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Study on Molecular Characterization and Plasmid Resistance Genes of Ear Bacteria Pathogens, among School Children in Nkpa, Abia State Nigeria

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Abstract:- The study was on molecular characterization and plasmid resistance genes of pathogenic bacteria isolated from the ear of school children. Ethical clearance was obtained and the consent of the Pupils and their Parents were sort for their inclusion in this study. The specimens were obtained from pupils aged 2 to 15 years. Aural swab was collected from pre-screened Pupils that complained of itching of the ear and had not taken any antibiotics prior to the specimen collection. The study area was Nkpa in Bende, Abia State. The Bacteria isolates were characterized using 16Subunit ribosomal Ribonucleic acid gene sequencing. at DNA laboratory Kaduna. The isolated Bacteria were), 1.0% *Comamonas aquatic,* 4.0% *Stenotrophomonas maltophilia, 5.5*0% *Delftia acidovorans,* 5.5% *Pseudomonas aeruginosa, 8.0*% *Achromobacter xylosoxidans.* The isolates were subjected to ascertain plasmid genes. From the 200 sampled population, plasmid resistant genes were isolated in 48 bacteria isolates. Of the statistical analysis done, 14.0% isolates were from males while the females children had 10.0%. Effect of age on the ear infection revealed that pupils of 2 to 3 of age had the major infection of 16 (8.00%), pupils of 4 to7 of age and 8 to 11 years had similar number of 13 (6.50%) infection while the least infected were pupils aged 12-15 years with 6 (3.00%). Plasmid resistance genes were identified from all the bacteria isolates, this result reveals the presence of resistant bacteria strains. Thus, going by this research, it is ideal to always carry out a surveillance on the bacteria ear infection and plasmid gene resistant before administering antibiotics among the pupils of this locality.

Keywords:- *Pathogens, Aural Swabs, Bacteria Plasmid Genes, Bacteria,16 Subunit Ribosomal Ribonucleic Acid, Inflammation, Ear Infection, Isolates, Pathogens, Labyrinthitis, Deoxyribonucleic Acid, Polymerase Chain Reaction, Electrophoresis, Gel Electrophoresis.*

I. INTRODUCTION

Ear is one of the sensory organ in the human body that aids in hearing and balancing. The impairment or inflammation of the hearing organ as a result of bacteria pathogens is known as bacteria ear infection. Currently, It is more comprehensible that ear infection has been predominantly among the tropical countries. Recent data showed that among all age groups, though often among in pupils (Brobby, 1992). Dhiangra (2004), posit that infant's Eustachian tube is narrow, more flat, having a more flaccid cartilage making its opening to damage easily and thus otitis media with effusion has a significant health issue. It Scientist believed that up to 65 to 330 million individuals worldwide had ear infection, 60% of this patients had hearing defect (Woodfield and Dugdale, 2008). As the children pharynx grows and develop from their adolescent age upward, the incidence reduces (Teele *et al.*,1989). Incidence of otitis could be more prevalence within individuals of low socioeconomic status, male sex, early marriage, daycare attendance, low birth weight, inadequate housing conditions, inadequate to health care, mothers that are exposed to smoke during pregnancy, and kitchen smoke (Strachan and Cook, 1998). It is evident that majority of otitis media do occur with no visible exudate (Raakheesagar and Unguturu, 2014), while in some others, the infection may even be totally without symptoms (Niemela *et al*., 1994). Otitis can emanate from the infection of outer layer of the auditory meatus (called as otitis externa), or the inflammation of the middle ear cavity, (called otitis media). in all the ear infections. Otolaryngologists has diagnosed more otitis media among Nigeria children (Okafor, 1984).

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Bacteria Ear inflammations has been linked as a significant factor for most hearing challenges with chronic effect effects, most of which can be controlled or treated, (Variya *et al*.,2002). The pathogen and existence of infection varies with climatic conditions and geographical areas (Muluye *etal*., 2013), evidently, some bacteria flora that colonizes the skin which include *Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli, and Proteus mirabilis* under antibiotic resistant bacteria and other certain conditions, were shown to be the most etiology of otitis (Abera and Kirbret, 2011). For Acute Otitis Media (AOM), the bacteria mostly isolated include nontypeable *Haemophilus influenza, Streptococcus pneumoniae,* and *Moraxella catarrhalis*. They cause more than ninety-five percent of all AOM issues of bacteria origin (Rosch, 2014).

Gram negative isolates like *Pseudomonas aeruginosa, Escherichia coli, Klebsiella* species, (Rosch 2014), *Achromobacter xylosoxidans* (Yabuchi and Yano, 1981), exert larger roles of about 20% of cases in children infants. *Haemophilus influenzae* and *Streptococcus pneumoniae* had the same cases among the infants. A few other researches also attributes *S. aureus* as pathogenic bacteria in under aged, however further studies opined that the flora in the children can be that of usual AOM in older children.

Many pundits had opined that the (MEE) middle ear effusion that were linked with (OME) otitis media with effusion should have no flora, since such cultures would be processed from fluids from the middle ear extracted by tympanocentesis mainly should no bacteria growth. The understanding has been revolutionized with evidence from current studies revealed the prevalence of positive results in thirty to fifty percent in bacteria culture of patients who had persistence MEE. The isolates can be cultured in a variety of aerobic and anaerobic media conditions. The common isolates are a group streptococci, *Streptococcus pneumonia, Moraxella catarrhalis, and Haemophilus influenzae*.

Acute otitis media can be caused by *M. catarrhalis* including some bacteria that vary differently. It is seen with a higher percentage of mix infections, at lower age groups, no mastoiditis, and the lower risk of perforation of the tympanic membrane (Broides *et al*., 2009). In order to prove the bacterial deoxyribonucleic acid is present in middle ear effusion patients -with otitis media with effusion, Polymerase chain reaction is applied by applying the middle ear effusion specimens which were certified to have no contaminants using a reference culture method. On the other hand , the bacteria pathogens that were often cultured in chronic suppurative otitis media, included *Corynebacterium* species, *Pseudomonas aeruginosa, Klebsiella pneumonia, and Staphylococcus aureus,* (Orji and Dike, 2015). More scientist have frequently identified *Helicobacter pylori* in children having middle ear infections (Yilmaz *et al.*, 2006). *H. pylori* present in middle ear infection with discharge may emanate from cultures involving adenoid tissue, tonsila, and middle ear in patients with OME. *Alloiococcus otitidis*- a G+ve bacteria isolate was shown a the main etiology in middle ear infection with effusion (Harimaya *etal*.,2006; De Miguel *et al*., 2008). It is also diagnosed among infants who have taken beta-lactam or erythromycin treatment, indicates that these medication may not be effective to eradicate this pathogen. Fliss, *et al.*, (1992) have it that major studies in microbiology on (CSO)- chronic Suppurative otitis identified *Staphylococcus aereus, Klebsiella* species, and *Pseudomonas aeruginosa*, to be the major aetiologic agents. In a similar study, Nwabuisi and Ologe, (2002), highlighted *Proteus* species and *Pseudomonas aeruginosa* as the commonest bacteria. Reports abounds that over 90% of otitis external are caused by bacteria (Clark *et al*.,1997). Roland and Stroman, (2002), also has it that the commonest isolates identified in otitis externa included *Staphylococcus* species, *Pseudomonas* species, together with other anaerobics and gram-negatives.

