American Journal of Information Science and Technology
2018; 2(1): 18-23
http://www.sciencepublishinggroup.com/j/ajist
doi: 10.11648/j.ajist.20180201.13

Plasmid Profile Analysis and Curing of Multidrug-Resistant Bacteria Isolated from Hospital Environmental Surfaces in Akure Metropolis, Ondo State, Nigeria

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To cite this article:
Anthony Kayode Onifade, Ogedegbe Gloria Palmer. Plasmid Profile Analysis and Curing of Multidrug-Resistant Bacteria Isolated from Hospital Environmental Surfaces in Akure Metropolis, Ondo State, Nigeria. *American Journal of Information Science and Technology*. Vol. 2, No. 1, 2018, pp. 18-23. doi: 10.11648/j.ajist.20180201.13

Received: April 19, 2018; **Accepted**: May 2, 2018; **Published**: May 24, 2018

Abstract: Plasmid profile analysis and curing of multidrug-resistant bacteria isolated from hospital environment in Akure metropolis, Ondo State Nigeria were investigated. *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Bacillus cereus* were the bacterial isolated in the course of the research. The result revealed that *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed multiple antibiotics resistance. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are rapidly increasing as multidrug resistant strains worldwide. The results of antibiotics sensitivity test on the microorganisms subjected to plasmid analysis indicated that they were more susceptible to antibiotics after the plasmid curing of the microorganisms. The results from this investigative study have shown that resistance in *S. aureus* and *P. aeruginosa* are plasmid based as a result of its loss after curing.

Keyword: Plasmid, Hospital, Environment, Multiple, Antibiotics

1. Introduction

Hospital acquired infections (HAIs) caused by antibiotic resistant bacteria are a significant cause of morbidity and mortality worldwide. In recent decades the incidence of HAI with antibiotic resistant bacteria has increased remarkably. Emerging trends in nosocomial infections signal high alerts towards multidrug resistant pathogens. Studies show that 70% of nosocomial infections are due to antibiotic resistant strains [1, 2]. Major causative agents include antibiotic resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The percentages of antimicrobial resistance continued to increase in Europe leading to mounting healthcare costs, failed treatment and deaths [3]. Some bacteria are naturally resistant to certain types of antibiotics. Recently, the probable involvement of surfaces and equipment from the hospital environment as a disseminating source of pathogens, including resistant bacteria, has been highlighted [4].

Antimicrobial resistance turns into a complex both ecological and clinical problems when considering the genetic variability in microorganisms. Its contention is one of the greatest challenges of the 21st century, and originates appeals from several international health organizations asking for regional data in bacterial susceptibility patterns, especially for strains of nosocomial circulation [5].

Most outbreaks of infection associated with inanimate objects are caused by items that should be sterile but have been contaminated [6]. The surfaces near colonized patients, or those touched frequently by health professionals and by those who move within the area, can become contaminated by antibiotic resistant bacteria, especially methicillin resistant *Staphylococcus aureus* MRSA [7, 8] and vancomycin resistant *enterococcus*-VRE [9].

The wide application of antimicrobial agents in clinical settings to treat infectious disease, as well as their use in aquaculture and veterinary medicine, is of great concern to public health as this can lead to the development and evolution of antibiotic resistant bacteria [10, 11, 12]. This occurs as a result of the selective pressure that antibiotics place on bacteria, resulting in the proliferation and subsequent dissemination of resistant bacteria. Resistance

genes can also be transferred between cells on plasmids or transposons by transductive or conjugative processes [13]. DNA elements which mediate integration of resistance genes (eg, integrons) may also be involved [14] resulting in the further spread of multidrug resistant bacteria.

The aim of this study was to examine plasmid profile analysis and curing of multidrug-resistant bacteria isolated from hospital environmental surfaces in Akure metropolis, Ondo State, Nigeria.

2. Materials and Methods

2.1. Description of Study Location

This research work was carried out from September 2016 to April, 2017 in Akure metropolis, Ondo state, Nigeria. Akure covers an area of 14,798.8,993.7 square kilometers and lies at latitude 7°15′0″N, 7^0 11′ N 5°11′42″E and longitude 5°11′42″E, 5^O35'E. Akure is one of the 18 local government areas of Ondo State with a population of 484,798 based on the 2006 population census. It is situated in the peripheral zone of the rainforest of Ondo state. Akure is the administrative capital of Ondo state. Akure lies about 70°15' north of the equator and 50°15' east of the Meridian. It is about 700 km Southwest of Abuja and 311 km north of Lagos State. The town is situated in the tropic rainforest zone in Nigeria.

