MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT *Staphylococcus aureus* FROM CLINICAL SPECIMENS AND ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS AGAINST THE ISOLATES.

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CERTIFICATION

This is to certify that that work 'Molecular Characterization of Methicillin Resistant *Staphylococcus aureus* from Clinical Specimens and Antimicrobial Activity of Plant Extracts, Against the Isolates' was carried out by Ifediora, Afoma Chinwe (FUTO/PG/20144940138 in partial fulfilment for the award of the degree of PhD in Medical Microbiology, in the Department of Microbiology of the Federal University of Technology, Owerri.

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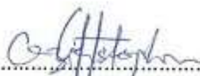

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DEDICATION

This work is dedicated to the loving memory of my beloved father, Sir Pius I. Ufudo.

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TABLE OF CONTENTS

Certification ii

Dedication iii

Acknowledgements iv

Table of Contents v

List of Tables xv

List of Figures xvi

Abstract xviii

CHAPTER ONE 1

INTRODUCTION 1

1.1 Background Information **1**

1.2 Problem Statement 3

1.3 Justification 4

1.4 Aim of the research 5

1.5 Specific Objectives 5

CHAPTER TWO 6

LITERATURE REVIEW 6

2.1 The Genus *Staphylococcus* 6

2.1.1 Scientific Classification 7

2.2 *Staphylococcus aureus* 7

2.2.1 *Staphylococcus aureus* cell wall 8

2.2.2 Important Properties of Staphylococci 10

2.2.3 Genome of *Staphylococcus aureus* 11

2.3 Virulence Determinants in *S. aureus* 13

2.3.1 Cell Surface Factors 14

2.3.2 Secreted Factors (Exotoxins) 15

2.3.2.1 Superantigens 15

2.3.2.2 Cytolytic (pore-forming) toxins 16

2.3.2.3 Various exoenzymes 16

2.3.2.4 Miscellaneous Proteins 17

2.4 Pathogenesis 17

2.5 Epidemiology of *S. aureus* infections 20

2.6 Bacterial Drug Resistance 22

2.6.1 Causes of Antibiotic Resistance 22

2.6.2 Antistaphylococcal Agents and Resistance 23

2.6.2.1 Beta-lactam Drugs 23

2.6.2.2 Macrolide, Lincosamide and Streptogramin 27

2.6.2.3 Aminoglycosides 27

2.6.2.4 Glycopeptide 28

2.6.2.5 Fluoroquinolone 30

2.6.2.6 Linezolid 31

2.6.2.7 Daptomycin 31

2.6.2.8 Ceftaroline 32

2.6.2.9 Rifampicin 32

2.7 Methicillin Resistant *Staphylococcus aureus* (MRSA) 33

2.7.1 Historical Evolution of MRSA 33

2.7.2 Mechanism of Methicillin Resistance 34

2.7.2.1 Regulation of PBP2a Expression 35

2.7.2.2 Staphylococcal Cassette Chromosome Mec (Sccmec) 36

2.7.2.3 Mec gene complex. 36

2.7.3 Healthcare-Associated MRSA (HA-MRSA) 37

2.7.4 Community-Associated MRSA strains 38

2.8 Diseases caused by MRSA 40

2.8.1 Bacteremia 41

2.8.2 Impetigo 41

2.8.3 Endocarditis 41

2.8.4 Toxic Shock Syndrome (TSS) 42

2.8.5 Food poisoning 43

2.8.6 Staphylococcal Scalded Skin Syndrome (SSSS) 44

2.9 Diagnostic Identification of MRSA from Clinical Specimens 45

2.9.1 Molecular identification and characterization assays of MRSA strains 46

2.9.2 Application of PCR assays for identification of MRSA 46

2.9.3 Plasmid profile analysis 47

2.9.4 Random Amplified Polymorphic DNA-PCR 48

2.9.5 Pulsed-field gel electrophoresis for genotyping of MRSA strains 50

2.9.6 Restriction Endonuclease Analysis (REA) of Chromosomal DNA 50

2.9.7 Staphylococcal Cassette Chromosome mec (SCCmec) typing 51

2.10 Treatment of *S.aureus* infections 51

2.11 Newer agents with MRSA Activity 52

2.11.1 Ceftaroline 52

2.11.2 Tedizolid 52

2.11.3 Telavancin 53

2.12 New Combination Therapy 53

2.13 Vaccine development for the prevention of *S. aureus* infections 54

2.14 Other Management Strategies: (Medicinal Plants) 55

2.14.1 Plant Derived Antimicrobials 56

2.14.2 *Acalypha wilkesiana* 58

2.14.2.1 Uses 59

2.14.2.2 Pharmacological Properties 59

2.14.3 *Alchornea cordifolia* (Euphorbiaceae) 60

2.14.3.1 Uses 60

2.14.3.2 Ethno-medicinal Properties of *Alchornea cordifolia* 61

CHAPTER THREE 63

3.1.0 MATERIALS 63

3.1.1 Study Site 63

3.1.2 Culture media 63

3.1.3 Chemical reagents 63

3.1.4 Glassware 63

3.1.5 Equipment 64

3.1.6 Reference bacterial strains 64

3.1.7 Antibiotics used 64

3.1.8 Polymerase Chain Reaction (PCR) Primers 64

3.1.9 Sample types 64

3.2 **Methods** 66

3.2.1 Isolation 66

3.2.2 Pure cultures 66

3.3 Identification Of bacterial Isolates 66

3.3.1 Gram stain 66

3.4 Biochemical Tests 67

3.4.1 Catalase Test 67

3.4.2 Coagulase Test 67

3.4.3 Deoxyribonuclease (DNAse) Test 67

3.4.4 Indole Test 68

3.4.5 Citrate Utilization tests 68

3.5 Antibiotic Susceptibility Tests 68

3.6 Detection of Methicillin Resistance 69

3.6.1 Cefoxitin disc diffusion 69

3.6.2 Latex Agglutination Test 69

3.7 Determination of inducible and constitutive MLSB resistance 70

3.8 Test for β-lactamase Production 70

3.9 Molecular Screening 71

3.9.1 DNA extraction (Boiling method) 71

3.9.2 DNA Quantification 71

3.9.3 Amplification of mecA genes 71

3.9.4 Amplification of ermB gene 72

3.9.5 Amplification of blaZ gene 72

3.9.6 Amplification of pvl genes 73

3.9.7 Amplification of the 16SrRNA of the bacterial isolates 73

3.9.8 Sequencing 74

3.9.9 Random Amplified Polymorphic DNA for Clonal Relatedness 74

3.9.10 Phylogenetic Analysis 74

3.10 Plant Antimicrobial Sensitivity Testing 78

3.10.1 Collection of Plant Material 78

3.10.2 Extraction of Plant Material 78

3.10.3 Antimicrobial Activity of Some Plant Extracts on MRSA Isolates 79

3.10.4 Minimum Inhibitory Concentration (MIC) 79

3.10.5 Minimum Bactericidal Concentration (MBC) 80

