tinnitus which is now known to be the most common childhood infections, lead annually to the death of over 50,000 children under 5 years (Rover et al., 2006) [6], in other cases nasal condition may be distressing, as in the case of nasal myiasis (Kuruvilla et al., 2006) [7]. Bacterial species such as *Staphylococcus aureuas*, *Streptococcus spp*, *Proteus* spp., *Haemophilus* and Coli forms were found to be responsible for most cases of ENT infections. Kumar *et al* [6] reported that *pseudomonas*, *Staphylococcus aureus*, *Proteus* and *Klebsiella* are the common bacteria that cause ENT infection in Japura India. In addition according to a study done on ENT infection in Benin City, Nigeria by Osozuwe *et al.* [7]. the following bacteria were found in 466 patients enrolled in the study; *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella spp., Streptococcus pneumoniae, E. coli* and *Citrobacter frenndi.* Based on their study, Pseudomonas aeruginosa was the most prevalent etiologic agent of ENT infections in Benin City.

P. aeruginosa is a well-known Gram-negative opportunistic human pathogen capable of causing both acute and chronic infections, being highly related to nosocomial infections according to Sharma *et al.* [8] The lungs are one of the most relevant niches for P. aeruginosa colonization, making it one of the most common pathogens isolated from respiratory infections, such as cystic fibrosis (CF) lung infection. Moreover, its great adaptability, phenotypic and genomic plasticity, ubiquity, and opportunistic sense enables *P. aeruginosa* association with other types of infection [9] *Staphylococcus aureus* colonises approximately 30% of the population [1] with the anterior nares historically considered the most frequent carriage site [2, 3]. Colonised persons carry the bacterium asymptomatically and may transmit it to susceptible persons either directly or through contact with fomites. While such colonisation often does not harm the host, it increases the risk of infections in both community and hospital settings [4], hence the need for this study to determine the Antibiogram and molecularly Characterized the *Staphylococcus* and *Pseudomonas* species in the Ear and Throat among Patients Attending Rivers State Teaching Hospital

MATERIALS AND METHODS

2.1 Study Area

Rivers State University Teaching Hospital is known to be a centre for massive influx of patients with one form of complications or another in Rivers State; hence its selection for this research study. Eighty patients who reported to ENT- in- patients unit of this hospital with various signs and symptoms of aural, nasal and throat related infections and diseases were selected randomly for this work. Patient's biodata was as well collected

2.2 Sample Collection

Total number of Eighty samples of throat swab, and aural (ear) swab were collected aseptically using sterile Evepon swab sticks and the sample collected were properly labelled with patients' number, date and the side (i.e. ear, and throat). The swabs were immediately transported with a transportation medium in sterile cotton plugged test tube to the microbiology laboratory for further analysis. Qquestionnaires designed with both open-ended questions such as age and closed ended questions with nominal categorical values such as gender were administered. Data including age, gender and symptoms were also collected [4].

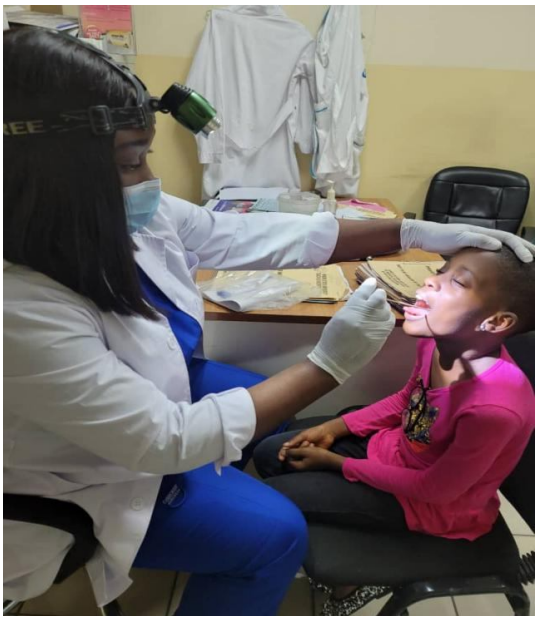


Plate 1: ENT Specialist collecting Throat Swab Sample

2.3 Isolation of *Staphylococcus* and *Pseudomonas* species

Each throat and aural swab sample was inoculated onto Mannitol salt agar and Cetrimide agar plates for isolation of *Staphylococcus* and *Pseudomonas* species respectively. The plates were incubated at 37°C and examined for growth after 24–48 hours. *S. aureus* were initially screened based on the presence of golden yellowish colonies on Mannitol salt agar while *Pseudomonas* species were primary screened based green pigmentation.