2.2. Collection of Samples

Swab Samples were collected by swab sticks from Male Accident and Emergency Bed, Female Accident and Emergency Bed, Male Toilet, Female Toilet, Male Surgical Ward Chair, Female Surgical Ward Chair, Male Medical Ward Floor, Female Medical Ward Floor, Male Ward Air flora, Female Ward Air flora, Theatre Couch, Injection Room Tables, Neonatal Ward Couch and Maternity Ward Couch from Health Centre FUTA, Don Bosco Catholic Hospital and State Specialist Hospital Akure. The date, time, conditions and sites of sampling were noted. Basically, swabs were used, at least, for each sampling site. For sampling, swabs were moistened in 2 mL sterile saline solution and rolled several times over a surface area of around 25 cm^2, and the swab sticks were transported to the laboratory. Sampling was always done between 8-10am

2.3. Isolation of Microorganisms from Hospital Environmental Surfaces

Isolation of microorganisms from hospital environmental surfaces was carried out as described by Bakkali *et al.* [15] with slight modification. Basically, swabs were used, at least, for each sampling site. For sampling, swabs were moistened in 2 mL sterile saline solution and rolled several times over a surface area of around 25 cm^2, and the swab sticks were transported to the laboratory. Sampling was always done between 8-10 am. A five-fold serial dilution was made and 0.1 mL of the 10^{-3} and 10^{-5} dilutions were uniformly pour-plated onto 14 cm diameter wide agar plates and of nutrient agar, Potato dextrose agar, MacConkey agar and EMB agar.

2.4. Characterization of Bacterial Isolates

The pure culture of each isolate was examined. Microscopic examination, staining techniques and biochemical tests were carried out on the isolates according to the methods described by Olutiola *et al.* [16] and Cheesbrough [17].

2.5. Identification of Fungal Isolates

Fungal isolates were characterized and identified based on macroscopic and microscopic details with reference to Barnett and Hunter [18].

2.6. Antibiotic Sensitivity Assay

2.6.1. Standardization of Inoculum

A pure bacterial colony was touched with a loop, and then transferred aseptically into a tube containing 4 to 5 mL of Mueller Hinton broth medium. The broth culture was incubated at 37°C until it achieved the turbidity of the 0.5 McFarland standards which took up to six hours.

2.6.2. Antibiotic Sensitivity Profile Test

The antibiotic sensitivity profile was investigated in order to compare the sensitivity of the microorganisms to the different conventional antibiotics. The disc diffusion method described by Bauer *et al*. [19] was used to determine the susceptibility and resistance of the organisms to the antimicrobial drugs.

2.7. Plasmid Analysis

2.7.1. Determination of Plasmid Profile of Multiple Antibiotics Bacterial Isolates

Plasmid extraction was carried out based on the methods of Molina-Aja *et al*. [20] with little modification. A single bacterial colony was picked up and grown in 5.0 mL of Muller Hilton broth overnight in an Eppendorf tube and centrifuged at 10,000 rpm for 2 min. The cell pellets obtained were re-suspended in 150 µl EDTA–Tris buffer and vortexes to mix. This was followed by the addition of 175 ul of 2% Sodium Dodecyl Sulphate (SDS) and 175ul of 0.4N NaOH. The tube was mixed vigorously, 250 ul of cold 5M potassium acetate was added vigorously, the tube was centrifuged at 12, 000 rpm for 5 min and the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and equal volume of cold isopropanol was added. After inverting gently, the mixture was immediately centrifuged at 12,000 rpm for 10 min and the DNA pellet was washed with 650 µl of cold (40°C) 70% ethanol by centrifuging at 12,000 rpm for 15 min. The supernatant was discarded and the pellet was dried for 30 min and re-suspended in 40 µl of sterile deionised water. Agarose Gel Electrophoresis was carried out by weighing 0.8g of agarose powder and 100 mL of 1X Tris Borate Buffer (TBE buffer) was added, the buffer was dissolved by boiling in a microwave oven and allowed to cool to about 60°C and then 10 ul of ethidium bromide was added and mixed by swirling.