3.10.6 Rate of Killing of the Organism by the Extracts 80

3.11 Phytochemical Analysis 80

3.11.1 Determination of Saponins 80

3.11.2 Determination of Tannins 81

3.11.3 Determination of Alkaloids 82

3.11.4 Determination of Flavonoids 83

3.11.5 Determination of Phenols 83

3.11.6 Determination of Glycoside 84

CHAPTER FOUR 85

RESULTS AND DISCUSSION 85

4.1 Results 85

4.1.1 Identification of *Staphylococcus aureus* 85

4.1.2 Prevalence of *S. aureus* among age and gender 85

4.1.3 Prevalence of *S. aureus* among clinical sample type 88

4.1.4 Antibiotic susceptibility profile of *S aureus* isolates 88

4.1.5 Phenotypic Detection of Methicillin Resistance 95

4.1.6 Prevalence of MRSA and MSSA from the 3 Zones 95

4.1.7 Antibiogram of MRSA and MSSA among the 3 Zones 95

4.1.8 Comparison of Antimicrobial Susceptibility Profiles of MRSA and MSSA 99

4.1.9 Resistance Profile of MRSA from Clinical Sites to Various Antibiotics 99

4.1.10 Prevalence of Inducible Clindamycin Resistance among isolates 103

4.1.11 Beta lactamase production among *S. aureus* isolates 106

4.1.12 Amplification of 16SrRNA 108

4.1.13 Phylogenesis obtained from 16SrRNA Sequencing 108

4.1.14 PCR Amplification of Antibiotic Resistance Genes 111

4.1.14.1 Amplification of *mecA* Gene 111

4.1.14.2 Amplification of *blaZ* gene 111

4.1.14.3 *ermB* gene amplification 111

4.1.15 Virulence gene profiling 115

4.1.15.1 Amplification of *pvl* gene 115

4.1.16 Gene Distribution among the MRSA Isolates 115

4.1.17 Occurrence of Genes among the Clinical Samples 115

4.1.18 RAPD Analysis 115

4.1.19 Molecular size of obtained RAPD Amplicons 116

4.1.20 Result of Antibacterial Activities of the Plant Extracts 123

4.1.21 MIC and MBC Obtained from the Two Plants 123

4.1.22 The rate of killing of the extracts on the strains 123

4.1.23 Quantitative Phytochemical Composition of the Two Plants 123

4.**2** **Discussion** 131

4.2.1 Prevalence of *S. aureus* and MRSA 131

4.2.2 Antibiotic resistance in *S.aureus* strains 132

4.2.3 Inducible clindamycin resistance and detection of *ermB* genes 134

4.2.4 Phenotypic and molecular detection of MRSA 137

4.2.5 Prevalence of *pvL* genes among the MRSA isolates 142

4.2.6 PCR-RAPD 143

4.2.7 Plant Antimicrobial and Phytochemistry 146

CHAPTER FIVE 150

5.0 Conclusion and Recommendations 150

5.1 Conclusion 150

5.2 Recommendation 151

5.3 Contributions to Knowledge 152

References 153

Appendix 1 177

LIST OF TABLES

Table	Title	Page
2.1	Prevalence of MRSA carriage in some countries across the world	39
3.1	Sequences and amplified product size of primers used in polymerase chain reaction assay	76
4.1	Prevalence of *S. aureus* among various age groups and genders	87
4.2	Distribution of staphylococcal isolates obtained from different clinical samples	89
4.3	Antibiotic susceptibility profile of *S. aureus* isolates from the 3 zones	93
4.4	Comparison of *S. aureus* resistance from various sample sites	94
4.5	Phenotypic detection of MRSA using oxacillin and cefoxitin discs	96
4.6	Prevalence of MRSA and MSSA from clinical samples from the 3 zones	97
4.7	Antibiotic resistance profile of MRSA and MSSA among the zones	98
4.8	Antibiotic sensitivity profile of MSSA and MRSA isolates	100
4.9	Resistance patterns of MRSA from clinical sites to various antibiotics	101
4.10	Prevalence of multiple-drug resistance among MRSA isolates	102
4.11	D test among methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) among the zones	104
4.12	Overall phenotypic pattern of inducible clindamycin resistance among MRSA and MSSA	105
4.13	Pattern of genes clustering among the isolates	119
4.14	Molecular Size of RAPD Amplicon (bp)	122
4.16	Antimicrobial effect of the ethanol extracts of *Alchornea cordifolia and Alcalypha wilkesiana* against MRSA	124
4.17	Antimicrobial effect of the aqueous extracts of *Alchornea cordifolia and Alcalypha wilkesiana* against MRSA	125
4.18	MIC and MBC of ethanol extract of *Alchornea cordifolia* against methicillin Resistant *Staphylococcus aureus*	126
4.19	MIC and MBC of ethanol extract of *Alcalypha wilkesiana* against methicillin resistant *Staphylococcus aureus*	127
4.20	Quantitative phytochemical composition of the plants	130

LIST OF FIGURES

Figures	Title	Page
2.1	Structure of *S. aureus*	9
2.2	Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin	26
4.1	Distribution of *S. aureus* by senatorial zone	86
4.2	Antibiotic susceptibility profile of *S. aureus* isolates from Umuahiasenotorial zone	90
4.3	Antibiotic susceptibility profile of *S. aureus* isolates from Aba senotorial zone	91
4.4	Antibiotic susceptibility profile of *S. aureus* isolates from Isiukwuato senotorial zone	92
4.5	β-lactamase production among the MRSA isolates	107
4.6	Agarose gel electrophoresis showing the 16rRNA gene of the bacterial isolates.	109
4.7	Phylogenetic tree showing the relationship between the query 16SrRNA nucleotide of *S. aureus* strains.	110
4.8	Agarose gel electrophoresis of the *mec* A gene of the *S. aureus* isolates.	112
4.9	Agarose gel electrophoresis showing the amplified *BlaZ* gene bands	113
4.10	Agarose gel electrophoresis of the *erm B* gene of the *S. aureus* isolates.	114
4.11	Agarose gel electrophoresis of the *pvl* gene of the *S. aureus* isolates.	117

4.12	Gene distribution among the MRSA isolates	118
4.13	Prevalence of genes among the clinical samples	120
4.14	Dendogram of genetic relationship of the isolates	121
4.15	Rate of kill of the Isolates by *Alchornea cordifolia*	128
4.16	Rate of kill of the Isolates by *Alcalypha wilkesiana*	129

