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# Plasmid Profile and antibiogram of Vancomycin and Methicillin Resistant Bacteria isolated from Wound Swabs from Hospital patients in Akure, Nigeria.

**Abstract:** A total of 347 clinical wound swabs were obtained in Ondo State Specialist Hospital. Isolated pure cultures of bacteria were subjected to various morphology and biochemical tests. The isolates were identified using Bergey's Manual of Systematic Bacteriology. Ten antibiotics (Oxoid UK) discs were used to determine the drug sensitivity pattern of the bacterial isolates. Plasmids were extracted by alkaline lysis method and electrophoresis of the DNA was carried out on a 0.8% agarose gel electrophoresis. This finding revealed that out of 501 bacterial isolates collected from 347 wound swabs of patients; *Staphylococcus aureus was the most predominant (*34.73%). *Pseudomonas aeuroginosa* (27.74%), *Streptococcus pyogenes* (15.57%), *Klebsiella pneumoniae* (8.18%), *Proteus mirabilis* (7.38%) and *Escherichia coli* (6.40%) were isolated respectively. Nineteen of the bacterial isolates showed a multiple resistance patterns to 6 or more antibiotics namely; Vancomycin, Methicillin, Cotrimoxazole, Gentamicin, Tetracycline, Netilmicin and Augmentin while all of the isolates were susceptible to Amoxicillin, Ofloxacin and Levoxin. *S. aureus* (1.4%) and *P. aeruginosa* (0.79%) were found to display high level of resistance to most tested antibiotics. Plasmid analysis shows that some multi drug resistant (MDR) bacterial isolates harbor one or more plasmids with different molecular weights while some (MDR) bacterial isolates harbor no plasmid. Gene coding for resistance were located on the plasmid while the bacterial isolates without plasmids have their gene coding located on their chromosomal DNA. It is therefore recommended that antimicrobials for the treatment of wound infections should be based on a recent antibiogram tests.

**Key words:** Antibiotics, Electrophoresis, Plasmids, Wound

## 1.0 INTRODUCTION

Nosocomial or hospitalized acquired infections are infections appearing in a patient in a hospital or other health care facility in whom the infection was not present or incubating at the time of admission. Such infection manifest within 72hrs or more after admission [1]. Nosocomial infections are estimated to cause or contribute to nearly 80,000 deaths annually in the United States [2] such infections are serious and public health hazard throughout the world. As a matter of fact, is the fourth leading cause of death [3]. Hospital-associated infection which has a worldwide distribution remains a major cause of deaths among hospitalized patients [4]. In view of that, [2] reported that the hospital is no longer a place where sick people recover from their illnesses but also where illnesses at times get complicated and healthy people get infected. Nosocomial infectious disease is a major problem in many health care systems. It has been reported that 10% of hospital patients will acquire an infection while in hospital [5]. Infections can complicate illness, cause distress to patients and family and can lead to death. Among nosocomial infections, there are main infections that has been reported such as blood stream infections (28%), ventilator-associated pneumonia (21%), lower respiratory infection (12%), urinary tract infection (12%), gastrointestinal, skin, soft tissue and cardiovascular infection (10%), surgical-site infection

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(7%) and ear, nose and throat infection (7%) [5]. Despite the extensive use of antibiotics and vaccine programs, infectious disease continue to be a leading cause of morbidity and mortality worldwide [6]. Emergence of resistant strains of pathogenic microorganism such as Methicillin - Resistant *Staphyloccocus aureus* (MRSA) and Vancomycin- Resistant *Staphyloccocus aureus* (VRSA) are very virulent in humans and are referred as professional pathogens. Which has also continued to pose a major health concern about the efficacy of several drugs, most importantly antibiotics in current use [7].

Methicillin resistant *S. aureus* (MRSA) is responsible for hospital-acquired (HA) infections [8, 9] and presently community acquired (CA) infections [10, 11, 12]. Hospital associated (nosocomial) staphylococcal infections have been reported to be resistant to as many as 20 antimicrobial compounds, including antiseptics and disinfectants. Resistance to penicillin among *S. aureus* strains appeared a few years after the introduction of penicillin therapy. Introduction of other antibiotics such as streptomycin, tetracycline and chloramphenicol, and the macrolides was similarly followed by emergence of resistant organisms. Resistant organisms that had acquired resistance to these antibiotics were reported to be usually resistant to penicillin through the production of penicillinase [13]. This resulted in the evolution of organisms with a wide spectrum of resistance and a marked ability to survive and spread in the hospital environment. Such multiple resistant *S. aureus* strains were of global significance as early as 1950s [14].

Paucible observations regarding drug resitance are available revealing the increased frequency of methicillin resistant *S. aureus* (MRSA) and VRSA by passage of time [15]. Pathogen developed differing mechanism and means to resist against different antibiotics depending on their mechanism of action. Enzymatic degradation of drug, structural modification of target and antibiotic efflux are mere strategies of resistance followed by the bacteria [16]. Drug resistant genes are either chromosomally located or plasmid lodged. However, conjugative plasmid are majorly accused for emerging resistance as it successfully transfer genetic material in both inter and intra-species [17, 18]. This confers a basis for spreading resistance genes among bacterial population quicker than native mutation and vertical evolution.

Two modes of vancomycin resistance have been identified in *S. aureus*. First type encountered in vancomycin intermediate is due to the piling up of additional peptidoglycan layer and second type detected in vancomycin resistance strains is resulted by conjugal transfer of responsible gene cluser [19]. Vancomycin molecules are trapped by more D-Ala-D-Ala residues, prohibiting them to reach their target on cytoplasmic membrane. Unlike former one, later is genetically driven replacement of lactate in place of alanaine during synthesis of peptidoglycan precursors. Besides, the organism is capable of synthesizing a sex pheromone, a facilating agent for conjugal transfer. The crucial point is the gene for sex pheromone is harboured in v*anA* plasmid [20].

Plasmid determination is very important in epidemiological studies. Moreover, it has proved to be the earliest DNA-based method implemented for analyzing resistance pattern, frequency and probable future status of the resistance in relation to certain parameter [21]. Molecular identification of genes provides the detail information about the pathways followed for resistance, potency of bacteria to drive this property in long run and genotypic view of resistance whereas curing of plasmid and transfer explains frequency of resistance transferred the extent of emerging resistance in new strains. The study was preceded with the objectives to determine plasmid mediated MRSA and VRSA along with plasmid profiling and detection of vancomycin resistance genes from VRSA.

