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Isolation and Characterization of Multidrug Resistant Bacterial Pathogens Present in Hospital Environment within Awka Metropolis

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ABSTRACT

The contamination of hospital environment by bacterial organisms plays a vital role in the spread of nosocomial infections. This study was conducted to determine bacterial contamination, bacterial profiles, antibiotic susceptibility pattern and plasmid profiles of bacterial isolates from hospital air, water and fomite environment within Awka. This cross-sectional study was conducted at Crest Specialist Hospital, Regina Caeli Specialist Hospital and Nnamdi Azikiwe University (UNIZIK) Medical Centre, Awka. Using random sampling techniques (surface, air and water), culturing, Gram staining, antibiotic susceptibility test, biochemical tests and molecular identification, bacterial organisms from surrounding air, water and fomites were isolated and identified. A total of 112 samples were collected from the three hospitals. Of all the samples, 110 (98.2%) showed positive for bacterial growth. A total of 40 bacterial isolates underwent antibiotic sensitivity testing, of which 6 (15%) showed multidrug resistance. Bacterial organisms isolated from Regina Caeli Hospital showed the most resistance to the antibiotic drugs (41.8%), followed by Crest Specialist Hospital (30.9%) and then UNIZIK Medical Centre (27.3%). Interestingly, there was 100% sensitivity of isolates to ciprofloxacin, levofloxacin, ofloxacin, augmentin (amoxicillin-clavulanate), rifampicin and septrin. Out of the six multidrug resistant isolates, *Staphylococcus hominis* strain PCP01, *Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6 and *Bacillus cereus* strain DS-2 were identified using DNA sequencing and PCR. The three identified microorganisms are opportunistic pathogens. While *S. hominis* has no plasmid, both *M. odoratimimus* and *B. cereus* showed the presence of plasmids. With proper prevention practices such as; disinfection of hospital surfaces, proper personal hygiene of health care workers, special air handling and ventilation and wearing of facemask within the hospital environment, possibilities of nosocomial infections can be reduced drastically, especially in Africa.

Keywords: Isolation, Characterization, Multidrug Resistant and Bacterial

INTRODUCTION

Hospital environment includes hospital buildings and healthcare settings with all indoor components that differentiate them: occupying people (sick people, visitors and hospital staff), indoor air, surfaces, medical equipment, drugs, medical devices, food and wastes [1]. All these components may potentially support survival and growth of biological agents. How microbial communities persist and change in indoor environments is of great concern to public health. Within hospitals, people can be exposed to bio-aerosols, particles of biological origin suspended in the air; and the potential for contracting a microbial pathogen is high [2]. The human exposure to pathogens may be associated with a wide range of major public health issues, such as infectious diseases, acute toxic effects and allergies. Hospital environments are characterized by high infection risk, mostly because of the compromised immunologic conditions of the patients making them vulnerable to bacterial, viral, parasitological and fungal opportunistic infections [3]. The potential transmission of biological matter during surgery operations and medical treatments of infected individuals make hospital environments strongly designated a place where contamination is easily spread [4].

Nosocomial infections, also known as hospital-acquired infections, are infections that are contracted within a hospital environment. Transmission usually occurs via healthcare workers, patients, hospital equipment, or interventional procedures. The most common sites of infection are the bloodstream, lungs, urinary tract and surgical wounds. Though any bacterium may

cause a nosocomial infection, there is an increasing incidence of multidrug-resistant (MDR) pathogens causing hospital-acquired infections [5]. This rise can be explained by indiscriminate use of antibiotics and lack of hygiene measures, especially among medical staff. Commonly seen multidrug-resistant pathogens include methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase-producing bacteria (ESBL), and vancomycin-resistant enterococci (VRE) [6]. The choice of antibiotic for treating infections with these pathogens is based on the individual resistance profile and often requires additional strict isolation methods for the patient (AMBOSS, 2021).Often, nosocomial infections are caused by pathogens acquired via invasive procedures, excessive or improper antibiotic use, and not following infection control and prevention procedures. In fact, many nosocomial infections are preventable through guidance issued by national public health institutes such as the Centers for Disease Control and Prevention (CDC) [7].

Furthermore, in the last decades, the use of antibiotics has been an excellent tool for preventing nosocomial infections. However, the extensive use of these drugs has inevitably led to the insurgence of antibiotic resistance events [8]. Hospital-acquired infections are emerging as important cause of morbidity and mortality in immunocompromised patients with severe underlying illnesses. Each year, 2 million patients suffer from hospital-acquired infections and nearly 100,000 of them die [8]. In hospital rooms, the surfaces are frequently contaminated with pathogens which are able to survive for a long time on room surfaces (beds, sheets, floors, walls and furniture) and medical equipment [9,10,11]. Biological agents may be transmitted to the patients by personnel gloves and visitor hands or through dust that, once deposited on the surfaces, contaminates them and then re-suspends by natural convection or conditioning air systems. Airborne hospital microorganisms are apparently harmless to healthy people. Nevertheless, they can cause adverse health effects in immunocompromised individuals. The hospital itself and its technological systems can offer detrimental particles to the indoor air quality. Air-conditioning systems and aeraulic plants can become contaminated over time and trap various contaminants such as dust and biological organisms. Moisture from them can condense within the ducts and support microbial growth. Thus in hospitals, special air handling and ventilation are required to prevent airborne transmission [12]. Inadequate ventilation is implicated in the airborne transmission of bacteria [12]. Factors that increase the risk of nosocomial infection include increasing age, length of hospitalization, excessive or improper use of broad-spectrum antibiotics, and the number of invasive devices and procedures (Cheung, 2021). In addition, most patients often have accompanying conditions such as diabetes, chronic lung disease, renal insufficiency, or malnutrition [13].

Though various bacteria, viruses, and fungi can cause nosocomial infections, the most common is the bacterium *Staphylococcus aureus*. Other common pathogens like *Escherichia coli*, enterococci, and *Candida* species are common culprits, and all can be normally found on the skin and mucous membranes [14]. Prevention of nosocomial infections begins with health-care facilities implementing infection control protocols to reduce exogenous and endogenous transmission [15]. Exogenous transmission occurs due to person-to-person interactions and through environmental cross-contamination. Frequent hand hygiene is the most important preventive measure to limit the spread of pathogens [16]. Other measures include compliance with isolation precautions and proper use of personal protective equipment (PPE) [17]. Additionally, health-care providers should avoid unnecessary use of indwelling devices, and remove them. Reducing environmental transmission involves practicing proper aseptic and/or sterile techniques during insertion and maintenance of devices. For instance, the routine disinfection of surfaces, patient equipment, and medical devices—as well as appropriate waste management—are important measures in preventing the exogenous transmission of nosocomial infections [18]. Endogenous transmission comes from excessive and improper use of broad-spectrum antibiotics [19]. For instance, vancomycin affects the normal balance in the patient's own endogenous bacterial flora, ultimately leading to an overgrowth of some bacteria. Endogenous transmission can also occur as a transfer from one part of the body to the other (as with urinary tract infections), or if the patient's immune system is depressed by factors like malnourishment or chemotherapy. Appropriate antimicrobial use with the correct agent, dose, and duration is needed to minimize the growth of antibiotic-resistant pathogens [20]. The

discovery (and development) of antibiotics was undoubtedly one of the greatest advances of modern medicine. It is difficult to appreciate fully the impact that antibiotics have had in alleviating human suffering and prolonging life over the past years. A discovery of comparable magnitude in current times would be that of the cure for cancer. Unfortunately, the emergence of antibiotic-resistant bacteria, particularly over the last decade, is now threatening the effectiveness of many antimicrobial agents. Nowhere is this threat greater than in hospitals where most sick persons are congregated, the majority of invasive procedures are done, and the greatest amounts of antimicrobials are used [21].

When a single bacterium is resistant to more than one antibiotic, it is said to be multi-drug resistant. This can occur in two ways; A bacterium can have several different resistance genes, each providing resistance to a particular antibiotic. Accumulation of resistance genes often takes place on the plasmid. The other possibility is that a single resistance mechanism gives resistance to more than one antibiotic [22]. For example, one resistance strategy used by bacteria is to pump the antibiotic out of the cell (efflux). Sometimes such pumps can recognize many different molecules, including different types of antibiotics causing cross resistance. Infections with multidrug-resistant bacteria are hard to treat since few or even no treatment options remain. In some cases, health care providers have to use antibiotics that are more toxic for the patient [23]. Multidrug resistance facilitates spread of antibiotic resistance. When multi-drug resistance plasmids are transferred to other bacteria, they become resistant to many antibiotics at once. Multidrug resistance complicates efforts to reduce resistance. When many different antibiotics select for the same resistant bacteria or plasmids, reducing use of one type of antibiotic is not enough to reduce resistance to that antibiotic [24].

AIM OF THE STUDY

This research was designed to isolate and characterize multidrug resistant bacterial pathogens present in hospital environment within Awka metropolis.