ABSTRACT

Staphylococcus aureus is a major bacterial pathogen that causes different community and hospital-acquired infections. *S. aureus* resistant to methicillin has become a big and expanding problem of concern in many developing countries. This study examined the molecular characterization and the effect of plant extracts on methicillin resistant *Staphylococcus aureus* (MRSA) from clinical specimens in Abia State, Nigeria using standard recommended procedures. Conventional cultural, morphological and biochemical methods were used to identify the isolates, while the antimicrobial susceptibility testing was performed by the disc diffusion method. Methicillin resistance was detected phenotypically using cefoxitin 30μg disc and oxacillin 1μg disc. Inducible clindamycin resistance was evaluated by the D-test. Polymerase chain reaction (PCR) was used to amplify genes for methicillin resistance (*mecA*), clindamycin resistance (*ermB*), beta-lactamase production (*blaZ*), Panton Valentine leukocidin (*pvl*) with 16SrRNA gene being the internal control. Sequencing was carried out on the amplified isolates. The Random Amplified Polymorphic DNA (RAPD) was implemented on the *mecA* strains isolated using three randomly selected oligonucleotide primers. Plant antimicrobial assay was done using the agar well technique and phytochemicals detected in the two plants tested. A total of 750 clinical specimens of blood, urine samples, wound, ear, nasal, high vaginal, urethral and ear swabs were collected from three major health facilities located in the three senatorial zones of Abia State, Nigeria. A total of 265 (35.3%) *S. aureus* isolates were recovered, out of which 126(47.5%) were from males and 139(52.5%) were from females, however there was no association between the prevalence and gender (p-value = 0.05) and also prevalence and age (p-value = 0.52). Phenotypic detection of MRSA using cefoxitin disc diffusion gave an MRSA prevalence of 164(61.9%) with 65(39.6%) being from urine, 3(23.1%) from wound, 31(18.9%) from high vaginal swab, 22(13.4%) from urethral swab, 4(2.4%) from ear swab, 3(1.8%) from nasal swab and 1(0.6%) from blood samples. All (100%) of the MRSA were susceptible to vancomycin, 120(73.2%) to clindamycin, 92 (56.1%) to gentamycin. All were resistant to ceftazidine, 157(95.7%) to cloxacillin, 146(89.0%) to augmentin, 136(82.9%) to ceftriaxone and 103(61.6%) to erythromycin. The MRSA strains showed much higher resistance rate than their methicillin susceptible *Staphylococcus aureus* (MSSA) counterparts to all tested antibiotic except clindamycin. Exactly 64(39.0%) of the MRSA were resistant to 4 classes of antibiotics indicating multi drug resistance (MDR). The overall prevalence of inducible clindamycin resistance among methicillin resistant isolates was 29(17.7%) while 66.5%

produced beta-lactamase. Out of 40 cefoxitin positive isolates, 12 (30%) possessed *mecA* gene, 17.5% harboured the β-lactamase (*blaZ)* gene, 20% and 10% possessed the *pvl* gene. Dendogram analysis of RAPD-PCR amplification of *mecA* positive strains showed three different clones in circulation in the state. The plant extracts showed varied levels of antimicrobial activity against the MRSA isolates. The growth of the microorganisms used for the test was inhibited by the ethanolic extracts of the leaves of *Alchornea cordifolia* and *Acalypha wilkesiana* at concentrations of 50mg/ml to 200mg/ml. The inhibition zones ranged from 9.0mm to 21.0mm whereas the water extracts showed moderate activity against the isolates. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Alchornea cordifolia* ranged from 12.5-50mg/ml and 25-100mg/ml for *Acalypha wilkesiana.* The results of the rates of kill revealed a gradual reduction in the total viable count of bacteria from 1hr to 24hrs in all the test isolates. The phytochemical screening of the ethanol extract revealed the presence of tannins, flavonoids, glycosides, resins and carbohydrates but in variable degrees. The percentage yields of phytochemical content of the leaves of the *Alchornea cordifolia* plants were as follows: alkaloids (1.85%), flavonoids (1.08%), Glycosides (1.05%), saponins (4.13%), and tannins (0.70%). The use of phenotypic and molecular methods in this study provided useful information on antibiotic resistance and genetic diversity of *S. aureus* isolates from clinical specimens in Abia State of Nigeria. The information provided could help in monitoring the evolution of *S. aureus* strains in Nigeria over time.

Key words; *Staphylococcus aureus,* MRSA, D-Test, RAPD, *Alchornea cordifolia, Acalypha wilkesiana,*

CHAPTER ONE

1.1 INTRODUCTION

Staphylococcus aureus is a Gram positive, non sporing, facultative anaerobe that belongs to the family Staphylococaceae. The organism often colonizes the skin, skin glands and mucous membranes especially the anterior nares of healthy individuals (Plata *et al*., 2009). Around 20-30% of the populations permanently carry *S. aureus* asymptotically in their nose (Sakr *et al.,* 2018). Colonization by a strain of *S. aureus* has the adverse effect of making the individual vulnerable to subsequent infection by the colonizing strain (Gordon & Lowy, 2008).

Staphylococcus aureus has been one of the leading causes of human infection throughout history. From 1997-1999, it was implicated as the most abundant cause of skin and soft tissue, bloodstream and lower respiratory tract infections in the United States, Europe, Canada, Latin America and Western Pacific Coast (Diekema *et al*.,2001). In Nigeria and Africa, a high proportion of *S. aureus* is reported from urinary tract infections (Ayepola *et al*., 2015). Other infections associated with *S. aureus* include endocarditis, mastitis, meningitis, osteomyelitis, phlebitis, pneumonia as well as toxic shock syndrome and food poisoning (Bhatia & Zahoum, 2007; Shittu *et al*., 2011). These infections caused by *S. aureus* can manifest in virtually all the organs and tissues of the human body with the skin reportedly the most affected part of the body (Daum, 2007).

The pathogenesis of infection caused by this organism is attributed to the expression of an array of virulence determinants. Reports from different authors have assigned over 50 potential virulence factors that include toxins and enzymes to these organisms and these are carried either on the chromosomes or on mobile genetic elements found in the organisms (Bal & Gould, 2005).

Treatment of *S. aureus* infections was originally carried out with the antibiotic penicillin but resistance soon followed and it was discovered that the organism produced penicillinase; an enzyme capable of hydrolyzing penicillin (Rayner & Munckhof, 2005). By 1960, about 80% of all strain of *S. aureus* had acquired resistance and in recent decades there is increase in resistance due to evolution of the bacteria and drug abuse (Deurenberg & Stobberingh, 2008; Guo *et al*., 2020).

By 1960, penicillin therapy was replaced with methicillin (a semi- synthetic penicillin) and resistance to it was also reported in less than 1 year (Chambers, 2001; Lowy, 2003). This group of *S. aureus* resistant to methicillin was termed Methicillin Resistant *Staphylococcus aureus* (MRSA) and they are grouped into Hospital Acquired Methicillin Resistant *Staphylococcus aureus* (HA-MRSA) and Community Acquired Methicillin Resistant *Staphylococcus aureus (*CA-MRSA).