**1.1 Wound**

A wound is a breach in skin or sub cutaneous tissues. It happens accidentally or during surgery, sutures or stitches. This results to loss of integrity of the skin. The wound

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sites becomes moist, warm and nutritive for microbes proliferation. Infections can therefore set in. Bacteria such as: *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella* spp*., Proteus mirabilis, Pseudomonas aeruginosa, Bacteroides fragilis, Peptostreptococcus* and *Propionibacterium* spp. are mostly implicated in wound infections [22].

## 2.0 MATERIALS AND METHODS

### 2.1 Collection of Clinical Samples

A total of three hundred and forty-seven (347) septic wound swabs were collected from patients at the accidental clinic of Ondo State Specialist Hospital Akure between October 2015 and March, 2016.

### 2.2 Bacterial Isolation and Identification

#### 2.2.1 Isolation of Bacteria

The plate streaking technique was used for isolation of bacteria. Swab sticks were used to streak the samples on the already solidified nutrient agar plate, blood agar and chocolate agar, and incubated at 37$^{o}$C for 24hour. Pure cultures of isolate were obtained by sub-culturing unto freshly prepared plates as appropriate [23].

### 2.3 Identification and Characterization of Bacterial Isolates

The isolated bacteria were identified by using their cultural and morphological characteristics in different media. Distinct colonies of isolates that grew on the culture plates were observed for their cultural and morphological features, this was followed by microscopic examination of the bacterial isolates on the glass slide placed under the microscope. The cultural features examined included shape elevation, surface edge and consistency. Physiological and biochemical test were employed to confirm their identification [24].

### 2.4 Standardization of bacterial inoculum

A loop full of test organism was inoculated on nutrient broth and incubated for 24 hours. Exactly 0.2 ml from the 24hours culture of the organisms was dispensed into 20 ml sterile nutrient broth and incubated for 3-5hours to standardize the culture to 0.5 McFarland standards ($10^{6}$cfu/ml) before use according to the method of [25].

### 2.5 Antibiotic sensitivity of Bacterial Isolates

Antibiotic sensitivity tests were performed on the isolates using disc diffusion technique as described by Clinical Laboratory Standard Institute [26]. All susceptibility tests were carried out using overnight culture. Muller-Hinton sensitivity test agar plates were prepared according to manufacturer's specification. The isolates were sub-cultured onto freshly prepared nutrient agar plates and incubated at 35$^{o}$C for 18hour. A 1ml suspension of each bacterial isolates, equivalent to McFarland standards was aseptically seeded into Muller-Hinton agar plate respectively. This was allowed to stand for 1 hour to solidify. The antibiotic paper disc containing Vancomycin (30µg) (VAN), Methicillin (MET) (1µg), Cotrimoxazole (25µg) (COT), Gentamicin (10µg) (GEN), Tetracycline (30µg) (TET), Netilmicin (30µg) (NET), Amoxicillin (30µg) (AMC), Augmentin (30µg) (AUG), Ofloxacin (5µg) (OF) and Levoxin (5µg) (LE), were aseptically placed on the surface of the molten Muller-Hinton agar and allowed for 30 minutes to pre-diffuse. The set up was done in triplicates for each isolate, with a control plate containing no antibiotic disc. These were incubated for 18-24hours at 37$^{o}$C after which the diameter of zone of inhibition was recorded and the results interpreted using standard interpretative charts as recommended by Clinical Laboratory Science Institute [27]. In this study, multiple antibiotic resistance were indicated by resistance to a minimum of 5 different antibiotics.

### 2.6 Plasmid analysis

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Plasmid analysis was carried out on bacterial isolates that showed multiple resistances to antibiotics. This was carried out in order to know the molecular basis of resistance in some bacterial isolates.

**2.6.1 Extraction of plasmid**

A 2µl 1x PBS buffer was added into the eppendorf tube, an overnight culture was carefully picked into the buffer and then was vortexed at high speed to re-suspend cells completely then spinned for 5 minutes in a micro-centrifuge at 10,000rmp to pellet cells. The supernatant was gently decanted, for Gram-positive bacteria; 2µl of lysozymes was added to break the peptidoglycan cell wall and incubated for 1 hour at 37$^{o}$C. After incubation, bacterial cells were revoltex and then spinned for 5 minute in a micro-centrifuge at 10,000rmp. Palleted bacterial cells was re-suspended in 300µl Lysis Buffer by pipetting and then mixed by inverting the tubes for 3-4 times, immediately a slimy solution was observed, meaning lysis has occurred. 300µl of Neutralization buffer (containing RNase A) was added and mixed gently by inverting the tubes 4-6 times and centrifuged at 10,000rmp for 5 min at room temperature in a micro-centrifuge. Change in colour of the mixture to bright yellow indicates a pH of 7.5 required for optimal DNA binding. A spin Column was placed into a 2ml collection tube and 100µl of Activation Buffer was added into the spin Column and then centrifuged at 10,000rpm for 30sec in a micro-centrifuge. The supernatant was decanted into the activated Spin column and centrifuged at 10,000rmp for 30sec and the flow through was discarded. 500µl of washing Buffer (containing Ethanol) was added to the Spin Column, and centrifuged at 10,000rmp for 30sec and discard the flow through. The Spin Column was placed into a clean 1.5ml microtube and 50µl Elution solution to the center of the column membrane and incubated for 1 min at room temperature, and centrifuged at 10,00rmp for 1 min to elute DNA [28].

**2.6.2 Agarose gel electrophoresis**

Agarose gel Electrophoresis is a separation method that can be used to separate DNA based on their molecular weight. The concentration of Agarose used is dependent on the size of the DNA to be separated but 0.8% Agarose is basically used for plasmid DNA. A 0.8g portion of agarose powder (for plasmid DNA) was weighed and mixed with 100ml of 0.5X TAE (Tris and Acetic EDTA) buffer was added to it, dissolved by boiling using a microwave oven, and then allowed to cooled to about 60$^{o}$C and 10µl of ethidium bromide was added. This was mixed by swirling gently and poured into electrophoresis tank with the comb in place to obtain a gel thickness of about 4-5mm. This was allowed to solidify for about 20mimutes, the comb was carefully removed, the tray was placed in the electrophoresis tank and the 0.5X TAE (Tris and Acetic EDTA) buffer was poured into the tank. Ten microlitre 10µl of the sample was mixed with 2µl of the loading dye, the samples were carefully loaded into the wells created by the combs, the electrodes were connected to the power pack and electrophoresis was at 60-100v until the loading dye had migrated about 3- quarter of the gel. The electrode was disconnected and the gel was observed on a uv-transilluminator/photo documentation system [29].