MATERIALS AND METHODS

Study Area

The study was carried out in Awka, the Capital of Anambra state. The city has an estimated population of 301,667 as of the 2006 Nigerian census, and over 2.5 million as at 2018 estimate. Awka is located at 199.1 kilometers by road, directly north of Port Harcourt in the center of the densely populated Igbo heartland in South East Nigeria. Awka is sited in a fertile tropical valley but most of the original rain forest has been lost due to clearing for human settlement. The temperature of Awka is generally 27 °C-30°C between June and December but rises to 32 °C-34°C between January and April.

Site Mapping

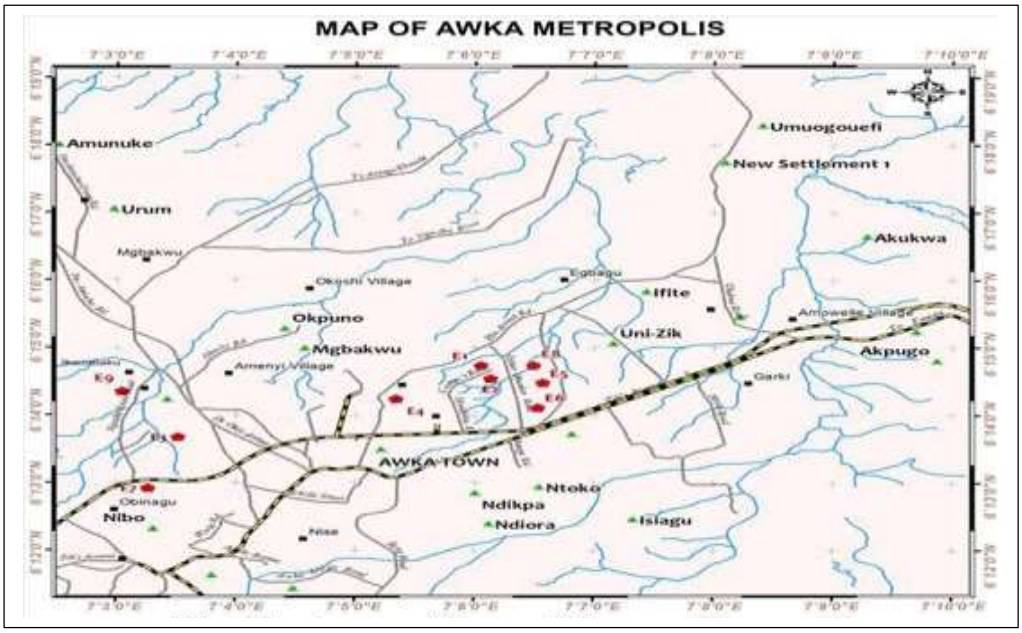

Figure 1. The study area (Awka)
Source: Anambra State Geographic Information System (2018).

Study design

Three hospitals located within Awka were considered in this research; Crest Specialist Hospital, Regina Caeli Specialist Hospital and Nnamdi Azikiwe University Medical Centre. A cross-sectional study was conducted at Crest Specialist Hospital, Regina Caeli Specialist Hospital and Nnamdi Azikiwe University (UNIZIK) Medical Centre; all in Awka, Anambra state, Nigeria, from January to June, 2022.

Selection of hospital

We have three categories of hospitals in Awka, they include; Public, Private and Missionary Hospitals. This research is geared towards sampling one hospital from these three categories. These hospitals were selected because, they are major referral center for other hospitals in Awka. Each has beds and provides care for approximately 5,000–10,000 patients per year.

Sample Size

Bacterial organisms from surrounding air, water and fomites (bed spread, rails, doorknobs, table, chair, curtain, fan switch, light switch, drawer and clothing worn by healthcare personnel) were isolated. A total of 112 samples (100 swab samples, 9 air samples and 3 water samples) were collected from the three hospitals [25].

Sterilization of materials

All glass wares were washed thoroughly with detergent, rinsed with water and then sterilized in hot air oven at 160^{o}C for 1hour. The workbench was disinfected with 70% ethanol and inoculating loops sterilized by flaming until they were red hot before each use. The culture media was prepared according to the manufacturer's instructions, then sterilized in an autoclave at 121°C for 15 min and allowed to cool down to about 40-45°C before use.

Surfaces sampling

The collection of samples was performed using the swab method from surfaces and medical devices. All samples were collected in the morning after the cleaning of the hospital environment was completed (Dancer, 2004). Sampling sites around a bed in each unit were chosen based on the frequency with which the surfaces are touched. Sterile swabs were used to swab commonly touched hospital fomites including bed spread, rails, doorknob, table, chair, curtain, fan switch, light switch, drawer and clothing worn by healthcare personnel [26].

Air Sampling

Using passive sampling, settle plates (Petri dishes) were opened and exposed to the air for one hour, one metre above the floor and one metre away from walls or any major obstacles to determine what microbiological particles may be present in the environment, as they may settle out of the ambient air, and onto the media surface of the Petri Dish. These plates were then incubated and analyzed [27].

Water Sampling

A 1 litre sample in a sterilized plastic bottle is required for this analysis. Sampling was from Hospital tap.

Sampling techniques (Tap)

i. The tap was checked for leakages.
ii. The tap was then opened and water was allowed to run for 2-3 minutes to permit clearing of the service line.
iii. The flow was reduced to permit filling the bottle without splashing.
iv. The bottle was tightly capped [28].

The water samples were inoculated using pour plate method after serial dilutions.

Microbiology Analysis

Each sample (swab, water and air) was inoculated on a Nutrient agar and incubated for 18-24 hours. Discrete colonies were sub-cultured to obtain pure cultures. Gram-negative and Gram-positive bacteria were further identified by Gram stain and standard biochemical tests like methyl red, voges-proskaur, indole, motility, oxidase, catalase, coagulase, and sugar fermentation tests as described by hand book of Clinical Microbiology Procedures [29].

The media used;

- Nutrient agar is a general purpose media for bacterial cultivation.
- Mueller Hinton agar is a general purpose media for determination of susceptibility of microorganisms to antimicrobial agents.
- Eosin methylene blue contains dyes that are toxic for Gram-positive

bacteria. It is the selective and differential medium for coliforms.
- MacConkey agar is a selective media for Gram-negative bacteria.
- Mannitol salt agar is selective media for gram-positive bacteria and differential for mannitol.

Preparation of the Media used

Preparation of Nutrient agar

Twenty-eight grams of nutrient agar powder was suspended in 1L of distilled water, mixed and dissolved completely, sterilized by autoclaving at 121°C for 15 minutes and poured the liquid into the petri dish and wait for the medium to solidify.

Preparation of Mueller Hinton agar

Thirty-eight grams of Mueller Hinton agar powder was suspended in 1L of distilled water, mixed and dissolved completely, sterilized by autoclaving at 121°C for 15 minutes, poured the liquid into the petri dish and waited for the medium to solidify.

Preparation of Eosin Methylene Blue agar

Thirty-six grams of EMB agar was suspended in 1L of distilled water, heated to dissolve the medium completely, dispensed and sterilized by autoclaving at 121 °C for 15 minutes. Overheating was avoided and it was cooled to 50 °C and shook in order to oxidize the methylene blue (i.e. to restore its blue color) and to suspend the flocculent precipitate.

Preparation of MacConkey agar

Fifty-two grams of MacConkey agar powder was suspended in 1 litre of distilled water, boiled to dissolve completely and sterilized by autoclaving at 121°C for 15 minutes.

Preparation of Mannitol Salt Agar

One hundred and eleven grams of Mannitol Salt Agar was suspended in 1L of distilled water, boiled to dissolve the medium completely and sterilized by autoclaving at 121°C for 15 minutes. All the media were prepared in clean environment to prevent any contamination.

Identification of bacteria

After incubation, bacterial isolates were identified according to the morphological appearance, cultural characteristics and biochemical reactions. Bacterial colonies were viewed under a microscope after Gram staining. The procedure for Gram staining that was followed are outlined below; A slide of cell sample is made to be stained, heat fixed the sample to the slide. Crystal violet is added to the slide and incubated for 1 min. The slide is then rinsed with a gentle stream of water for 5s. Gram's iodine is added for 1 min, then the slide is rinsed again with water. Again, the slide is rinsed with alcohol for 3s and with then water. The secondary stain, safranin, is added to the slide and incubated for 1 min. The slide is rinsed with gentle stream of water for 5s. Gram positive bacteria retains the primary stain (Crystal violet) and appeared violet under the microscope. Gram negative, loses the primary stain and took the secondary stain, causing it to appear red when viewed under a microscope [30].