2.4 Identification of the Isolates

Pure culture of the isolates were primarily identified biochemically and confirmed using molecular approaches.

2.4 .1 Bacterial **genomic DNA extraction**

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95^{0}C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^{o}C for other downstream reactions.

2.4.2 DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

2.4.3 16S rRNA Amplification

The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3’ and 1492R: 5'-CGGTTACCTTGTTACGACTT-3’ primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

2.4.4 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final

volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

2.4.5 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA X (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

2.5 Antimicrobial Susceptibility Testing

Isolates confirmed were subjected to antimicrobial susceptibility testing to 15 antibiotics. Profiling was performed by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute guidelines [9]. An inoculum for each isolate was prepared by emulsifying colonies from an overnight pure culture in sterile normal saline (0.85%) in test tubes with the turbidity adjusted to 0.5 McFarland standard (0.5 mL of 1% *w*/*v* $BaCl_2$ and 99.5 mL of 1% *v*/*v* H_2SO_4), equivalent to 1.0×10^8 cfu/mL. The bacterial suspension was uniformly streaked on Mueller Hinton agar plates using sterile swabs and left for 3 min prior to introduction of the antibiotics. Antibiotics commonly used for treatment of *S. aureus* and *Pseudomonas* species infections were selected for this assay, Plates were incubated at 35 °C for 24 h, and the diameters of zone of inhibition were measured and results interpreted according to Clinical Laboratory Standards institute

2.6 Calculation of Multiple Antibiotic Resistances Index (MAR)

Multiple antibiotic resistance (MAR) index was calculated using the formula, a/b (where, a=number of antibiotics to which the organism was resistant and b = total number of antibiotics to which the organism was tested [18]

2.7 Statistical Analysis

Statistical analysis was carried out on the data obtained during the study using a computer-based program SPSS version 20 for Analysis of Variance (ANOVA)

3. Results and Discussion

A total number of 50 ear swabs and 30 throat swabs were collected from the patients attending Rivers State University Teaching Hospital. Out of these, ear swabs were positive of *Pseudomonas* spp. 17 (34%) and 8 (26.7%) in throat swabs. While *Staphylococcus* spp. 8 (16%) out of the 50 ear swabs and 4 (13.7%) out of 30 throat swabs were positive (Figure 1). Female patients recorded the percentage of positive for both *Staphylococcus* spp and *Pseudomonas* spp. In ear and throat swabs (Table 1 and 2) respectively. The molecular results of the isolates show that *Pseudomonas fluorescens* which has NCBI accession number NR_115715.1. and *Pseudomonas aeruginosa* which has NCBI accession number NR_113599.1. were the predominates species isolated while *Staphylococcus aureus* which has NCBI accession number NR_113956.1.

and *Staphylococcus aureus* which has NCBI accession number NR_037007.2.

Table 1: Prevalence of *Pseudomonas* species Based on Sex and sample

Sample	Sex	Negative	Positive	Total sample Examined
Ear Swabs	Male	10 (62.5)	6 (37.5)	16
	Female	23 (67.6)	11(32.3)	34
Total		**33 (66)**	**17 (34)**	**50**
Throat Swabs	Male	8 (72.7)	3 (27.3)	11
	Female	14 (73.7)	5 (26.3)	19
Total		**22 (73.3)**	**8 (26.7)**	**30**

Table 2: Prevalence of *Staphylococcus* species Based on Sex and sample

Sample	Sex	Negative	Positive	Total sample Examined
Ear Swabs	Male	13 (81.3)	3 (18.7)	16
	Female	29 (85.3)	5(14.7)	34
Total		**42 (84)**	**8 (16)**	**50**
Throat Swabs	Male	10(90.9)	1(9)	11
	Female	16 (84.2)	3(15.8)	19
Total		**26 (86.7)**	**4 (13.7)**	**30**