**2.6.3 Curing of plasmid DNA**

Curing of the Plasmid was done to determine whether or not a plamid encodes a trait that codes for antibiotics resistance or multi resistance. Curing was done using the ethidium bromide. The isolates that showed multiple resistances to different antibiotics due to plasmid bands were subjected to plasmid curing. 0.1mg/ml of ethidium bromide was added into 100ml of Luria Bertaru broth. The solution was autoclaved at 121$^{o}$C at 15psi for 15min. An overnight culture of the sample was standardized according to 0.5 McFarland standard and 0.5ml from the standardized solution was pipette using Pasteur pipette into the 100ml sterile Luria Bertaru broth. The solution was incubated at 37$^{o}$C for 4hours. After incubation, the isolates was re-inoculated into a sterile nutrient broth and incubated for 24hours [30].

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**2.7 Post Curing Sensitivity Testing**

The Plasmid cured isolates were tested against those antibiotics to which they were previously resistant. Post curing sensitivity tests were performed on the isolates using standardized disc agar diffusion technique as described by Clinical Laboratory Standard Institute [26] using overnight culture. Muller-Hinton sensitivity test agar plates were prepared according to manufacturer's specification. The isolates were subcultured onto freshly prepared nutrient agar plates and incubated at 35$^{o}$C for 18hours. A 1ml suspension of each bacterial isolates, equivalent to McFarland standards was aseptically seeded into Muller-Hinton agar plate respectively. This was allowed to stand for 1 hour to solidify. The antibiotic paper disc containing Vancomycin (30µg) (VAN), Methicillin (MET) (1µg), Cotrimoxazole (25µg) (COT), Gentamicin (10µg) (GEN), Tetracycline (30µg) (TET), Netilmicin (30µg) (NET), Amoxicillin (30µg) (AMC), Augmentin (30µg) (AUG), Ofloxacin (5µg) (OF) and Levoxin (5µg) (LE), were aseptically placed on the surface of the molten Muller-Hinton agar and allowed for 30 minutes two pre- diffuse. The set up was done in triplicate for each isolates, with a control plate containing no antibiotic disc. These were incubated for 18-24hours at 37$^{o}$C after which the diameters of zone of inhibition were measured using meter rule in mm and zones were compared with standard antibiotics chart.

**2.8 Data Analysis**

All the experiments were carried out in triplicate and data obtained from the study were subjected to analysis of variance. Treatment means were compared using Duncan's New Multiple Range Test (DNMRT) at 5% level of significance using SPPS version 21.

## 3.0 RESULTS

**Incidence of wound infection among the patients examined**

A total of three hundred and forty-seven (347) septic wound swabs were collected from patients including males and females attending Ondo State Specialist Hospital Akure between October 2015 and March, 2016. The following bacteria were isolated from the samples namely; *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis* and *Streptococcus pyogenes.* The profile of the wound swabs collected for the investigation based on age and sex of the patients can be seen in Table 1 while the morphological and biochemical characteristics of the bacterial isolated from wound swabs can be seen in Table 2. Table 3 shows the occurrence of bacterial isolates examined from wound samples of patients. While figure 1 shows their frequency from different regions of the body parts.

Table 4 Shows Susceptibility and Resistant rate of the bacterial isolates to Vancomycin and Methicillin antibiotics. Table 5 shows the Frequency of resistant of the bacterial isolates to Vancomycin and Methicillin. Table 6 shows resistance pattern of the bacterial isolates to conventional antibiotics.

Out of five hundred and one (501) bacterial isolates collected from three hundred and forty-seven (347) wound swabs of patients attending Ondo State Specialist Hospital, *Staphylococcus aureus* (174, 34.73%), *Pseudomonas aeuroginosa* (139, 27.74%), *Streptococcus pyogenes* (78, 15.57%), *Klebsiella pneumoniae* (41, 8.18%), *Proteus mirabilis* (37, 7.38%) and *Escherichia coli* (32, 6.40%) were isolated respectively.

Antibiotics sensitivity test were carried out on the bacterial isolates from wound swabs using conventional sensitivity discs containing Vancomycin (30mg) (VAN), Methicillin (MET) (1mg), Cotrimoxazole (25mg) (COT), Gentamicin (10mg) (GEN), Tetracycline (30mg) (TET), Netilmicin (30mg) (NET), Amoxacillin (30mg) (AMC), Augmentin (30mg) (AUG), Ofloxacin (5mg) (OF) and Levoxin (5mg) (LE). *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Streptococcus pyogenes* were resistant to, (MET), (GEN), (TET), (COT) (NET)

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and (AUG) while The Pre and Post sensitivity curing of multiple antibiotics resistant bacteria isolated from wound patients were shown in Table 7.

**Table 1: Wound swabs collected based on Age and Sex from patients attending Ondo State Specialist Hospital Akure, Ondo State.**

| Age-group (years) | Males (%) | Female (%) | Total (%) |
|---|---|---|---|
| **< 10** | 10(2.88) | 6(1.73) | 16(4.61) |
| **10-19** | 17(4.90) | 12(3.46) | 29(8.36) |
| **20-29** | 38(10.95) | 57(16.43) | 95(27.38) |
| **30-39** | 76(21.90) | 43(12.39) | 119(34.29) |
| **40-49** | 32(9.22) | 18(5.19) | 50(14.41) |
| **50-59** | 16(4.61) | 10(2.88) | 26(7.49) |
| **> 60** | 7(2.02) | 5(1.44) | 12(3.46) |
| **Total** | **196(56.5)** | **151(43.5)** | **347(100)** |

| No | Colour | Elev | Edg | Cell shape | Arrang. | Gram | CAT | CAO | IN | MP | VP | CI | UR | GLU | LAC | SUC | MAL | GAS | PROBABLE ISOLATE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | Pink | Raised, flat | Serrated | Short Rod | Single | - | + | - | [+] | + | - | - | - | AG | AG | A+/- | + | + | ***Escherichia coli*** |
| **2** | Yellow | Convex | Round | Coccus | Irregular clusters | + | + | + | - | - | + | D | + | + | + | + | + | - | ***Staphylococcus aureus*** |
| **3** | Pink | Raised/smooth | Serrated | Rod | Single | - | + | - | - | [-] | + | + | + | + | + | + | + | + | ***Klebsiella pneumoniae*** |
| **4** | Colourless, Beta haemolytic colonies | Raised | Round | Coccus | Pairs/ Chains | + | - | - | - | + | - | - | - | + | + | + | + | - | ***Streptococcus pyogenes*** |
| **5** | Blue/green | Flat | Round | Rod | Single | - | + | - | - | - | - | + | D | - | - | - | - | - | ***Pseudomonas aeruginosa*** |
| **6** | Pink | | | Rod | Single | - | + | - | - | + | [-] | D | + | + | - | - | + | + | ***Proteus mirabilis*** |