Biochemical Tests

Biochemical reactions carried out on the isolates are as follows;

Catalase test

Many aerobic bacteria and most of those which are facultatively anaerobic produce the enzyme catalase. The function of this enzyme is to detoxify hydrogen peroxide (H_2O_2), which is formed from the superoxide radical by superoxide dismutase. Many aerotolerant anaerobic bacteria have peroxidase (which is not the same enzyme as cytochrome c oxidase) instead of catalase. Obligate anaerobic bacteria lack superoxide dismutase and catalase. Catalase contains a heme group at the active site and it is catalyzing the following reaction with a very high turnover number:

Method

1. Bacterial organism was incubated on an agar plate overnight (18-24 hours) under appropriate conditions.
2. A loop full of the overnight culture was used to make a smear on a microscopic slide.
3. A drop of 3% H_2O_2 was added and mixed with the bacteria before observation.

- **Positive test result:** Gas formation (O_2) in the form of bubbles shows that the bacterium has a catalase.
- **Negative test result:** No gas formation [31].

Coagulase test

Some bacteria produce coagulase, which is an enzyme that converts fibrinogen to fibrin, which means that it can coagulate plasma. The ability to produce coagulase is assumed to be associated to the virulence of staphylococci. The test is used to distinguish between coagulase positive and coagulase negative staphylococci

Method

1. A colony from the suspected pure culture was suspended in 0.5 ml of plasma from horse, rabbit or man and incubated at 37ºC.
2. The test was read after 4 h. If the result was negative, incubation continued.
3. The final read was performed after 24 hours.

- **Positive reaction** if the plasma coagulates and the coagulate is stable. It must not be dissolved upon stirring.
- **Negative reaction** if the plasma does not coagulate or if the coagulate is dissolved again upon stirring [31].

Indole test

Bacteria, which express the enzyme tryptophanase can hydrolyze the amino acid tryptophan to indole, pyruvic acid and ammonia. Presence of indole can be shown by means of Kovác's reagent or by spot indole test. In the spot test indole reacts with p-dimethylaminocinnamaldehyde to produce a blue to blue-green product. Used for the confirmation of suspected *Escherichia coli* strains and in typing (species determination) of *Brachyspira* spp. in combination with other tests.

Method; Kovac´s reagent

1. A colony from a pure culture of the bacterium to be investigated was suspended in tryptophan medium and incubated at 37°C for 20-28 hrs.
2. Few drops of Kovác's reagent was added.

- **Positive test result:** The indole reagent change colour to cerise red.
- **Negative test result:** The indole reagent remains pale yellow [31].

Oxidase test

Bacteria, which have aerobic respiration, often have cytochrome c and a cytochrome c oxidase. The presence of these components can in combination with other methods be used for typing. Cytochrome c oxidase is a transmembrane protein complex (Complex IV), which is also present in the cytoplasmic (inner) membrane of mitochondria. The oxidase test is used for identification of gram negative bacteria. For instance, to identify members of the family *Enterobacteriaceae,* which are oxidase negative, except members of the genus *Plesiomonas* (oxidase positive).

Method

1. Two drops of the oxidase reagent were applied on a piece of filter paper.
2. A bacteria colony was transferred with a platinum loop onto the spot with the oxidase reagent and the colonies were incubated at 37°C for 18-24 hours.

- **Positive test result:** Dark blue-purple colour change within 10-30 secs.
- **Negative test result:** No colour change or colour change after more than 30 secs [31].

Voges-Proskauer (VP) test

The VP test shows if the bacterium has butanediol fermentation and can split glucose to acetoin via pyruvate and further to 2,3-butanediol according to:
2 pyruvates + NADH $\longrightarrow$ $2CO_2$ + 2, 3-butanediol.
If KOH (potassium hydroxide) is added, acetoin will be converted to diacetyl (=2, 3-butanedione), which reacts with alpha-naphtol and forms a pink complex. *Klebsiella* spp. and *Enterobacter* spp. has the capacity to perform butanediole fermentation in contrast to *Escherichia coli*, *Salmonella* spp. and *Shigella* spp.

Method

1. A colony from the pure culture was suspended in VP/MR medium and incubated at 30-37ºC for 24-48 hours.
2. 0.2 ml of 40% KOH and 0.6 ml of alpha-naphtol solution were added.

Positive test result: colour change to pink.
Negative test result: no colour change [31].

Motility test

Motility has long been recognized as an important taxonomic tool and biological characteristic of microorganisms. The presence of flagella occurs primarily in bacilli but there are a few flagellated cocci, thus motility is a very important means of identification in the family Enterobacteriaceae.

Method

1. A colony of a young (18- to 24-hours) culture growing on agar medium was touched using a straight needle and stabbed once to a depth of only ⅓ to ½ inch in the middle of the tube containing Sulphide Indole Motility medium, incubated at 35°-37°C and examined daily for up to 7 days, as described by [32].

Methyl Red (MR) test

Methyl red test, commonly known as MR test is used to determine the ability of an organism to produce and maintain stable acid end products from glucose fermentation. MR test along with the VP test is performed simultaneously because they are physiologically related and are performed on MR/VP broth. All members of the Enterobacteriaceae can convert glucose to pyruvic acid by the Embden-Meyerhof pathway, but bacteria can further metabolize pyruvic acid by two different pathways.

Method

1. Methly-red/Voges-Proskauer (MR/VP) broth was inoculated with a pure culture of the organism and incubated at 35°-37°C for a minimum of 48 hours in ambient air.
2. 5 or 6 drops of methyl red reagent were added per 5 mL of broth and observations were made for colour change in the broth medium, as described by [32].

Carbohydrate Fermentation test

Carbohydrate fermentation is the process by which the microorganism utilizes to produce energy in the form of ATP, the ultimate energy source of the organism. Glucose after entering a cell can be catabolized either aerobically (in the presence of O_2), where molecular oxygen serves as the final electron acceptor (oxidative pathway), or anaerobically (in absence of O_2) in which inorganic ions can serve as the final electron acceptor (fermentative pathway). The metabolic end products of a carbohydrate fermentation can either be organic acids (lactic, formic, acetic acid) or organic acid and gas (hydrogen or carbon dioxide). Fermentative degradation of the carbohydrates (monosaccharide, disaccharide, and polysaccharide) by microorganisms under the anaerobic condition is carried out in the fermentation tube, which comprises of Durham tube for the detection of the gas production.

Method

1. Trypticase, sodium chloride, and phenol red were weighed and dissolved in 100 ml distilled water and transfer into conical flasks.
2. A 0.5% to 1% of the desired carbohydrates were added into all flasks.
3. Inverted Durham tubes were inserted into all tubes; the Durham tubes were fully filled with broth and sterilized in an autoclave at 112°C for 15 minutes.
4. Each tube was inoculated with 1 loop of an 18 hour or 24-hour culture in aseptic condition and incubated at 18-24 hours at 37°C.
5. The tubes were examined for acid and gas production [32].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the isolates were performed using antibiotics (Oxoid, UK) based on the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) (Oxoid, UK) (CLSI, 2018). An inoculum for each isolate was prepared by emulsifying colonies from an overnight pure culture in sterile normal saline (0.85%) in test tubes with the turbidity adjusted to 0.5 McFarland standards. The bacterial suspension was uniformly streaked on MHA plates using sterile swabs and left for 3 minutes prior to introduction of the antibiotic discs. For Gram-negative bacteria, the following antibiotics were used (in μg/disk); ampicillin (30), ceporex (10), gentamicin (10), ciprofloxacin (10), augmentin (30), pefloxacin (10), ofloxacin (10), Nalidixic acid (30), septrin (30) and streptomycin (30) based on Clinical Laboratory Standards Institute (CLSI) [33]. On the other hand, for Gram-positive bacteria, antibiotics (in μg/disk) selected for susceptibility testing include; norfloxacin (10), gentamicin (10), erythromycin (30), ciprofloxacin (10), amoxicillin (20), ampiclox (20), streptomycin

(30), levofloxacin (20), rifampicin (20) and chloramphenicol (30). The plates were incubated at 35oC for 24 hours, and the diameters of zone of inhibition were measured with Vernier caliper; Results were reported as susceptible (S), intermediate (I), or resistant (R), according to CLSI guidelines [33].

DNA Extraction

Protocol

Two hundred microlitre of each sample was added to a microcentrifuge tube. Two hundred microlitre of BioFluid & Cell Buffer and 20 µl of Proteinase K was added to it and mixed thoroughly using a vortex for 10-15 seconds and then incubated the tube at 55ºC for 10 minutes on a heating block. Genomic Binding Buffer (i.e. 420µl) was added to the digested sample and mixed thoroughly with a vortex mixer for 10-15 seconds. The mixture was then transferred to a Zymo-Spin™ IIC-XL Column in a Collection Tube and centrifuged at 12,000 x g for 1 minute. The collection tube was discarded with the flow through. Four hundred microlitre DNA Pre-Wash Buffer was added to the spin column in a new Collection Tube and centrifuged at 12,000 x g for 1 minute. The collection tube was emptied and 700 µl g-DNA Wash Buffer was added to the spin column and centrifuged at ≥ 12,000 x g for 1 minute. The spin column was then transferred to a clean microcentrifuge tube. Fifty microlitre of DNA Elution Buffer was added directly on the matrix and incubated for 5 minutes at room temperature, then centrifuged at maximum speed for 1 minute to elute the DNA. The eluted DNA was stored ≤ -20ºC for future use.