As the bacterial aetiologic agents of ear infection changes periodically, understanding the local variety of the infection is very crucial for proper medication of these diseases. (Oni *etal*.,2001). If there is very low drug susceptibility, it may arise from bacteria plasmids genes (Levy, 2002), or biofilms (Lewis, 2005) in many of disorder of the ear. In medicine, bacteria plasmids which frequently carry Multiple-Antibiotic-Resistance genes (M.D.R Plasmids) are vital as it can be transmitted to a vast bacteria (Mollenkopf *et al*., 2017). As a matter of fact, one plasmid that is transferred, can make a bacterium that is susceptible to a given drug to turn around and become resistant to such drug, such consequent could be seen in that of a rapid worldwide plasmid-mediated spread of antibiotic resistant of Colistin drug. This drug was once seen as utmost antibiotic (Liu *et al*., 2016). Study results by Venekamp *et al* (2016) revealed a short term hearing defect due to general use of antibiotic medication on middle ear infection. It also lead to complications and occurrence of bacteria pathogens that are no longer susceptible to antibiotics. Generally, low performing antibiotics have cause a significant problems to the health of the masses leading deficiencies in universal health at which aim result are in there need. These involve finding complementary and alternative therapeutic principles (Tiara *et al*., 2013).

A plasmid gene is a small circular DNA molecule found in bacteria and some other microorganisms. Bacterial plasmids are small, circular deoxyribonucleic acid molecules that can be separated from the rest of the chromosomal components. They replicate independently of the bacterial chromosome. Plasmid genes can be physically separated from chromosomes DNA and they replicate independently. Resistance plasmids do carry one or more antibiotic resistant genes. They are accompanied with genes encoding virulence determinants (Hughes and Datta, 1983).

Plasmids often carry multiple antibiotic resistant genes, which contribute to the spread of multidrug-resistance (MDR) (Nikado, 2009). It is also prominent that antibiotic resistant, mediated by multi drug resistance (MDR) plasmids severely hampers treatment options for the infections of by Gram-negative bacteria origin, especially the family of Enterobacteriaceae (Schultsz and Geerlings,

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2012). The global spread of MDR plasmids has been enhanced by selective pressure from antimicrobial medications used in medical facilities and when raising animals for food (Mathers et. Al,. 2015).

The aim of this research is therefore to carry out molecular characterization of bacteria pathogens from the ear of school children as well as to profile the resistance plasmid gene on the bacteria isolates.

The specific objectives

- To collect ear exudates using sterile swab sticks
- To carryout bacterial culture and identification using bacteriological technique
- To further identify the bacteria species and strains using 16S Rrna. Gene sequencing.
- To isolate plasmid genes of the bacteria isolates
- To identify the plasmid resistant genes

II. MATERIALS AND METHODS

A. Area of Study

A conglomerate community called Nkpa which have seven communities, situated in Bende the apex of the northern part of Abia state served as the study area. The natives of these communities were occupied with Farmings, Fishing, Livestock production, Trading and a few Civil service activities.

B. SAMPLE COLLECTION

Firstly, a letter for ethical approval was applied, approved and collected with registration number MOE/PSI/123/T/133 from the Education ministry, Abia State. The parents and pupils were acknowledged with the objectives of conducting the research and in return, they obliged participating in the studies. About two hundred aural swab samples were collected with sterile aural swab sticks from children aged 2 to 15 years who had either itching, pains or ear discharge and who have neither taken antibiotic nor any herbal mixture for the past 5 days. The samples were from community primary schools of Ugwu Nkpa, Eluama Nkpa, Amaegbuato Nkpa, Amaohoro Nkpa, and Amaokpu Nkpa. They were well labeled with gender, age group, and name of school. The samples were transferred to microbiology laboratory, Michael Okpara University of Agriculture, Umudike.

➢ Media Sterilization and Preparation

Detergent was used to thoroughly wash the heat resistant wares such as glass-petri -dishes, flasks, test tubes and other glass ware, air dried on the tube racks, sterilize by stacking them into a hot air oven, at 170^{o}C for holding duration of 1hour. Cetrimide agar, Nutrient medium, Mueller-Hinton agar Mac Conkey agar, blood agar, and Eosin methylene blue media was well processed by adhering to the different manufacturers guidelines and were all sterilized in autoclave at 121^{o}C (at 1.05kg/Cm^{3} pressure) for 15 minutes. Each medium was dispensed in 9cm wide petri dishes. Sterility of the media was determined using a control set up by incubating two sets of the uninoculated media at 37 ^{0}C for 24 hour.

C. Culturing and Identification of Bacteria

From aural swabs collected were inoculated by streaking onto nutrient agar, Mac Conkey agar, Eosin methylene blue Cetrimide agar and Blood agar media plates. The streaked plate's media were also incubated at 37 ^{0}C for about 24 hours. Plate with visible growths was further streaked on nutrient medium to obtain enhanced colonies. The pure colony were preserved slants of nutrient agar at 4^{0}C for subsequent use.

D. Isolates Characterizations and Identifications

Preliminary bacteria isolate features and identification was done by the help of Cheesbrough, (2002) identification schemes on the basis of looking at the cultural morphological characteristics of the isolates together with Gram staining.

E. Gram Staining

Principle of Gram staining procedure relies the retention of the basic dye (crystal violet) by Gram positive isolates and retention secondary dye, which was carbol-fuchsin by Gram negative bacteria isolate to retaining the (Jawetz, *et al*., 1985). An inoculums was prepared on a clean grease free glass slide using an inoculating loop. The films were air dried by waving for a period of 20 minutes, the air-dried films were heat-dried by gently waving over a green fire. At this point,the heat0fixed smears were allowed to cool for 1 minute before flooding with crystal-violet solution for 60 seconds, the crystal violet stain was washed off gently using clean water for a period of 5 secs. Grams iodine was used to flood the smears for the next 60 seconds. Again, the slides were rinsed in slowly using clean water. Three drops of ethanol solution (95% concentration) was applied to decolorize the preparation, they were covered with carbolfuchsin (0.2%) for the next 30 secs. Then, the slides were rinsed slowly under running water, and were then air dried. The prepared slides were viewed with X100 objective lens using oil immersion on the compound light microscope. Gram negative isolates appeared pink and Gram positive isolates appeared purple.

F. Identification of the Bacteria Pathogens by Molecular Process

The technique used was the 16 Subunit ribosomal Ribonucleic-Acid Gene Sequencing method. After the letter for a bench Space Reservation was submitted and approved. The pre identified bacteria cultures were sent to DNA research laboratory in Kaduna State Nigeria.

The principle objective by utilizing the hyper variable regions present in the 16 sub unit ribosomal ribonucleic acid gene to identify isolates specific signatures sequences (Valerie *et al*., 2008).

G. Extraction of Deoxyribonucleic Acid

Materials such as the preserved bacterial isolate, Lysozyme solution, Proteinase K, Tris buffer: Ethylene diaminetetra Acetic acid(TE), 10%, 1% of sodium dodecyl sulfate(SDS), Phenol, Sodium acetate, Chloroform, Isoamyl alcohol, Micropipettes, Water bath, Absolute ethanol, Vortex and Centrifuge. Prior to starting, the heating blocks

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were set at 60^0C. from 24 hr isolate slant pallets of six pre identified bacteria specimen originating from gram stains and microbiologil procedures. With inoculation loop, small particles was picked and incerted in a 1.5 ml eppendorf plastic microtube that is already containing 400ul sodium dodecyl sulfate lyses buffer and mixed with 10ul of protenase K. the preparations was immersed in the heating block set at a temperature of 60^0C allowing it to reaction on a I hour mark.

To each separated top clear layer, was introduced 400ul chloroform, they were vortexed short while to mix it thoroughly. The solution was centrifuged at 13000 rpm for a period of five 300 seconds for each phase to separate from the mixture. The top layer was gently removed with a micropipette. and was transferred to a fresh 1.5ml Eppendorf tube. Leaving behind the white interphases.