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**Table 3: Occurrence of Different Bacteria Collected From Wound Swabs of Patients Attending Ondo State Specialist Hostipal Akure**

| S/N | BACTERIA | NUMBER OF ISOLATES | PERCENTAGE OCCURRENCE (%) |
|---|---|---|---|
| **1** | ***Escherichia coli*** | 32 | 6.40 |
| **2** | ***Staphylococcus aureus*** | 174 | 34.73 |
| **3** | ***Pseudomonas aeruginosa*** | 139 | 27.74 |
| **4** | ***Klebsiella pneumoniae*** | 41 | 8.18 |
| **5** | ***Proteus mirabilis*** | 37 | 7.38 |
| **6** | ***Streptococcus pyogenes*** | 78 | 15.57 |
| | **TOTAL** | **501** | **100** |

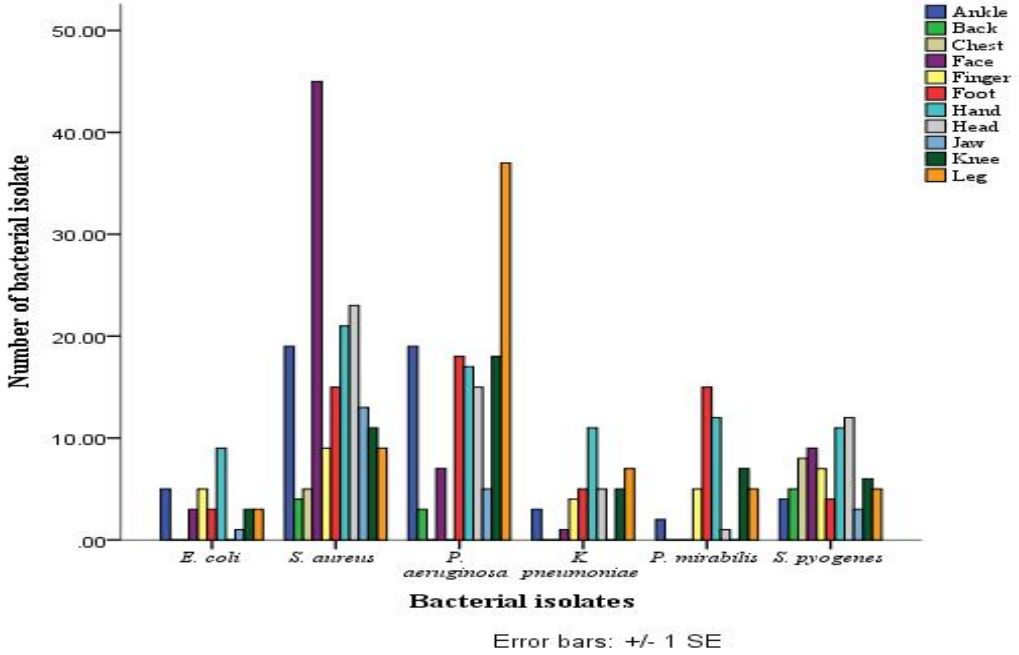


**Figure 1: Frequency of occurrence of different bacteria collected from wounds on different body sites of examined.**

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**Table 4: Susceptibility and Resistant of the Bacterial isolates to Vancomycin and Methicillin antibiotics**

| Bacteria | Susceptible (%) | Resistant (%) |
|---|---|---|
| ***Escherichia coli*** | 29(5.78) | 3(0.60) |
| ***Staphylococcus aureus*** | 167(33.33) | 7(1.40) |
| ***Pseudomonas aeruginosa*** | 135(26.94) | 4(0.79) |
| ***Klebsiella pneumonia*** | 39(7.78) | 2(0.40) |
| ***Proteus mirabilis*** | 35(6.98) | 2(0.40) |
| ***Streptococcus pyogenes*** | 77(15.37) | 1(0.20) |
| **Total** | **482(96.20)** | **19(3.80)** |

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**Table 5: Frequency of resistant of the bacteria to Vancomycin and Methicillin**

| Bacteria isolated | Methicillin | Vancomycin |
|---|---|---|
| ***Escherichia coli*** | 2 | 1 |
| ***Staphylococcus aureus*** | 3 | 4 |
| ***Pseudomonas aeruginosa*** | 2 | 2 |
| ***Klebsiella pneumonia*** | 0 | 2 |
| ***Proteus mirabilis*** | 1 | 1 |
| ***Streptococcus pyogenes*** | 1 | 0 |
| **Total** | **9** | **10** |

**Table 6: Shows the resistance pattern of the bacterial isolate tos conventional antibiotics.**

| No | Bacterial isolates | Resistant pattern |
|---|---|---|
| **1** | $S_1$ | VAN, COT, AMC, NET, TET, AUG |
| **2** | $STREP_4$ | MET, COT, AMC, TET, AUG , GEN |
| **3** | $S_5$ | VAN, AMC, COT, NET, TET, AUG |
| **4** | $P_6$ | MET, VAN, AUG, NET, TET, COT |
| **5** | $P_7$ | MET, COT, GEN, AUG, TET, NET, AMC |
| **6** | $K_{11}$ | VAN, TET, NET, AMC, NET, LE |
| **7** | $P_{12}$ | VAN, COT, GEN, NET, TET, AMC |
| **8** | $E_{14}$ | MET, AMX, COT, LE, GEN, NET |
| **9** | $S_{15}$ | MET, COT, AMC, NET, OF, TET, |
| **10** | $E_{39}$ | MET, COT, TET, GEN, NET, AUG |
| **11** | $K_{40}$ | VAN, COT, GEN, TET, AUG, AMC |
| **12** | $PRO_{45}$ | VAN, TET, COT, LE, GEN, NET, AUG |
| **13** | $E_{50}$ | VAN, COT, TET, NET, AMC, AUG |
| **14** | $S_{83}$ | MET, AUG, COT, OF, TET, AMC |
| **15** | $S_{112}$ | VAN, NET, TET, COT, AMC, AUG |
| **16** | $S_{302}$ | VAN, MET, AMC, NET, GEN, AUG, TET, COT |
| **17** | $PRO_{320}$ | MET, COT, GEN, TET, AMC, AUG, NET, |
| **18** | $P_{492}$ | VAN, AMC, COT, GEN, NET, TET, AUG |
| **19** | $S_{499}$ | VAN, GEN, NET, TET, COT, AUG |