PCR Protocol

A 12.5µl of One Taq Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs Inc.); 0.5µl each of forward and reverse primers (27F: 5'-AGAGTTTGATCGTGGCTCAG -3' and 5'-1492R TACGGTTACCTTGTTACGACTT -3'); 8.5µl of Nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to thermalcycler. Amplification conditions for the PCR was as follows: Initial denaturation for 30secs at 94°C, followed by 35 cycles of denaturation at 94°C for 20secs, primer annealing at 56°C for 45secs and strand extension at 68°C for 1 min. Final extension at 68°Cfor 5 min on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 2% agarose gel and DNA bands were visualized with Ethidium bromide.

Agarose Gel Electrophoresis

Two percent agarose gel was prepared by dissolving 1.2g of Agarose in 60ml of 1X TAE Buffer. The mixture was heated to a clear solution using a microwave oven and allowed to cool to about 50 ºC. Three microlitre of Ethidium Bromide was added into the solution and mixed thoroughly. The agarose preparation was carefully poured into a gel tray, with the gel comb in place and allowed to solidify. The tray was loaded into the gel tank and 1X TAE Buffer was poured into the tank, making sure that the gel was properly submerged. The gel comb was carefully removed. Five microlitre of amplicon was loaded into the wells. The tank was connected to the power pack and set to run at 100volts for 20 minutes after which it was viewed on a gel documentation system.

Sequencing Protocol
PCR products were cleaned using ExoSAP Protocol as follows

The Exo/SAP master mix was prepared by adding the following to a 0.6ml micro-centrifuge tube:
a. Exonuclease I (Catalogue No. NEB M0293L) 20 U/ul 50 µl
b. Shrimp Alkaline Phosphatase (Catalogue No. NEB M0371) 1 U/ul 200 µl.
The following reaction mixture were prepared:
Amplified PCR Product 10 µl
ExoSAP Mix (step 1) 2.5 µl
They were mixed well and incubated at **37˚C** for **15 min**
The reaction was stopped by heating the mixture at **80˚C** for 1**5 min**
Fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions: https://www.nimagen.com/products/Sequencing/Capillary-Electrophoresis/BrilliantDye-Terminator-Cycle-Sequencing-Kit/
The labelled products are then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053): http://www.zymoresearch.com/downloads/dl/file/id/52/d4052i.pdf
The cleaned products are injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50cm array, using POP7: https://www.thermofisher.com/order/catalog/product/4406016
Sequence chromatogram analysis is performed using FinchTV analysis software: https://www.softpedia.com/get/Science-CAD/FinchTV.shtm

Plasmid Extraction Protocol

Five hundred microlitre of each sample was added to a microcentrifuge tube and centrifuged at full speed for 20 seconds and the resulting supernatant was discarded. The bacteria pellets were re-suspended with 250µl of ZymoPURETM P1 (red) and completely mixed by vortexing. Two hundred and fifty microlitre of ZymoPURETM P2 (green) was added and immediately mixed by gently inverting the tube 6-8 times and allowed to sit at room temperature for 2-3 minutes. Two hundred and fifty microlitre of ice cold ZymoPURETM P3 (yellow) was added and mixed thoroughly by inversion. The sample was then left to incubate on ice for 5 minutes. The sample was then centrifuged at 16000xg for 5 minutes. Six hundred microlitre of the supernatant was carefully transferred into a new 1.5ml microcentrifuge tube. Two hundred and seventy-five microlitre of ZymoPURETM binding buffer was added to the transferred supernatant and mixed thoroughly by inverting the tube 8 times. The entire mixture was then transferred to a Zymo-spinTM II-P column in a collection tube and incubated at room temperature for 2 minutes before centrifuging at 5000xg for 1 minute after which the flow through was discarded. Eight hundred microlitre of ZymoPURETM wash 1 was added to the Zymo-spinTM II-P column and centrifuged for 5000xg for 1 minute after which the flow through was discarded. Eight hundred microlitre of ZymoPURETM wash 2 was added to the Zymo-spinTM II-P column and centrifuged for 5000xg for 1 minute after which the flow through was discarded. 200µl of ZymoPURETM wash 2 was added to the Zymo-spinTM II-P column and centrifuged for 5000xg for 1 minute after which the flow through was discarded. The Zymo-spinTM II-P column was centrifuged at ≥10000xg for 1 minute in order to remove any residual wash buffer. The Zymo-spinTM II-P column was transferred to a clean 1.5ml tube and 25µl of ZymoPURETM Elution Buffer was added directly to the column matrix and allowed to incubate at room temperature for 2 minutes and then centrifuged at ≥10000xg for 1 minute. The eluted plasmid DNA was then stored at ≤ -20ºC. For DNA extraction, sequencing and plasmid profiling, samples were assayed under the auspices of Molecular Research Foundation for Students and Scientists, Nnamdi Azikiwe University Awka (MRFSS).

Ethics approval

Prior to sample collection, ethical approval was obtained from administrative unit of Crest Specialist Hospital, Regina Caeli Specialist Hospital and Nnamdi Azikiwe University Medical Centre.

RESULTS

Sample Collection Result

During the six months' studies, a total of 112 samples (100 swab samples, 9 air samples and 3 water samples) were collected from the three hospitals. There are about 5 (12.5%) isolates from air, 1 (2.5%) from water, 13 (32.5%) from bedspreads, 4 (10%) from tables, 3 (7.5%) from drawers, 2 (5%) from clothing of healthcare personnel, 1 (2.5%) from curtain, 2 (5%) from rails, 4 (10%) from chairs, 1 (2.5%) from fan switch, 1 (2.5%) from light switch and 3 (7.5%) from doorknobs making a total of 40 isolates (Tables 1 and 2).

Culture Result

Of all the samples, 110 (98.2%) showed positive for bacterial growth (swab samples, 99 (99%) were positive for bacterial, air samples showed 100% (9) positive for bacterial growth while water samples showed 66.7% (2) positive for bacterial growth. Of the 110 bacterial growth, a total of 40 (36.4%) bacterial species were identified and tested for multidrug resistance.

Gram Staining Result

Of the 40 bacterial isolates, 11 (27.5%) were Gram positives while 31 (72.5%) were Gram negative. From all the isolates, 9 (22.5%) were cocci, 2 (5%) were coccobacilli and 29 (72.5%) were rods. Amongst the 11 Gram positives, 7 (63.6%) were from UNIZIK Medical Centre, 3 (27.3%) were from Crest Specialist Hospital and 1 (9.1%) was isolated from Regina Caeli Specialist Hospital. Meanwhile, for Gram negatives, 3 (10.3%) were from UNIZIK Medical Centre, 10 (34.5%) from Crest Specialist Hospital and 16 (55.2%) from Regina Caeli Specialist Hospital (Table 1).

Table 1: Sample collection, Culture and Gram stain results (sorted according to environment).

Isolates ID	Gram reaction	Shape	Growth on Selective media			Environment	Hospital
			MA	EMB	MSA		
P1	+	Cocci	-	-	+^{o}	Air	CSH
P2	-	Rod	+	+		Air	CSH
P3	-	Rod	+	+		Air	CSH
P4	-	Rod	+	+		Air	RCH
P36	+	Cocci	-	-	+^{o}	Air	UMC
P30	-	Rod	+	+		Water	RCH
P6	-	Rod	+	+		Bedspread	CSH
P7	-	Rod	+	+		Bedspread	CSH
P8	-	Rod	+	+		Bedspread	CSH
P9	-	Rod	+	+		Bedspread	RCH
P10	+	Cocci	-	-	+^{o}	Bedspread	CSH
P16	-	Rod	+	+		Bedspread	RCH
P21	-	Rod	+	+		Bedspread	RCH
P22	-	Rod	+	+		Bedspread	CSH
P28	-	Rod	+	+		Bedspread	CSH
P29	-	Rod	+	+		Bedspread	CSH
P32	+	Rod	-	-		Bedspread	UMC
P33	+	Cocci	-	+	+^{o}	Bedspread	UMC
P40	+	Cocci	+	-	+	Bedspread	UMC
P14	-	Rod	+	+		Table	RCH
P15	-	Rod	+	+		Table	RCH
P34	+	Cocci	-	-	+	Table	UMC
P37	-	Rod	+	+		Table	UMC

Isolates ID	Gram reaction	Shape	Growth on Selective media			Environment	Hospital
			MA	EMB	MSA		
P5	-	Rod	+	+		Drawer	RCH
P26	-	Rod	+	+		Drawer	RCH
P27	-	Rod	+	+		Drawer	RCH
P12	+	Cocci	-	-	+^{o}	Clothing of Healthcare Personnel	RCH
P24	+	Rod	+	-		Clothing of Healthcare Personnel	CSH
P25	-	Rod	+	+		Curtain	RCH
P13	-	Rod	+	+		Rail	CSH
P18	-	Rod	+	+		Rail	RCH
P17	-	Rod	+	+		Chair	RCH
P19	-	Rod	+	+		Chair	RCH
P20	-	Rod	+	+		Chair	RCH
P23	-	Rod	+	+		Chair	CSH
P39	-	Coccobacillus	-	-	+	Fan switch	UMC
P38	+	Cocci	-	+	+^{o}	Light switch	UMC
P11	-	Rod	+	+		Door	RCH
P31	-	Coccobacillus	-	+	+	Door	UMC
P35	+	Cocci	-	-	+^{o}	Door	UMC

RCH: Regina Caeli Specialist Hospital; CSH: Crest Specialist Hospital; UMC: Nnamdi Azikiwe University Medical Centre; ^{o}: media colour changes to yellow.