Generally typical CA-MRSA strains display important differences from HA-MRSA strains including production of Panton-Valentine Leukocidin (*pvl*) toxin, low level susceptibility to non beta-lactam antibiotics and carriage of SCC*mec* types IV or V.

Currently MRSA has become a leading cause of hospital acquired infections worldwide accounting for more than 60% of *S. aureus* isolates in hospitals in the United States (Baranovich *et al.,* 2010), *S. aureus* becomes resistant to methicillin when it acquires the gene *mecA* which encodes for the altered penicillin binding protein, PBP2a. This low affinity penicillin binding protein is not inactivated by the drug methicillin and other beta-lactam drugs (Gaze *et. al*., 2008). Following resistance to methicillin, other antibiotics related to penicillin were developed including vancomycin, oxacillin and ampicillin and eventually methicillin was removed from the market. Patients with MRSA infections are treated with

vancomycin while those intolerable to vancomycin are treated with ciprofloxacin, clindamycin and tetracycline but surprisingly reports of resistance have also been observed.

Recently there has been alternative therapeutic options for parenteral treatment of invasive MRSA infections which include newly licensed drugs from new antibiotic classes—linezolid, the first oxazolidinone; daptomycin, the first lipopeptide; and tigecycline, the first glycylcycline. Randomised controlled trials have shown these drugs to be equivalent to vancomycin in the treatment of skin and soft tissue infections involving MRSA (Falagas *et al.,* 2008; FlorescuI *et al.,* 2008; Bamigboye *et al.,* 2018)

Various techniques have been implemented for accurate identification and characterization of MRSA strains including antimicrobial susceptibility testing, agglutination tests, molecular typing techniques including Polymerase Chain Reaction (PCR), Random Amplified Polymorphic DNA (RAPD) etc. However, despite all the advancements in medical sciences, antibiotic resistance is still a huge global concern and this has necessitated the search for new antimicrobials from medicinal plants. It is on records that medicinal plants especially those with antimicrobial properties are largely used in Africa due to their availability, ease of access and such plants could also serve as sources of new antimicrobials and other drugs (Akinyemi *et al*., 2005). For this reason, researchers are steadily focusing their attention to herbal products, seeking for new leads to develop better drugs against multi drug resistant microbial strains.

1.2 Problem Statement

The rise in the prevalence of MRSA worldwide is a growing public health concern; moreover, infections from these resistant organisms are becoming more difficult to treat as a result of restricted spectrum of antimicrobial drugs of proven efficacy. In Abia state there is paucity of data on the prevalence of MRSA, their genotypes or molecular epidemiology. The

few available data was derived from studies based on conventional phenotypic methods and clinical presentation of diseases associated with staphylococci.

1.3 Justification

The increasing prevalence and transmission of MRSA all over the globe constitute a major clinical challenge in the management of serious infections. In Abia State, preliminary studies conducted among health care workers showed inadequate knowledge of MRSA, its virulence and resistance pattern. Furthermore, recent evidence shows that these organisms are becoming increasingly resistant to glycopeptides and newer therapies. Consequently, the determination of the different antibiotypes will help in healthcare workers' education, monitoring of the resistance profile trends which in turn will aid in the correct implementation of antibiotic regimens for MRSA infections.

There are few reports on phenotypic expression of MRSA from the region and no reported cases of detection of *mecA* and *pvl* genes from the State to the best of our knowledge hence the need for characterization studies. Characterizing MRSA will help to identify the different strains, understand their evolution and further compare the genetic variation in different location.

The prevailing resistance trends for existing antimicrobials highlight the urgent need for new and potent antimicrobial hence the the testing of plant extracts on test isolates.

Finally, it is commendable to have a good knowledge of prevalence and molecular evolution of an organism in order to develop a good strategy for its control.

The above reasons prompted the research to determine the prevalence, molecular characterization and antibiotic pattern of MRSA in Abia State, Nigeria so that the use of

appropriate antimicrobial therapy would be undertaken to ensure the effective management of MRSA in Abia state, Nigeria.

1.4 Aim of the research

To molecularly characterize Methicillin Resistant *Staphylococcus aureus* from clinical specimens and determine the antimicrobial activity of plant extracts against the isolates.

1.5 Specific Objectives

I. To determine the antibiotic susceptibility profile of *S. aureus* strains isolated from clinical sources in Abia state.

II. To estimate the proportion of the inducible macrolide-lincosamide-streptogramin B (imlsB) resistance among the isolates.

III. To identify and investigate the prevalence of methicillin resistant *S. aureus* strains and subsequently determine their genetic characteristics.

IV. Molecular characterization of MRSA isolates with respect to virulence-associated genes, antibiotic resistance genes.

V. To determine the clonal relatedness of methicillin resistant *Staphylococcus aureus* isolates from the various hospitals.

VI. To evaluate the antibacterial activity of *Alchornea cordifolia* and *Acalypha wilkesiana* extracts against the MRSA isolates.

VII. To identify the phytochemicals associated with the test plant extracts

CHAPTER TWO

LITERATURE REVIEW

2.1 The Genus *Staphylococcus*

The microorganisms belonging to the genus *Staphylococcus* were first identified in pus by Koch in 1878. The bacterium was successfully cultivated in a liquid medium by a man named Pasteur prior to the designation of the name *Staphylococcus* by Sir Alexandar Ogston in 1881 (Bergdoll & Lee Wong, 2006). The name *Staphylococcus* was coined from Greek words "staphyle" meaning a bunch of grapes and "kokkos" meaning grain or berry (Etymologia, 2013). Organisms in this genus have been described as specifically gram-positive bacteria ranging in size from 0.5-1.5µm, appearing as small circular shapes that separate in greater than one plane to give rise to grape-like clusters. These bacteria are characterized by their non-sporing, non-motile, and non-capsulated nature. They are generally facultative anaerobes with diverse growth requirement (Plata *et al.,* 2009). Genetically, members of this genus have been shown to possess a low G+C content in their DNA (in the range of 30-40 mol %) and the ability to withstand a high concentration of salt and resistance to heat (Plata *et al.,* 2009).

The staphylococci are classified into the family Staphylococcaceae in the order Bacillales and are categorized according to their clinical significance: *Staphylococcus aureus*, which gives a positive reaction to coagulase; and a heterogeneous group of staphylococci that, give a negative reaction with coagulase test, the coagulase-negative staphylococci (CoNS). *S. aureus* has the ability to cause acute pyogenic infections whereas CoNS cause infections in susceptible hosts with certain predisposing conditions. A prominent species of CoNS that often translates to infection is *Staphylococcus epidermidis*. This species (*Staphylococcus epidermidis*) also differs from *S. aureus* by its inability to ferment mannitol (Waldvogel, 2000).