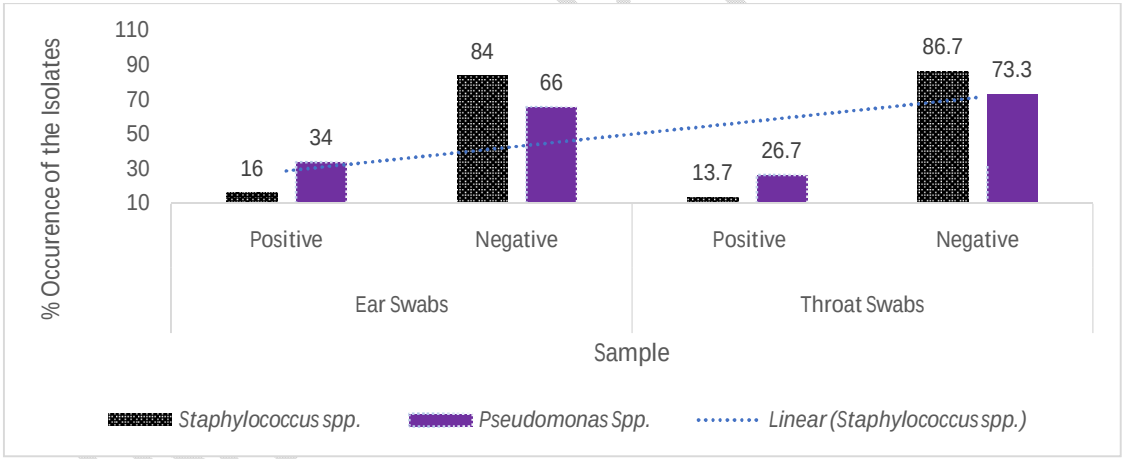


Figure 1 : Percentage Occurrence of *Staphylococcus* and *Pseudomonas* species in the Ear and Throat Swab.

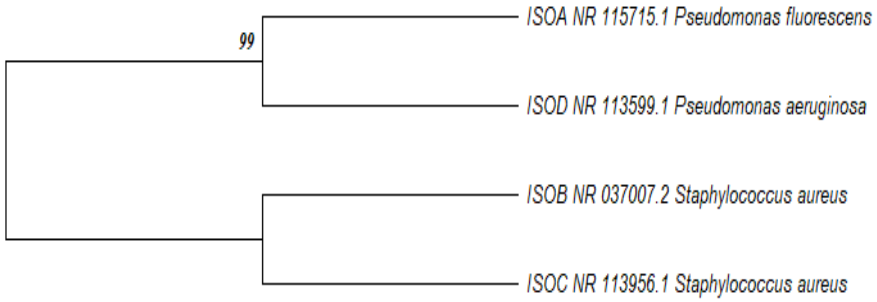


Figure 2: Phylogenetic tree showing the Evolutionary Distance between the Bacterial Isolates

Generally, the most prevalent organisms was *Pseudomonas aeruginosa* (NR_113599.1) while the least was *Staphylococcus aureus* (NR_037007.2.). Nevertheless, both stains of *Staphylococcus aureus* and species of *Pseudomonas* were isolated in both ear and throat swabs.

The finding in this study is in agreement with the report of Kumar *et al.* [23], who find out that *Pseudomonas spp*., *S. aureus*, *Proteus spp.* and *Klebsiella spp*., are the common bacteria that cause ENT infection in Japura India, similarly Osazuwa *et al.* (2011) find out that *P. aeruginosa, S. aureus, Klebsiella spp, S. pneumoniae, E. coli* are the bacteria associated with ENT infection and *Pseudomonas aeruginosa* was the most prevalent aetoliogic agent of ENT infection in Benin city, El-Mohmood *et al.* [24], These study collaborate the above findings that *P. aeruginosa, Pseudomonas fluorescens* and *Staphylococcus aureus* were found in both Ear, and throat infection.