Biochemical Tests Result

The biochemical result amazingly showed that all the isolates tested positive for catalase and majority (85%) actually ferment glucose. Meanwhile, oxidase test showed the least positive result (15%), followed by indole test (17.5%). The following species were identified via this test; 8 (20%) *Staphylococcus aureus*, 10 (25%) *Klebsiella* species, 7 (17.5%) *Escherichia coli,* 4 (10%) *Acinetobacter* species, 4 (10%) *Proteus* species, 2 (5%) *Bacillus* species, 2 (5%) *Haemophilus influenzae* and 1 (2.5%) *Staphylococcus* species (Table 2).

Isolates Distribution

Of the 40 isolates examined for multidrug resistance, Regina Caeli Specialist Hospital has 17 (42.5%), Crest Specialist Hospital has 13 (32.5%) while UNIZIK Medical centre has 10 (25%) (Figure 1). In UNIZIK Medical Centre, the organism with the highest occurrence is *Staphylococcus aureus* (50%), followed by *Haemophilus influenzae* (20%). In Regina Caeli Specialist Hospital, the organism with the highest occurrence is *Escherichia coli* (41.2%), followed by *Klebsiella* sp. (35.3%). While in Crest Specialist Hospital, the organism with the highest occurrence is *Klebsiella* sp. (30.8%), followed by *Proteus* sp. (23.1%) (Table 3). The distribution of the isolates based on environment showed that in air, the organism with the highest occurrence is *Klebsiella* species (60%), followed by *Staphylococcus aureus* (40%). On drawer, the organism with the highest occurrence is *Escherichia coli* (66.7%), followed by *Acinetobacter* species (33%). Equal presence of *Klebsiella* sp. and *Staphylococcus aureus* was found on bedspread (23%). Equal presence of *Klebsiella* sp., *Acinetobacter* sp., *Proteus* sp. and *Staphylococcus* sp. was found on table (25%). The clothing of healthcare personnel showed equal presence of *Staphylococcus aureus* and *Bacillus* species (50%). Also, doorknobs showed the equal presence of *Staphylococcus aureus*, *Escherichia coli* and *Haemophilus influenzae*. Water showed the presence of *Acinetobacter* species, curtain and rail showed the presence of *Klebsiella* species, chair showed the presence of *Escherichia coli*, fan switch showed the presence of *Haemophilus influenzae* whilst light switch showed the presence of *Staphylococcus aureus* (Table 4).

Table 2: Biochemical tests result of isolates

								Sugar Tests			
ID	**Cat**	**Coag**	**Ind**	**MR**	**VP**	**Oxi**	**Mot**	**Glu**	**Suc**	**Mal**	**Probable Organism**
P1	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*
P2	+	-	-	-	-	-	-	+º	+	+º	*Klebsiella* sp.
P3	+	-	-	-	-	-	-	+	+	+	*Klebsiella* sp.
P4	+	-	-	-	±	-	-	+º	+º	+	*Klebsiella* sp.
P5	+	-	-	-	-	-	-	-	-	-	*Acinetobacter* sp.
P6	+	-	-	-	-	+	+	-	-	-	*Pseudomonas* sp.
P7	+	-	-	-	-	+	+	-	-	-	*Pseudomonas* sp.
P8	+	-	-	-	-	-	-	-	-	-	*Acinetobacter* sp.
P9	+	-	-	-	-	-	-	+	+	+	*Klebsiella* sp.
P10	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*
P11	+	-	+	+	-	-	+	+	-	-	*Escherichia coli*
P12	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*
P13	+	-	-	-	-	-	-	+	+	+	*Klebsiella* sp.
P14	+	-	-	-	-	-	-	+	+	+	*Klebsiella* sp.
P15	+	-	-	+	-	-	+	+	-	-	*Proteus* sp.
P16	+	-	-	-	-	-	-	+	+	+	*Klebsiella* sp.

P17	+	-	+	+	-	-	+	+	-	-	*Escherichia coli*
P18	+	-	-	-	-	-	-	+	+	+	*Klebsiella* sp.
P19	+	-	+	+	-	-	+	+	-	-	*Escherichia coli*
P20	+	-	+	+	-	-	+	+	-	-	*Escherichia coli*
P21	+	-	+	+	-	-	+	+	-	-	*Escherichia coli*

								Sugar			
					Tests						
ID	**Cat**	**Coag**	**Ind**	**MR**	**VP**	**Oxi**	**Mot**	**Glu**	**Suc**	**Mal**	**Probable Organism**
P22	+	-	-	+	-	-	+	+	-	-	*Proteus* sp.
P23	+	-	-	+	-	-	+	+	-	-	*Proteus* sp.
P24	+	+	-	-	±	±	+	+	+	+	*Bacillus* sp.
P25	+	-	-	-	±	-	-	+º	+º	+	*Klebsiella* sp.
P26	+	-	+	+	-	-	+	+	-	-	*Escherichia coli*
P27	+	-	+	+	-	-	+	+	-	-	*Escherichia coli*
P28	+	-	-	-	±	-	-	+º	+º	+	*Klebsiella* sp.
P29	+	-	-	+	-	-	+	+	-	-	*Proteus* sp.
P30	+	-	-	-	-	-	-	-	-	-	*Acinetobacter* sp.
P31	+	-	-	-	-	+	-	+	-	+	*Haemophilus influenza*
P32	+	+	-	-	±	±	+	+	+	+	*Bacillus* sp.
P33	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*
P34	+	-	-	-	+	-	-	+º	+º	+º	*Staphylococcus* sp.
P35	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*
P36	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*
P37	+	-	-	-	-	-	-	-	-	-	*Acinetobacter* sp.
P38	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*
P39	+	-	-	-	-	+	-	+	-	+	*Haemophilus influenza*
P40	+	+	-	+	+	-	-	+	+	+	*Staphylococcus aureus*

+: positive result; -: negative result; ±: weak positive result; º: gas production; MR: Methyl red; VP: Vogues Proskaur; Glu: Glucose; Suc: Sucrose; Mal: Maltose; Cat: Catalase; Coag: Coagulase; Ind: Indole; Oxi: Oxidase; Mot: Motility.

Table 3: Distribution of the Isolates from the Three Hospitals

Isolates	Regina Caeli Specialist Hospital n (%)	Crest Specialist Hospital n (%)	UNIZIK Medical Centre n (%)	Total n (%)
Staphylococcus aureus	1 (12.5%)	2 (25%)	5 (62.5%)	8 (100%)
Klebsiella sp.	6 (60%)	4 (40%)	0 (0%)	10 (100%)
Acinetobacter sp.	2 (50%)	1 (25%)	1 (25%)	4 (100%)
Pseudomonas sp.	0 (0%)	2 (100%)	0 (0%)	2 (100%)
Escherichia coli	7 (100%)	0 (0%)	0 (0%)	7 (100%)
Proteus sp.	1 (25%)	3 (75%)	0 (0%)	4 (100%)
Bacillus sp.	0 (0%)	1 (50%)	1 (50%)	2 (100%)
Haemophilus influenza	0 (0%)	0 (0%)	2 (100%)	2 (100%)
Staphylococcus sp.	0 (0%)	0 (0%)	1 (100%)	1 (100%)
Total	**17 (42.5%)**	**13 (32.5%)**	**10 (25%)**	**40**

Table 4: Distribution of the Isolates based on environment

Isolates	Air	Water	Bedspread	Table	Drawer	Clothing of Healthcare Personnel	Curtain	Rail	Chair	Fan switch	Light switch	Doorknob	Total
Staphylococcus aureus	2 (25%)	0 (0%)	3 (37.5%)	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (12.5%)	1 (12.5%)	8 (100%)
Klebsiella sp.	3 (30%)	0 (0%)	3 (30%)	1 (10%)	0 (0%)	0 (0%)	1 (10%)	2 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	10 (100%)
Acinetobacter sp.	0 (0%)	1 (25%)	1 (25%)	1 (25%)	1 (25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (100%)
Pseudomonas sp.	0 (0%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (100%)
Escherichia coli	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)	2 (25%)	0 (0%)	0 (0%)	0 (0%)	4 (50%)	0 (0%)	0 (0%)	1 (12.5%)	8 (100%)
Proteus sp.	0 (0%)	0 (0%)	2 (66.7%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (100%)
Bacillus sp.	0 (0%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (100%)
Haemophilus influenza	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	0 (0%)	1 (50%)	2 (100%)
Staphylococcus sp.	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Total	**5**	**1**	**13**	**4**	**3**	**2**	**1**	**2**	**4**	**1**	**1**	**3**	**40**