About the same quantities of 20ul of 3M sodium acetate and 100% ethanol was transferred in the eppendorf tubes, and mixed by turning up and down each tube severally. Incubation was done by setting the tubes at a temperature of -20^0 C for the next 24 hrs. The reaction tubes were then centrifuged in a refrigerated centrifuge at a vey high revolution for a period of 1800 seconds. The ethanol solution was removed and a fresh 400ul of 70% ethanol was introduced to each before centrifuging on a very velocity for 5 minutes at 4^0C. The steps was repeated to remove all the salt.

The eppendorf tubes was centrifuged for about 30 seconds intervals at high velocity, to remove all the ethanol traces. The obtained deoxyribonucleic acid was dried out by allowing the tubes open for 10 minutes. The deoxyribonucleic acid pellets was re-suspended in 50ul sterile water. 1ul to 5ul of the deoxyribonucleic was test- run on an agarose gel to estimate integrity and quantity.

H. Polymerase chain reaction

1µL of Template deoxyribonucleic acid, 2µL dNTP's, 37.5 µL of RNAse DNAse free water, 2µL of Forward primer, 5µL Taq deoxyribonucleic acid polymerase, 2 µL of Reverse primer and 5µL of Taq assey buffer(x10) was mixed in a sterile 0.5-ml microfuge tube.

The solution mixtures was overlaid with 1 drop (approximately 50 µl) of light mineral oil inside the thermal cycler, the tubes was placed inside the thermal cycler. The deoxyribonucleic acids was amplified using the denaturation procedure at 94^0C for 2 mins followed by 30cycles at 94 ^{0}C for 30 seconds,, annealing at 52 ^{0}C for 30 seconds and 72 ^{0}C for 2mins, and a final extension polymerization step at 72 ^{0}C for 300 seconds. About 10 µl of each sample was taken off from the test reaction mixtures and the four control reactions. They were analyzed using gel electrophoresis. The gel was stained with ethidium bromide as to visualize the deoxyribonucleic acid. Visible deoxyribonucleic acid fragments was produced at the end of the process (amplification).

I. Gel-Electrophoresis

The reagents and wares included: Ethidium bromide, Agarose powder, deoxyribonucleic acid Ladders, Electrophoresis buffer, deoxyribonucleic acid samples, a gel casting tray and Transilluminator (fotodyne, foto/wv 15 model 33017USA). Agarose gel specification used:. Lot: 5KR081612. W230-500, 500 grams; QD LE Agarose high resolution Quick dissolve, gel strength= 1200g/cm3 (1.0%); low EE: ≤0.13; moisture: ≤10%; gel-point: 36^0C ± 1.5^0C (1.5%); DNase and RNase: None detectable, meting point 88^0C±1.5^0C (1.5%); sulfate: about 0.14%. separation range: 50 bp – 20 kb; 3g of agarose was used for 1.5% preparation.

- **Principle**: DNA fragments of different sizes can be separated by an electrical field applied to a "gel". Based on the fact that the negatively charged DNA do migrates away from the negative electrode and to the positive electrode. In addition, the smaller the fragment the faster it migrates.

The Polymerase chain reaction amplicons was separated and visualized using the following steps: The solutions was heated inside a microwave for 60 seconds until the agarose was completely dissolved, and was allowed to cool in a water bath set at 55^0 C. The gel casting tray was set by sealing the ends of gel chamber with masking tape. Then 2 combs was carefully placed in the gel tray.

Up to 5ul of ethidium bromide was introduced to the cooled gel and was poured into the agarose gel tray. It gently cooled for 30 minutes at room temperature. After the combs were removed, the gel was placed inside the electrophoresis chamber before it was covered with TAE buffer. using pipette, the standard ladder, the Negative control that contain water and primer together with the extracted deoxyribonucleic acid was loaded onto gel starting from left to right of the wells. The switch was on, setting the voltage at 111 for a period of 1 hour and the deoxyribonucleic acid bands was visualized using Ultraviolet light box. The images was captured.

J. Elution Stage of DNA

The reagents and machines included: Agarose, Micropipettes, Elution buffer, , Microfuge, incubator, Centrifuge, tubes, Dry bath, N-Butanol, 70% and 95% Ethanol, Cryo box, Cyclomixer, TE buffer; -20ºC freezer, Ultraviolet light, and -70ºC freezer. The agarose gel with deoxyribonucleic acid bands which has a low melting point was observed using ultra violet light transilluminator, the right bands of deoxyribonucleic acid identified and sliced out. The agarose gel piece was introduced into microfuge tubes.

The sliced gel was covered with the elution buffer in the microfuge tubes.

The gel slices was heated at 65ºC until they all melted. The melted agarose gel containing deoxyribonucleic acid was freeze by placing in -70ºC freezer for about 600 seconds. After this, it was centrifuged for about 600 seconde, and then supernatants was transferred into a another empty microfuge tubes.

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Again, about half amount of elution buffer used in the previous step was introduced into each pellet, heated at 65°C until the agarose dissolved. At this point, the dissolved gel containing the deoxyribonucleic acid was freeze by placing in the -70°C freezer for about 10 minutes duration. The tubes was centrifuged again for 10 minutes, and then supernatant was introduced into the previous microfuge tube with supernatant. After that, the tubes containing pellets was then discarded safely.

The supernatant was weighed and introduced into them an equal volume of n-Butanol. The tubes were vortex for a period of 15 minutes in just to remove the Ethidium bromide. The upper phase of butanol was removed and the process was repeated by addition of n-butanol again for 1 extra time. Two times the volume of 95% ethanol was introduced and mixed thoroughly, and was conserved for precipitation in -70°C freezer for 30 minutes. Thereafter, the reaction tubes were centrifuged for about 15 minutes and the supernatants were removed, while a 200µl, 70% ethanol was mixed with the pellets, the solution was centrifuged for up to 5 minutes and the supernatants were again discarded. At this point, the pellets were allowed to dry well (Hongli, *et al*., 2011). Furthermore, the pellets were suspended in 20µl of TE buffer. The deoxyribonucleic acid recovered was preserved in -20°C freezer for further process.

K. Radio-Labeling

Reagents and equipments: 2µL Klenow enzyme, 4 µl bacteria DNA (product of polymerase chain reaction), 10 µl of Labeling buffer, Micropipettes, 4 µL of ATP, 6 µL of 32P dCTP, -4°C freezer and a -20°C freezer, Water bath.

The reagents was prepared using a clean 0.5ml eppendorf tube, the mixtures were denatured allowing them to remain in a bath of water at a temp., of 37 degree Celsius for a period of 4 hrs. After incubation, the solutions were conserved in an ice cubes for period of 5 minutes. About 30 µl of the marker solution was introduced in a refrigerated centrifuge at -20°C. The solution was then centrifuged at a precision of 1000 rpm, 2 minutes. Thereafter, the solution was allowed to settle in the bath at 37°C for a period of 600 seconds. Then, the top layer obtained was further stored inside a -20°C freezer.

L. Restriction-Digestion

Reagents and wareables: Micro-centrifuge tubes, 0.5 µL of EcoRI enzyme, Micropipettes, Incubator, 3µL of deoxyribonucleic acid, Centrifuge,. 0.5 µL BamH1 enzyme, 1µL of 10x Buffer and 5µL dH_2O was mixed thoroughly (to bring total volume to 10µL). The solution was transferred in a micro centrifuge tube. The mixture was incubated at 37°C for a period of 1 hr. The 1.5mL microtubes was preserved in -4°C freezer immediately the incubation was over.

