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Study on Bacteriology of Ear Infection among School Children and the Susceptibility of Isolates to Antimicrobials

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Abstract

The study on the bacteriology of ear infection of school children and the susceptibility of isolates to extracts of *Chromonaela odorata, Cleome viscosa, Jatropha curcas* and *Sansevieria trifasciata* as well as commercial antibiotics were determined. The study was conducted on 200 school children of age range 2 to 15 years from five selected schools in Nkpa, Bende local government area Abia state, from August 2015 to February 2017. Aural swab samples were taken from pre-screened school children who had not taken antibiotics within the period of the sampling. Standard bacteriological methods were adopted for the isolation of bacteria. Further characterization of isolates was performed using 16 subunit ribosomal ribonucleic acid genes sequencing for the identification of isolates. Agar well diffusion of water and ethanolic extracts of the leaves were done to determine their antimicrobial activity. Antibiotic sensitivity test of isolates and their biofilms were determined using the *Kirby-bauer* disc diffusion and Khalid method respectively. Plasmid analysis of isolates was determined to plasmid genes. The major isolates included *Achromobacter xylosoxidans* (8.00%), *Delftia acidovorans* (5.50%), *Pseudomonas aeruginosa* (5.50%), *Stenotrophomonas maltophilia* (4.00%) and *Comamonas aqua ica* (1.00%). Among the sample population, 48 positive specimens were obtained, out of which 14.00% were from males while 10.00% from females. Age range of 2-3 years had the highest infection of 16 (8.00%), followed by 4-7 years and 8-11 years with the same rate of 13 (6.50%) infection while 12-15 years had the least infection of 6 (3.00%). Yield of ethanolic extracts was 23.5%, while that of water extracts was 19.0%. *In vitro* testing revealed no antimicrobial activities of the extracts, except ethanolic *C. odorata* (250 mg) extract with slight activity (3 mm) and (2 mm) zones of inhibition of *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* respectively. Commercial antibiotics showed range of 2 mm to 13 mm on the isolates and the range of 2 mm to 7 mm biofilm zone of inhibitions. *Stenotrophomonas maltophilia* was the most susceptible (13 mm) to Levofloxacin (30 µg) and to Ampiclox (20 µg) while *Comamonas aqua ica* was the least susceptible to all the tested antibiotics especially with no zone of inhibition by Amoxicilline (20 µg). Plasmid existed in each of the 5 representative bacteria. *Achromobacter xylosoxidans* was the most common isolated bacteria from the pupils in this locality and was highly susceptible to levofloxacin, ampiclox and ciprofloxacin. The target specificity of the tested extracts was among the least susceptible antimicrobials. In this era of increasing resistance to antimicrobial agents by bacteria, a periodic surveillance of bacteriological and susceptibility profiles is therefore essential for effective management of bacterial ear infection among school children.

Keywords: Otitis; Antimicrobials; Antibiotics; Plasmid; Susceptibility; Biofilm; Bacteriology; 16 subunit ribosomal Ribonucleic acid; Inflammation; Pathogens; Infection; Extract; Bacterial DNA; Ear infection; Isolates, *Jatropha curcas; Sansevieria trifasciata*; *Cleome viscose*; *Chromolaene odorata*; Otitis externa; Otitis media; Gram stain; Indole test; Methyl orange; Voges proskauer; Urease; Labyrinthitis; Citrate; Catalase; Coagulase; Oxidase; Hydrogen sulphide; Motility test; Deoxyribonucleic acid; Polymerase chain reaction; Electrophoresis; Mc-farland standard; Antibacterial; Agarose gel electrophoresis

Introduction

Ear is a sensory organ of hearing and balancing. It is progressively more understandable that ear infection has been more common problem in the tropics. It is seen in all age group but is more prevalent in infants and children. According to Dhiangra, the children's Eustachian tube is shorter, more horizontal with a more flaccid cartilage which can easily impair its opening and hence infection of the ear together with discharge is a major health problem [1]. Universally, about 65-330 million persons are infected with ear infection and 60% of this patient had loss of hearing. It's decreasing incidence during and after adolescent stage is due to growth and development of the pharynx. Ear infection is more common among those with poor socioeconomic status, younger maternal age, male sex, low birth weight, daycare attendance, inadequate housing conditions, lack of access to health care, exposure to cigarette smoking and mothers who smoke during pregnancy. Although most ear infections may occur without visible ear discharge, in some cases, ear infection can even be totally asymptomatic. Otitis may arise from the inflammation of external auditory meatus and is known as otitis externa or the

inflammation of the middle ear cavity, known as otitis media [2]. Among all the ear inflammations, otitis media has been commonly diagnosed by otolaryngologists among children in Nigeria.

Bacteria ear infections are attributed as a major factor for the bulk of hearing problems with lifelong consequences, many of which are preventable and treatable. The etiology and prevalence of infection differs with geographical areas and climate conditions, however colonizing normal flora of the skin such as *Pseudomonas aeruginosa, Staphylococcus aureus, Proteus mirabilis, Klebsiella pneumoniae* and *Escherichia coli* under certain circumstances and also by antibiotic resistant bacteria, have been reported as the main agents of ear infection [3]. In Acute Otitis Media (AOM), the pathogens mostly isolated include *Streptococcus pneumoniae, Nontypeable haemophilus in luenzae* and *Moraxella (Branhamella) catarrhalis*. They are the causes of over 95% of all AOM cases with a bacterial etiology.

Gram negative bacteria such as *Achromobacter xylosoxidans, Escherichia coli, Klebsiella species, and Pseudomonas aeruginosa*, play larger roles and up to 20% of cases among infants. *Streptococcus pneumoniae* and *Haemophilus in luenzae* pathogens had similar casesamong the infants [4]. Some studies also *found Staphylococcus aureus* as a pathogen in little children, but subsequent studies suggested that the flora in these young infants may be that of usual AOM in older children.

Many pundits had suggested that the Middle Ear Effusion (MEE) that was linked with Otitis Media with Effusion (OME) had no contaminants because cultures that were prepared from middle ear fluid obtained by tympanocentesis mostly had no bacteria growth. This scope is changing as recent researches showed the incidence of positive results in 30%-50% cases of bacterial cultures in patients that were chronic to middle ear effusion. These cultures grow a wide range of aerobic and anaerobic bacteria. The common isolates are *Streptococcus pneumoniae, Haemophilus in luenzae, Moraxella catarrhalis and* group-A streptococci [5].

Acute otitis media induced by *M. catarrhalis* and that of other bacterial pathogens differ in many ways. It is associated with a higher fraction of mixed infections, tender age at the time of identification of pathogens, the absence of mastoiditis and the decreased risk of perforation of the tympanic membrane. Polymerase chain reaction was further used to buttress the presence bacterial Deoxyribo Nucleic Acid (DNA) in MEE patients with OME by using MEE samples that were determined to be sterile with standard bacterial culture techniques. In chronic supportive otitis media, pathogens isolated most often include *Pseudomonas aeruginosa*; others are *Staphylococcus aureus*, *Corynebacterium* species, and *Klebsiella pneumoniae*. Many researchers increasingly observed the activity of *Helicobacter pylori* among infants with otitis media with effusion. The evidence of *H. pylori* associated with otitis media with effusion might originate from the isolation from tonsilar, adenoid tissue and middle ear in patients with OME. Gram positive bacterium such as *Alloiococcus o i idis* has been identified as pathogen in OME [6]. This pathogen has been mostly isolated in acute otitis media and otitis media with effusion. As well as being diagnosed among infants who had been treated with erythromycin or beta-lactam, which indicates that these antibiotics might not be effectively eliminate this pathogen. According to Fliss et al., most microbiological researches on Chronic Suppurative Otitis Media (CSOM) have revealed that are *Pseudomonas aeruginosa*, *Staphylococcus aereus* and *Klebsiella* species were the most frequently isolated pathogens. Nwabuisi and Ologe, noted *Pseudomonas aeruginosa* and *Proteus* species as the most observed pathogens in a similar research. Reports abounds that over 90% of otitis externa were caused by bacteria [7]. In a similar study by Roland and Stroman, the most common isolates observed was *Pseudomonas* species followed by *Staphylococcus* species, other anaerobes and gram-negative organisms in otitis externa.