The agarose was then poured into electrophoresis tank with the comb in place to obtain a gel thickness of about 4-5 mm and was allowed to solidify for about 20 minutes and the comb was removed, the tray was then placed in the electrophoresis tank. This was followed by the addition of 1X TBE buffer; this was then poured into the tank ensuring that the buffer covered the surface of the gel. The sample 15 ul was mixed with 2 ul of the loading dye and was carefully loaded into the wells created by the combs (marker was loaded in line 1). Electrodes were connected to the power pack in such a way that the negative terminal is at the end where the sample was loaded; electrophoresis was run at 60-100 V until loading dye has migrated about three-quarter of gel. Electrodes were disconnected and gel was removed from the tank and visualized in UV- trans-illuminator.

2.7.2. Procedure for Plasmid Curing

This was carried out based on the method described by Akinjogunla and Enabulele [21] with slight medication. Fifty microliter (50 µl) of Acridine orange (0.10 mg/mL) was added to 5 mL of Lysogeny broth (LB) followed by subsequent culture inoculation of resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* with plasmid into separate LB broth having acridine orange. These were then incubated at 37°C for 24 hrs in a shaker. After incubation, the cultures were swabbed in to the Muelller Hinton Agar (MHA) plates for confirmatory antibacterial assay.

2.8. Post Curing Susceptibility Testing

After incubation, the standardized inocula of these bacteria were swabbed in to the Muelller Hinton Agar (MHA) plates and incubated at 37°C for 18hrs as a confirmatory antibacterial assay. The plates were examined and the diameters of the zones of inhibition measured to the nearest whole millimeter with a ruler. The sizes of the zone of inhibition were then juxtaposed with those obtained before curing.

2.9. Statistical Analysis of Data

All experiments were carried out in triplicate, and data obtained were subjected to one way analysis of variance, while the means were compared by Duncan's New Multiple Range Test at 95% confidence interval using Statistical Package for Social Sciences version 16.0. Differences were considered significant at $p \leq 0.05$.

3. Results

Table 1. *Rate of occurrence of different bacteria isolated from FUTA Health Centre, State Specialist hospital Akure and Don Bosco Hospital Akure.*

Bacteria	Number of surfaces Tested Positive	Percentage positivity (%)
Staphylococcus aureus	39	22.81
Streptococcus pyogenes	24	14.04
Escherichia coli	21	12.28
Pseudomonas aeruginosa	27	15.79
Klebsiella pneumonia	33	19.30
Bacillus cereus	27	15.79
Total	171	100.01

Table 1: The rate of occurrence of different bacteria isolated from different hospital environmental surfaces is presented in Table 1. It was observed that *Staphylococcus aureus* had the highest rate of occurrence, while *Esherichia coli* had the lowest rate of occurrence out of the bacteria isolated for different hospital environment surfaces.

Table 2. *Rate of occurrence of different fungi isolated from FUTA Health Centre, State Specialist hospital Akure and Don Bosco Hospital Akure.*

Fungi	Number of surfaces Tested Positive	Percentage positivity (%)
Aspergillus fumigatus	21	36.84
Aspergillus flavus	18	31.58
Candida albicans	18	31.58
Total	57	100

Table 2: The rate of occurrence of different fungi isolated from different hospital environmental surfaces is presented in Table 4. It was observed that *Aspergillus fumigatus had* the highest rate of occurrence followed by *Candida albicans* and *Aspergillus niger* which share the same number percentage positivity.

Table 3. *Susceptibility pattern of bacteria isolated from hospital environmental surfaces.*