M. Southern Blotting

Reagents and equipments used were: Autoradiography cassette, neutralizing solution, agarose gel electrophoresis, Nylon membranes, 20 µl of probe solution, Whatman filter paper, Plastic wrapper, Tris- Ethylene diamin tetra acetic acid (TE) buffer, Stack of paper, Hybridization buffer, Large glass slide of 2kg, Transfer buffer, UV transilluminator, Prehybridization buffer, hybridization oven, 0.1% sodium dodecyl sulfate(SDS), and X-ray film. The agarose gel was transferred in to the denaturing solution and was carefully vortex for about 5 minutes, denaturing solution was carefully discarded and was washed in a neutralizing solution for a period of 5 minutes. Blot transfer was set up as prescribed by Terry, (1999): The gel containing the deoxyribonucleic acid was transferred into a wet whatman-filter paper. The whole set up were done in a reservoir filled with transfer buffer. The gel was covered with nylon membranes. While the filter papers were placed over the nylon membrane.

The filter papers were flooded with TE buffer solution while ensuring that no air bubbles was formed.

The reservoir was then covered with plastic wrapper, followed by, placing a stack of paper over the gel, then applying hand pressure on it gently.

An additional weight of 2 kg was placed over the stack of paper together with a large glass slide to keep the blot in good position. It was then left undisturbed for 12 hours.

After the incubation, the glass slide, weight and stacks of paper were carefully removed.

Then, the nylon membrane was carefully pulled off and transferred to a tray containing 6x transfer buffer after a while the nylon membrane was placed in the ultraviolet transilluminator. Then membrane was transferred to a white paper. At this point, the pre-hybridization buffer was introduced into the hybridization tube containing the nylon membrane.

The hybridization tube was transferred to a hybridization oven were the content was incubated at 42°C for a period of 2 hours. Thereafter, the used pre-hybridization buffer was carefully discarded while a hybridization buffer was now introduced together with 20 µl of the probe in the hybridization tube (Terry, 1999).

The hybridization tubes were transferred to the hybridization oven and was incubated at a temperature of 42°C for 4 hours. The used hybridization buffer was then carefully discarded and 4ml of 0.1% Sodium-dodecyl sulphate was mixed with the solution in the hybridization tube, was incubated at a temp. of 52°C for a period of 30 minutes.

At this point, the nylon membrane was gently pulled off from the hybridization tube and conserved on the autoradiography cassette to read the bands that corresponds to the 16S ribosomal ribonucleic acid gene sequence of the bacteria isolates.

The RNA gene sequences obtained was matched by aligning the outcomes with Embank using a computer program called Basic Local Alignment Search Tool (BLAST) search engine (Altschul, *et al*., 1990). The

acronym: BLAST stands for Basic Local Alignment Search Tool. It is an algorithm for marching primary nucleotides sequence information, such as nucleotides of deoxyribonucleic acid sequences or amino acid sequences of proteins. Each of the nucleotide sequences that were obtained was compared to a reference nucleotide in the Gen-Bank to identify the bacteria isolates. With a computer connected to the internet, the examined codon sequences were transferred into the search engine www.blast.nbi.nlm.nih.gov/Blast.cgi a website for biotechnology "the natural centre for biotech information" (NCBI). Then, the outcome from the cearch engine is recoded as the name of the bacteria isolates.

N. Bacteria Plasmid Gene Profiling:

Here, our target was to characterize and possibly identify the constituent codons that carry the special genes which are evidence of virulent factors of these bacterial isolates from aural swabs as pathogens and not the ordinary ear flora.

O. Plasmid Gene Extraction.

By adopting the method of Stanisich, (1988) with slight modifications. From the pallets of 24 hour cultured bacterial of *Achromobacter xylosoxidans, Comamonas aquatica, Delftia acidovorans, Pseudomonas aeruginosa, and Stenotrophomonas maltophilia*, a 5ml of each pallet, was prepared by centrifugation at a specification of 800 revolution per minute for a period of 3 minutes at a temperature of 25 ^{0}C. The pallet cells were re-suspended in 250ul phosphate-buffered saline (PI) and were transferred to a microfuge tube. A 250ul buffer P2 was introduced to each and were mixed very well by inverting each tube 6 times, until the reaction becomes clear. The lyses reaction was allowed to proceed for 5 minutes. A 350ul buffer N3 was then mixed with the lyses solution quickly and thoroughly by inverting the tube 6 times. Then mixtures were centrifuged for a period of 10 minutes at a 13000 rpm (17900 x g) on a table-top micro-centrifuge. At this point, 800 ul supernatants that were centrifuged were applied to the QIAprep 2.0 spin column using micropipette. Thereafter, the mixture was centrifuged for a period of 60 seconds, the flow-through was then discarded. This step is only required when using end A+ strains or other bacteria strains with high nuclease activity or carbohydrate content. The QIA prep 2.0 spin columns were washed by mixing with a 0.75ml buffer PE. It was centrifuge for a period 60 seconds, then the flow-through was discarded from each column and the connection tube was then switched off. In addition, the preparations were centrifuged for 1 minute to evaporate the residual wash buffer. The QIA-prep 2.0 columns were placed in a clean 1.5ml centrifuge tubes. To elute deoxyribonucleic acid, A 50ul buffer EB (10m MTrisCl, pH8.5) was introduced to the middle of the QIA-prep 2.0 spin column, the preparation was untouched for 1 minute duration and was centrifuged for 1 minute. 1 volume of loading dye was introduced to 5 volumes of the purified deoxyribonucleic acid. The solution was mixed by pipetting it up and down before loading in agarose gel.

P. Agarose Gel-electrophoresis

The application of electrical field on the gel, tend to dissociate the charges thus making the negatively charged DNA to migrate away from the negative electrode and to the positive electrode.

The agarose gel-electrophoresis was applied for analyzing and separating the extracted plasmid genes (DNA). The aim of using the gel was to identify the plasmid deoxyribonucleic acid, and to measure their sizes and possibly to examine each band type. The addition of ethidium bromide marker to the gel makes the plasmid deoxyribonucleic acid more visible. The proprietary dyes bind to deoxyribonucleic acid making it to be fluorescent, meaning that they absorb invisible ultra violet light and transmit the same energy as a visible light.

- **Step one: using a 50X stock solution of** Ethylene-diaminetetra-acetic acid **(TAE) buffer in a 1000 ml of distilled water was prepared by measuring** 242 g of Tris base buffer using a chemical balance. The solution was transferred to a 1000ml beaker. Ethylene-diaminetetra-acetic acid (EDTA) solution (pH 8.0, 0.5M) was prepared by weighing 9.31g of EDTA and dissolved in 40ml distilled water. The pH was checked using pH meter. Make the solution 100ml by adding distilled water. 57.1 ml of glacial acetic acid was pipetteout.A Mixed solution of the Tris base, EDTA solution and glacial acetic acid were made by adding distilled water to make the volume to 1000ml.
- **Step two: sufficient electrophoresis buffer (1x TAE) was used to fill the electrophoresis tank and to dispense the agarose gel by measuring a** 2ml of TAE stock solution into clean Erlenmeyer flask making up the volume to 100ml by adding up a 98ml of distilled water. The 1X working solution was 4 mM Tris-acetate/ mM ethylene diamine tetra acetic acid (EDTA).
- **Step three: Solution of agarose-gel in electrophoresis buffer at the right concentration was prepared by mixing** 2 grams of agarose gel to 100 ml of electrophoresis buffer. before heating the slurry in a microwave oven, the neck of the Erlenmeyer flask was slightly plugged, until the agarose was well dissolved within a period of 45 seconds.