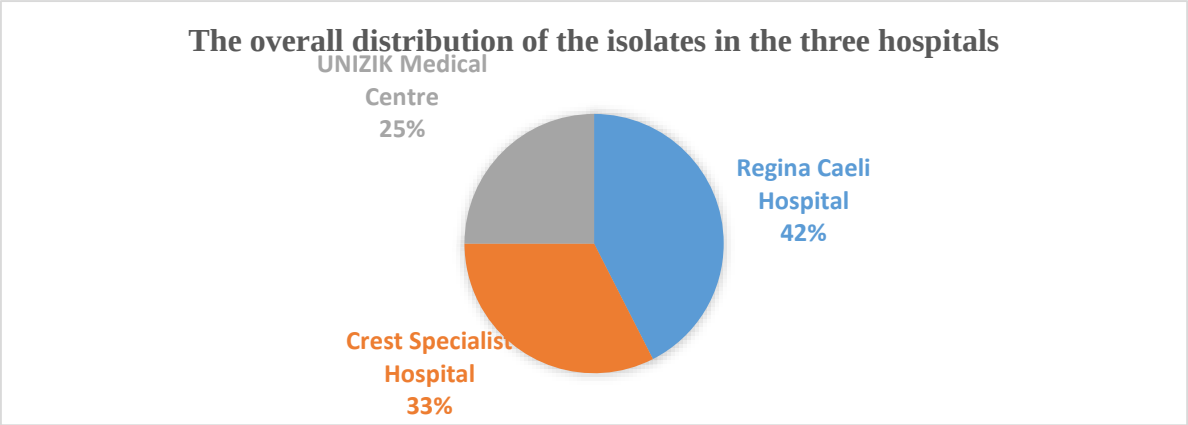

Figure 1: The overall distribution of the isolates in the three hospitals

Antibiogram Profile for Gram negative isolates

The proportion of antibiotic resistance among Gram negative bacteria was high for nalixidic acid (544.8%), ceporex (32.3%) and ampicillin (29%). Low level of resistance was recorded for streptomycin (3.2%) and gentamicin (3.2%). However, there were no resistance to ofloxacin, pefloxacin, augmentin, ciprofloxacin and septrin. Among the Gram negative isolates, *Klebsiella* spp. and *Acinetobacter* sp. showed multidrug resistance (Table 5).

Antibiogram Profile for Gram positive isolates

Most of the Gram positive bacteria exhibited significantly resistance to some of the tested antibiotics, for example; norfloxacin (88.9%), amoxicillin (44.4%) and ampiclox (44.4%). Low level resistance was recorded for chloramphenicol (22.2%) and erythromycin (11.1%). Meanwhile, there was no resistance to the following drugs; ciprofloxacin, gentamicin, streptomycin, rifampicin and levofloxacin. Among the Gram positive isolates, *Bacillus* sp. and *Staphylococcus* sp. showed multidrug resistance (Table 6).

Table 5: Antimicrobial susceptibility pattern of Gram-negative bacteria

	Antimicrobial agents									
Isolates	**OPX**	**PEF**	**CPX**	**AU**	**CN**	**S**	**CEP**	**NA**	**SXT**	**PN**
Klebsiella sp.	-	-	-	-	-	-	+	+	-	+
Klebsiella sp.	-	-	-	-	-	-	+	+	-	+
Klebsiella sp.	-	-	-	-	+	+	+	+	-	+
Acinetobacter sp.	-	-	-	-	-	-	-	+	-	-
Pseudomonas sp.	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	-	-	-	-	-	-	-	+	-	-
Klebsiella sp.	-	-	-	-	-	-	-	+	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-
Klebsiella sp.	-	-	-	-	-	-	-	+	-	-
Klebsiella sp.	-	-	-	-	-	-	+	+	-	-
Proteus sp.	-	-	-	-	-	-	-	-	-	-
Klebsiella sp.	-	-	-	-	-	-	+	+	-	-
Escherichia coli	-	-	-	-	-	-	+	+	-	-
Klebsiella sp.	-	-	-	-	-	-	+	+	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-
Proteus sp.	-	-	-	-	-	-	-	-	-	-
Proteus sp.	-	-	-	-	-	-	-	-	-	-
Klebsiella sp.	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	+	-	-
Escherichia coli	-	-	-	-	-	-	-	+	-	+

	Antimicrobial agents									
Isolates	**OPX**	**PEF**	**CPX**	**AU**	**CN**	**S**	**CEP**	**NA**	**SXT**	**PN**
Klebsiella sp.	-	-	-	-	-	-	-	+	-	-
Proteus sp.	-	-	-	-	-	-	-	+	-	-
Acinetobacter sp.	-	-	-	-	-	-	-	-	-	-
Haemophilus influenzae	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	-	-	-	-	-	-	+	+	-	+
Haemophilus influenzae	-	-	-	-	-	-	-	-	-	-

+: resistant; -: sensitive; OFX: Ofloxacin; PEF: Pefloxacin; AU: Augmentin; CPX: Ciprofloxacin; CN: Gentamicin; S: Streptomycin; CEP: Ceporex; NA: Nalixidic acid; SXT: Septrin; PN: Ampicillin.

Table 6: Antimicrobial susceptibility pattern of Gram-positive bacteria

	Antimicrobial agents									
Isolates	**CPX**	**NB**	**CN**	**AMX**	**S**	**RD**	**E**	**CH**	**APX**	**LEV**
Staphylococcus aureus	-	+	-	-	-	-	-	-	+	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	+	-	+	-	-	-	-	-	-
Bacillus sp.	-	+	-	+	-	-	-	+	+	-
Bacillus sp.	-	+	-	-	-	-	-	-	-	-
Staphylococcus sp.	-	+	-	+	-	-	+	-	+	-
Staphylococcus aureus	-	+	-	+	-	-	-	-	-	-
Staphylococcus aureus	-	+	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	+	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	+	+	-	-

+: resistant; -: sensitive; CPX: Ciprofloxacin; NB: Norfloxacin; CN: Gentamicin; AMX: Amoxicillin; S: Streptomycin; RD: Rifampicin; E: Erythromycin; CH: Chloramphenicol; APX: Ampiclox; LEV: Levofloxacin.

Multidrug Resistant Isolates

From Table 5 and 6 above, there are six isolates that have demonstrated multidrug resistance (i.e., acquired non-susceptibility to at least one agent in three or more antimicrobial categories**)** and they are; *Klebsiella* spp., *Bacillus* sp., *Staphylococcus* sp. and *Acinetobacter* sp. The class of antibiotic they show resistance to can be seen below;

Klebsiella sp. = Cephalosporin, Quinolone and Penicillin

Klebsiella sp. = Cephalosporin, Quinolone and Penicillin

Klebsiella sp. = Aminoglycosides, Cephalosporin, Quinolone and Penicillin

Bacillus sp. = Quinolone, Penicillin and Chloramphenicol

Staphylococcus sp. = Quinolone, Penicillin, Macrolide and Chloramphenicol

Acinetobacter sp. = Cephalosporin, Quinolone and Penicillin.

Klebsiella spp. were isolated from air sample of the female ward in Crest Specialist Hospital, *Klebsiella* sp. was also isolated from air sample of the male ward in Regina Caeli Specialist Hospital, *Bacillus* sp. was isolated from clothing of a Health Personnel in Crest Specialist Hospital, *Acinetobacter* sp. and *Staphylococcus* sp. were isolated from table surfaces of the female ward and annex building of Nnamdi Azikiwe University Medical Centre. Table 7 shows the Multidrug Resistant Index (MRI) of the isolates. MRI was calculated using the formula, MRI=a/b, where "a" is the number of antibiotic resisted and "b" is the total number of antibiotic used in the study. Klebsiella species has the highest MRI (50%) followed by Bacillus species (40%) and Staphylococcus species (40%). It is worthy to note that two (33.3%) of the multidrug resistant were Gram positive while the other four (66.7%) were Gram negative bacterial organisms. Also, three (50%) were cocci and three (50%) were rods.