As predominance of isolates from the ear infection change over time, the knowledge of the local pattern of infection is therefore essential to enable efficacious treatment of this disorder. When drug resistance is suspected, it could be due to various limitations including biofilms or bacteria plasmids, on most of the ear infections. The range plasmids that often carry multiple antibiotic resistance genes (MDR plasmids) are very important in medicine because they can be transferred to a wide range of bacterial species. Consequently, a single plasmid transfer event can turn a drug sensitive bacterium into a multiple drug resistant strain; such include the rapid worldwide plasmid mediated spread of antibiotic resistance to colistin, an antibiotic of last resort. Research findings by Venekamp, et al. showed that impact of antibiotics on short term hearing loss was uncertain due to widespread use of antibiotics for treatment of otitis media causing adverse effects and emergence of bacterial resistance. Altogether, the issue of antibiotic resistance has posed serious public health challenge and has increased the global health predicaments for which purpose answers are being expected of including of finding alternative and complementary therapeutic modalities. Applications of herbal preparations not only help in preventing ear infections, but also prevent a complication that arises from ear infections [8].

Materials and Methods

Area of study

The study area was Nkpa with 7 autonomous communities located in Bende local government area, at the Northern apex of Abia state. Most of the residents are Farmers, Traders and a few Civil servants.

Sample collection

Approved ethical clearance was first obtained from Abia state ministry of education, thereafter, the purpose of the study was explained to the parents/teachers and their consents were also obtained for their inclusion in this study [9]. Two hundred (200) aural swab samples were collected with sterile aural swab sticks from pupils (2 to 15 years) who had either itching, pains or discharge and who had neither taken antibiotic nor herbal remedy for the past 5 days, They were collected from Ugwu Nkpa, Amaohoro Nkpa, Amaokpu Nkpa, Amaegbuato Nkpa, and

Eluama Nkpa community primary schools and were labeled properly with age group, gender, and school name and sent to the Microbiology laboratory at Michael Okpara university of agriculture, Umudike.

Sterilization and media preparation

Petri dishes (glass), bottles and other glass wares that were heat resistant were washed thoroughly with detergent, dried on the racks and packed into the hot air oven, at 170°C for holding period of 1 hour. Nutrient medium, Mac Conkey agar, Eosin methylene blue, blood agar, Cetrimide agar and Mueller-hinton agar media were prepared according to their manufacturers' standards and autoclaved at 121°C (at 1.05 kg/cm^3 pressure) for 15 minutes. The sterilized media were dispensed per sterilized 9 cm diameter petri dishes.

Culture and isolation

The aural swabs collected were inoculated by streaking on to nutrient agar, Mac Conkey agar, eosin methylene blue, Blood agar and Cetrimide agar media plates. And were incubated at 37°C for 24 hours [10]. Discrete colonies were sub-cultured repeatedly by streaking on nutrient agar plates to obtain pure colony. The pure colony was stored in nutrient agar slant at 4°C for further use.

Characterization and identification of isolates

Preliminary bacteria characterization and identification were carried out with the aid of identification schemes by Cheesbrough, based on examining the morphological cultural characteristics of the isolates, gram reaction and motility and biochemical reactions of the isolates.

Gram stain reaction: The method of Jawetz, et al. was adopted based on ability of the gram positive bacteria to retain the basic dye (crystal violet), while gram negative bacteria retain secondary dye (carbol-fuchsin) [11]. A smear of the culture was made on a clean grease free slide using a wire loop. The film was air dried by waving around for a while and the smear was heat fixed by passing over a Bunsen flame. The smear was covered with crystal violet reagent for one minute and the slide was rinsed in slow running tap water for 5 seconds. The slide was then rinsed with grams iodine and the slide was flooded with the same for 1 minute. The slide was rinsed again in slowly running tap and 95% ethanol reagent was applied drop wise until no more dye runs off from the smear and it was covered with 0.2% carbol-fuchsin reagent for 30 seconds. The slide was rinsed slowly under running water, and was then air dried. The prepared slide was observed using the x 100 oil immersion lens of the binocular microscope. Gram positive cells stained purple or violet while gram negative cells stained pink or red.

Indole test: The test was to determine the ability of the test bacterium to convert tryptophan into indole. The unknown bacterium was inoculated into a bijoux bottle of Sulphide Indole Motility (SIM) media with a needle, all the way to the bottom. The inoculated test tubes were incubated at 37°C for 48 hours, and thereafter 8 drops of Kovac's reagent was added to the layer within 5 minutes. A ring layer indicated positive test while the absence of a red layer indicated a negative test [12]. The results of the reactions of the unknown bacterial were recorded.

Methyl red: The test determines the utilization of glucose with subsequent production of acid. The Methyl Red Voges Proskauer (MRVP) broth was prepared by mixing 1 gram of peptone water and I gram of Di-Potassium Hydrogen Phosphate (K_2HPO_4) with 200 ml of distilled water and was dispensed into 5 ml volume bijoux bottle and sterilized. After sterilization, 0.25 ml of glucose solution sterilized by steaming was added into each of the bijoux bottles. The test bacterium was inoculated into the MRVP broth and well labeled, as described by Cowan and Steel [13]. It was Incubated at 37°C for 48 hours, and after incubation: 1/3 of the suspension were poured into a clean sterile tube and added 8 drops of methyl red reagent. The presence of red-pink color of acid within a few seconds was recorded as positive, while a yellow colour was recorded as negative methyl red test.

Voges-Proskauer test: The method of Cruickshank et al. was adopted to determine glucose utilization and production of a neutral product called acetoin/acetyl methyl carbinol. The MRVP broth was prepared by mixing 1 gram of peptone water and I gram of Di-Potassium Hydrogen Phosphate (K_2HPO_4) with 200 ml of distilled water and was dispensed into 5 ml volume bijoux bottle and sterilized. After sterilization, 0.25 ml of glucose solution sterilized by steaming was added into each of the bijoux bottles. The MRVP broth was dispense in the open tube and the bacterium was inoculated and incubated for 5 days, after incubation, 1 ml of the culture was transferred to sterile test tubes and 12 drops of Barritt's A, and 4 drops of Barritt's B were added, and mixed gently for 1 minute to aerate the solution (the reaction utilized O_2) [14]. The reaction was allowed undisturbed, for 20 minutes. Bacterial that produce a neural end product from glucose fermentation gave a pink colour at the inter phase was recorded as VP positive and where no colour formation occurred at the inter phase was recorded as negative result.

Citrate test: Citrate utilization test was used to determine the ability of bacteria to utilize sodium citrate as its only carbon source. It was employed as part of a group of tests, the IMViC (Indole, Methyl Red, VP and Citrate) tests. Simmons citrate agar was incubated lightly on the slant by touching the tip of a needle to a colony that was 24 hours old at 37°C. It was incubated for 24 hours at 37°C [15]. The development of blue colour was noted, which denotes alkalization. A positive citrate test was noted when the growth was visible on the slant surface and the medium was an intense Prussian blue. A negative citrate test showed neither visible growth nor colour change; the medium remained the deep forest green colour of the inoculated agar.

Catalase test: These tests demonstrate the presence of catalase, an enzyme that catalyzes the release of oxygen from Hydrogen Peroxide (H_2O_2). Using the tube method, 2 ml of 3% hydrogen peroxide solution was poured into a test tube. A glass rod was used to take several colonies of the 24 hours test organism and immerse in the hydrogen peroxide solution. Immediate bubbling was observed and recorded as catalase positive [16].

Coagulase test: Coagulase test was used to differentiate *Staphylococcus aureus* (positive) which produce the enzyme

coagulase, from *Staphylococcus epidermis* and *Staphylococcus saprophy icus* (negative) which do not produce coagulase. Coagulase (free and bound coagulase/clumping factor) is an enzyme produced by *S. aureus* that converts (soluble) fibrinogen in plasma to (insoluble) fibrin). Slide test (to detect bound coagulase): A drop of physiological saline was placed on each end of a slide. With the loop, a portion of the isolated colony was emulsified in each drop [17]. A drop of human plasma was added to one of the suspensions, and mixed gently, while no plasma was added to the second suspension. Clumping of the organisms within 10 seconds was looked out for as positive test whereas no clumping indicated a negative test.

Oxidase test: This test depends on the presence in bacteria that catalyzes the transport of electrons between electron donors in the bacteria and a redox dye tetramethyl-p-phenylene-diamine. Cytochrome C oxidases, an artificial electron acceptor. This test was used in detection of *Pseudomonas aeruginosa, Aeromonas* which gave positive reactions and for exclusion of *Entero bacteriaceae* [18]. In this reaction, A few drops of 1% aqueous solution of tetra methyl-p-phenlene-diamine hydrochloride reagent were added to a piece of filter paper in a petri dish. A smear of the bacteria isolate was made onto the impregnated filter paper using a sterile glass rod. The reaction was observed after 5 minutes. Purple colouration produced within 10 seconds indicated oxidase positive cultures. Absences of colour indicate negative result.