The percentage of ENT patients attending Rivers State University Teaching Hospital who are currently taking antibiotics before seeking specialist advise is significant high as shown in (Figure 3)

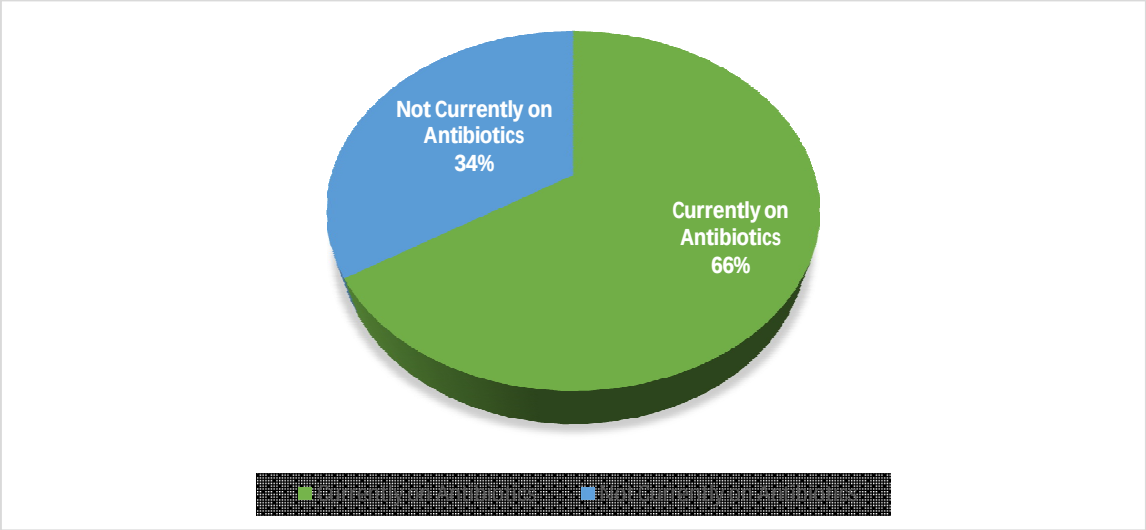
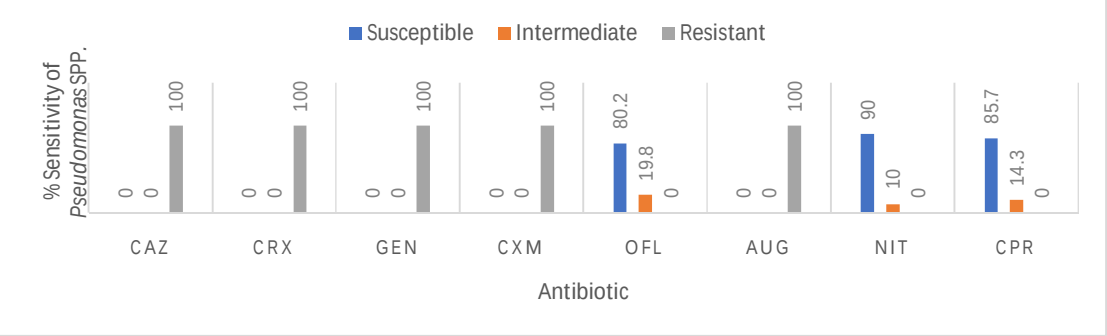