2.1.1 Scientific Classification

Dormain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Notable among the 41 species that cause diseases in humans are *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus and S. lugdunensis* (Trülzsch *et al*., 2007). Amongst all the species, *Staphylocuccus aureus* has been reported as the most virulent (Murray *et al*., 2005). The other species of *Staphylococcus* are mostly commensals that hardly result in diseases (Murray *et al*., 2005). An earlier study by Trülzsch *et al.* (2007) described a novel coagulase negative *Staphylococcus* species, *Staphylococcus pettenkoferi*, isolated from blood specimens in Belgium and Germany.

2.2 *Staphylococcus aureus*

S. aureus is the most pathogenic species of the genus *Staphylococcus* and is described as spherical coccus of about 0.8 to 1.0µm in diameter. They are arranged in characteristic grape-like clusters and have been associated with both hospital-acquired and community-acquired infections. Healthy carriers are colonized by this organism at different parts of the body including the skin and mucous membranes especially the anterior nares where they exist asymptomatically (Wertheim *et al.*, 2005; Sakr *et al.,* 2018). In separate studies by Peacock *et al*. (2001) and Wertheim *et al*. (2005), it was reported that this organism permanently

colonized over 30% of the population while another 30% are transient carriers (Peacock *et al.*, 2001, Wertheim *et al.*, 2005). A negative effect of this colonization is the elevated risk of contracting infection as the carriers provide a reservoir from which the bacteria can take over the patients' system when the host defense becomes compromised (Powers & Wardenburg, 2014).

2.2.1 *Staphylococcus aureus* Cell Wall

The cell wall network of most Gram-positive organisms comprises of diverse structures. The cell wall structural design of staphylococci is different from the walls of other gram positive microorganisms in the level of cross linking and quantity of mucopeptide fraction of these walls (Boris *et al.,* 2004).

The cell walls of gram positive bacteria which serve the role of protection exhibit a wide diversity from simple to very complex structures. These cell walls have also been implicated in the ability of the organism to cause infections and enhance pathogenicity (Van Heijenoort & Gutmann, 2000). The cell wall envelope serves as a mechanical barrier that shields the bacteria from their environment and the rigid exoskeletal element prevents the organism from lysis in low osmolar environments such as host tissues. Structurally, the cell wall of *S. aureus* is composed of murein, techoic acid and wall-associated surface proteins (Tomasz, 2000; Mazmanian *et al*., 2001).

Murein which provides the structural integrity of the cell is composed of a network of glycan strands which are held together by peptide bridges. The glycan strands of peptidoglycan of members of *Staphylococcus* comprises of disaccharide subunits, N-acetylglucosamine and N-acetylmuramic acid. The glycan chains are enmeshed in short peptides and create a three-dimensional molecular network that maintains the integrity of the organism. Also, it has been reported previously by Perry *et al.* (2002) that penicillin binding proteins (PBP) catalyze the

polymerization of lipid II subunits through trans-glycosylation and trans-peptidation reactions, thus generating the cross-linked peptidoglycan that constitutes the main component of the bacterial cell wall (Perry *et al*., 2002). The teichoic acid in *Staphylococcus* is a polymer of N-acetylglucosamine and polyribitol phosphate. It plays a crucial role amongst gram positive bacteria during adherence to mucosal surfaces. The cell wall of *Staphylococcus* is also made up of protein components. Notable amongst them is protein A which reacts with IgG of human serum and is believed to protect the organism from host immune system (Fabiana *et al*., 2013). This same protein is often affected by treatment with lysostaphin (a glycylglycine endopeptidase that cleaves the pentaglycyl cross – bridge of the cell wall) resulting in its release from the bacterial surface.

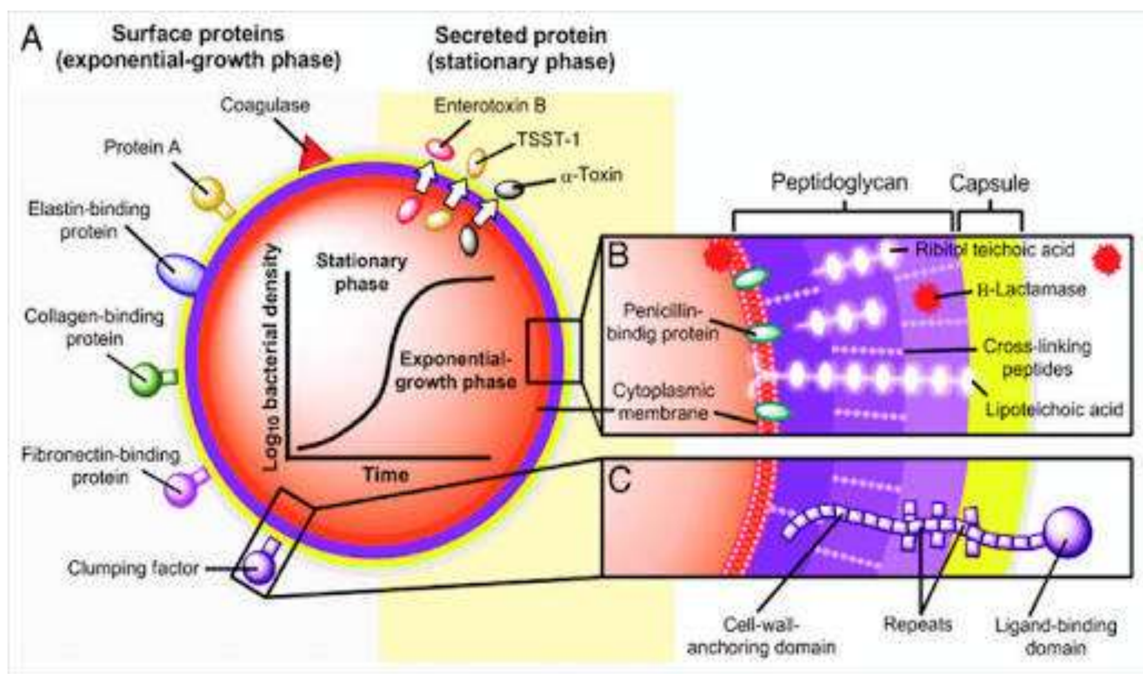

Figure 2.1: Structure of *S. aureus* (Gordon & Lowy, 2008) Pathogenic factors of *Staphylococcus aureus*, with structural and secreted products both playing roles as virulence factors. A, Surface and secreted proteins. B and C, Cross- sections of the cell envelope. TSST-1, toxic shock syndrome toxin 1.

2.2.2 Other Important Properties of Staphylococci

Macroscopically, *S. aureus* being a facultative anaerobic bacterium grows luxuriantly on blood agar and other non-selective media including nutrient agar under both aerobic and anaerobic conditions. The resultant colonies appear as smooth, convex and sharply defined on blood agar plates when grown at room temperature. The colonies are gold pigmented due to carotenoids but this may not be apparent under certain conditions, such as anaerobic conditions or in liquid medium (Waldvogel, 2000). *Staphylococcus aureus* usually produces beta-haemolysis on horse, human or sheep blood agar plates, whereas *S. epidermidis* is non-haemolytic on blood agar plates when grown at 37°C (Todar, 2005).