Urease test: This test was carried out using urea medium (modification of Christensen's test medium). This test was aimed at observing the ability of the organisms to split urea by means of urease (an enzyme) produced by microorganism. The medium was prepared according to manufacturers' standard and 5 ml of phenol red solution was added. The medium was dispensed into bijoux bottles and sterilized using an autoclave. The bottles after sterilization were allowed to cool and solidified. The isolates were inoculated by stabbing the solid medium with sterile inoculating needles. The cultured medium was incubated at 37°C for 3 days. A change to pink colour was a positive indicating urea hydrolysis, while a negative test showed no pink colour.

Hydrogen Sulphide (H_2S) production test: Hydrogen Sulphide (H_2S) production test is used for the detection of hydrogen sulphide gas produced by an organism. An inoculum from each isolate was transferred into Sulphide Indole Motility Medium (SIM) slant. It was incubated at 35°C for 24 hours. The presence of H_2S produced by organism is detected by the turning of the agar slant to black colour as positive test whereas no colour change was recorded as negative test.

Motility test: Sulphide Indole Motility (SIM) agar slant was prepared according to manufacturers' standard and the test organism was inoculated with a sterile needle, to the bottom of the slant and incubated at 35°C for 24 hours. Positive motility test was recorded when there was a dispersed growth in the slant, whereas the organism remained stable at the original position it was inoculated.

Molecular characterization of the bacteria using the 16S subunit Ribosomal Ribonucleic Acid (16S rRNA) gene sequence

This procedure was done using the method of Valerie, et al. at the DNA laboratories Kaduna state Nigeria after bench space reservation was granted. The aim was to use the presence of hyper variable regions in the 16S rRNA gene to identify species specific signature sequence of isolates [19].

Deoxyribonucleic acid extraction

The materials include: Bacterial culture, Tris Ethylene Diamine Tetra Acetic acid (TE) buffer, lysozyme solution, 10% and 1% of Sodium Dodecyl Sulfate (SDS), proteinase K, phenol, chloroform, isoamyl alcohol, sodium acetate, absolute ethanol, micropipettes, water bath, vortex and centrifuge. Before beginning, the heating block was set at 60°C. The 24 hour pure culture of the 6 representative bacteria isolates on nutrient agar medium were scraped using a wire loop and suspended each in a 1.5 ml eppendorf tube containing 400 µl of SDS lysis buffer and 10 µl of protenase K was added to each. The 6 tubes were placed on heat block at 60°C for minimum 1 hour.

400 µl of chloroform was added to the recovered clear layer, and vortex briefly homogenize. The reactions tubes were spanned at 13000 rpm for 5 minutes to separate the phases. The upper layer was carefully removed with a pipette and each transferred to a new 1.5 ml eppendorf tube. The white interphase was not extracted.

Equal volumes of 100% ethanol and 20 µl of 3 M sodium acetate were added and mixed by inverting the tube several times. The 6 tubes containing the solutions were incubated 20°C for 24 hours. The tubes were then spanned at maximum speeds for 30 minutes (in refrigerated centrifuge) in the same orientation. The ethanol was removed and fresh 400 µl 70% ethanol was added and spanned at maximum speed for 5 minutes at 4°C. The step was repeated to ensure that the salt is gone.

The tubes were spanned for 30 seconds intervals at high speed, to remove all traces of ethanol. The DNA were dried out by leaving the tube open for 10 minutes. The DNA pellets were re suspended in 20-50 µl sterile water of appropriate volume. 1 µl to 5 µl of the DNA was run on an agarose gel to estimate quantity and integrity.

Polymerase chain reaction

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

The reaction mixture was over laid with 1 drop (approx. 50 µl) of light mineral oil in the thermal cycler, the eppendorff tubes were placed in the thermal cycler. The nucleic acids was amplified using the initial denaturation step at 94°C for 2 mins followed by 30 cycles at 94°C for 30 sec, annealing at 52°C for 30 sec and 72°C for 2 mins, and a final extension (polymerization)

step at 72°C for 5 minutes. 10 µl of each sample was withdrawn from the test reaction mixture and the four control reactions, and were analyzed by gel electrophoresis through an agarose gel, and stain the gel with ethidium bromide to visualize the DNA. Visible DNA fragment was produced at the end of the amplification.

Electrophoresis

Materials include: Agarose powder, ethidium bromide, electrophoresis buffer, DNA samples, DNA ladders, Gel casting trays and trans illuminator (foto/wv 15 models 33017, fotodyne, USA). Specification of agarose gel used: W230-500. Lot: 5KR081612. QD LE Agarose 500grams; high resolution quick dissolve, gel strength=1200 g/cm^3 (1.0%); low EE: ≤ 0.13; gel point: 36°C ± 1.5°C (1.5%); moisture: ≤ 10%; meting point 88°C ± 1.5°C (1.5%); sulfate: ≤ 0.14%. separation range: 50 bp–20 kb; background very low; DNase and RNase: None detectable. 3 g of agarose was used for 1.5% preparation.

PCR products (amplicons) were separated and visualized as follow: The solution was heated in a microwave for 60 seconds until agarose was completely dissolved, and allowed to cool in a water bath set at 55°C. The gel casting tray was prepared by sealing ends of gel chamber with tape. And 2 combs were placed in the gel tray. 5 µl of ethidium bromide was added to the cooled gel and poured into the gel tray. It was allowed to cool for 30 minutes at room temperature. The combs were removed, and the gel was placed in electrophoresis chamber and was covered with buffer (TAE or TBE as used previously). The standard (Ladder), the extracted DNA and the Negative control (that contain water and primer) were loaded onto gel from left to right of the wells using pipette. It was Electrophorese at a voltage of 111 for 1 hour and the DNA bands were visualized using Ultraviolet (UV) light box and image was captured

Elution of Deoxyribonucleic Acid (DNA)

Materials include: Elution buffer, agarose, micropipettes, dry bath, incubator, microfuge tubes, centrifuge, N-Butanol, cryo box, cyclomixer, Ethanol (70% and 95%),TE buffer; -20°C freezer, -70°C freezer and Ultraviolet UV light. The low melting point agarose gel with DNA bands was visualized under a UV trans illuminator and the desired DNA band were located and cut around carefully using a scalpel blade. The gel piece was transferred into a microfuge tube. Elution buffer was added into the microfuge tube until the level of buffer was just above the level of gel slice.

The gel slice was heated at 65°C until it melts, and the melted gel with DNA was freeze by placing in a 70°C freezer for 10 minutes. After freezing, it was centrifuged for 10 minutes and the supernatant was transferred into a new microfuge tube.

Again, half amount of elution buffer used in the previous step was added into the pellet and was heated at 65°C until the agarose melts. The melted gel with DNA was freeze by placing in a 70°C freezer for 10 minutes, the tube was centrifuged again for 10 minutes and the supernatant was added up into the previous microfuge tube with supernatant. The tubes with pellets were then discarded.

An equal volume of n-Butanol was added to the supernatant, the tube was vortex for 15 minutes in order to remove the Ethidium bromide, the upper phase of butanol was discarded and the process was repeated by adding n-butanol again for one more time. 2 times volume of 95% ethanol was added and mixed thoroughly.

It was kept for precipitation in 70°C freezers for 30 minutes. After precipitation, the tube was centrifuged for 15 minutes and the supernatant was discarded into a waste beaker while a 200 µl of 70% ethanol was added to the pellets, the solution was centrifuged for 5 minutes and the supernatant was again discarded. The pellets were allowed to dry well. The pellets were suspended in 20 µl of TE buffer. The recovered DNA was stored in -20°C freezer for further process.

Radiolabeling

The materials include: 4 µL of Isolated DNA (PCR Product), 2 µL of Klenow enzyme, 6 µL of 32P dCTP, 4 µL ofd ATP, 10 µL of labeling buffer, water bath, micropipettes, 4°C freezer and a 20°C freezer.

In a sterile 0.5 ml microfuge tube, the reagents were mixed, the solution was denatured by keeping it in a water bath at 37°C for 4 hours and after incubation, it was kept in the ice cubes for 5 minutes. 30 µl of the radio labeling mixture was transferred into a spin column and kept in spin column in 20°C freezers. It was later centrifuged at 1000 rpm for 2 minutes. After centrifugation, it was kept in the water bath 37°C for 10 minutes. After the incubation, the spin column was kept in 20°C freezers [20].

Restriction digestion

The materials include: Micro-centrifuge tubes, micropipettes, centrifuge, and incubator. 3 µL of DNA, 1 µL of 10x buffer, 0.5 µL of *EcoRI* enzyme, 0.5 µL Bam H_1 enzyme were mixed with 5 µL d H2O (to bring total volume to 10 µL). The solution was transferred in a micro centrifuge tube. The mixture was incubated at 37°C for 1 hour. The 1.5 mL tubes were kept in 4°C freezers after the incubation.