Figure 3: **Percentage of patients based on History Antibiotic Uses**

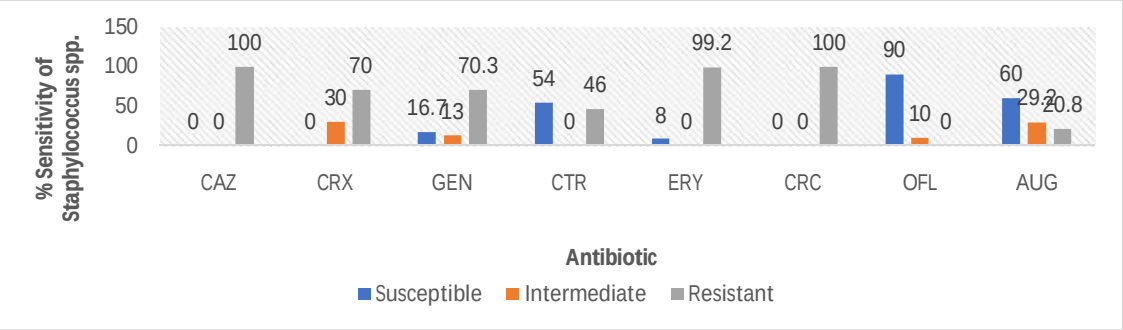
The results of antibiogram of the *Staphylococcus* and *Pseudomonas* species isolated in this study from ear and throat swab is presented in Figure 4 and 5 respectively. The *Staphylococcus* and *Pseudomonas* species were subjected to eight different types of antibiotics such as Ceftazidime (30µg) Cefuroxime (30µg), Gentamicin (10µg), Cloxacillin (5µg), Ofloxacin (5µg), Augmentin (30µg), Ceftriaxone (30µg) Erythromycin (5µg), and Nitrofurantoin (30µg) out of which only Ofloxacin, Ceftriaxone and Nitrofurantoin were susceptible to the isolates with a percentage of 80.2%, 85.7% and 90% for the *Pseudomonas* species respectively (Fig 3) while for the *Staphylococcus* species the percentage susceptibility ranged from Erythromycin (8%), Gentamicin (16.7%), Ceftriaxone (50%), Augmentin (60%) to Ofloxacin (90%), as shown in (Figure 4). Other commonly uses antibiotics especially Ceftazidime, Cefuroxime, Cloxacillin Gentamicin and Augmentin were 100% resistant to the isolates. The high percentage resistant recorded in this study could be as a results of overuse and frequent misuse of antibiotics by patients who observed little symptoms in their ear, nose and throat

which have resulted in changes of antibiotic resistance profiles of microorganisms amongst bacterial populations.The high rate of resistance of the isolates to Cefuroxime, Ceftazidime and Augmentin is of concern as most physicians are quick to prescribe these drugs especially Augmentin in the treatment of bacterial infections according to Ogbonna and Azuonwu [24]. Resistance of bacteria to Cefuroxime has been reported by Harrison and Bratcher [25] who investigated the susceptibility of some microorganisms to antibiotics such as cefuroxime. Study carried by Oparaodu *et al* [27] on Antibiogram of Methicillin Resistant *Staphyloccocus aureus* isolated from Nasal Carriage of Some University Students of Rivers State University, Port Harcourt, Nigeria also revealed high percentage resistant of commonly uses antibiotic as obtained in this study.



Key: Ceftazidime (CAZ), Cefuroxime (CRX) Gentamicin (GEN) Cloxacillin (CXM) Ofloxacin (OFL) Augmentin (AUG) Ceftriaxone (CTR) Nitrofurantoin (NIT)

Figure 4 Sensitivity profile of *Pseudomonas* species Isolated from Ear and Throat Swab



Key: Ceftazidime (CAZ), Cefuroxime (CRX) Gentamicin (GEN) Cloxacillin (CXM) Ofloxacin (OFL) Augmentin (AUG) Ceftriaxone (CTR) Erythromycin (ERY)

Figure 5 Sensitivity profile of *Staphylococcus* species Isolated from Ear and Throat Swab

CONCLUSION AND RECOMMENDATIONS

The percentage of *Pseudomonas fluorescens* (NR_115715.1). *Pseudomonas*(NR_113599.1.),*Staphylococcus aureus* (NR_113956.1). and *Staphylococcus aureus* (NR_037007.2.) isolated from ear and throat swabs in this study revealed that *Staphylococcus and Pseudomonas* species are the main causative agent of ENT infection. The findings of this study shown low percentage of the *Staphylococcus* and *Pseudomonas* species in the ear and throat of patients as points to the high

level of frequent and indescrimate use of antibiotics which in turn resulted to high level of resistant of common uses antibiotics such as Augmentin, Gentamicin, Erythromycin and Ceftazidime among patient suffering from symptoms of ENT resulting to difficulty in the treatment of infections using the common antibiotics. It is strongly recommended that self medical practices by patient be discouraged. Continuous monitoring of patients is also required for preparing antibiograms. These susceptibility results will help the physician for deciding the empirical regime for the patients.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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