**KEY = S- *S. aureus*, P- *P. aeruginosa*, E- *E. coli*, PRO- *P. mirabilis*, K- *K. pneumoniae*, STREP- *S. Pyogenes***

Plate 1 reveals the plasmid profiling of some isolated bacteria resistant to some commercially produced antibiotics lanes (1-6). *Staphylococcus auerus* in lane 1 showed 2 different bandswith molecular weights of 23130bp and 9,416bp when compared with Hind III lambda as a molecular marker. *Escherichia coli* in *lanes* 2 showed no plasmid bands lanes. *Streptococcus pyogenes* in lane 3 had no plasmid band. *Pseudomonas aeruginosa* in Lane 4, showed a single plasmid band with molecular weight of 2,027bp when compared with Hind III lambda as a molecular marker. *Proteus mirabilis* in lane 5 showed a single plasmid band with molecular weight of 2,027bp when compared with Hind III lambda as a molecular marker. Finally, *Klebsiella pnuemoniae* in lane 6 had no plasmid band. The plasmid analysis shows that some multiple resistant bacterial isolates harbor one or more plasmid with different molecular

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weight while some multiple resistant bacterial isolates harbor no plamid which denote their resistance is chromosomal borne. The molecular weight of plasmid haboring bacterial isolates was determined using standard molecular Hind III marker.

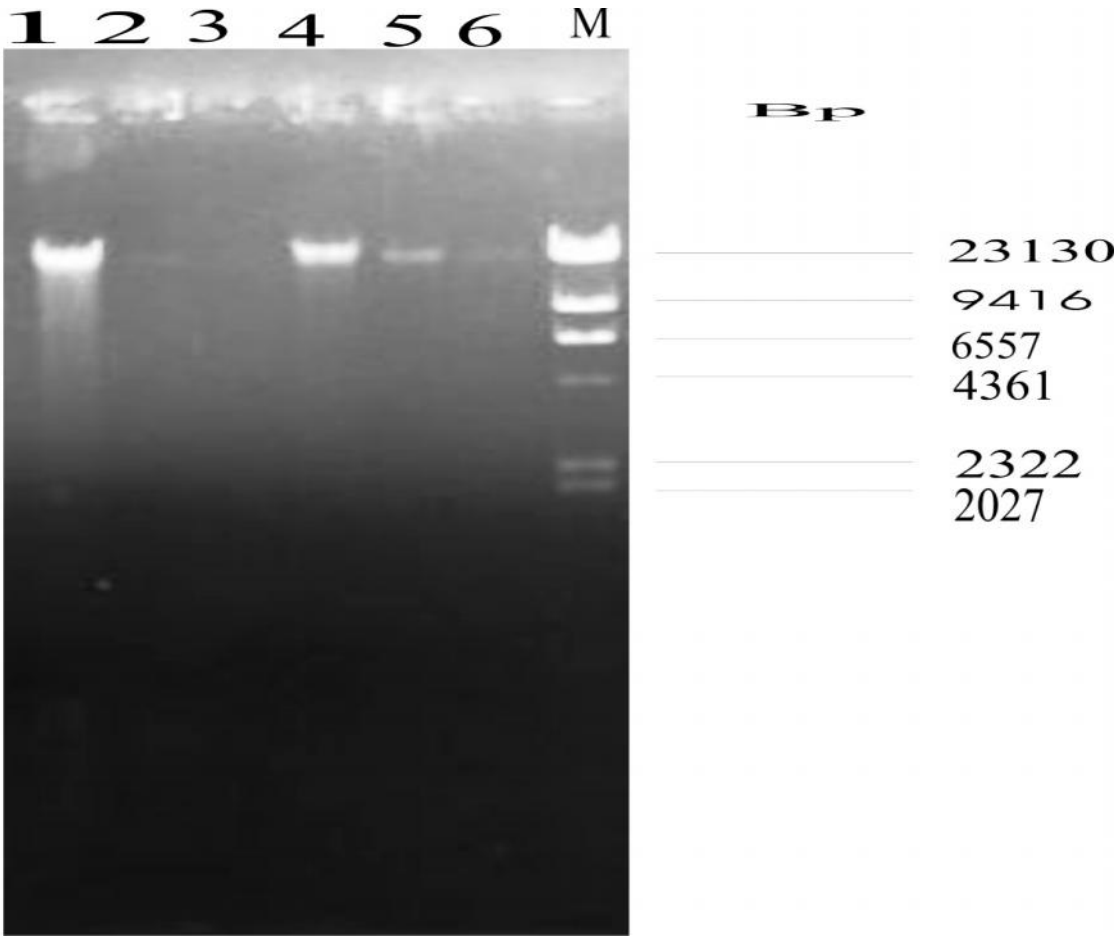


**Plate 1: Photograph showing plasmid profile of some multiple antibiotics resistant** bacterial **isolates from patients with wound infections.** Plasmid DNA on 0.8 Agarose, M-Molecular marker (M, λDNA Hind III digest marker). Lane 1-6 indicates bacterial isolates. The unit of molecular weight of plasmid DNA in base pair

