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Full Length Research Paper

Characterization and antibiotic-resistant status of pathogenic bacteria from middle ear of children in Udi, south-eastern Nigeria

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ABSTRACT

Children's middle ear diseases, especially chronic otitis media with effusion (OME) which are difficult to treat are still prevalent in developing and emerging countries. This study characterized and tested the antibiotic susceptibility of pathogenic bacteria from middle ear of 105 children (56.2% boys; 43.8% girls) aged between 1 and 13 years in Udi, south-eastern Nigeria. Swabs of both external auditory canals were inoculated in duplicates onto differential culture media. A set was incubated aerobically and other anaerobically at 37ºC for 24 and 48 hours respectively. Bacteria isolates were identified by standard microbiological, biochemical, and PCR methods. Frequency percentages of the 113 bacterial isolates obtained were *Staphylococcus* species coagulase negative (74; 65.5%), *Streptococcus pneumoniae* (15; 13.3%), *Staphylococcus aureus* (10; 8.8%), *Pseudomonas aeruginosa* (9; 8.0%) and *Haemophilus influenzae* (5; 4.4%). Generally the isolates were higher in age group 6-13 years (68%) than in those 1-5 years old (31%), as well as in boys (60.2%) than girls (39.8%). *Staphylococcus aureus* and *streptococcus pneumoniae* isolates were both 65% susceptible to Ceftriaxone while *Pseudomonas aeruginosa* were 33% and 22% susceptible to Ciprofloxacin and Amikacin, respectively. All the isolates were 100% resistant to Septrin, Sparfloxacin and Ofloxacin, with strong evidence that all bacterial isolates encountered were developing varying levels of resistance to Chloramphenicol, Amikacin, Streptomycin and Tetracycline. Further studies are therefore needed from different parts of Nigeria to confirm the antibiotic resistance status of these pathogenic bacteria from middle ear of children nation-wide.

Keywords: Children, middle ear, bacteria, characterization, antibiotic resistance

INTRODUCTION

Otitis media is a group of inflammatory diseases of the middle ear (Qureishi *et al*., 2014). There are two main types, acute otitis media (AOM) and otitis media with effusion, OME (Chris Stockmann *et al*., 2013). AOM is an infection of abrupt onset that usually presents with ear pain. In young children this may result in pulling at the ear, increased crying, and poor sleep. Decreased eating and fever may also be present. OME is typically not associated with symptoms (Lieberthal *et al*., 2013). Sometimes a feeling of fullness is described. It is characterized by the presence of non-infectious fluid in the middle ear for more than three months. Chronic suppurative otitis media, CSOM is middle ear inflammation of greater than two weeks that results in episodes of discharge from the ear. It may be a complication of acute otitis media. Pain is rarely present (Minovi *et al*., 2014). All the three may be associated with hearing loss (Bhatt *et al*., 1991; Qureishi *et al*., 2014). The hearing loss in OME, due to its chronic nature, may affect a child's ability to learn (Bidaldi *et al*., 2008; Da Costa *et al*., 2009; Gouma *et al*., 2011).

The cause of AOM is related to childhood anatomy and immune function. Bacteria (*Streptococcus* species, *Haemophilus influenza,* etc.) or viruses may be involved. Risk factors include: exposure to smoke, use of pacifiers, and attending daycare. OME frequently occurs following AOM but may also be related to viral upper respiratory infections, irritants such as smoke, or allergies (Qureishi *et al*., 2014). A number of measures decrease the risk of otitis media including: pneumococcal and influenza vaccination, exclusive breastfeeding for the first six months of life, and avoiding tobacco smoke. Worldwide AOM affect about 11% of people a year i.e. about 710 million cases (Coker et al., 2010; Monasta et *al*., 2012). Half the cases involve children less than five years of age and it is more common among males. Of those affected about 4.8% or 31 million develop chronic suppurative otitis media (Monasta *et al*., 2012). Before the age of ten, OME affects about 80% of children at some point in time. Otitis media resulted in 2,400 deaths in 2013 down from 4,900 deaths in 1990 (GBD, 2013). The aim of this study was to characterize and test for antibiotic-resistant status of pathogenic bacteria from middle ear of children in Udi, south-eastern Nigeria

MATERIALS AND METHODS

Study Population

A total of 105 children age between 1 and 13 years were used as subjects of this study, with informed consent of their parents or guardians. Swabs of their left and right external auditory canals were taken using sterile swab sticks. The swabs were immediately transferred to the laboratory for microbiological examination.