Antibiotics	*S. aureus*	*S. pyogenes*	*B. cereus*	*P. aeruginosa*	*E. coli*	*K. pneumonia*
COT	4.67 ± 0.58^{c}	14.33 ± 0.58^{b}	15.67 ± 0.58^{e}	0.00 ± 0.00^{a}	15.67 ± 0.58^{e}	10.33 ± 0.58^{b}
CXC	0.00 ± 0.00^{a}	15.33 ± 0.58^{c}	18.00 ± 1.00^{f}	0.00 ± 0.00^{a}	16.67 ± 0.58^{ef}	13.33 ± 0.58^{c}
ERY	0.00 ± 0.00^{a}	17.67 ± 0.58^{ef}	10.33 ± 0.58^{ab}	3.33 ± 0.58^{cde}	13.33 ± 0.58^{d}	10.67 ± 0.58^{b}
GEN	8.67 ± 0.58^{e}	15.33 ± 0.58^{bc}	15.67 ± 0.58^{e}	2.00 ± 1.00^{b}	17.33 ± 0.58^{fg}	18.67 ± 1.15^{g}
AUG	0.00 ± 0.00^{a}	21.00 ± 1.00^{g}	17.33 ± 0.58^{f}	4.33 ± 0.58^{e}	18.33 ± 0.58^{gh}	14.67 ± 0.58^{d}
STR	0.00 ± 0.00^{a}	20.67 ± 0.58^{g}	9.33 ± 0.58^{a}	2.33 ± 0.58^{bc}	3.00 ± 0.00^{b}	15.67 ± 0.58^{e}
TET	8.00 ± 0.00^{d}	9.33 ± 0.58^{a}	10.67 ± 0.58^{b}	0.00 ± 0.00^{a}	15.67 ± 1.15^{e}	13.67 ± 0.58^{c}
CHL	3.67 ± 0.58^{b}	10.33 ± 0.58^{a}	12.33 ± 0.58^{c}	5.67 ± 1.15^{f}	9.67 ± 0.58^{c}	18.33 ± 0.58^{g}
AMX	0.00 ± 0.00^{a}	16.67 ± 0.58^{de}	10.67 ± 0.58^{b}	0.00 ± 0.00^{a}	14.00 ± 1.00^{d}	13.33 ± 0.58^{c}
NIT	4.00 ± 0.00^{b}	16.33 ± 0.58^{cd}	10.00 ± 0.00^{ab}	3.67 ± 0.58^{de}	10.67 ± 0.58^{c}	17.33 ± 0.58^{f}
NAL	0.00 ± 0.00^{a}	10.33 ± 0.58^{a}	14.33 ± 0.58^{d}	9.33 ± 0.58^{g}	19.00 ± 0.00^{h}	17.00 ± 0.00^{f}
OFL	10.67 ± 0.58^{f}	18.00 ± 0.00^{f}	13.33 ± 0.58^{cd}	2.67 ± 0.58^{bcd}	0.00 ± 0.00^{a}	3.00 ± 0.00^{a}

Data are presented as Mean±S.D (n=3). Values with the same superscript letter(s) along the same column are not significantly different ($P<0.05$).
LEGEND: COT= Cotrimazole (25µg), CXC= Cloxacillin (5µg), ERY= Erythromycin (5µg), GEN= Gentamycin (10µg), AUG= Augmentin (30µg), STR= Streptomycin (10µg), TET= Tetracycline (10µg), CHL= Chloramphenicol (10µg), AMX= Amoxacillin (10µg), NIT= Nitrafuratoin (10µg), NAL= Nalixidic acid (30µg), OFL= Ofloxacin (10µg).

Table 3: The susceptibility pattern of gram positive bacteria isolated from hospital environmental surfaces is presented in Table 3. It was observed that *S. aureus* and *P. aeruginosa* had multiple resistant against antibiotics.

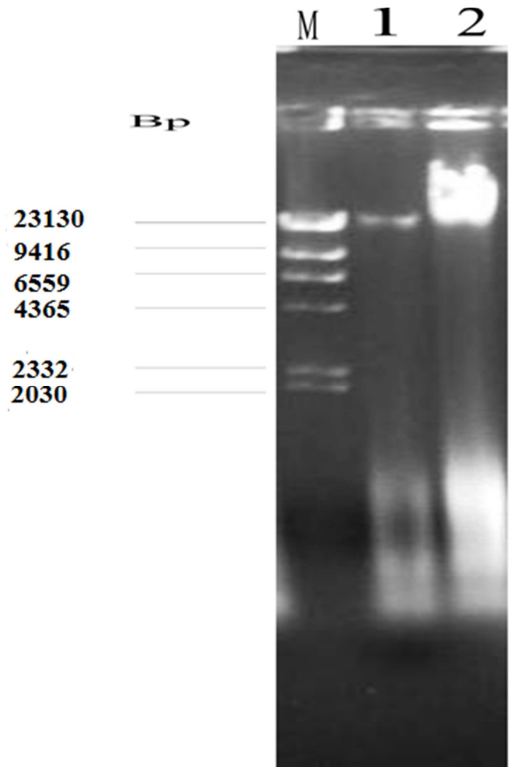

Figure 1. *Electrophoretic patterns for plasmid profile of bacterial isolates from hospital environmental surfaces.*

Keys: M=Molecular weight marker, 1= *Staphylococcus aureus,* 2= *Pseudomonas aeruginosa*, bp= basepair, 1bp= 3.4Angstrom (Å) while 1000bp=1kilo base pairs.