Infections by *Staphylococcus aureus* can manifest in virtually every organ and tissue of the human body with the skin reportedly being the part most affected (Daum, 2007). Other more elaborate and significant infections associated with *S. aureus* infections include endocarditis, mastitis, meningitis, osteomyelitis, phlebitis (inflammation of veins) and pneumonia (Bhatia & Zahoum, 2007). A number of severe acute food poisoning outbreaks reported worldwide has been linked to production of very heat-stable enterotoxin B that are pre-produced in the food by the bacterium (Le Loir *et al*., 2003). Various other diseases linked to *S. aureus* specific toxins include staphylococcal scalded skin syndrome (SSSS) and toxic shock syndrome (TSS) (Salyers & Whitt, 2002).

Other species such as *Staphylococcus epidermidis* cause infections associated with indwelling medical devices (Vadyvaloo & Otto, 2008). Urinary tract infections which are a common phenomenon among young girls is caused by *Staphylococcus saprophyticus* (Horowitz & Cohen, 2007) while others such as *Staphylococcus lugdunensis, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus schleiferi* and *S intermedius* have been implicated with pathogenesis in health-care settings. Amongst the staphylococcal species, coagulase-positive *Staphylococcus aureus* and coagulase-negative (CNS)

Staphylococcus epidermidis (*S. epidermidis*) are of clinical importance (Waldvogel, 2000). The transmission of *S. aureus* in hospitals is often a result of exposure of patients to health-care workers who are *S. aureus* carriers or from infected patients. *Staphylococcus aureus* and *S. epidermidis* are both important causes of nosocomial infections (Ziebuhr, 2001), with CNS accounting for 50% of catheter related infections worldwide (Murray *et al*., 2005).

2.2.3 Genome of *Staphylococcus aureus*

Kuroda *et al.* (2001) reported the genomic sequences of two related *S. aureus* strains (N315 and Mu50) as were obtained using the shot-gun random sequencing method. The size of the genome of strain N315 as was obtained by Kuroda *et al.* (2001) was 2,813,641 mbp. The *S. aureus* genome consists of a singular circular chromosome of about 2.7 to 2.9 mbp containing about 2,600 genes composed of core and auxiliary (accessory) genes and it has a G+C content of 33% (Kuroda *et al.,* 2001). In silico analysis suggests that the core genome makes up about 75% of any *S. aureus* genome and is highly conserved between isolates. Most of the genes comprising the core genome are those associated with central metabolism and other housekeeping functions. Supplementing these are genes that are associated with common species functions but that are not essential for growth and survival, including virulence genes. Also included are surface binding proteins, exoenzymes, toxins, and the capsule biosynthetic cluster (Lindsay & Holden, 2004). The accessory genome accounts for about 25% of any *S. aureus* genome, and mostly consists of mobile genetic elements that transfer horizontally between strains. These genetic elements include bacteriophages, chromosomal cassettes, pathogenicity islands, genomic islands, plasmids and transposons. Accessory genes typically have a different G + C content than those in the core genome, often because they are obtained from other species of bacteria. Many of these genetic elements are known to carry genes associated with virulence, drug and metal resistance, to substrate utilization and miscellaneous metabolism. Therefore, the distribution and horizontal spread of these

elements can have important clinical implications. The identification and characterization of these elements provide insights into how *S. aureus* cause disease, and their diversity (Shittu *et al*., 2007).

There are different ways bacteria obtain genetic information from other cells or the surrounding environment and they include:

(1) Uptake of free DNA from the environment (transformation),

(3) Direct contact between bacterial cells (conjugation) and

(3) Bacteriophage transduction.

Bacteriophages which are bacterial viruses seem to have the biggest impact on staphylococcal diversity and evolution. They are known to transfer genes such as lukF-PV and lukS-PV that encode the Panton-Valentine leukocidin (*pvl*) components which is strongly associated with severe forms of pneumonia (necrotic pneumonia) caused by community-acquired *S aureus* strains; the staphylokinase gene (*sak*) gene, which is a potent plasminogen activator that could facilitate bacterial spreading through its fibrin-specific blood clotting activities and the enterotoxin genes (Shittu *et al*., 2007; Malachowa & DeLeo, 2010).

Majority of the antibiotic resistance genes are carried either by plasmids or by mobile genetic elements including a unique resistance island. Three groups of new pathogenicity islands (*S. aureus* pathogenicity islands {SaPIs}) have been identified in the genome: a toxic-shock-syndrome toxin island family, exotoxin islands and enterotoxin islands (Shittu *et al*., 2007). All the sequenced *S. aureus* strains are known to carry one or more free or integrated plasmids. All types of *S. aureus* plasmids frequently carry antibiotic resistance genes, or resistance to antiseptics or heavy metals. Some virulence genes are also known to be carried on plasmids, such as exfoliative toxin B and some superantigens (Lindsay & Holden, 2004).

Other mobile genetic elements found in the *S. aureus* chromosome include staphylococcal cassette chromosomes (SCCs), which encode antibiotic resistance and/or virulence determinants. SCCs encode the methicillin resistance gene (*mecA*) (Shittu *et al.,* 2007; Malachowa & DeLeo, 2010). Repeated duplication of genes encoding super-antigens is responsible for capability of *S. aureus* to infect humans and cause severe immune reactions (Kuroda *et al*., 2001). This organism manufactures a wide variety of exoproteins that enable it to successfully colonize and consequently cause disease in humans. Majority of the strains secrete a group of cytotoxins and enzymes which includes four haemolysins (alpha, beta, gamma, and delta), nucleases, proteases, hyaluronidase, lipases, and collagenase. The main function of these proteins may probably be to convert local host tissues into nutrients needed for bacterial growth. Some strains equally produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins, exfoliative toxins and leukocidin (Dinges *et al*., 2000).

2.3 Virulence Determinants in *S. aureus*

S. aureus has been implicated in many important infections in humans. Expression of a vast number of virulence determinants has been reported to contribute in the pathogenesis of infections caused by this group of organisms. The mechanisms employed by these virulence determinants to cause infection include but are not limited to mediating colonization of the host organism, invasion of damaged skin and mucosa, evasion of the host cells primary defense system as well as dissemination through the body. Several authors in their separate studies have reported that in *Staphylococcus aureus*, there are over 50 potential virulence factors with a plethora of biological activities (Dinges *et al.,* 2000). These factors are grouped as either cell-surface-associated (adherence) or secreted (exotoxins) factors. The virulence factors of *Staphylococcus* are carried either on the cell's chromosomes or on mobile genetic

elements including transposons, plasmids and bacteriophages. Acquisition of genes mediating antibiotic resistance, such as the *mecA* gene conferring resistance to methicillin, can further favors epidemic spread by promoting acquisition of additional virulence factors. Expression of most virulence factors in *S. aureus* is controlled by the accessory gene regulator (*agr)* locus, which encodes a two-component signaling pathway whose activating ligand is a bacterial-density sensing peptide (autoinducing peptide) also encoded by *agr* (Justyna *et. al.,* 2011).