Sample Processing

The swabs were inoculated in duplicate onto MacConkey agar, Nutrient agar, Blood *agar* and Chocolate agar. A set of the inoculated plates were incubated aerobically in the incubator at 37ºC, while the other set was incubated anaerobically at the same temperature. After 24 hours, the aerobic cultures were read. The anaerobic cultures were incubated for 48 hours before reading - using anaerobic jar and Oxoid gas generating kit. The inoculated Petri dishes were packed in a 3.5 liters capacity anaerobic jar. A corner of a sachet of Oxoid gas generating kit was cut out and10 ml of water added to, following Manufacturer`s instruction. The sachet was kept upright in the anaerobic jar and the lid closed immediately and incubated at 37ºC for 48 hours.

Identification of bacterial isolates

All the bacteria isolates were identified by standard microbiological methods (i.e., appearance in differential culture media), Gram reactions and biochemical tests such as Bile solubility, Catalase, Coagulase, Oxidase and Optochin sensitivity tests (Cheesbrough, 2006), as well as polymerase chain reaction, PCR (NEB, 2018) which involved DNA extraction, PCR, and DNA Electrophoresis.

In DNA extraction (Queipo-Ortuño1 *et al*., 2017), 500 µl of 48 hours broth cultures were put into micro-centrifuge tubes, and 50 µl of sterile distilled water added to each and centrifuged at 12,000 rpm for five minutes and supernatants were gently decanted. Equal 50 µl of sterile distilled water was added to each cell pellet and heated in heating block at 99ºC for 10 minutes to lyse the cells and release the DNA. The cells were subjected to "cold shock" treatment for 10 minutes by placing the micro-centrifuge tubes in ice block, and spun at 12, 000 rpm for 5 minutes before the supernatants were transferred into micro-centrifuge tubes for PCR reaction. Following the PCR procedure of NEB (2018), each mini micro-centrifuge tube was labeled on top with sample number and name of the primer by the side of the tube. Then, 35.8 µl of sterile de-ionized water was added to each tube. To these were added sequentially, 5 µl of PCR buffer ($MgCl_2$), followed by the additions of 1 µl dNTPs, 3 µl of Primers (F), 3 µl of Primers (R), 0.2 µl of Taq DNA polymerase, and 2 µl Template DNA. Then, 2 µl sterile distilled water and 2 µl of standard DNA were respectively added to the negative and positive controls. The tubes were Vortex at low speed, Pulse centrifuge and placed in Thermal cycler (Techne Thermal Cycler Bibby Scientific UK) for two hours under the following PCR conditions: Initial denaturation at 95ºC for 5 minutes, thirty five cycles of denaturation at

95ºC for 30 seconds, annealing at 58ºC for 30 seconds, and extension at 72ºC for 45 seconds, final extension at 72ºC for five minutes and hold at 4ºC. In DNA electrophoresis, 10 µl of ethidium bromide was added to each tube (PCR). Then 3 µl of bromophenol blue was added to samples, negative control and Positive. Then 15 µl of PCR amplified DNA, negative control and positive control (100bp DNA Ladder NORGEN Biotek Corporation) were added to agar wells and electrophorosed (Minigel Horizontal Electrophoresis kit, Cleaver Scientific, USA) for 45 minutes before the agarose gels were viewed under ultra violet (u-v) light.

Susceptibility Testing

The bacterial isolates (24-hour old) were tested for susceptibility to antimicrobial agents by disc diffusion method (Bauer *et al*., 1966) using commercial discs from Maxicare Medical Laboratories and Mayo Diagnostic Lab).