The flask was transferred with a tong to a water bath at a temperature of 55°C. When the molten gel has cooled, a 0.5µg /ml of ethidium-bromide was mixed with gel solution thoroughly by carefully swirling. (The preparation of the Ethidium bromide was done by adding 1g of ethidium-bromide to a 100 ml of water, a magnetic stirrer was used to stir the solution for 2 hours to ensure that the dye has dissolved. 10 mg/ ml of the solution was transferred to amber bottle and preserved at a room temperature). A suitable comb was selected to form the sample slots in the gel, while the agarose gel solution was cooling, the warm agarose gel solution was poured in the mold. **(The gel used was 4 mm thickness and there were no air bubbles between the teeth of the comb or under).** The agarose-gel was allowed to settle completely for 45 minutes at a temperature of 25^{0}C. A small quantity of electrophoresis buffer solution was then poured on the top of the gel, and

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the comb gently removed. The electrophoresis buffer solution was then carefully poured off. The agarose gel was the mounted in the electrophoresis tank. Large electrophoresis buffer that that was enough to cover the gel to a depth of 1 mm were introduced. Thereafter, deoxyribonucleic acid samples from the sample bacteria isolates were mixed with 0.20ml of the desired 6x gel-loading buffer. Then sample mixtures were carefully and slowly loaded in the slots of the submerged agarose gel using an automatic micropipette. The size standards were loaded into slots on both the right and left sides of the gel, and the lid of the gel tank was closed, and the electrical Leeds were attached, so that the deoxyribonucleic acid will move toward the positive pole (anode red lead). A specific voltage of 5 V/cm was applied (measured as the distance between the negative electrodes and positive). Finally, the gel was allowed to run until the xylenecyanol FF and the bromophenol-blue moved to the right distance via the agarose gel. Thereafter, the agarose gel tray was carefully removed and placed directly on a transilluminator machine. Finally, the Ultra Violet light was switched on, and the orange bands of the deoxyribonucleic acid were captured a computer scanner.

III. RESULTS

A. Characterization and Identification of Isolates

The first stage of bacteria identification was by applying microbiological cultural features and Grams reactions as shown in Table 1. The results of the molecular sequencing of the specimens are as follows: Figures 1a. and 1b. Were the extracted deoxyribonucleic acid in agarose gel to estimate the quantity and integrity before carrying out amplification and elution for the final 16S rRNA gene sequencing.

Table 2 shows the confirmatory identification of the sampled pathogens using 16S rRNA gene sequences to obtain the nucleotide sequences that were used in Basic local alignment search tool. The study was conducted on aural swabs samples from two hundred pupils from five selected Nkpa Nursery and primary schools in Bende, Nigeria, starting from August 2015 to February 2017. Bacterial ear infection was ascertained from 48 (24%) children which consist of 28 (14%) Males and 20(10%) Females and resulted to overall ratio of 1.40 Male to 1.00 of Female. This is fully displayed in Table 3. Five different bacteria pathogens were isolated in different proportions as shown in table 4, in the results, there was 16 (8%) of *Achromobacter xylosoxidans* (the highest percentage occurrence), while 2(1%) of *Comamonas aquatica* was the lowest pathogen in this locality. Evaluation of Table 5, showed that the mean age of the research population was 7.1 ± 3.6 years (Appendix iv), with a rang 13 years. The pupils of age group of 2.0 to 3.0 years were mostly infected 16(8.00%), this was followed by pupils of age group 4.0 to 7.0 years and 8.0 to 11.0 years which had the same prevalence of 13 (6.50%). While the pupils of age group 12.0 to 15.0 years had the lowest ear infection of 6(3.00%), thus the ear infection decreases with increase in age.

Table 1: The preliminary characterization based on Cultural features and Grams stain reactions

Isolate codes	**AF**	**BF**	**CF**	**DF**	**EF**	**FF**
Grams reaction	G **-ve** Light red rods	G **-ve**, Non spore forming rods	G **-ve** Aerobic, Non fastidious	G **-ve**, Aerobic rods	G**-ve** rods	G**-ve**, Aerobic bacilli, Non fastidious

KEY: ND → G+ve →gram positive and **G-ve** →gram negative

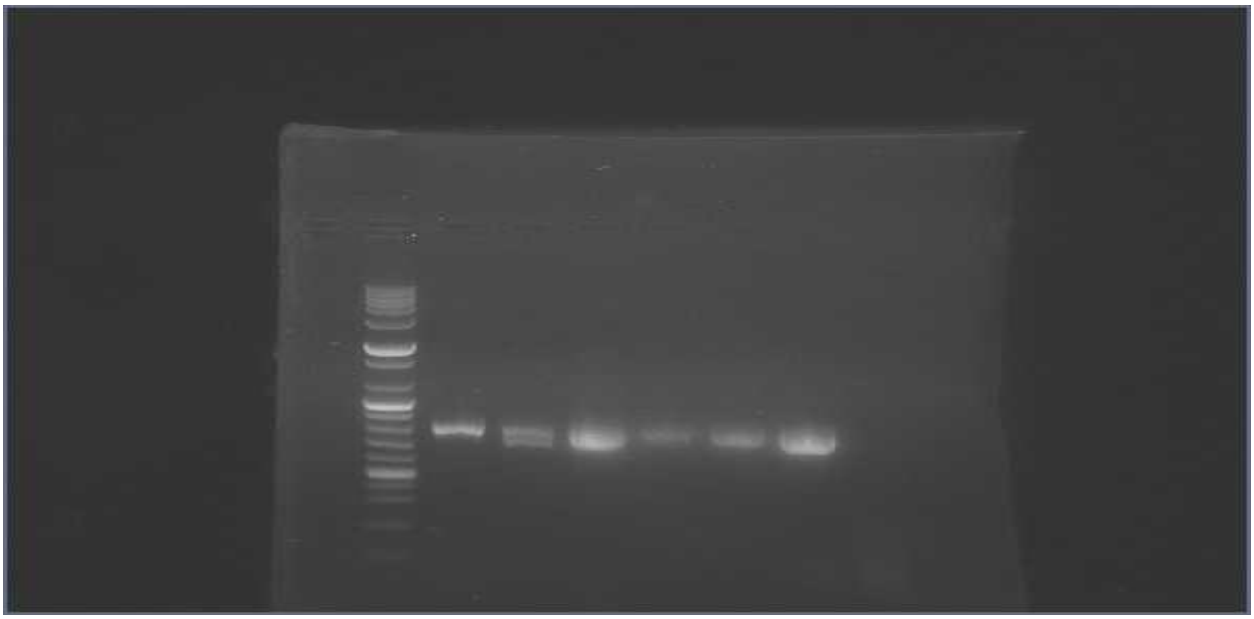

Fig. 1(a): Showing the isolated DNA to ascertain the sizes and integrity. Keys: stating left to right indicate DNA markers, AO; BO; CO; DO; EO; and FO DNAs and a negative control