Southern blotting

The materials include: Gel (subjected to agarose gel electrophoresis), neutralizing solution Whatman filter paper, nylon membranes, Tris-Ethylene diamin tetra acetic acid (TE) buffer, plastic wrapper, stack of paper, large glass slide of 2 kg, transfer buffer, UV trans illuminator, pre-hybridization buffer, hybridization buffer, hybridization oven, 20 µl of probe solution, 0.1% Sodium Dodecyl Sulfate (SDS), X-ray film and autoradiography cassette. The gel (subjected to agarose gel electrophoresis) was transferred into the denaturing solution and was gently vortex for 5 minutes. The denaturing solution was discarded and then washed in neutralizing solution for 5 minutes. The blot transfer was set up as follows: The gel containing the DNA was transferred into a wet Whatman filter paper. The whole set up was carried in a reservoir which was filled with transfer buffer. Nylon membranes were placed over

the gel. Whatman filter paper was placed over the nylon membrane.

The paper was flooded with TE buffer without forming any air bubbles. Then, the reservoir was covered with plastic wrapper, after which, a stack of paper was placed over the gel and was pressed gently.

A large glass slide and an additional weight of 2 kg were placed over the stack of paper to keep the blot in good position. It was kept undisturbed for 12 hours.

After the incubation, the glass slide, weight and stacks of paper were carefully removed. Then, the nylon membrane was carefully taken and kept in the tray containing 6X transfer buffer and the nylon membrane was placed in the UV trans illuminator. The membrane was transferred to a white paper. The pre-hybridization buffer was added into the hybridization tube and the nylon membrane kept in the hybridization tube.

The hybridization tube was kept in the hybridization oven and incubates at 42°C for 2 hours. The pre-hybridization buffer was discarded and hybridization buffer added together with 20 µl of probe into the hybridization tube.

The hybridization tube was transferred into the hybridization oven and incubated at 42°C for 2 to 4 hours. The hybridization buffer was then discarded and 4 ml of 0.1% sodium dodecyl sulfate was added into the hybridization tube. It was incubated at 52°C for 30 minutes.

The nylon membrane was carefully taken from the hybridization tube and placed on the autoradiography cassette to observe the band that corresponds to the 16S ribosomal RNA sequence of the test organism.

The gene sequences obtained were compared by aligning the results with GenBank using the Basic Local Alignment Search Tool (BLAST) search program. BLAST for Basic Local Alignment Search Tool is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of proteins or the nucleotides of DNA sequences. Each of the nucleotide sequences that were obtained was compared with existing reference nucleotide in the GenBank to identify the bacteria samples. The test nucleotide sequence was copied and paste in the black space of the website of Natural Centre for Biotech Information (NCBI) the sequence result was generated by the search engine.

Plant leaves extraction

Collection of plant leaves: The selected local leaves which include: *Cleomeviscosa*, Sansevieria trifasciata leaves, Jatropha curcas leaves and *Chromolaena odorata* leaves were sourced locally from Nkpa bushes in Bende LGA, Abia State Nigeria in August, 2015. They were identified by plant taxonomist in the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike Abia State, in accordance to Dutta.

Preparation of the water extracts: The leaves were washed in clean water and allowed to air dry. Then Cleome viscosa, *Chromolaena odorata* and Jatropha curcas leaves were dried on sunny day light, while Sansevieria trifasciata leaves being succulent was oven dried at controlled temperature of 45°C ± 5°C until they become crispy. They were ground into fine powder with an Arthur thamus milling electric machine (Arthur Thamus model). Approximately 100 g of the powdered leave was placed into a conical flask and 250 ml of distilled water was added into it. The flask was sealed and was shacked at 30 minutes interval for 4 hours. It was filtered using Buchner funnel through Whatman-No.1 filter paper. The filtrate was centrifuged, decanted and the concentrate was vacuum dried at 50°C in a water bath to obtain the crude extract. While the used filter paper and the residue were discarded. The concentrated extract was weighed, recorded and stored in a vial container at 4°C. 0.5 grams of extract was re-solubilized in 2 ml of distilled water to obtain 250 mg/ml crude extract for susceptibility test. The Water extracts was calculated as shown below;

$$\% \text{ Extraction} = ((W_2 - W_l)/W) \times 100.$$

Where:

W=Weight of sample used,

W_1=Weight of empty evaporated flask,

W_2=Weight of dish + Water extract.

Preparation of ethanol extracts: Fifty gram (50 g) of the powdered leave was placed into a conical flask and 150 ml of ethanol was added. The flask was sealed and incubated at room temperature on a rotator shaker for 4 hours. It was vacuum filtered using Buchner funnel through Whatman-No.1 filter paper. The filtrates obtained was concentrated using a water bath at 50°C under vacuum. The concentrate was air dried. The extract was weighed, recorded and stored in a vial container at 4°C. The Ethanol extracts was calculated as shown below;

$$\% \text{ Extraction} = (W_2 - W_l)/W) \times 100.$$

Where:

W=Weight of sample used,

W_1=Weight of empty evaporated flask,

W_2=Weight of dish+Ethanol extract.

Using weighing balance and measuring cylinder, 0.5 grams of extract was re-solubilized in 2 ml of distilled water to obtain 250 mg/ml crude extract for susceptibility test [21].

Preparation of 0.5 Mcfarland turbidity standards: McFarland turbidity standard was a turbid solution that contains a mixture of barium salt, distilled water and tetra oxo sulphate (VI) acid (H_2SO_4). It was used to compare the turbidity of both the test and control. 1 ml of concentrated H_2SO_4 was added to 99 ml of distilled water in a beaker and mix well to obtain, a 1 % v/v solution of H_2SO_4.

With a weighing balance, 0.5 g of dehydrated Barium Chloride salt ($BaCl_2.2H_2O$) was dissolved in 50 ml of distilled water. In this way a 1 % w/v of $BaCl_2$ was prepared. 0.6 ml of $BaCl_2$ solution was added to 99.4 ml of H_2SO_4 solution to make up to 100 ml. The solution was mixed well and it served as the stock solution of the 0.5 McFarland turbidity standards. 3 ml of the solution

was transferred into a capped tube and stored at room temperature until its use.

Antibacterial activity of the plant extracts using agar well diffusion: Antimicrobial screening of the four water and four ethanolic extracts were carried out using agar well diffusion method. The inocula were prepared by growing the various isolates on separate agar plates and colonies from the plate were transferred with inoculating loop into 3 ml of normal saline in a test tube. The density of these suspensions was adjusted to 0.5 McFarland standards equivalent to 1×10^6 CFU/mL. Three round holes of 7 mm diameter per plate, was made on the solidified Mueller hinton plates using a sterile cork borer. Three holes per plate and for the eight extracts and six text organisms, a total of 48 plates were prepared. The test organism was evenly inoculated on Muller-Hinton agar using a sterile swab. The experiment was performed in duplicates. The swab was dipped into the suspension and pressed against the side of the test tube to remove excess fluid. The wet swab was then used to inoculate the Muller-Hinton agar by evenly streaking across the surface. 0.05 ml of 250 mg/ml of each extract was added into each agar well. The plates were incubated for 24 hours at 37°C. The each zone of inhibition was recorded.

Determination of antibiotic sensitivity of the isolates

Antibiotic sensitivity test was also carried out on the bacteria isolates, using commercially manufactured 6mm multidisc containing Ampiclox (20 µg), Ciprofloxacin (10 µg), Amoxicillin (20 µg), Gentamicin (10 µg), Erythromycin (30 µg), Chloramphenicle (30 µg) and Levofloxacin (20 µg) antibiotic concentrations.

Kirby Bauer method was adopted. Mueller-hinton agar plate was prepared according to manufacturer's directives and poured into petri dishes. Each plate was labeled with the bacterium to be tested. The bacteria were dispersed in a broth culture and adjusted to 0.5 McFarland and 1:100 diluted in Muller Hinton broth to obtain approximately 1×10^6 CFU/mL. Using the streak technique, a wire loop was used to streak the plates. The agar plates were allowed to stand for 10 minutes. The 6 mm multi discs were aseptically placed on the inoculated plates. A pair of forceps was sterilized, and allowed to cool and used to gently tap each disc to ensure even contact with the agar surface. The plates were incubated at 37°C for 24 hours. Zones of inhibition were recorded.

Biofilm formation and biofilm antibiotic sensitivity testing

Khalid, et al., method of Biofilm Antibiotic Sensitivity Testing (BAST) was employed. Colony of biofilm was first developed on a cellulose filter/membrane disk, over which an antibiotic disc was imposed. Zone of inhibition were measured after incubation on a nutrient agar.