Table 7: Multidrug Resistant Index for Isolates

ID	Isolates	Hospital (Environment)	Number of drugs shown to be resistant	Multidrug Resistance Index (MRI)
P2	*Klebsiella* sp.	CSH (Air)	3	0.3
P3	*Klebsiella* sp.	CSH (Air)	3	0.3
P4	*Klebsiella* sp.	RCH (Air)	5	0.5
P24	*Bacillus* sp.	CSH (Clothing of Health Care Provider)	4	0.4
P34	*Staphylococcus* sp.	UMC (Table)	4	0.4
P37	*Acinetobacter* sp.	UMC (Table)	3	0.3

Distribution of Antibiotic Resistance and Bacterial Isolates in the Three Hospitals

The distribution of antibiotic resistance can be seen in Figure 2 below. Organisms isolated from Regina Caeli Specialist Hospital showed more resistance to the antibiotic drugs (41.8%), followed by Crest Specialist Hospital (30.9%) and then UNIZIK Medical Centre (27.3%). The isolates from Regina Caeli Specialist Hospital demonstrated more resistance to gentamicin, streptomycin, ceporex, nalixidic acid and ampicillin. Those from Crest Specialist Hospital were more resistant to

ampiclox whereas those from UNIZIK Medical Centre were more resistant to norfloxacin, amoxicillin and erythromycin. There was 100% sensitivity of isolates to ciprofloxacin, levofloxacin, ofloxacin, augmentin (amoxicillin-clavulanate), rifampicin and septrin.

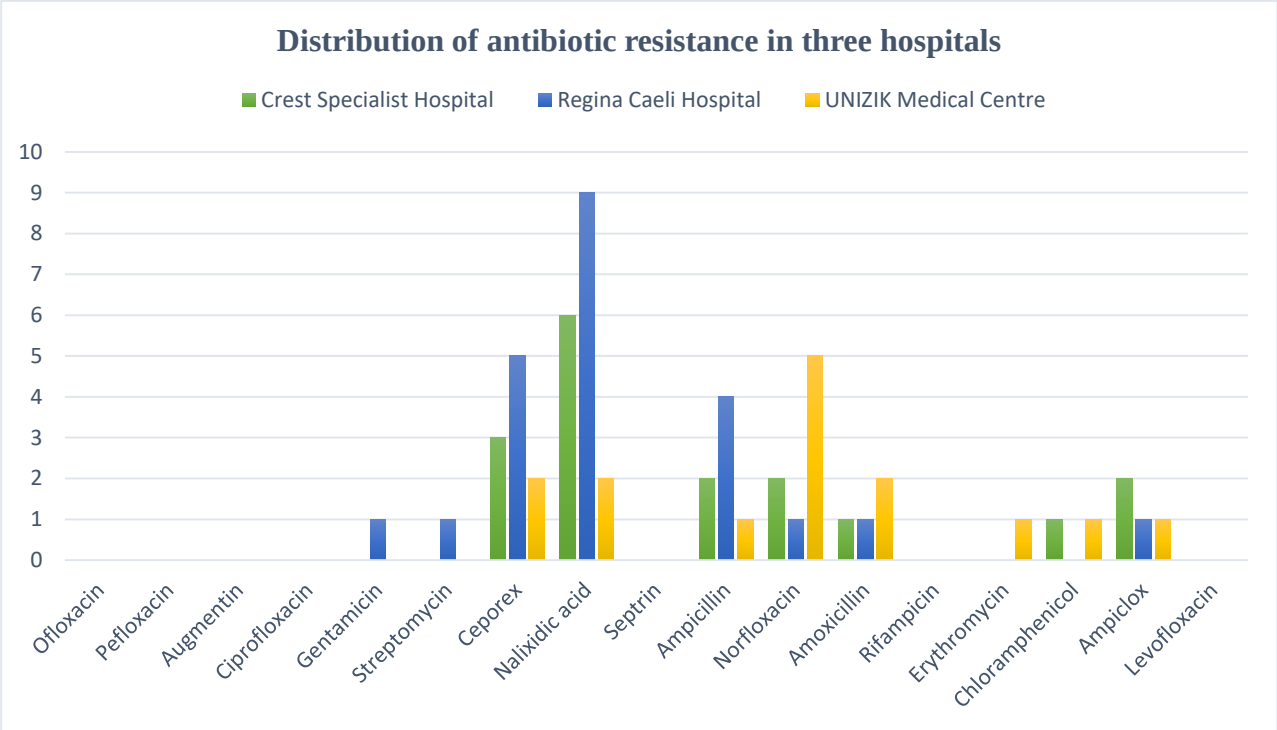

Figure 2: Distribution of antibiotic resistance in three hospitals Molecular Identification Result

DNA extraction and sequencing was carried out on three of the six multidrug resistant isolates as shown in the table above. These three isolates were selected on the basis; (i) their multidrug resistance index and (ii) as a representative from the hospitals. The FASTA sequences of the three selected multidrug resistant species were subjected to Basic Local Alignment Search Tool (BLAST). *Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6 having 620bp sequence, showed 97.58% identity in a query coverage of 100%. *Bacillus cereus* strain DS-2 having 649bp sequence, showed 100% identity in a query coverage of 100%. *Staphylococcus hominis* strain PCP01 having 872bp sequence, showed 77.64% identity in a query coverage of 89% (Table 8). When comparing the result of biochemical and cultural test with molecular test, the identity of *Staphylococcus* species and *Bacillus* species corresponded. Although their biochemical test results are similar, the identity of Klebsiella species was wrongly indicated as it was different from the molecular test result (*Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6) (Table 9).

Plasmid Extraction and Profiling Result

Plasmid extraction and profiling was carried out on the isolates; P4 (G2), P24 (G3) and P34 (G1). *Staphylococcus hominis* strain PCP01 (G1) showed absence of plasmid while *Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6 (G2) and *Bacillus cereus* strain DS-2 (G3) showed the presence of plasmid (Figure 3).

Table 8: The BLAST outputs of total score, query coverage, e-value, percentage identity and accession number obtained from the isolates' sequence.

Isolates IDs	Sequence length (bp)	Top Hit from NCBI database	Accession No	% Query coverage	E-value	% Identity	Total score
P4	620	*Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6	MK606113.1	100	0.0	97.58	1066
P24	649	*Bacillus cereus* strain DS-2	ON005108.1	100	0.0	100	1199
P34	872	*Staphylococcus hominis* strain PCP01	MT705968.1	89	4e-125	77.64	462

Table 9: Comparison of the Biochemical and Cultural Tests Identification with Molecular Identification.

ID	Initial Identification	Molecular Identification
P4	*Klebsiella* sp.	*Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6
P24	*Bacillus* sp.	*Bacillus cereus* strain DS-2
P34	*Staphylococcus* sp.	*Staphylococcus hominis* strain PCP01

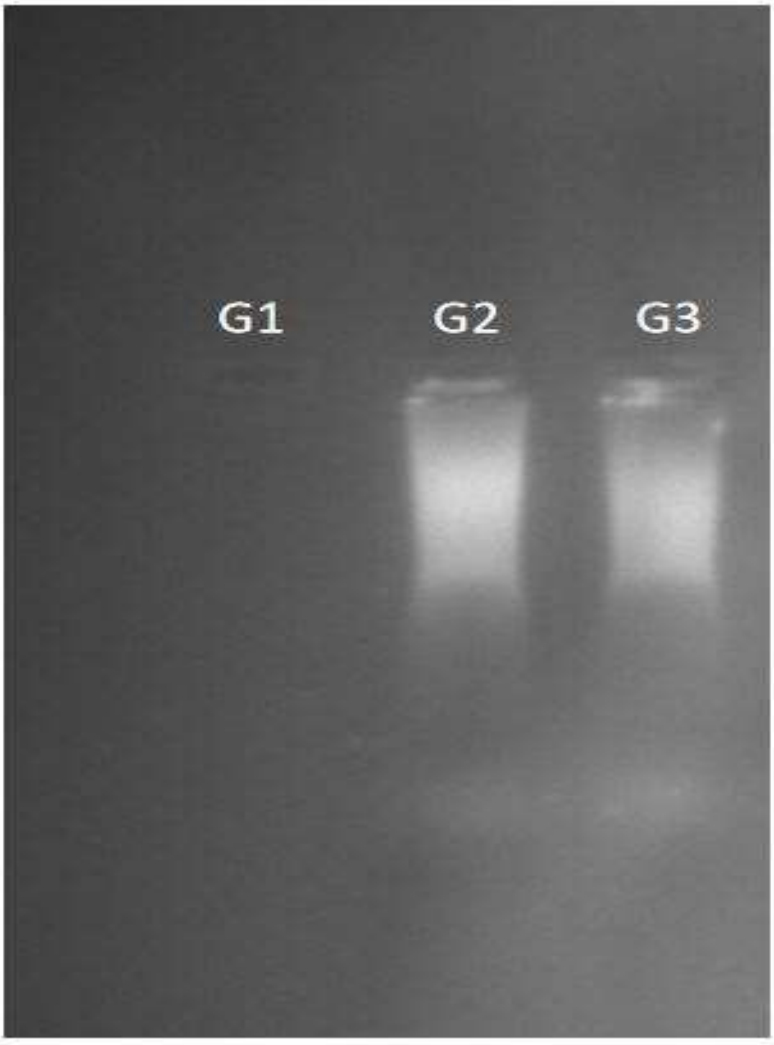

Figure 3: Electrophoretic plasmid profile of isolates

DISCUSSION

Healthcare associated infections appear in patients under medical care in the hospital or other health care facility which was absent at the time of admission [34]. The hospital environment is widely and naturally contaminated with microorganisms of specifically human or environmental origin [35]. This contamination varies qualitatively and quantitatively in time, from one institution to another and within the same institution, based on the services, patients, treatments and techniques used [35]. There is paucity of data regarding multi-antibiotic resistant bacterial organisms in health care setup not only in Awka metropolis but also in Nigeria.