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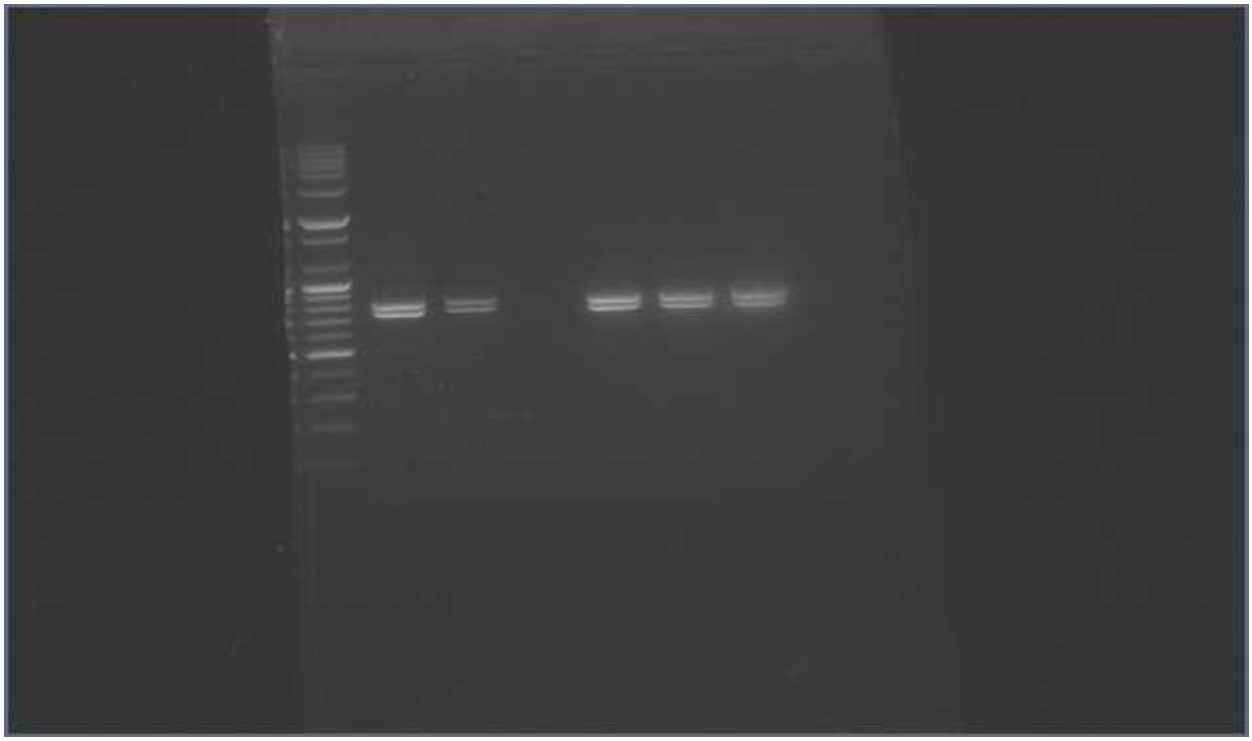

Fig. 1(b): Showing the extracted DNA to ascertain the sizes and integrity. Key: starting left to right indicates DNA markers, optimized BO, with double bands, and a negative control. .

Table 2: Showing the 16S rRNA gene sequencing.

Code	Nucleotide sequences	BLAST
AO	CTGGCAAGTCGAACGGAACAGTCTTCGTATGCTGACTAGTGGCGAACGGGTGAGTATACTCGAAA CGTGCCTATAGCGGGGATAACTACTCGAAAGAGTGGATATACCGCATGACATCTACGGATGAAA GCAGGGATCGCAAGACCTTGTGCTACTATAGCGTGCGATGGCAGATTAGGTAGTGGCGGGATAA AAGCTTACCAGGCCGACGATCTGTACCTGGCTGAGAGGACGATCAGCCACACTGGGACTGAGAC ACGGCCCAGACTCCTACGGAGGCAGAGGGGGAATTTTGGACAATGGGGCAAGCCTGATCCAGCA ATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAGCCCTGG GTTAATACCCTGGCATGACGACCGAAGAATAAGCACCGCTAACTACGTGCCAGCACGCGAATAC GTAGGGGCGAGCGTAATCGGAATTACTGGCGTAGCGTGCGCAGGCGCTTTGTRAGACAGAGGTG AAATCCCCG	***Comamonas aquatica***
BO	CGGTCTGATGGCGAGTGGCGAACGGGTGAGTCATGTATCGGAACGTGCCCAGTAGCGGGGGATA ACTACGCAAAGCGTAGTAATACCGATACG	*Achromobacterxylosoxidans*
CO	TACCATGCAGTCGAACGGACAGGTCTTCGGACGCTGACGAGTGGGGAACGGGTGAGTAATACAT CGGAACGTGCCCAGTCGTGSGGGATATCTACTCGAAAGAGTACTAATACCGCATACGATCTGAGG ATGAAAGCGGGGGACCTTCGGGCCTCGCGCGATTGTAGCGGGCGATGGAGATTAGTATTGGGGG ATAAAAGTTACCAAGCCGACGATCTGTAGCTGGGCTGAGAGGACGACCAGCCACACTGGACTGA GACACGGGCCAACTCCTACGGGAGGSAGCAGGGGGAATTTTGACAATGGGCGAAAGCTGATCCA CAATGCGCGTGCAGGATGAAGCTTCGGGTTGTAAACTGCTTTTGTACGGAACGAAAAACTCCTTC TAATACAGGGCCCATGAGGACCGCAGAATAAGCACCGTACTACGTGCCAGCAGCCGCGGTAATA CGTAGGTGCGAGCGTAATCGGAATTACTGGGGCGTAGCGGCGCACGCGTATGGTAAGACAGATG TGAAATC	*Delftiaacidovorans*
DO	CATGCAGTCGAACGGCAGCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGTGAGGA ATACATCGGAATCTACTCTGTCGTGGGGGATAACGTASGGAAACTTACGCTAATACCGCATACGA CCTACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCT AGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCA CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG GCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGT TGGGAAAGAAATCCAGCTGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGG CTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGT AAAGCGYGCGTAGGT	
EO	ATGCAGTCGAACGGCAGCACAGAGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGTGAGGAAT ACTCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCT	*Pseudomonas*

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	ACGGGTGAAAGAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTT GGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTACTGGACTGAGAGGATGATCAGCCACACTG AACTGAGACACGCCAGACTCCTACGGGAGGCAGCAGGGGAATATTGGACAATGGGCGCAAGCCT GATCCAGCCATACCGCGWGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGA AATCCACTGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCCCGGCTAACTTCGTGC CAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAGAGCGTGCGT AG	*aeruginosa*
FO	CACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACT CTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGSTGAARGCAG GGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGG CCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACAC GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAG CCATACCGCGTGGGYGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGC TGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAG CCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGG TCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTA GARTGTGGTAGAGGGTAGCGRAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAAC ATCCATGGCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGT	*Pseudomonas aeruginosa*

Keys Code AO, BO, CO, DO, EO and FO were the 6 representative bacteria samples with similar cultural characteristics and grams' stains used. BLAST→ Basic Local Alignment Search Tool

Table 3: Effects of Gender on Isolation

Sex	Samples	Cases	%= Cases /sum total X100
Male	108	28	14
Female	92	20	10
Total	200		

Table 4: Showing the % frequency Different Isolates.

Bacteria	Samples	Cases	%
Stenotrophomonas maltophilia	200	2	1.00
Achromobacter xylosoxidans	200	16	8.00
Delftia acidovorans	200	11	5.50
Comamonas aquatica	200	2	1.00
Pseudomonas aeruginosa	200	11	5.50
Total positive		**48**	**24.00**

Table 5: Effect of differences in age on % frequency of Bacteria pathogens

Pathogens	2.0 – 3.0 yrs		4.0 – 7.0 yrs		8.0 – 11.0 yrs		12.0 – 15.0 yrs	
	P	%	P	%	P	%	P	%
Comamonas aquatic	0	0.00	0	0.00	2	1.00	0	0.00
Achromobacter xylosoxidans	5	2.50	5	2.50	5	2.50	1	0.50
Delftia acidovorans	4	2.00	5	2.50	1	0.50	1	0.50
Stenotrophomonas maltophilia	3	1.50	1	0.50	1	0.50	3	1.50
Pseudomonas aeruginosa	4	2.00	2	1.00	4	2.00	1	0.50
Total	**16**	**8.00**	**13**	**6.50**	**13**	**6.50**	**6**	**3.00**