RESULTS AND DISCUSSIONS

Figure 1 and Table 1 show the appearances and results of the bacterial isolates (A, B, C, D, and E) after Gram staining reactions. Similarly, biochemical tests for the isolates are shown in Tables 2.

Table 1: Gram reactions

Isolate	Gram reaction
A	Gram +ve
B	Gram +ve
C	Gram +ve
D	Gram -ve
E	Gram -ve

Table 2: Biochemical tests

Isolate	BiL	CaT	CoG	OxD	OpC
A	–	+	–	–	–
B	–	+	+	–	–
C	+	–	–	–	+
D	–	–	–	–	–
E	–	–	–	+	–

BiL= Bile solubility, CaT= Catalase, CoG= Coagulase, OxD= Oxidase, OpC= Optochin

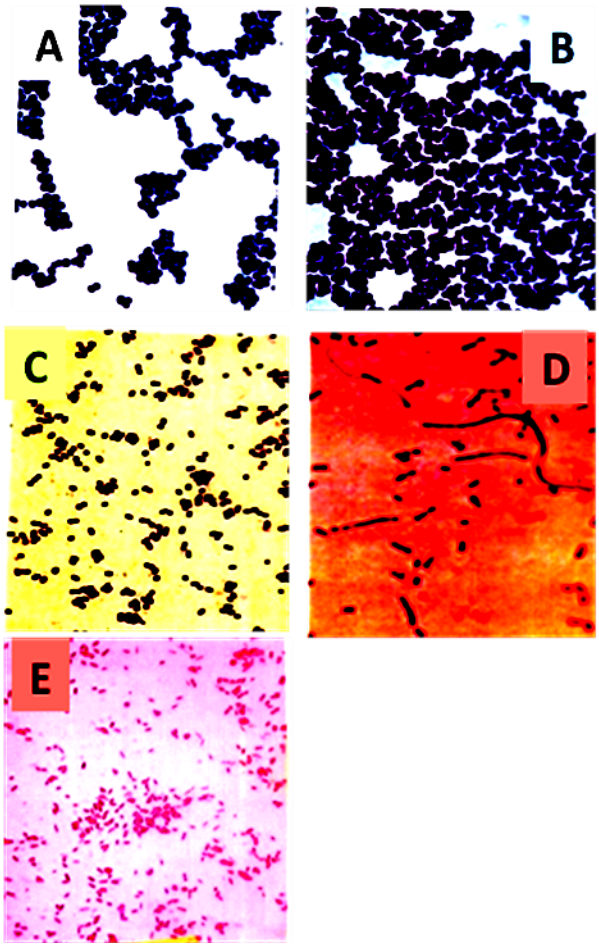

Fig. 1: Appearance of smears of bacterial isolates after Gram staining reactions. A= Cocci in clusters, B= Cocci in clusters, C= Cocci in chains, D= Coccobacilli, E= Bacilli

From appearances of isolates in Figure 1, and reactions in Tables 1 and 2, the bacterial isolates were characterized as:

A=*Staphylococcus* spp. Gram +ve Cocci in clusters, coagulase negative, bile solubility negative, catalase positive, oxidase negative and optochin sensitivity negative.

B=*Staphylococcus aureus* Gram +ve cocci in clusters, coagulase +ve, bile solubility negative, catalase +ve, oxidase negative and optochin sensitivity negative.

C=*Streptococcus pneumaniae* Gram +ve cocci in chains, *coagulase* negative, bile solubility +ve, *catalase* negative, *oxidase* negative and *optochin sensitivity* +ve.

D=*Haemophilus influenza*e Gram negative coccobacilli, coagulase negative, bile solubility negative, catalase negative, oxidase negative and optochin sensitivity negative.

E=*Pseudomonas aeruginosa* Gram negative bacilli, coagulase negative, bile solubility negative, catalase negative, oxidase +ve, and optochin sensitivity negative.