**Table 7: Pre and Post sensitivity curing of multiple antibiotics resistant bacterial isolates from wound patients**

| Antibiotics | Zone of Inhibition (diameter in mm) | | | | | |
|---|---|---|---|---|---|---|
| | *Escherichia Coli* | *Staphylococcus aureus* | *Pseudomonas aeruginosa* | *Klebsiella pneumoniae* | *Proteus mirabilis* | *Streptococcus Pyogenes* |
| **VAN bf** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 21.50±0.12$^{b}$ |
| **VAN af** | 0.00±0.00$^{h}$ | 25.24±0.21$^{g}$ | 25.18±0.19$^{g}$ | 0.00±0.00$^{e}$ | 23.08±0.45$^{f}$ | 23.42±1.10$^{b}$ |
| **MET bf** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **MET af** | 0.00±0.00$^{a}$ | 27.00±0.45$^{h}$ | 33.87±0.69$^{h}$ | 0.00±0.00$^{a}$ | 22.26±0.30$^{f}$ | 0.00±0.00$^{a}$ |
| **LE bf** | 23.23±0.25$^{e}$ | 20.87±0.70$^{e}$ | 22.13±0.71$^{g}$ | 18.93±0.21$^{d}$ | 24.52±0.43$^{d}$ | 13.57±0.40$^{d}$ |
| **LE af** | 23.79±0.51$^{e}$ | 21.19±0.27$^{e}$ | 25.87±0.85$^{g}$ | 22.80±0.53$^{d}$ | 25.36±1.19$^{g}$ | 17.11±0.29$^{e}$ |
| **COT bf** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **COT af** | 0.00±0.00$^{a}$ | 12.52±0.32$^{c}$ | 2.81±0.26$^{b}$ | 0.00±0.00$^{a}$ | 11.56±0.43$^{c}$ | 0.00±0.00$^{a}$ |
| **GEN bf** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **GEN af** | 0.00±0.00$^{b}$ | 14.34±0.46$^{d}$ | 12.35±0.32$^{c}$ | 0.00±0.00$^{a}$ | 14.06±0.66$^{d}$ | 0.00±0.00$^{a}$ |
| **AUGbf** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **AUG af** | 0.00±0.00$^{a}$ | 2.62±0.10$^{b}$ | 3.15±0.16$^{b}$ | 0.00±0.00$^{a}$ | 3.76±0.12$^{b}$ | 0.00±0.00$^{a}$ |
| **TET bf** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **TET af** | 0.00±0.00$^{a}$ | 2.62±0.10$^{b}$ | 3.15±0.16$^{b}$ | 0.00±0.00$^{a}$ | 3.76±0.12$^{b}$ | 0.00±0.00$^{a}$ |
| **NET bf** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **NET af** | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **AMC bf** | 7.09±0.16$^{b}$ | 2.83±0.25$^{b}$ | 3.05±0.05$^{b}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **AMC af** | 18.70±0.44$^{c}$ | 14.14±0.41$^{d}$ | 14.94±0.81$^{e}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| **OF bf** | 15.30±0.30$^{d}$ | 12.80±0.20$^{c}$ | 14.50±0.20$^{d}$ | 12.27±0.21$^{b}$ | 11.37±0.31$^{b}$ | 12.80±0.26$^{c}$ |
| **OF af** | 24.52±0.44$^{f}$ | 21.99±0.95$^{f}$ | 23.87±0.31$^{f}$ | 20.81±0.53$^{c}$ | 20.41±0.52$^{e}$ | 22.26±0.40$^{g}$ |

**Data are presented as Mean±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05). KEY: Bf** = Before plasmid curing, **Af** = After plasmid curing.

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## 4.0 DISCUSSION

Findings from this study have revealed the rate of occurrences of wound infection among patients attending Ondo State Specialist Hospital Akure. A wound is a breach in skin or sub-cutaneous tissues. It happens accidentally or during surgery, sutures or stitches. The wound sites become moist, warm and nutritive for microbes proliferation.

In the present study, the peak for occurrence of pathogenic bacteria was found in the age group 30 – 39year with (34.29%) which was noted in male gender while the least occurrence was found in age > 60years with (3.46%). The results showed that there was significant difference in the prevalence of wound infection between age groups at P<0.5 (Table 1). Male child between age group 30-39years happen to be the most active in the family where they engaged in lot of activities such as farming, forestry, transports to provide for the family; in this process they are exposed to lost hazards such as accidents, surgery, sutures and stitches etc. which results in wound infections when not properly managed. This finding is in agreement with workdone by Ajao and Yakubu [2] which reported that wound infections happen accidentally.

In this study, the bacteria; *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Streptococcus pyogenes* were isolated from wound infections as shown on Table 2. This is in agreement with previous study of Church *et al.* [22] where the named bacterial isolates were the mostly implicated in wound infections.

Table 3 revealed occurrences of bacterial isolates collected from wound swabs of Patients attending Ondo State Specialist Hospital Akure. It is observed in this study that out of five hundred and one (501) bacterial isolates collected from three hundred and forty-seven (347) wound swabs of patients attending Ondo State Specialist Hospital, Akure. *Staphylococcus aureus* (174, 34.73%) has the highest percentage frequency in wound infection which is in agreement with the work done by Akpaka *et al.* (31], Haque e*t al.* [32] and Den Heijer *et al.* [33] which reported that *S. aureus* is the major predominant causative agent of wound infections. Most times they can exist as normal flora of the skin, however if there is a break in the skin from a wound or surgery, or if there is a suppression of a person's immune system, then the presence of *S. aureus* in the skin can cause an infection [34].

In this study, Out of 10 antibiotics used for testing namely; Vancomycin (30µg) (VAN), Methicillin (MET) (1µg), Cotrimoxazole (25µg) (COT), Gentamicin (10µg) (GEN), Tetracycline (30µg) (TET), Netilmicin (30µg) (NET), Amoxicillin (30µg) (AMC), Augmentin (30µg) (AUG), Ofloxacin (5µg) (OF) and Levoxin (5µg) (LE). This study showed that Levoxin, ofloxacin and Amoxicillin had the highest inhibition against the bacterial isolates which is in agreement with workdone by [13, 35, 2].

Eugene *et al.* [36] reported that resistance shown to antibiotics by the bacteria might be due to various reasons such as gross misuse of these drugs, over production of target sites, inactivation of the antibiotic, alteration of membrane permeability and spontaneous mutation through DNA transfer amongst others.

In this study, plasmid analysis shows that some multiple resistant bacterial isolates harbor one or more plasmids with different molecular weights while some multiple resistant bacterial isolates harbor no plasmid which denoted that their resistant is chromosomal borne. This study is in agreement with workdone by Van Hal *et al.* [37] which reported that Multi Drug Resistance plasmids could be acquired by susceptible bacteria during treatment with antibiotics that can induce and select for horizontal transfer. Agbagwa *et al.* [38] also reported that the possession of plasmids may have acquired their resistance through selective pressure from increased use and misuse of antimicrobial agents. Oleghe *et al.* [39] presumed that the acquisition of resistance

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may b e due to chromosomal mutations or through plasmids that are capable of transfer from one strain of organism to another even across the species in addition to environmental influence. Thus, the gene coding for antibiotics resistance may either coded on the plasmid and chromosomal DNA.

In this study, multiple antibiotics resistant *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* that were resistant before plasmid curing became sensitive to the prescribed antibiotics after curing with ethidium bromide. This showed that the resistance is plasmid borne not chromosomal. Multiple antibiotics resistant *Klebsiella pneumoniae*, *Escherichia coli* and *Streptococcus pyogenes* were resistant before plasmid curing still retain their resistant to prescribed antibiotics after curing with ethidium bromide. This showed that the resistance is chromosomal borne not plasmid borne.

It has been documented that plasmid have encoded gene that provides resistance to naturally occurring antibiotics in competitive environmental niche [40, 41]. Findings from this study is in agreement with the work done by [39, 42] which reported that resistance shown by some bacterial isolates was mainly plasmid mediated e.g *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, resistances shown by some of the bacterial isolates are chromosomal mediated e.g *Klebsiella pneumoniae*, *Escherichia coli* and *Streptococcus pyogenes*. Resistance to bacterial organisms not due to plasmid or chromosome might be due to efflux pump mechanism [43] or other factors like mutation of gene encoding ribosomal protein which decrease permeability of the cell envelope in enteric bacteria [44]. The screening of the bacterial isolates with ethidium bromide resultantly suggest that the resistance markers were stably lost which is in line with previous studies of [45, 46, 46].