Membrane filters with 0.22 um pore size and 7 mm diameter were sterilized using autoclave for 15 minutes at 121°C. The disc was soaked for 24 hours in phosphate buffered saline. The discs were then dipped into the 24 hour bacterial cultures that have been diluted to optical density of 0.05 at 600 nm. The filters were placed carefully on the surface of the nutrient agar plates and were incubated at 37°C for 48 hours. The discs were then carefully dipped in 4% sheep plasma in order to promote the adherence of the bacterial to the membranes. After dipping, the membranes were placed on new plates. The same antibiotic discs used in the antibiotic susceptibility test were superimposed over the membrane filters and zones of inhibition of bacterial growth were measured after 48 hours.

Plasmid profiling

The aim of the plasmid profiling was to ascertain the various isolates markers, coded genes for antibiotic resistance as well as their virulent factors.

Plasmid extractions: The method of Stanisich, was adopted with slight modifications. Pallet 5 ml of each of the 24 hour bacterial cultures of *Comamonas aqua ica*, *Achromobacte rxylosoxidans*, *Delftia acidovorans*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, were prepared by centrifugation at 800 rpm for 3 minutes at room temperature (25°C). The pallet cells were re suspend in 250 µl phosphate buffered saline (PI) and transferred to a micro-centrifuge tube. 250 ul buffer P2 was added and mixed thoroughly by inverting the tube 6 times, until the solution becomes clear. The lyses reaction was not allowed to proceed for more than 5 minutes. 350 µl buffer N3 was then added and mixed immediately and thoroughly by inverting the tube 6 times. The reaction mixture was centrifuged for 10 minutes at 13000 rpm (17900 x g) in a table top micro-centrifuge. At this point, 800 ul supernatant from the centrifuged reaction mixture was applied to the QIAprep 2.0 spin column using pipette. After that, it was centrifuged for 60 seconds and the flow-through was discarded. This step is only required when using end A+strains or other bacteria strains with high nuclease activity or carbohydrate content. The QIAprep 2.0 spin column was washed by adding 0.75 ml buffer PE. It was centrifuge for 60 seconds and the flow-through was discarded and the connection tube was then switched off. Furthermore, the preparation was centrifuged for 1 minute to remove residual wash buffer. The QIAprep 2.0 column was placed in a clean 1.5 ml centrifuge tube. To elute DNA, 50 µl buffer EB (10 mMTrisCl, pH 8.5) was added to the center of the QIAprep 2.0 spin column, allowed to stand for 1 minute and centrifuged for 1 minute. 1 volume of loading dye was added to 5 volumes of purified DNA, the solution was mixed by pipetting up and down before loading the gel.

Agarose gel electrophoresis: Agarose gel electrophoresis was used for separating and analyzing the extracted plasmid Dioxy ribo Nucleic Acid (DNA). The purpose of the gel was to look at the plasmid DNA, to quantify it and possibly to isolate a particular band. The plasmid DNA was visualized in the gel by addition of ethidium bromide, ethidium bromide and the proprietary dyes bind to DNA and are fluorescent, meaning that they absorb invisible UV light and transmit the energy as visible light.

First step: A 50x stock solution of tris base Acetate Ethylene diamine tetra acetic acid (TAE) buffer in 1000 ml of distilled H_2O was prepared by weighing 242 g of Tris base using a chemical balance. The solution was transferred to a 1000 ml beaker.

Ethylene Diamine Tetra Acetic Acid (EDTA) solution (pH 8.0, 0.5 M) was prepared by weighing 9.31 g of EDTA and dissolved in 40 ml distilled water. The pH was checked using pH meter. Make the solution 100 ml by adding distilled water. 57.1 ml of glacial acetic acid was pipette out. A Mixed solution of the tris base, EDTA solution and glacial acetic acid were made by adding distilled water to make the volume to 1000 ml.

Second step: Sufficient electrophoresis buffer (1xTAE) was prepared to fill the electrophoresis tank and to cast the gel by weighing 2 ml of TAE stock solution in an Erlenmeyer flask and made the volume to 100 ml by adding 98 ml of distilled water. The 1x working solution was 40 mM tris-acetate/1 mM EDTA

Third step: A solution of agarose in electrophoresis buffer at an appropriate concentration was prepared by adding 2 grams of agarose to 100 ml of electrophoresis buffer. The neck of the Erlenmeyer flask was loosely plugged, before heating the slurry in a microwave oven until the agarose dissolved for 45 seconds.

A tong was used to transfer the flask into a water bath at 55^{o}C. After the molten gel has cooled, 0.5 µg/ml of ethidium bromide was added and the gel solution was thoroughly mixed by gentle swirling. (Ethidium bromide was prepared by adding 1 g of ethidium bromide to 100 ml of H_2O, stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. 10 mg/ml of the solution was transferred to a dark bottle and stored at room temperature.). A suitable comb for forming the sample slots in the gel was selected, while the agarose solution was cooling, the warm agarose solution was poured into the mold. (The gel used was 4 mm thickness and there were no air bubbles under or between the teeth of the comb). The gel was allowed to set completely for 45 minutes at room temperature. A small amount of electrophoresis buffer was then poured on the top of the gel, and the comb carefully removed [22]. The electrophoresis buffer was poured off. The gel mounts in the electrophoresis tank. Enough electrophoresis buffers that covered the gel to a depth of 1 mm were added. Thereafter, DNA samples from the bacteria were mixed with 0.20 volumes of the desired 6x gel-loading buffer. Then sample mixtures were slowly loaded into the slots of the submerged gel using an automatic micro pipette. 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 DNA would migrate toward the positive anode (red lead). A voltage of 5 V/cm was applied (measured as the distance between the positive and negative electrodes). Finally, the gel was allowed to run till the bromo phenol blue and xylene cyanol FF migrated to an appropriate distance through the gel. Then, the gel tray was removed and placed directly on a trans illuminator. At this point, the Ultra Violet light was switched on in order to view the orange bands of DNA and then, capture the image using a computer scanner.

Results

Characterization and identification of isolates

Preliminary characterization and identification of isolates were based on cultural, microscopic and biochemical characteristics as seen in Table 1. Figures 1 and 2 are the extracted DNA in agarose gel to estimate the quantity and integrity before carrying out amplification and elution for the final 16S rRNA gene sequencing.

Table 1: Preliminary characterization using biochemical 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
Motility	+	+	+	+	+	+
Citrate	+	+	+	+	+	+
Oxidase	-	+	+	+	+	+
Indole	-	-	-	-	-	-
Methyle red	+	ND	ND	-	-	-
Vogesproskauer	+	ND	ND	-	-	-
Citrate	ND	+	+	+	+	+
Hydrogen sulphide	-	ND	-	-	ND	-
Urea hydrolysis	-	ND	-	+	+	+
Coagulase	ND	ND	ND	ND	+	ND

Key: ND: Not Determined; G-ve: Gram Negative and G+ve: Gram Positive.

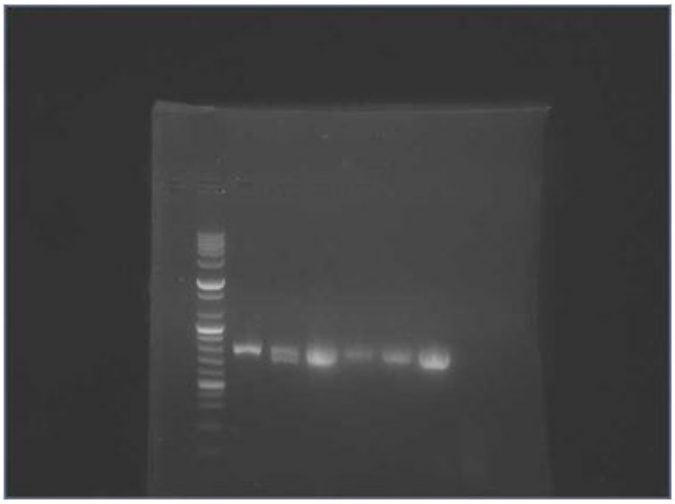

Figure 1: Extracted DNA in agarose gel to estimate the quantity and integrity.

Key: From left to right indicates DNA markers, AF, BF, CF, DF, EF, and FF DNA and a negative control.