The molecular confirmation of *S. aureus* in the agarose gels viewed under u-v light is shown in Figure 2 in which the gel shows 2 panels (top and bottom). In the top panel, lanes 1 and 7 contained molecular weight standard; lane 6 is negative control while lanes 2 to 5 contained DNA of *S. aureus*. Lanes 2 to 5 (top), and lane 5 (bottom) show positive detection of the 750bp amplicon of the 16s rDNA which confirmed those isolate to be *Staphylococcus aureus.*

The overall frequency percentages of bacterial isolates from middle ear of the children are shown in Tables 3 while the total frequency percentages of the different bacterial isolates are shown in Figure 3.

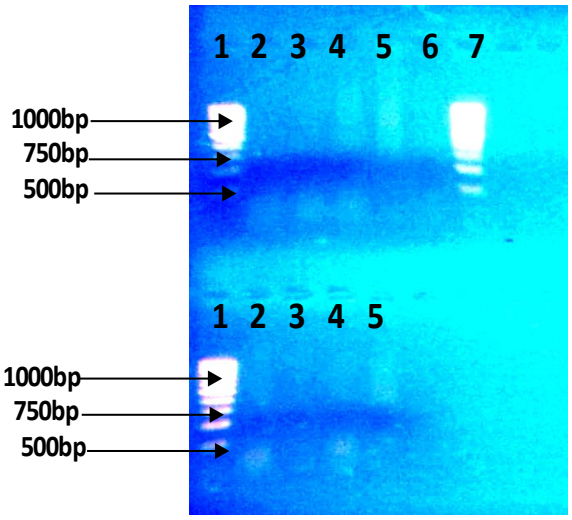

Fig. 2: Polymerase Chain Reaction (PCR) Agarose gels under u-v light.

Staphylococcus spp. (coagulase negative) was most frequently isolated, being significantly different ($p<0.05$) from the others. However, Figure 4 shows the frequency distribution of the isolates by gender and age of the children examined. Generally, boys had more frequent isolates than girls, but 6 to 13 year olds of both sexes experienced more bacterial isolates than those less than 5 years old. Monasta *et al.* (2012) had also reported otitis media more commonly in males. Presence of the bacteria in children is of public health concern because their immune system may not be fully developed to challenge the condition (Bernstein *et al.*, 1987). Inner ear infections may arise as a secondary effect of otitis media or bacterial meningitis - an inflammation in the meninges of the brain – leading to hearing loss. *Streptococcus pneumoniae* and *H. influenzae* are the most frequent bacteria that cause acute otitis media and subsequently Otitis media with effusion leading to hearing loss (Bhatt *et al.*, 1991; Qureishi *et al.*, 2014).

Table 3: Overall frequency of bacterial isolates by gender and age

Gender	Age (y)	Examined No. (%)	All isolates *f* (%)
Boys	1-5	28 (26.7)	18 (16.0)
	6-13	31(29.5)	50 (44.2)
Girls	1-5	19 (18.1)	17 (15.0)
	6-13	27 (25.7)	28 (24.8)
Total		**105 (100)**	**113 (100)**

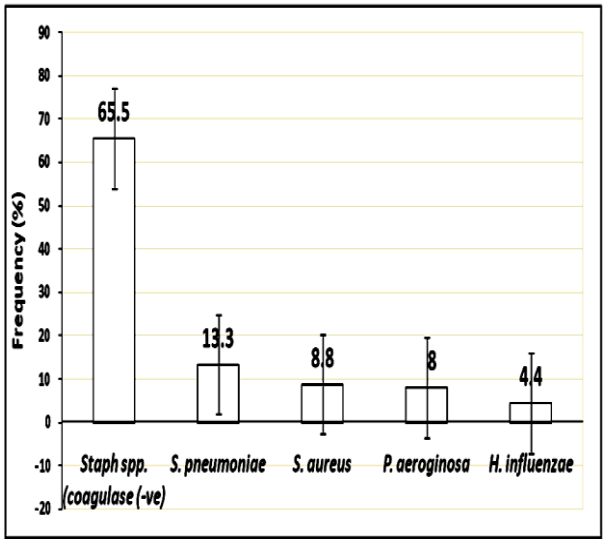

Fig. 3: Overall Frequency of bacterial isolates from middle ear of children studied.