2.3.1 Cell Surface Factors

Numerous cell surface factors are regularly expressed by *S. aureus* which contribute to their virulence. Aside the cell surface factors, they also possess other secreted as well as surface anchored proteins which they (*Staphylococcus aureus)* use to cling onto their host cells extracellula*r* matrix and plasma components. Prominent among these surface components are Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) adhesins which are glued to the cell wall peptidoglycan covalently.

Another protein, staphylococcal protein A (SpA) has been reported to interfere with the process of phagocytocis and opsonization by binding to IgG. Similarly, Fibronectin-binding proteins (FnbpA and FnbpB) are known to mediate attachment to fibronectin and plasma clot. Collagen-binding protein on the other hand has been described to facilitate adhesion of the cells to collagenous tissues and cartilage whereas clumping factor proteins (ClfA and ClfB) have been reported to promote clumping and adherence to fibrinogen in the presence of fibronectin.

Other notable cell surface factors include capsular polysaccharides and staphyloxanthin which are responsible for reduction of phagocytosis by neutrophils; enhancing bacterial

colonization, persistence on mucosal surfaces and resistance to neutrophil reactive oxidant-based phagocytosis respectively.

2.3.2 Secreted Factors (Exotoxins)

Another mechanism devised by *S. aureus* in taking over host immunity aside the cell wall associated virulence determinants is the secretion of exotoxins. These toxins disrupt host cells and tissues, making the host immunocompromised causing them to release nutrients that trigger spread of the bacterium. These secreted factors are categorized into four major groups: superantigens, cytolytic (pore-forming) toxins, various exoenzymes and miscellaneous proteins (Lin & Peterson, 2010).

2.3.2.1 Superantigens

Superantigens are a group of powerful secreted immune-stimulatory proteins capable of inducing a variety of human diseases, including toxic shock syndrome (TSS). These superantigens also known as pyrogenic exotoxin genes are very peculiar to *S. aureus*, with greater than 73% of *S. aureus* possessing at least one of the genes encoding a classic pyrogenic exotoxin. However, the distribution of these superantigens has been reported to differ amongst different clones (Xu & McCormick, 2012).

Different super antigenic exotoxins numbering nineteen has been described to exist in *S. aureus*. They comprise toxic shock syndrome toxin-1(TSST-1, encoded by the *tst* gene) and staphylococcal enterotoxins (SE) A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R, and U, encoded by *sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser,* and *seu*, respectively. These proteins are potent mitogens, pyrogenic, and studies have shown them to enhance lipopolysaccharide lethality in various studies involving animal models. They stimulate large populations of T cells expressing a particular Vβ element in their T-cell receptor, leading to massive T-cell proliferation and uncontrolled release of pro-inflammatory

(Th-1) cytokines such as interferon-γ, TNF-α, interleukin (IL)-1β and IL-6 (Ferry *et al*., 2005). The super antigens can also manifest a sepsis-like syndrome by starting excessive synthesis of cytokines in a process called cytokine storm (Dinges *et al*., 2000; Prevost *et al*., 2003).

2.3.2.2 Cytolytic (pore-forming) toxins

S. aureus also secretes a group of structurally diverse cytolytic toxins with different target specificity but share similar roles on the host cells. These toxins produce pores in the cytoplasmic membranes of target cells with resultant leakage of the cells content and cell lysis. These toxins include cytolysins with α-hemolysin as a notable example which induces lysis on a wide spectrum of cells, mainly platelets and monocytes and leukocidin which includes Panton-Valentine leukocidin (*pvl*) (Lin & Peterson, 2010; Foster, 2005).

The toxin *pvl* is a staphylococcal bi-component cytotoxin, which has been shown in vitro, to cause destruction of a number of cells including human polymorphonuclear neutrophils (PMNs), macrophages, and monocytes. It may also inactivate mitochondria resulting in apoptosis (Genestier *et. al*., 2005).

Furthermore, *pvl* also induces pore formation in cells when two components of the toxin LukS-PV and LukF-PV are present (Justyna *et al*., 2011). Meanwhile *pvl* and leukocidin D-E (LukD-E) function as superantigens and thus manipulate the immune system by hyper stimulating the release of cytokines (SEB, SEA and TSST) (Mertz *et al*., 2007).

2.3.2.3 Various Exoenzymes

The disruption of host tissues and inactivation of host antimicrobial mechanisms (e.g., lipids, defensins, antibodies and complement mediators) for easy acquisition of nutrients and increased dissemination of bacterial growth is also another means employed by *S. aureus* through secretion of various exoproteins (Dinges *et al.,* 2000). These exoenzymes include

lipases, nucleases, proteases (serine, cysteine (e.g., staphopain), aureolysin), hyaluronidase, coagulase and staphylokinase (SAK) (Dinges *et al.,* 2000; Bokarewa, 2006).

Lipases exert their effect on the host immune response through inactivation of the fatty acids meant for disruption of the bacterial cell; Proteases act by inactivating the host defence peptides as well as blocking antibodies while staphylokinase is a plasminogen activator. Hyaluronidase digests hyaluronic acid present in the skin, bone, umbilical cord, vitreous body of the eye and synovial fluid whereas coagulase shields the bacteria against host defence by clotting fibrin around a focal infection.

2.3.2.4 Miscellaneous Proteins

Other specific proteins produced by *S. aureus* have been noted to impact on the innate and adaptative immune system of the host cell. These proteins include staphylococcal complement inhibitor (SCIN) which inhibits complement activation (Rooijakkers *et al.,* 2005), extracellular fibrinogen binding protein (Efb) (Lee *et al.,* 2004; Rooijakkers *et al.,* 2005), chemotaxis inhibitory protein of *S. aureus* (CHIPS) which inhibit chemotaxis and activation of neutrophils, formyl peptide receptor-like-1 inhibitory protein (FLIPr) (Prat, 2006), and extracellular adherence protein (Eap).

2.4 Pathogenesis

Staphylococcus aureus is a commensal organism with the capability of becoming pathogenic resulting in a vast array of diseases in human through the expression of various virulence factors that facilitate colonization of the host cells, invasion of damaged skin and mucosa, spread across the body and evasion of the defense set up of the host (Chanda *et al.,* 2010). It has been reported that 30% of healthy individuals are colonized by *S. aureus* usually in the anterior nares, vagina and peri-anal area. When certain conditions in the body including pH, temperature and nutrient availability are altered; this can facilitate the growth of

Staphylococcus aureus which may become pathogenic (Mims *et al*., 2004). The result of this colonization by *S. aureus* is the increased risk of infection which facilitates the transmission of this bacterium in both community and hospital settings.