Keys .P→ positive cases; %→percentage frequency; yrs→years

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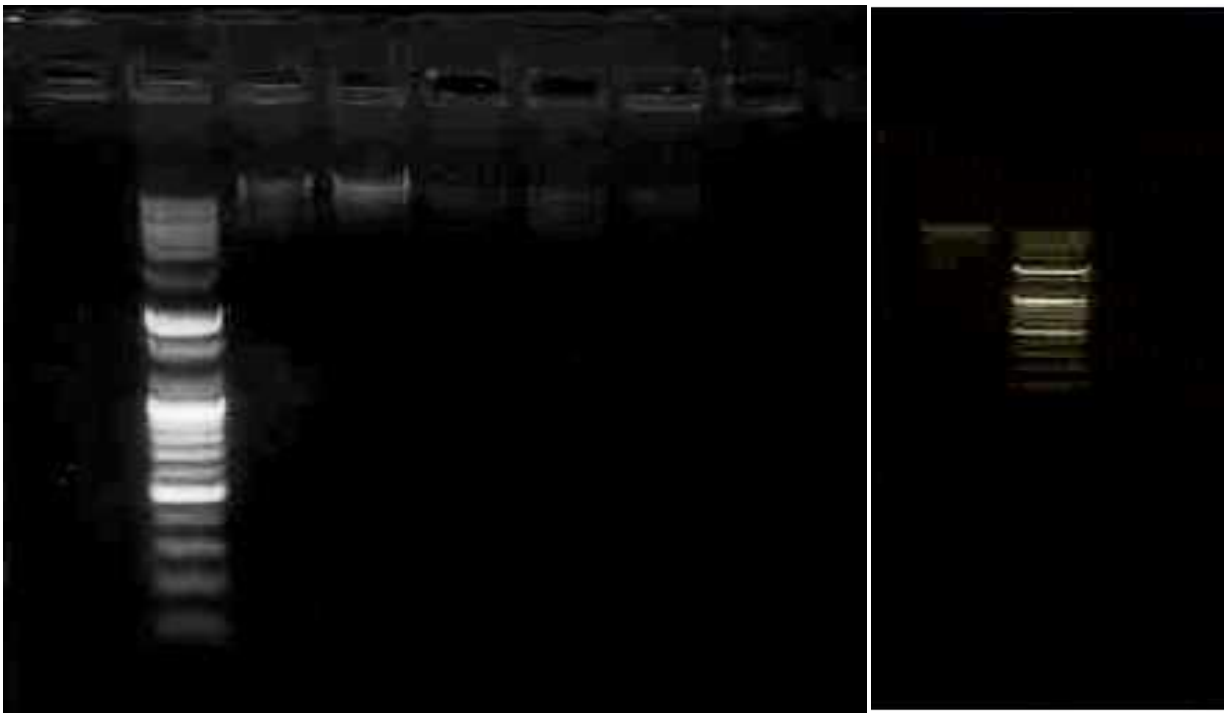

Fig. 2: Films of Plasmids genes. Keys: starting left to right are DNA marker, bands of bacteria plasmid resistant gene AO; BO; CO; DO; EO; and FO.

Table 6: Isolated Bacteria Plasmids Gene Profiles

Code	Isolates	Molecular weight	Occurrence
AO	*Comamonasaquatica*	approx.1600bp	1
BO	*Achromobacterxylosoxidans*	approx.1600bp	1
CO	*Delftiaacidovorans*	approx.1600bp	1
DO	*Stenotrophomonasmaltophilia*	approx.1600bp	1
EO	*Pseudomonasaeruginosa*	approx.1600bp	1
FO	*Pseudomonasaeruginosa*	approx.1600bp	1

Keys: The bp → base pair; approx. → approximation

IV. DISCUSSIONS

The research was the 1st attempt to carry out the culture and identification of possible pathogens on aural swabs using of the 16S rRNA genomic sequencing technique, on children from Nkpa in Bende, Abia State of Nigeria. The bacteria which were characterized using gram stain were majorly purple stained isolates (100%), the outcome on bacteriological features that shifted to Gram negative pattern was revealed in the reports of Sharma *et al*., (2004). From 48 (24%) of the children cases, Males had preponderance of 28 (14%) when compared with 20 (10%) of the Females at 1.40 : 1.00. ratio. Such higher ratio of Male to Female were revealed in Shamboul and Omdurman,(1992) and Olubanjor *et al*.,(2008). This assertions was not in agreement with Adeyemi and Hassan (2007), they noted that, Females had higher otitis. However (Yilikal and Areya, 2015) study outcome reported no disparity on the Males versus Females compares. There are currently no clear reasons behind gender predominance. The identified bacteria genes were from two and above out of the forty-eight positive reports, *Comamonas aquatica* 2(1%) being the lowest; while *Achromobacter xylosoxidans* 16 (8%) was the most frequent isolate. Others include others were *Pseudomonas aeruginosa* 11(5.50%); *Stenotrophomonas maltophilia* 8(4.00%); *Delftia acidovorans* 11(5.50%), these out comes showed that, there was variety of isolates at varying numbers on the experimental study, the outcomes were in tandem with the study results of Sweeney, *et al., (*2002). More so, of the forty-eight bacterial pathogens identified, *Achromobacter xylosoxidans* with (8.00%) was shown to occur most, followed by 5.50% of *Delftia acidovorans and Pseudomonas aeruginosa,* these observations were not in line when compared to summary made in the same research in Loy et al., (2002) and Indudharan *et al*.,(1998), that identified *P. aeruginosa* to be the highest pathogen causing otitis media. While, Oni *et al.,* (2001) summarized that predominance changes over time. With this evidence, it could be that *A. xylosoxidans* has overtaken *P. aeruginosa* in this study area. In addition, *A. xylosoxidans* characterized herein research were in agreement that of Yano and Yabuchi, (1981) which showed *A. xylosoxidans* were predominantly from middle ear infections. This report could infer that the school pupils were at higher risk of otitis media. *P. aeruginosa and S. maltophilia* had nine point five percent of the sum-total isolates, which was among the identified etiology of illness from recreational water like swimmer's ear, that is clinically known as otitis external, such similar report has been made elsewhere by Heaney *et*

al.,(2009) who showed that pupil younger than 10 years have more tendency to contract illness from recreational water because they play on sand and in shallow waters that are mostly contaminated with these bacteria. *C. aquatica*, had only 1.00% incidence, *C. aquatica* is a pathogen of water origin. It is characterized with a pungent odour. It was cultured from two children aged 8 to 11 years. This bacteria may be gotten from contaminated shallow rivers. Zhu et al.,(1986) reported that this *Pseudomonad*-like strain induces clumping of blood in mammals. *D. acidovorans* originally known as *Comamonas acidovorans* had 5.50% isolates of the aural samples from the infants in this study locality. There exist some experimental results on *D. acidovorans* pathogen from debilitating inflammations such as empyema, Acute Suppurative otitis media, bacteremia, and corneal ulcers diseases (Perla and Knutson, 2005; Kawamur *et al*., 2011; Horowitz *et al*., 2009; Chotikanatis *et al*., 2011; and Chun, *et al.*, 2012). The isolates can be found mostly in the soil and water as saprophytic bacteria (Gilligan and Whittier 1999). This bacteria isolated shows that the children involved enjoyed playing play on humid soils and shallow waters, unknown to them to have the bacteria contaminates.

Our result showed that the average age of the studied population was 7.1 ± 3.6 years (Appendix iv), with a range of 13 years. Out of the 48(24%) infected pupils, 29(14.5%) ear infection was from children that were below eight yrs. While 19 (9.5%) ear infection was from children of eight and fifteen of age, which were in the ratio of 1.5 : 1.0. it could be therefore be depicted that otitis eases as the child grows older, which is in tandem to the summary of such experiment carried out in Poorey and Lyer (2002), and Teele *et al*.,(1989) this could be due to their eusterchian tubes were more level and smaller as such it becomes a problem for fluids formed in the middle ear to find its way out, and in infant when the system is functioning well which late enlarges with time.