## 5.0 CONCLUSION

There is an alarming increase of infections caused by antibiotic-resistant bacteria. This study has highlighted diverse plasmid profiles and wide spread antimicrobial resistance patterns of some clinical bacterial isolates from wound swabs in Akure Nigeria. Therefore the rational use of antibiotics must be a priority. Public health policy on appropriate prescribing and use of antibiotics must be instituted and affected based on recent antibiogram tests. This study showed that the genetic basis for antibiotic resistance in the study area is not entirely plasmid mediated. Resistances seen in the bacterial isolates were either chromosomal mediated or plasmid mediated.

## 7.0 REFERENCES

1. David, O. M. and Famurewa, O. (2010). Toward effective management of nosocomial infections in Nigerian Hospitals-A review. *Academic Arena,* **2**(5): 1-7.

2. Ajao, T. and S. E. Yakubu (2015). Identification, Characterization and Plasmid Profiling of Multi Drug Resistant Nocomial Pathogens Isolated from Selected Hospitals in Ilorin Metropolis. *British Microbiology Research Journal,* **5**(1): 33-43.
3. Alvarado, C. J. (2000). The Science of Hand Hygiene: A Self-Study Monograph. University of Wisconsin Medical School and Sci-Health Communications. Cairo, Egypt. pp. 142-146.
4. Olajubu, F. O., Osinupebi, O. A., Lawal, I., Obadina, B. and Deji-Agboola, A. M. (2012). Pattern of hospital associated infections in a teaching hospital in Nigeria. *Asian Pacific Journal of Tropical Disease*, S869-S873.

UNDER PEER REVIEW

5. Asefzadeh, M. (2005). The risk of nosocomial infections for NCD patients. *NCD Malaysia*, **4**: 8-12.

6. Bloom, B. R. (2000). Microbial infections and immune defence. *Nature*, **406**:759.

7. Timothy, O. and Idu, M. (2011). Preliminary phytochemistry and in vitro antimicrobial properties of aqueous and methanol extracts of *Icacina trichantha. Olive Leaf*, **1**(3): 184–188.

8. McDougal, L. K., Fosheim, G. E., Nicholson, A., Bulens, S. N., Limbago, B. M., Shearer, J. E. S., Summers, A. O. and Patel, J. B. (2010). Emergence of Resistance among USA300 methicillin resistant *Staphylococcus aureus* isolates causing invasive disease in the United States. *Antimicrobial Agents and Chemotherapy*, **54**(9): 3804–3811.

9. Hena, J. V. and Sudha, S. S. (2011). RFLP analysis of clinical MRSA isolates. *International Journal of Pharmacy and Biological Sciences,* **2**(1): 637-645.

10. Khadri, H. and Alzohairy, M. (2010). Prevalence and antibiotic susceptibility pattern of methicillinresistant and coagulase-negative staphylococci in a tertiary care hospital in India. *International Journal of Medicine and Medical Sciences*, **2**(4): 116-120.

11. Okwu, M., Bamgbala, S. and Aborisade, W. (2010). Prevalence of nasal carriage of community associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) among healthy primary school children in Okada, Nigeria. *Journal of Natural Sciences Research*, **12**(4): 61-65.

12. Moussa, I. M., Al-Qahtani, A. A., Gassem, M. A., Ashgan, M. H., Ismail, D. K., Ghazy, A. I. and Shibl, A. M. (2011). Pulsed-field gel electrophoresis (PFGE) as an epidemiological marker for typing of methicillin-resistant *Staphylococcus aureus* recovered from KSA. *African Journal of Microbiology Research*, **5**(12): 1492-1499.

13. Chibuike, I., Reginald, A. O. and Solomon, C. U. (2014). Antibiotic Resistance Patterns and Plasmid Profiles of Methicillin Resistant *Staphylococcus aureus* Isolates from Human Samples. *British Microbiology Research Journal,* **4**(2): 185-194.

14. Olowe, O. A., Eniola, K. I. T., Olowe, R. A. and Olayemi, A. B. (2007). Antimicrobial susceptibility and betalactamase detection of MRSA in Osogbo, SW Nigeria. *Nature and Science*, **5**(3): 44-48.

15. Dubery, D., Rath, S., Sahu, M. C., Pattnaik, L., Debata, N. K. and Padhy, R. N. (2013). Surveillance of infection status of drug resistant *Staphylococcus aureus* in an Indian teaching hospital. *Asian Pacific Journal Tropic Disease,* **3**(2): 133-142.

16. Lowy, F. D. (2003). Antimicrobial resistance: the example of *Staphylococcus aureus. Journal Clinical Investment,* **111**: 1265-1273.

17. Grohmann, E., Muth, G. and Espinosa, M. (2003). Conjugative plasmid transfer in Gram-positive bacteria. *Microbiology Molecular Biology Reviews,* **67**(2):277-301.

18. Kalpana, C. and Harish, K. B. (2015). Plasmid Mediated Methicillin and Vancomycin Resistant *Staphylococcus aureus* Isolated From Northern India. *Journal of Agricultural and Biological Science*, **10**(3): 1990-6145

19. Benjamin, P., Howden, B. P., John, K., Davies, J. K., Johnson, P. D. R. and Stinear, T. P. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clinical Microbiology Reviews,* **1**: 99-139.