Figure 1: The bacterial were investigated for the presence of DNA plasmids using Agarose-gel electrophoresis. The results obtained revealed the presence of plasmid bands of different molecular weights. The molecular weights of the plasmids were determined using DNA- Hind III molecular weight marker (Figure 1).

Table 4. *Post-curing antibiotics sensitivity patterns of selected Gram positive bacteria.*

Antibiotics	*Staphylococcus aureus*
COT bf	4.67 ± 0.58^{c}
COT af	12.33 ± 0.58^{i}
CXC bf	0.00 ± 0.00^{a}
CXC af	11.00 ± 0.00^{gh}
ERY bf	0.00 ± 0.00^{a}
ERY af	11.67 ± 0.58^{hi}
GEN bf	8.67 ± 0.58^{ef}
GEN af	17.67 ± 0.58^{jk}
AUG bf	0.00 ± 0.00^{a}
AUG af	6.67 ± 0.58^{d}
STR bf	0.00 ± 0.00^{a}
STR af	8.67 ± 0.58^{ef}
TET bf	8.00 ± 0.00^{e}
TET af	17.33 ± 0.58^{j}
CHL bf	3.67 ± 0.58^{b}
CHL af	9.67 ± 1.15^{g}
AMXbf	0.00 ± 0.00^{a}
AMXaf	9.00 ± 0.00^{g}
NITbf	4.00 ± 0.00^{bc}
NITaf	10.67 ± 0.58^{h}
NALbf	0.00 ± 0.00^{a}
NALaf	6.33 ± 0.48^{d}
OFLbf	10.67 ± 0.58^{h}
OFLaf	18.33 ± 0.58^{k}

Data are presented as Mean±S.D (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05).
LEGEND: bf = before curing, af = After curing, COT = Cotrimazole (25µg), CXC = Cloxacillin (5µg), ERY = Erythromycin (5µg), GEN = Gentamycin (10µg), AUG = Augmentin (30µg), STR = Streptomycin (10µg), TET = Tetracycline (10µg), CHL = Chloramphenicol (10µg), AMX = Amoxacillin (10µg), NIT = Nitrafuratoin (10µg), NAL = Nalixidic acid (30µg), OFL= Ofloxacin (10µg).

Table 4: Shows the comparison of antibiotics susceptibility pattern of selected Gram positive bacteria isolate before and after plasmid curing. It was observed that the bacteria were more susceptible to antibiotics after plasmid curing.

Table 5. *Post-curing antibiotics sensitivity patterns of selected Gram negative bacteria.*

Antibiotics	*Pseudomonas aeruginosa*
AMX bf	0.00±0.00a
AMX af	3.33 ± 0.58^{cde}
COT bf	0.00 ± 0.00^{a}
COT af	6.00 ± 1.00^{g}
NIT bf	3.67 ± 0.58^{def}
NIT af	6.67 ± 0.58^{h}
GEN bf	2.00 ± 1.00^{b}
GEN af	4.67 ± 1.15^{fg}
NAL bf	9.33 ± 0.58^{ij}
NAL af	17.33 ± 0.58^{l}
OFL bf	2.67 ± 0.58^{bcd}
OFL af	8.33 ± 0.58^{i}
AUG bf	4.33 ± 0.58^{ef}
AUG af	9.33 ± 0.58^{ij}
TET bf	0.00 ± 0.00^{a}
TET af	8.33 ± 0.58^{i}
CXCbf	0.00 ± 0.00^{a}
CXCaf	5.67 ± 0.58^{gh}
ERYbf	3.33 ± 0.58^{cde}
ERYaf	10.33 ± 0.58^{j}
STRbf	$2.33\pm0.58b^{c}$
STRaf	6.33 ± 0.58^{h}
CHLbf	5.67 ± 1.15^{gh}
CHLaf	15.67 ± 0.58^{k}

Data are presented as Mean±S.D (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05).
LEGEND: bf= before curing, af = After curing, AMX = Amoxacillin (10µg), COT = Cotrimazole (25µg), NIT= Nitrafuratoin (10µg), GEN = Gentamycin (10µg), NAL= Nalixidic acid (30µg), OFL = Ofloxacin (10µg), AUG = Augmentin (30µg), TET = Tetracycline (10µg), CXC = Cloxacillin (5µg), ERY = Erythromycin (5µg), STR= Streptomycin (10µg), CHL = Chloramphenicol (10µg).