In this experimental result on plasmid genes, the bacterial plasmid DNA weighed about 1600 base pairs which resembles resistant plasmids genes to antibiotics, having band of single strands.. Wilkins, (1995), agued that this type of mobilizable bacteria plasmid resistance gene are usually smaller in sizes, ranging from 10 kbp to lower. Which code just a few DNA, such as genes that are resistant to antibiotic treatment, compared to very large plasmids called conjugative plasmids that weighs from 30 kbp and above. The bacteria plasmid genes identified herein could be more probably the emerging strains that are low susceptibility to antibiotics (Levy, 2002).

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APPENDIX 1: AN APPROVED ETHICAL CLEARANCE

GOVERNMENT OF ABIA STATE OF NIGERIA

OFFICE OF THE PERMANENT SECRETARY
MINISTRY OF EDUCATION
NNAMDI AZIKIWE SECRETARIAT
P. M. B. 7217
UMUAHIA

MOE/PSI/123/T/133
Our Ref:.....

Yours Ref:.....
(All replies to be made to Honourable Commissioner)

19th October, 2015.
Date:.....

OGBUKA, CHIBUISI FRANCIS
MOUAU/PG/M.SC/13/2852
DEPARTMENT OF MICROBIOLOGY
COLLEGE OF NATURAL SCIENCE
MICHAEL OKPARA UNIVERSITY OF AGRICULTURE
UMUDIKE

ETHICAL CLEARANCE TO COLLECT AURAL SWAB SAMPLES FOR THE PURPOSE OF ACADEMIC RESEARCH WORK

With reference to your letter dated 29th September, 2015 on the above subject, I am directed to convey the approval of the Permanent Secretary for you to carry out the research or collect aural swab sample from pupils in Nkpa Bende LGA.

Kindly accept the assurance of our esteemed regard.

ONYEASO A. N. (LADY)
For: Permanent Secretary

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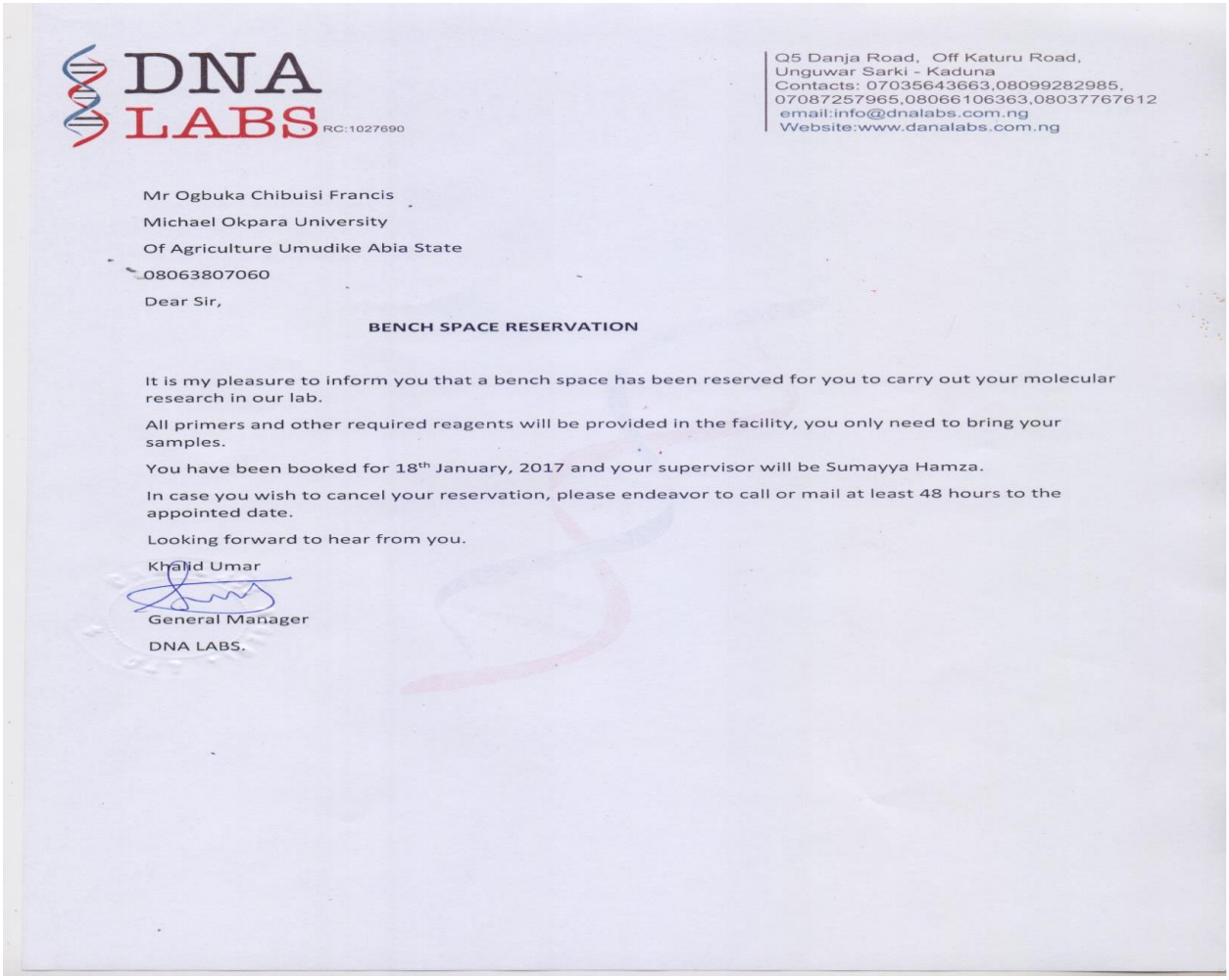
APPENDIX 2: DNA LABS KADUNA ACCEPTANCE LETTER FOR BENCH WORK



DNA LABS RC:1027690

Q5 Danja Road, Off Katuru Road,
Unguwar Sarki - Kaduna
Contacts: 07035643663,08099282985,
07087257965,08066106363,08037767612
email:info@dnalabs.com.ng
Website:www.danalabs.com.ng

Mr Ogbuka Chibuisi Francis
Michael Okpara University
Of Agriculture Umudike Abia State
08063807060

Dear Sir,

BENCH SPACE RESERVATION

It is my pleasure to inform you that a bench space has been reserved for you to carry out your molecular research in our lab.

All primers and other required reagents will be provided in the facility, you only need to bring your samples.

You have been booked for 18th January, 2017 and your supervisor will be Sumayya Hamza.

In case you wish to cancel your reservation, please endeavor to call or mail at least 48 hours to the appointed date.

Looking forward to hear from you.

Khalid Umar
General Manager
DNA LABS.

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APPENDIX IV: STATISTICS ON AGE DISTRIBUTION OF SAMPES COLLECTED

AGE(YEARS) (X)	MALE	FEMALE	TOTAL SAMPLES(F)	Mid X	FX	$F(X-\mu)^2$
2-3	22	26	48	2.5	120	48x21.16=1015.68
4-7	34	31	65	5.5	357.5	65x2.56=166.40
8-11	36	24	60	9.5	570	60x5.76=345.60
12-15	16	11	27	13.5	365.5	27x40.96=1105.92
Total	108	92	200		1412	2633.60

Mean age = $\sum FX / \sum F = 1412/200 = 7.06 = 7.1 \pm 3.6$

Standard deviation = $\sqrt{(\sum F(X - \mu)^2/F} = \sqrt{2633.60/200} = 3.6$