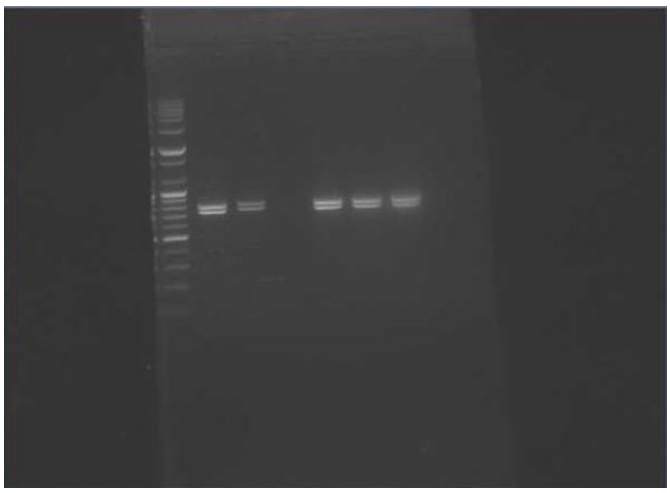

Figure 2: Extracted DNA on agarose gel to estimate the quantity and integrity.

Key: From left t o r ight i ndicates D NA m arkers, optimized BF with double bands, and a negative control.

Table 2 was the confirmatory identification of the isolates by 16S rRNA gene sequences to obtain the nucleotide sequences that was used in Basic local alignments search tool.

The study population was a total of 200 pupils from 5 selected Nkpa nursery/primary schools in Bende local government area, from August 2015 to February 2017.

Ear infection was recorded in 48 (24%) pupils, consisting of 28 (14%) males and 20 (10%) females which resulted to overall cases of male to female ratio of 1.4 : 1.0. as in Table 3.

Table 2: 16S subunit ribosomal ribonucleic acid gene sequences.

Code	16S rRNA gene sequences	Blast
AF	CTGGCAAGTCGAACGGAACAGTCTTCG TATGCTGACTAGTGGCGAACGGGTGAG TATACTCGAAACGTGCCTATAGCGGGGA TAACTACTCGAAAGAGTGGATATACCGC ATGACATCTACGGATGAAAGCAGGGATC GCAAGACCTTGTGCTACTATAGCGTGC GATGGCAGATTAGGTAGTGGCGGGATA AAAGCTTACCAGGCCGACGATCTGTAC CTGGCTGAGAGGACGATCAGCCACACT GGGACTGAGACACGGCCCAGACTCCTA CGGAGGCAGAGGGGGAATTTTGGACA ATGGGGCAAGCCTGATCCAGCAATGCC GCGTGCAGGATGAAGGCCTTCGGGTT GTAAACTGCTTTTGTACGGAACGAAAAG	*Comamonas aquatica*

	CCCTGGGTTAATACCCTGGCATGACGA CCGAAGAATAAGCACCGCTAACTACGT GCCAGCACGCGAATACGTAGGGGCGA GCGTAATCGGAATTACTGGCGTAGCGT GCGCAGGCGCTTTGTRAGACAGAGGT GAAATCCCCG	
BF	CGGTCTGATGGCGAGTGGCGAACGGG TGAGTCATGTATCGGAACGTGCCCAGT AGCGGGGGATAACTACGCAAAGCGTAG TAATACCGATACG	*Achromobacter xylosoxidans*
CF	TACCATGCAGTCGAACGGACAGGTCTT CGGACGCTGACGAGTGGGGAACGGGT GAGTAATACATCGGAACGTGCCCAGTC GTGSGGGATATCTACTCGAAAGAGTACT AATACCGCATACGATCTGAGGATGAAAG CGGGGGACCTTCGGGCCTCGCGCGAT TGTAGCGGGCGATGGAGATTAGTATTG GGGGATAAAAGTTACCAAGCCGACGAT CTGTAGCTGGGCTGAGAGGACGACCA GCCACACTGGACTGAGACACGGGCCA ACTCCTACGGGAGGSAGCAGGGGGAAT TTTGACAATGGGCGAAAGCTGATCCAC AATGCGCGTGCAGGATGAAGCTTCGGG TTGTAAACTGCTTTTGTACGGAACGAAA AACTCCTTCTAATACAGGGCCCATGAGG ACCGCAGAATAAGCACCGTACTACGTG CCAGCAGCCGCGGTAATACGTAGGTGC GAGCGTAATCGGAATTACTGGGGCGTA GCGGCGCACGCGTATGGTAAGACAGAT GTGAAATC	*Delftia acidovorans*
DF	CATGCAGTCGAACGGCAGCACAGAGG AGCTTGCTCCTTGGGTGGCGAGTGGC GGACGGTGAGGAATACATCGGAATCTA CTCTGTCGTGGGGGATAACGTASGGAA ACTTACGCTAATACCGCATACGACCTAC GGGTGAAAGCAGGGGATCTTCGGACCT TGCGCGATTGAATGAGCCGATGTCGGA TTAGCTAGTTGGCGGGGTAAAGGCCCA CCAAGGCGACGATCCGTAGCTGGTCTG AGAGGATGATCAGCCACACTGGAACTG AGACACGGTCCAGACTCCTACGGGAG GCAGCAGTGGGGAATATTGGACAATGG GCGCAAGCCTGATCCAGCCATACCGCG TGGGTGAAGAAGGCCTTCGGGTTGTAA AGCCCTTTTGTTGGGAAAGAAATCCAG CTGGCTAATACCCGGTTGGGATGACGG TACCCAAAGAATAAGCACCGGCTAACTT CGTGCCAGCAGCCGCGGTAATACGAAG GGTGCAAGCGTTACTCGGAATTACTGG GCGTAAAGCGYGCGTAGGT	*Stenotrophomonas maltophilia*
EF	ATGCAGTCGAACGGCAGCACAGAGAG CTTGCTCCTTGGGTGGCGAGTGGCGG ACGGTGAGGAATACTCGGAATCTACTCT GTCGTGGGGGATAACGTAGGGAAACTT ACGCTAATACCGCATACGACCTACGGGT GAAAGAGGGGATCTTCGGACCTTGCGC GATTGAATGAGCCGATGTCGGATTAGCT AGTTGGCGGGGTAAAGGCCCACCAAG	*Pseudomonas aeruginosa*

	GCGACGATCCGTACTGGACTGAGAGGA TGATCAGCCACACTGAACTGAGACACG CCAGACTCCTACGGGAGGCAGCAGGG GAATATTGGACAATGGGCGCAAGCCTG ATCCAGCCATACCGCGWGGGTGAAGAA GGCCTTCGGGTTGTAAAGCCCTTTTGT TGGGAAAGAAATCCACTGGCTAATACCC GGTTGGGATGACGGTACCCAAAGAATA AGCCCGGCTAACTTCGTGCCAGCAGCC GCGGTAATACGAAGGGTGCAAGCGTTA CTCGGAATTACTGGGCGTAGAGCGTGC GTAG	
FF	CACAGAGGAGCTTGCTCCTTGGGTGGC GAGTGGCGGACGGGTGAGGAATACATC GGAATCTACTCTGTCGTGGGGGATAAC GTAGGGAAACTTACGCTAATACCGCATA CGACCTACGGSTGAARGCAGGGGATCT TCGGACCTTGCGCGATTGAATGAGCCG ATGTCGGATTAGCTAGTTGGCGGGGTA AAGGCCCACCAAGGCGACGATCCGTAG CTGGTCTGAGAGGATGATCAGCCACAC TGGAACTGAGACACGGTCCAGACTCCT ACGGGAGGCAGCAGTGGGGAATATTGG ACAATGGGCGCAAGCCTGATCCAGCCA TACCGCGTGGGYGAAGAAGGCCTTCG GGTTGTAAAGCCCTTTTGTTGGGAAAG AAATCCAGCTGGCTAATACCCGGTTGG GATGACGGTACCCAAAGAATAAGCACC GGCTAACTTCGTGCCAGCAGCCGCGGT AATACGAAGGGTGCAAGCGTTACTCGG AATTACTGGGCGTAAAGCGTGCGTAGG TGGTCGTTTAAGTCCGTTGTGAAAGCC CTGGGCTCAACCTGGGAACTGCAGTG GATACTGGGCGACTAGARTGTGGTAGA GGGTAGCGRAATTCCTGGTGTAGCAGT GAAATGCGTAGAGATCAGGAGGAACAT CCATGGCGAAGGCAGCTACCTGGACCA ACATTGACACTGAGGCACGAAAGCGT	*Pseudomonas aeruginosa*
Key: Code AF, BF, CF, DF, EF and FF were the 6 representative bacteria samples with similar grams' reaction and biochemical reactions used for the 16S rRNA gene sequences; BLAST: Basic Local Alignment Search Tool.		

Table 3: Gender distribution in 200 school children.