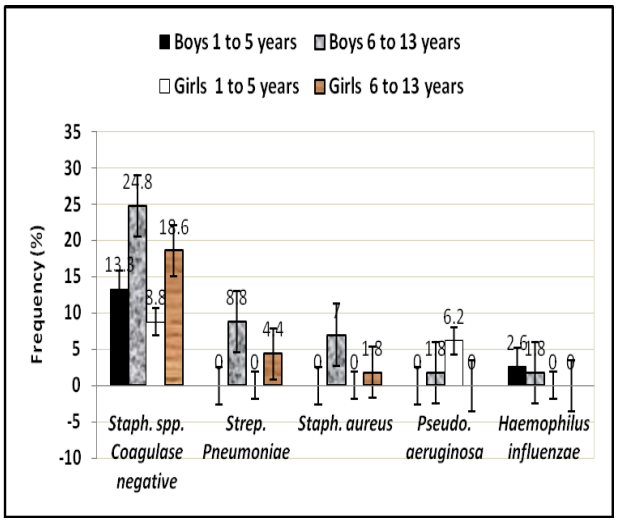

Fig. 4: Frequency of different bacterial isolates from middle ear of children studied.

Similarly, *Pseudomonas aeruginosa* has been reported in acute external otitis (swimmer's ear) and sometimes *S. aureus* and *Streptococci* species (Todar, 2008).

Table 4 shows the susceptibility of the bacterial isolates to the 13 antibiotics tested.

These antibiotics were most readily available 'over the counter' in many medicine stores in Nigeria. The public health implication of observed developed 100% resistant by these bacteria to Septrin, Sparfloxin, and Ofloxacin need not be over emphasized. Many antibiotics are losing their efficacy, perhaps due to poor handling and storage, or are faked, expired or adulterated. However NAFDAC has been waging a relentless war to curb substandard, fake and adulterated drugs in Nigeria, and must be empowered to accomplish the task.

Table 4: Percentage susceptibility of the isolates to the antibiotics tested

Antibiotics	Susceptibility (%) of isolates			
	B	*C*	*D*	*E*
SXT	0	0	0	0
CH	0	0	0	30
SP	0	0	0	0
CPX	20	3	33	50
AMK	0	0	22	0
GN	25	5	22	0
PEF	30	0	0	0
OFX	0	0	0	0
S	25	10	0	0
E	0	0	0	20
TE	0	0	22	0
V	60	0	0	45
CFX	65	65	0	40

B = Staphylococcus aureus
C = Streptococcus pneumaniae
D = Pseudomonas aeruginosa
E = Haemophilus influenzae
SXT = Septrin
CH = Chloramphenicol
SP = Sparfloxacin
CPX = Ciprofloxacin
AMK = Amikacin
GN = Gentamicin
PEF = Peflacine
OFX = Ofloxacin
S = Streptomycin
E = Erythromycin
TE = Tetracycline
V = Vancomycin
CFX = Ceftriaxone

Conflicts of Interest

The authors declared no conflicts of interest.

Contributor's Statement

Multiple contributors were required to complete this study, and each author read and approved the final manuscript before submission.

Elomba, C. Chidozie developed the study design, collected bacterial samples from middle ear of subjects, conducted bacteriological, biochemical, and PCR tests, interpreted results, and wrote the manuscript.

Umedum, C.U participated in the conception of the study design, did the antibiotic susceptibility tests, and interpretation of the data.

Uboh, Ifiok Nse helped Elomba, C. Chidozie in carrying out the PCR, statistical analyses of the data, and interpretation of results.

Ikpeze, O.O provided input to the study design, produced all tables and figures, helped with the statistical analyses, accessed all the data in this study and is responsible their integrity, and accuracy, critically edited final version to be published.

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