Table 5: Shows the comparison of antibiotics susceptibility pattern of selected Gram negative bacteria isolate before and after plasmid curing. It was observed that the bacteria were

more susceptible to antibiotics after plasmid curing.

4. Discussion

Plasmid profile multidrug-resistant bacteria isolated from hospital environmental surfaces in Akure metropolis, Ondo State, Nigeria was investigated. Bacteria were found to be more predominant than fungi, the bacteria isolated from the hospital environmental surfaces were *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes* while fungi include *Aspergillus fumigatus*, *Aspergillus niger* and *Candida albicans*. *Staphylococcus aureus* was found to be predominant bacteria with the occurrence of 22.81%, this correlate with the report of Awosika *et al*. [22] who reported *Staphylococcus aureus* as the most frequently isolated bacterium from hospital surface. *Aspergillus funmigatus* was found to be predominant fungi with frequency occurrence of 36.84%, this correlate with the report of Cagginao *et al*. [23] who reported that *Aspergillus fumigatus* was the most commonly isolated (68.5%).

Staphylococcus aureus and *Pseudomonas aeruginosa* had high multiple resistances to antibiotics than other bacteria isolated in the course of the research, this correlate with report of Hauser and Sriram [24], Maltezou and Giamarellou [25] reported that Infections caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* are increasing both in hospitals and in general community. The efficacy of many antibiotics for treatment of severe infections has become quite limited due to the development of resistance. Seza and Fatma [26] reported that among the Gram positive bacteria, *Staphylococci* are the most frequently resistant pathogen to antibiotics. Infection caused by *P. aeruginosa* are often severe, life threatening and difficult to treat because of limited susceptibility to antimicrobial agents and high frequency of emergence of antibiotics resistance during therapy [27]. Multi Drug Resistance has usually been described as developing in a susceptible strain of *P. aeruginosa* exposed sequentially to various antibiotic agents [27]. According to the literature, the high level of antimicrobial resistance to drugs used in hospitals and in the community constitutes an important alert to this severe phenomenon, which is considered one of the great challenges to science and medicine in the 21st century [5]. The high levels of resistance shown by CNSs against penicillin, oxacillin, erythromycin, azithromycin and clindamycin are relevant since these antimicrobials are used in the hospitals and in the community. The *Staphylococcus aureus* and *Pseudomonas aeruginosa* were subject to plasmid curing, and the microorganisms were susceptible to antibiotics after plasmid curing. Plasmids frequently carry genes for antibiotic resistance, toxigenicity and can as well confer extremophily status on microorganisms. The functions of these plasmids have classically been correlated with phenotypical properties, including drug resistance, carbohydrate metabolism, amino-acid metabolism, carotenoids, cholic acid derivatives, organic acids and bacteriocins production. Plasmids are useful markers in Recombinant DNA technology and as such this makes plasmids indispensible tool in Molecular Biology [28, 29]. The results from this investigative study have shown that resistance in *S. aureus* and *P. aeruginosa* are plasmid based as a result of its loss after curing. The result showed by the isolates was plasmid mediated.

5. Conclusion

This research has been able to investigate, identify and prove the sensitivity patterns of microorganism isolated from hospital environmental surfaces, plasmid profile analysis and curing of multidrug-resistant bacteria isolated from hospital environmental in Akure metropolis, Ondo State Nigeria. *Staphylococcus aureus* and *Pseudomonas aeruginosa* have high multiple resistances to antibiotics than other bacteria isolated in the course of the research and after the *Staphylococcus aureus* and *Pseudomonas aeruginosa* were subject to plasmid curing the microorganisms were susceptible to antibiotics.

Regular surveillance of hospital and community associated *S. aureus* and *Pseudomonas aeruginosa* and their susceptibility to antibiotics is necessary to prevent an outbreak and spread of resistant strains in the locality.

Acknowledgements

The Author wish to acknowledge Mr. ADEGOKE, Tosin Victor for his contribution towards the research.

Competing Interests

The authors declare that they have no competing interests.

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