Gender	Number	Infected	%=Infected/Total × 100
Male	108	28	14
Female	92	20	10
Total	200		

There were 5 different bacteria pathogens isolated in different proportions as seen in Table 4, with *Achromobacter xylosoxidans* 16 (8%) having the highest percentage occurrence, while *Comamonas aquatica* 2 (1%) was the least isolate. From Table 5, the mean age of the study population was 7.1 ± 3.6 years (Supplementary Table 1), ranged 2 to 15 years. Age group of 2-3 years had the highest infection of 16 (8.00%), followed by 4-7 years and 8-11 years which had the same prevalence rate of 13 (6.50%). While group 12-15 years had the least infection of 6 (3.00%), thus infection decreases with increase in age.

Table 4: Percentage occurrences of the various isolate.

Bacteria	Number	Positive	%
Comamonas aquatica	200	2	1
Achromobacter xylosoxidans	200	16	8
Delftia acidovorans	200	11	5.5
Stenotrophomonas maltophilia	200	8	4
Pseudomonas aeruginosa	200	11	5.5
Total positive		48	24

Table 5: Percentage occurrence of isolates with respect of age differences.

Isolates	2-3 years		4-7 years		8-11 years		12-15 years	
	P	%	P	%	P	%	P	%
Comamonas aquatic	0	0	0	0	2	1	0	0
Achromobacter xylosoxidans	5	2.5	5	2.5	5	2.5	1	0.5
Delftia acidovorans	4	2	5	2.5	1	0.5	1	0.5
Stenotrophomonas maltophilia	3	1.5	1	0.5	1	0.5	3	1.5
Pseudomonas aeruginosa	4	2	2	1	4	2	1	0.5
Total	16	8	13	6.5	13	6.5	6	3

Key: NE: Number Examined; NP: Number Positive; %: Percentage occurrence. Age →Years.

Antibiotic susceptibility

Figure 3 shows *that Stenotrophomonas maltophilia* was most susceptible to Levofloxacin (30 µg) (19 mm) as well as Ampiclox (20 µg) (19 mm) while *Comamonas aquatica* has the least susceptibility profile to all antibiotic tested. Figure 4 shows zones of inhibition of antibiotics on biofilm. A comparison of Figure 2 and Figure 3 reveals that isolates were more susceptible to the antibiotics with a range of 8 mm to 19 mm than the biofilm counterparts ranged between 9 mm to 16 mm. This gives insight on the difficulty in the treatment of chronic superlative otits media.

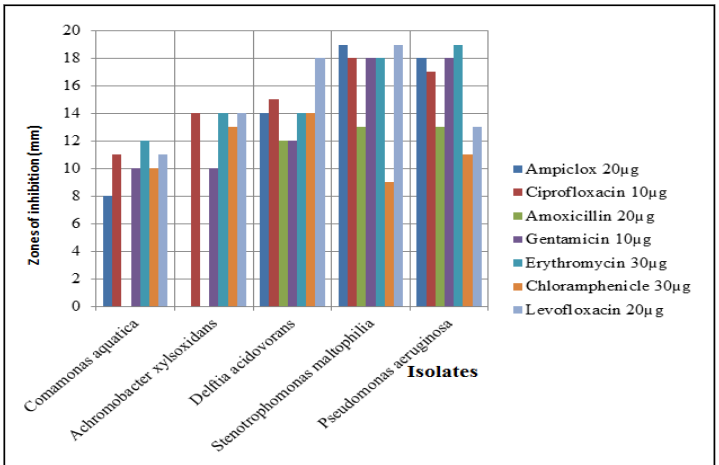

Figure 3: Susceptibility pattern of isolates to antibiotics. Size of discs→6 mm.

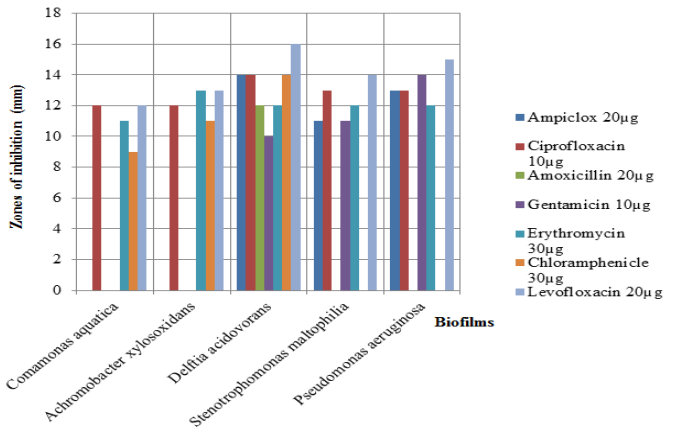

Figure 4: Biofilms antibiotic Susceptibility profiles. Size of discs→7 mm.

From Figure 5, there was higher yield of ethnol extract (23.5%) than water extract (19.0%). Figure 6 shows that agar well diffusion of extract had no antimicrobial activities of the extracts, except ethanolic *C. odorata* (250 mg) extract with slight activity (10 mm) and (9 mm) zones of inhibition of *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* respectively [23].

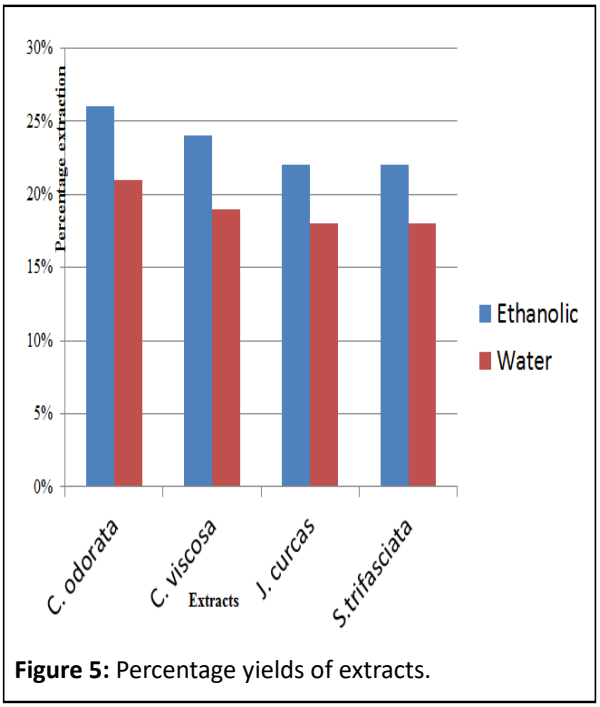

Figure 5: Percentage yields of extracts.

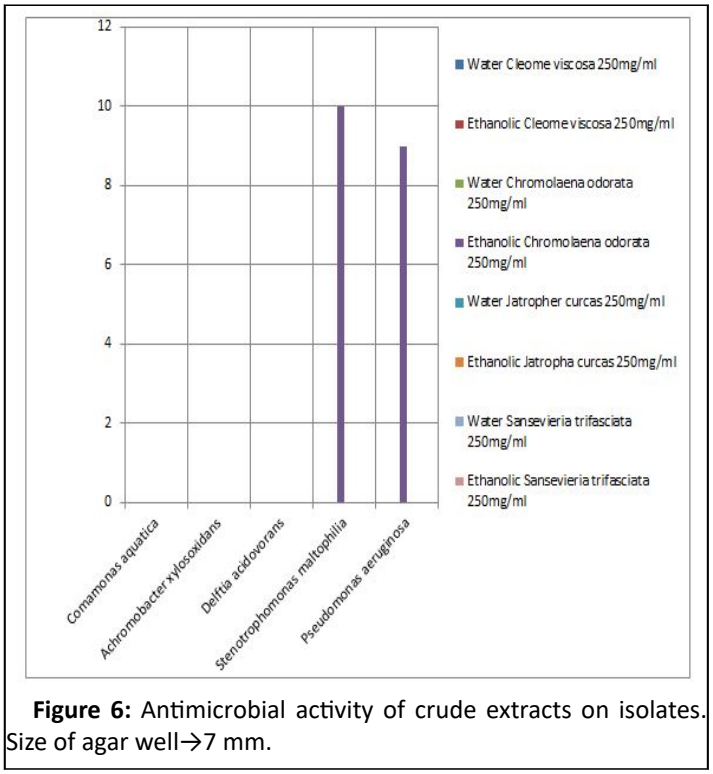

Figure 6: Antimicrobial activity of crude extracts on isolates. Size of agar well→7 mm.

Figure 7 reveals that plasmid DNA bands existed in each isolate and which were all nicked single stranded bands. From Table 6 are the molecular weights of each of the bacteria plasmid DNA weighed approximately 1600 base pairs. This showed that isolates possessed resistant plasmids.