In this study we performed a total of 112 samples, in the framework of microbiological monitoring of surfaces, water and air microbiota. 2 samples were found negative and 110 were identified contaminated, a percentage positivity of 98.2%. Previous study in a hospital in Fez city, Morocco, shows a percentage surface contamination of 96% [35]. Our result showed that Gram negative bacteria were responsible for 77.5% of contamination while Gram positive bacteria were responsible for 22.5%. This is similar to previous reports of 73.33% vs 26.67% in Morocco [35], 66.2% vs 33.82% in Zimbabwe [36], 51.6% vs 48.4% in Palestine [37] and 60.4% Gram negative bacilli in Egypt [38]. The dominance of Gram negative bacteria can be explained by the fact that they have an outer membrane and any alteration like changing the hydrophobic properties or

mutations in porins and other factors, can create resistance [39].

Meanwhile, in contrast to our result, there are studies that suggested that Gram positive isolates are more predominant; 56.3% vs 43.7% in Ethiopia [40], 52.2% vs 47.8% in Nigeria [42] and 81.6% vs 18.4% in Ethiopia [41]. These variations may be due to different sampling times (e.g. during endemic vs outbreak situations), the use of different sampling techniques, and variation in specific hospital sampling sites. In fact, in agreement to the latter reasoning, more Gram positive (63.6%) than Gram-negative ones were obtained from UNIZIK Medical Centre environment even from our finding. As can be seen in tables 1–12, in all the different hospital environments examined, the highest bacterial contaminated samples were taken from bed linens, with 32.5% of the entire isolates coming from there. This is similar to the observation from other study [43]. The reason of such level of bacterial contaminations could be as a result of cross-contamination from a patient's flora, health care workers' hands, or due to contamination during the washing process.

With regards to the antimicrobial resistance of the isolates, most isolates showed resistance to the quinolones; Nalixidic acid and Norfloxacin, possibly because they are first and second generation quinolones respectively. Similarly, some negative bacterial isolates were resistant to cephalosporin, while penicillin resistance was noted in some negative and positive isolates. This resistance may be caused by the presence of beta-lactamase enzyme in the bacteria or as a result of the reduction of affinity of existing Penicillin Binding Proteins. Interestingly, there was 100% sensitivity of isolates to ciprofloxacin, levofloxacin, ofloxacin, augmentin (amoxicillin-clavulanate), rifampicin and septrin.

In our study, we noticed that only few of our isolates were multidrug resistant (15%). This can be as a result of the fact that only environmental samples and not clinical samples were analyzed [44]. Amongst the 6 multidrug isolates, 3 (50%) were isolated from air, 2 (33.3%) were isolated from table surfaces while 1 (16.7%) was gotten from the clothing of a health care giver. This implies that the air is a very active route for the transmission of multidrug resistant bacterial organisms, in hospital settings. Special air handling and ventilation are required to prevent airborne transmission [45]. Inadequate ventilation is implicated in the airborne transmission of bacteria [46]. *Myroides odoratimimus* has formally been isolated from a range of bodily fluids, it is a rare opportunistic pathogen [47]. *Myroides* species are commonly found in the environment and infections can occur following contact with contaminated water [48]. *Myroides odoratimimus* has been associated with nosocomial infections where they affected immunocompromised patients [49, 50]. [51], reported a case of extensively drug-resistant *M. odoratimimus* responsible for urinary tract infection in an immunocompromised patient in a Romanian hospital. Soft tissue infection was noted by [52] whilst [53] reported bacteremia in a diabetic patient caused by *M. odoratimimus*. However, this is the first case whereby *M. odoratimimus* subsp. xuanwuensis strain M2S3B6 is being reported as a hospital pathogen.

It is worthy to note that; this is not the first time *Bacillus cereus* was isolated from a hospital environment. There has been a report of a multidrug resistant *B. cereus* isolated from health care workers and environmental surfaces in a hospital in Libya [54]. Similarly, the *B. cereus* isolated in this study was gotten from a health care worker. It is an opportunistic pathogen capable of causing a range of diseases, most prominently foodborne disease, due to the production of enterotoxins (diarrheal toxin) or a non-ribosomal peptide synthetase toxin (emetic or cereulide toxin) [55]. *Staphylococcus hominis* is normally found on human skin and is usually harmless, but can sometimes cause infections in people with abnormally weak immune systems. There are some research works that indicated the presence of nosocomial spread of a *Staphylococcus hominis* subsp. *novobiosepticus* strain, causing sepsis in a neonatal intensive care unit in Spain [56]. And also, a methicillin-resistant *S. hominis* been isolated from a blood infection [57]. However, recent findings suggest that *S. hominis,* which is a ubiquitous human skin commensal, protects the cutaneous barrier (skin) against opportunistic pathogens [50].

Using their FASTA Sequences, the molecular Identification result showed that *Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6 has 97.56 % identity from a query coverage of 100%, *Bacillus cereus* strain DS-2 has 100% identity from a query coverage of 100% whilst *Staphylococcus hominis* strain PCP01 has 77.64% identity from a query coverage of 89%. Meanwhile, with longer nucleotide sequence (bp), a 100% identity was gotten from a BLAST for both *Myroides odoratimimus* subsp. xuanwuensis strain

M2S3B6 and *Staphylococcus hominis* strain PCP01. After the BLAST for *Staphylococcus hominis* strain PCP01, the top hit organisms were uncultured organisms. *S. hominis* is the topmost known organism, hence its selection. When comparing the Biochemical and Molecular Identification results, it was noticed that one out of the three organisms did not match. This is primarily due to the similarity of the biochemical and cultural test results of *Klebsiella* sp. and *Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6. Resistance by *MecA* gene was reported by [54], [55], as the gene responsible for multidrug resistance in *Staphylococcus hominis.* This gene present in *S. hominis* is known to code for proteins that bind to penicillin or other such antimicrobial agents. This is to simply say that resistance in *S. hominis* is not plasmid-mediated, as can be proved from our result of plasmid profiling where the organism showed absence of plasmid. Plasmid was seen in *Myroides odoratimimus* subsp. xuanwuensis strain M2S3B6 and *Bacillus cereus* strain DS-2. Of which the plasmids might not be Resistance (R) plasmid. Further studies could help determine if the plasmids have any relationship with multidrug resistance or if they are for other essential functions in the bacterial organisms.

CONCLUSION

The issue of multidrug resistance has tremendously affected the world of science and medicine. This is firstly because of the difficulty these microbes pose in elimination and secondly, due to the pathogenic consequences of having them in the human body. Nosocomial infections caused by these resistant microorganisms affects the immunocompromised detrimentally, leading to increase in mortality rates across different parts of the world. This research, just as many others have highlighted the presence of multidrug resistant opportunistic pathogens in hospital environment that poses serious threat to the immunocompromised. However, with proper preventive measures being implemented and as well, concerted efforts geared towards sensitization, especially in the African continent, cases of outbreaks of hospital associated infections can be reduced to the barest minimum.

RECOMMENDATIONS

This research work has, in no little way, created an awareness especially with respect to the use of hospitals in different parts of the world, most especially in Nigeria. Amazingly, the multidrug resistant microbes are opportunistic pathogens, meaning that if given as opportunity, they can cause harm to man, as we have seen cases where diseases ensued from them. The immunocompromised are the worst hit in an event of an outbreak of these organisms, which is why proper attention should be paid in ensuring that preventive measures are adhered, in all hospitals in Nigeria and other parts of the globe. For purpose of emphasis, three of the six multidrug resistant isolates gotten from the three hospitals were from air samples, meaning that they are airborne pathogens. Thus, special air handling and ventilation are required to prevent airborne transmission and also the wearing of facemask within the hospital environment. Consequently, proper personal hygiene of health care workers is needful, as reported from the isolation of *Bacillus cereus* strain DS-2 from a health care giver in one of the hospitals. There is a great need for disinfection of hospital surfaces, especially tables, as two multidrug isolates were gotten from tables in one of the hospitals. The use of bactericidal detergents and antiseptics is required while washing bed linens as they showed the highest bacterial contamination. Finally, in future studies, we recommend that the plasmids be assayed to determine the true cause of resistance amongst the multidrug resistant isolates that showed the presence of plasmid.

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