UNDER PEER REVIEW

20. Hiramatsu, K. (2001). Vancomycin-resistant *Staphylococcus aureus*. A new model of antibiotic resistance. *Lancet Infectious Disease*, **1**: 147-155.
21. Nwoire, A, Madubuko, E. F., Eze, U. A., Oti-Wilberforce, R. O., Azi, S. O. and Ibiam, G. A. (2013). Incidence of *Staphylococcus aureus* in clinical specimens in Federal Teaching Hospital. Abakaliki, Ebonyi State. *Merit Research Journal of Medcine and Medical Sciences,* **1**(3): 043-046.
22. Church, D., Elsayed, S., Reid, O., Winston, B. and Lindsay, R. (2006). Burn wound infections. *Clinical Microbiology Reviews.* **19**(2): 403-434.
23. Cowon, S. T. and Steel, K. J. (2002). Manual for the identification of medical bacterial 2nd edition, Cambridge University Press, pp. 51-60.
24. Cheesbrough, M. (2010). District Laboratory practice in Tropical Countries, 3rd edition, Cambridge, University Press, United Kingdom. pp. 70-95,143
25. Oyeleke, S. B., Dauda, B. E. N. and Boye, O. A. (2008). Antibacterial activity of *Ficus capensis. African Journal of Biotechnology*, **7**(10): 1414-141.
26. Clinical Laboratory Standards Institute (CLSI) (2012). Performance Standards for Antimicrobial Susceptibility Testing: 20th informational supplement M100-S20. Clinical Laboratory Standards Institute, Wayne, PA., USA.
27. Clinical laboratory and standard institutes (2007). Performance standard for Antimicrobial susceptibility testing: seventeenth informational supplement, **27**(1): 1-182.
28. Chen, H., Hopper, S. L., Li, X., Ljungdahl, L. G. and Cerniglia, C. E. (2006). Isolation of extremely AT- rich genomic DNA and analysis of genes encoding carbohydrate-degrading enzymes from Orpinomyces sp. strain PC-2. *Current Microbiology,* **53**: 396-400.
29. Ezekiel, C. N., Olarinmoye, A. O., Oyinloye, J. M. A., Olaoye, O. B. and Edun, A. O. (2011). Distribution, antibiogram and multidrug resistance in Enterobacteriaceae from commercial poultry feeds in Nigeria. *African Journal of Microbiology Reviews*, **5**(3): 294–301,
30. Akinjogunla, O. J. and Enabulele, I. O. (2010). Virulent factors, plasmid and curing analysis of multi-drug resistant *Staphylococcus aureus* and Coagulase negative *Staphylococcus* species. Isolated from patients with acute otitis media. *Journal of American Science*, **6**: 1022-1033.
31. Akpaka, P. E., Kissoon, S., Swanston, W. and Monteil, M. (2006). Prevalence and antimicrobial Susceptibility pattern of methicillin resistant *Staphylococcus aureus* isolates from Trinidad and Tobago. *Annual Clinical Microbiology Antimicrobials*, **5**: 1476-0711.
32. Haque, M. E., Shahriar, M., Haq, A., Gomes, B. C., Hossain, M. M., Razzak, M. A. and Mazid, M. A. (2011). Prevalence of β-lactamase-producing and non-producing methicillin resistant *Staphylococcus aureus* in clinical samples in Bangladesh Prevalence of β-lactamaseproducing and non-producing methicillin-resistant *Staphylococcus aureus* in clinical samples in Bangladesh. *Journal of Microbiology and Antimicrobials,* **3**(5):112-118.
33. Den Heijer, C. D. J., van Bijnen, E. M. E., Paget, W. J., Pringle, M., Goossens, H., Bruggeman, C. A., Schellevis, F. G. and Stobberingh, G. E. (2013). The APRES Study Team. Prevalence and resistance of commensal *Staphylococcus aureus*, including methicillin-resistant *S. aureus*, in nine European countries: a cross-sectional study. *Lancet Infectious Diseases,* **13**: 409–415.

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34. Koneman, E. W., S. D. Allen, W. M. Janda, Schreckenberger, P. C. and W. C. Winn Jr., (1997). The Gram Positive Cocci. I. *Staphylococci* and Related Organisms. In: Color Atlas and Textbook of Diagnostic Microbiology, Koneman, E.W. (Ed.). 5th Edition, Lippincott/The Williams and Wilkins Co., Philadelphia, PA., USA., pp. 539-576.
35. Agbagwa, O. E. and C.E. Jirigwa, C. E. (2015). Antibiotics Resistance and Plasmid Profile of *Staphylococcus aureus* from Wound Swabs in Port Harcourt Nigeria. *Current Research in Bacteriology,* **8**: 70-76.
36. Eugene, O. T., Ekpo, M. A., Akinjogunla, O. J. and Idiong, D. F. (2013).Microorganisms associated with acute otitis media Diagnosed in Uyo City, *Journal of Nigeria Science Researcher*, **4**(6): 560-564.
37. Van Hal, S. J., Wiklendi, A., Espedido, B., Ginn, A. and Iredell, J. R. (2009). Immediate appearance of plasmid mediated resistance to multiple antibiotics upon antibiotics selection: an augument for systematic resistance epidemiology. *Journal of Clinical Microbiology*, **47**: 2325-2327.
38. Agbagwa, O. E., Otokunefor, T. V. and Frank- Peterside, N. (2012). Plasmid profile analysis of bacteria isolated from selected Honey samples in Nigeria. *Journal of Animal Science Advances*, **2**: 338-343.
39. Oleghe, P. O., Odimegwu, D. C., Udotia, E. and Esimore, C. O. (2011). Multidrug resistance bacterial isolates recovered from herbal medicinal preparations in Southeastern setting, Nigeria. *Journal of Rural and Tropical public health,* **10**: 70-75.
40. Lipps, G. (2008). Plasmid current research and future trends. *Caister Acadamic press*, **97**(8): 1-9.
41. Kroll, J., Klinter, S., Schneider, C. and Steinbucheal, A. (2010). Plasmid addition system. Prespective and application of biotechnology. *Microbiology Journal of Biotechnology,* **3**(6): 634-657.
42. Ojo, S. K. S., Sargin, B. O. and F. I. Esumeh, (2014). Plasmid curing analysis of antibiotic resistance in β-lactamase producing *Staphylococci* from wounds and burns patients. *Pakistan Journal of Biological Sciences*, **17**: 130-133.
43. Poole, K. (2004). ”Efflux-mediated multiple resistance in bacteria.”*Clinical Microbiology and infection,* **10**(1):12-26.
44. Isenberger, K., Hill, D. W., Jenkins, I. E. and Mager, J. T. (2002). Clinical signs of the emergence of bacterial resistance in the hospital environment. *Journal of Applied Microbiology*, **92**:908-978.
45. Akortha, E. E. and Filogona, J. (2009). Transfer of gentamycin resistance gene among enterobacteriaceae isolated from the outpatients with urinary tract infections attending 3 hospitals in mubi, Adamawa State. *Scientific Research Essay*, **4**: 745-752.
46. Gad, G. F. M., Abd El-Ghafar, A. E. G. F., El- Domany, R. A. and Hashem, Z. S. (2010). Epidemiology and antimicrobial resistance of *Staphylococci* isolated from different infectious diseases. *Brazil Journal of Microbiology*, **41**:333-344.
47. Adegoke, A. A. and Okoh, A. I. (2011). The *in vitro* effect of vancomycin on multi-drug resistant *Staphylococcus aureus* from hospital currency notes. *African Journal Microbiology Research*, **5**: 1881-1887.