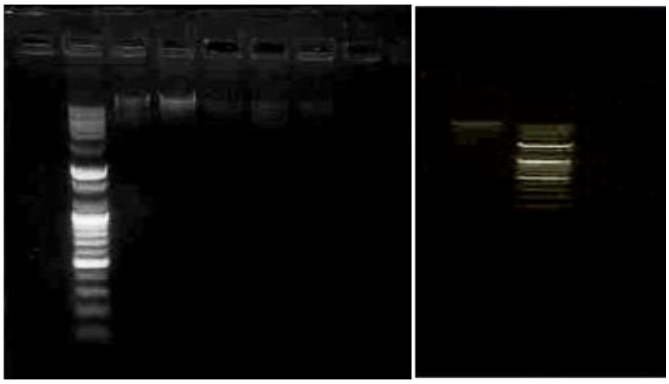

Figure 7: Plasmids in agarose gel.

Key: from left to right is DNA marker, plasmid resistant gene bands of AF, BF, CF, DF, EF and FF bacteria isolates.

Table 6: Extracted bacteria plasmids profiles.

Code	Isolates	Molecular weight	Occurrence
AF	*Comamonas aquatica*	approx.1600 bp	1
BF	*Achromobacter xylosoxidans*	approx.1600 bp	1
CF	*Delftia acidovorans*	approx.1600 bp	1
DF	*Stenotrophomonas maltophilia*	approx.1600 bp	1

EF	*Pseudomonas aeruginosa*	approx.1600 bp	1
FF	*Pseudomonas aeruginosa*	approx.1600 bp	1

DISCUSSION

The present study is the first to attempt to isolate and characterize the possible etiological bacteria ear pathogens using the 16S rRNA gene sequences, among school pupils from Nkpa, Bende Local Government Area, Abia state Nigeria The pathogens that was isolated were predominantly gram-negative isolates (100%), similar observation of bacteriological characteristics of which shift toward Gram negative pattern has been made by Sharma, et al. Out of the 48 (24%) pupils infected, there was 28 (14%) males preponderance to 20 (10%) females in the ratio of 1.4:1.0. cases. Similar male preponderance was noted by Olubanjor, et al. and Shamboul and Omdurman. While it was not in agreement with Hassan and Adeyemi, who observed that, females were more affected by ear infection. But Areya and Yilikal showed no differences between male and female. The reasons for this gender predominance are not well understood. The 16S rRNA gene of each bacteria was isolated more than ones in the 48 cases, with *Achromobacter xylosoxidans* 16 (8%), *Stenotrophomonas maltophilia* 8 (4.00%), *Delftia acidovorans* and *Pseudomonas aeruginosa* 11 (5.50%), while *Comamonas aqua ica* 2 (1%) was the least isolate, this results connotes that were various bacteria pathogens in different proportions in the studied population, which was in agreement with the observations of Sweeney. In the same vein, of the 48 total bacterial isolates, *Achromobacter xylosoxidans* (8.00%) was observe as the most occurrence bacteria pathogen and closely followed by 5.50% each of *Pseudomonas aeruginosa* and *Delftia acidovorans*, these observation was in contrast with a conclusion drawn by another similar study in Indudharan, et al. and Loy, et al. who found *Pseudomonas aeruginosa* as the most common microorganism in otitis media. However, with reference to Oni, et al. who reported that predominance changes over time, as such, *A. xylosoxidans* have overtaken *P. aeruginosa* in this locality [24]. More so, *Achromobacter xylosoxidans* identified in our study was in tandem with their port made by Yabuchi, and Yano that's howed *Achromobacter xylosoxidans* was originally isolated from patient with otitis media. This observation could mean that the school children were at higher risk of otitis media. *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* account for 9.50% of the total bacterial isolates, and were part of known causes of Recreational Water Illness such as the swimmer's ear, known medically as otitis externa, similar observation has been made elsewhere by Heaney, et al. who observed that children younger than 10 years of age contract more illness from recreational water because they play in shallow water and sand which are most contaminated with these organisms. *Comamonas aqua ica*, accounts for 1.00% case, *Comamonas aqua ica* is a pathogen in water sources with a foul smell and was isolated from two patients between ages of 8-11, could be contracted from swimming or bathing in polluted streams. Zhu, et al. observed this *Pseudomonas*-like strain to induce agglutination in Human and various animals. *Delftia acidovorans* formally called *Comamonas acidovorans* accounts for 5.50% cases of ear infection of pupils in this locality, reports of isolation of this pathogen from serious infections like acute Suppurative otitis media, corneal ulcers, empyema, bacteremia and intravenous catheter related infections exists. These bacteria are common in water and soil saprophyte. These isolations could reveal that the infected pupils do play on shallow waters and humid soils which are bacterial contaminated. The mean age of the studied population was 7.1 ± 3.6 years (Supplementary Figures 1-3), ranged 2 to 15 years. From the 48 (24%) infected pupils, pupils younger than 8 years, had 29 (14.5%) ear infection. While pupils between 8 to 15 years had 19 (9.5%) ear infection, in the ratio of 1.5:1.0. Thus ear infection decreases with increase in age. These observations were in agreement with similar studies made elsewhere by Teele, et al., and Poorey and Lyer, this may be because their euster chian tubes were smaller and more level and makes it difficult for fluid to drain out of the ear, or even under normal conditions in their tender age and expands as they grow up. The isolates were more susceptible to the antibiotics than their biofilm counterparts, with *Stenotrophomonas maltophilia* being most susceptible (19 mm) to Levofloxacin (30 µg) and to Ampiclox (20 µg). Levofloxacin (30 µg) discs yielded highest inhibitory activity against all the isolates and the biofilm antibiotic sensitivity test, ranged 11 mm to 19 mm zone of inhibition on the bacteria while 12 mm to 16 mm on biofilm zone of inhibition, which was in agreement with the findings of Dalilly-Tariah. *Comamonas aquatica* had the least antibiotic susceptibility activity against all the antibiotics tested 10 mm inhibition of isolate and 9 mm inhibition of biofilm. The observed biofilm formations and susceptibility patterns were a micro colony that developed resistant to the antimicrobials tested and causes most of the persistent otitis media known as chronic suppurative otitis media and the swimmers ear called otitis externa according to Lewis and Walters, et al. The observed biofilms signified the potentials of the pathogens to cause complications such as necrotizing otitis externa and chronic suppurative otitis media. In the plant leave extractions; there was high yield of ethnol extract (23.5%) than water extract (19.0%). This was in agreement with the observation of Ibekwe, et al. *In vitro* testing revealed no antimicrobial activities of the extracts, except ethanolic *Chromolaena. Odorata* (250 mg) extract with slight activity (10 mm) and (9 mm) zones of inhibition of *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* respectively. Several investigators had reported that plants contain antimicrobial substances. The result of selected antimicrobial susceptibility in our study was not in full agreement with the reports of the previous researchers. The crude extracts of *Cleome viscosa, Jatropha curcas and Sansevieria trifasciata* were unable to inhibit the growths of the various bacteria pathogens except a slight inhibition exerted by the crude extract diluted to 250 mg/ml of *Chromolaena odorata* on *Pseudomonas aeruginosa* (9 mm) and *Stenotrophomonas*

maltophilia (10 mm). The antimicrobial activity of the *Chromolaena odorata* could be due to higher volatility of ethanol, which tends to extract more active compounds from the herbs than water. This was in agreement with the observation by Ibekwe, et al. Therefore, the observation on *Chromolaena odorata* was in tandem with Schmidt and Schilling, who reported that freshly crushed *Chromolaena odorata* was used in the treatment of skin wounds. However, our findings were not in tandem with Arekemase, et al. *Jatropha curcas* plant was employed to cure various infections in traditional medicine of bacteria origin. Although it was confirmed that Juice made from the fresh leaves of *cleomeviscosa* to remove pus from ear during the experiment, as reported by Nadkararni, but the *in vitro* method failed to yield positive result [25].

Conclusion

Also our present study could not reveal the claim made elsewhere by Aliero, et al., and Philip, et al., on *Sansevieria trifasciata* used in treatment of wounds of the foot and cough, of bacteria origin. Plasmid Deoxyribonucleic Acid (DNA) bands existed in each isolates and which were all nicked single stranded bands. The molecular weight of each of the bacteria plasmid DNA weighed approximately 1600 base pairs which were inferred as scaffold of resistant plasmid, in reference to Wilkins, who observed that mobilizable resistance plasmids tend to be relatively small, often less than 10 kb in size, encoding only a handful of genes including the resistance gene (s), whereas conjugative plasmids tend to be somewhat larger, 30 kb or more with rare exceptions. The resistance bacteria observed especially on the tested plant extract could be as a result of used herbs and the more the use, the greater the likelihood that resistant strains of the bacterial pathogens would emerge, which was in agreement with that of Levy.

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