Other factors like breach in host defenses through either shaving, aspiration, insertion of an indwelling catheter or surgery promotes the inoculation of this organism into the skin of a host cell resulting in infection. The establishment of an infection by *S. aureus* involves the adherence of this organism to the host tissues by means of several surface proteins collectively referred to as "Microbial Surface Components Recognizing Adhesive Matrix Molecules" (MSCRAMMs) (Gordon & Lowy, 2008). The adherence of *S. aureus* to host tissue is an important step in pathogenesis as well as in colonization. The MSCRAMMs bind molecules such as collagen, fibrinogen and fibronectin, and contribute to initiation of infections of the endovascular cavity, bone and joint infections as well as infections involving prosthetic devices (Tung *et al*., 2000; Menzies, 2003). The MSCRAMM protein A, also binds the Fc portion of immunoglobulin which prevents opsonization by immune cells (Foster, 2005).

The pathogenicity of *S. aureus* has also been attributed to the secretion of toxins, including the 33-kd protein-alpha toxin, exfoliatin A, exfoliatin B and Panton-Valentine leukocidin (*pvl*) toxins. These toxins expose the host to potential skin diseases ranging from carbuncles, boils, folliculitis and impetigo. Other complications attributed to the actions of these toxins include endocarditis, meningitis as well as toxic shock syndrome (TSS) (Mims *et al*., 2004).

On adherence to host tissues or prosthetic materials, this bacterium is able to grow and persist in various ways. Biofilm formation on the host cell as well as on other prosthetic surfaces has been reported as one of the very important mechanisms used by this group of bacteria to evade the host defences and several antimicrobial agents (Donlan & Costerton, 2002).

Several other *in vitro* studies have further revealed that *S. aureus* can invade and survive inside epithelial cells, including endothelial cells, contributing to its evasion of host defences, particularly in endocarditis (Moreillon *et al*., 2002). Aside from biofilm formation, *S. aureus* has been reported to form small-colony variants (SCVs), which may contribute to persistent and recurrent infection. SCVs are slowly growing organisms that exhibit small, non-pigmented, non-haemolytic colony morphology. The growth of these variants is favoured by supplementation with several substrates including thymidine, haemin and menadione.

Studies carried out in the past two decades have led to an increased understanding of the importance of the SCV phenotype as a general strategy adopted by the bacterium for survival and intracellular persistence. Though S. *aureus* grows extracellularly, it adopts this lifestyle change by conversion of its metabolism which affects the host- pathogen interplay. This can lead to dire clinical consequences such as treatment failures and chronic infections (Kahl *et al.,* 2016)

Anti-phagocytic microcapsule called zwitterionic capsule (both positively and negatively charged) is another important mechanism employed by *S. aureus* in the evasion of host immune system during an infection through the induction of abscess formation (Foster, 2005). It is further alleged that the secretion of chemotaxis inhibitory protein or the extracellular adherence protein by *S. aureus* causes an interference with neutrophil extravasation and chemotaxis to the site of infection (Foster, 2005). Furthermore, another important protein leukocidin facilitates leukocyte destruction by the formation of pores in the cell membrane. This is a prominent feature of CA-MRSA strains (Foster, 2005; Gordon & Lowy, 2008).

In the course of an infection, various enzymes which are associated with *S. aureus* notably proteases, lipases, and elastases also contribute to the invasion and destruction of host tissues

(Gordon & Lowy, 2008). The organism is capable of invading endovascular tissue and this encourages its dissemination to other tissues.

Acquisition of nutrients from the host is also another step towards survival within the human host. Iron is a very indispensable nutrient required for growth and *S. aureus* acquires iron from its host cell through the secretion of high affinity iron-binding compounds, aureochelin and staphyloferrin (Liu, 2009). These compounds make iron readily accessible to *S. aureus* by unbinding serum iron which is mostly bound to the host proteins (Liu, 2009).

The dissemination of this organism in the bloodstream facilitates spread to other peripheral sites in various organs resulting in septic shock. Peptidoglycan, lipoteichoic acid, and α-toxin have been implicated in the initiation of sepsis. Other specific staphylococcal infections including endocarditis, osteomyelitis, renal carbuncle, septic arthritis, or epidural abscess most often occur following invasion of the bloodstream.

2.5 Epidemiology of *S. aureus* infections

The reports of infection caused by *S. aureus* from around the world are on the increase. Most of these infections arise from medical developments, such as the use of catheters, joint prostheses, and immunosuppressants. Infections arising from these organisms add to the burden on health care resources as well as increasing the rate of morbidity and mortality. Researchers have identified *S. aureus* as the main causative agent of different bacterial infections in sub-Saharan Africa, and equally among the most frequently encountered pathogens in many Microbiology laboratories in Nigeria (Esan *et al*., 2009; Shittu *et al*., 2011).

Individuals with a particular profession including health-care workers, nursing home inhabitants, prison inmates, military recruits and children are more susceptible to *S. aureus* colonization than others (CDC, 2003; Zinderman *et al*., 2004; Cardoso *et al*., 2007; Chen *et*

al., 2007; Ho *et al*., 2008). In an earlier study, by Albrich & Harbath (2008), health-care workers accounted for 93% of personnel to patient transmission of MRSA. Previously several outbreaks have been reported in Northern-Taiwan in 1997 that suggested MRSA transmission associated with health-care workers, including surgeons (Wang *et al*., 2001). Grundmann *et al*. (2006) reported a prevalence of >50% in countries such as Singapore (1993-1997), Japan (1999-2000) and Colombia (2001-2002) while countries with a prevalence of 25% to 50% included South Africa (1993-1997), Brazil (2001), Australia (2003), Mexico and the United States (Grundmann *et al*., 2006). The lowest prevalence of less than 1% was found in Norway, Sweden and Iceland (1993-1997). In 2007, a prevalence of more than 50% of MRSA strains isolated from Cyprus, Egypt, Jordan and Malta was reported by Borg *et al.* (2007). This high prevalence was attributed to overcrowding and poor hand-hygiene facilities in the hospitals.

The spectrum of *S. aureus*-related infections differs as regards to data from different parts of the world, with a higher proportion of pyomyositis, of up to 27%, among cases with bone, skin and soft tissue infection, and up to 21.7% among all *S. aureus*-related infections. Multifocal lesions are also encountered frequently, and affect mostly the immune-compromised patients, and rarely affect immunocompetent individuals (Ntusi & Khaki, 2011). Additionally, some studies have reported a higher proportion of *S. aureus* in urinary tract infections: 6.3– 13.9% of urinary tract infections are caused by *S. aureus* in Senegal (Dromigny *et al*., 2002), Nigeria (Otajevwo, 2013), and Ghana (Adjei & Opoku, 2004), as compared with 1.06% in Brazil and Europe (Naber *et al*., 2008). In general, *S. aureus* is a major pathogen in bloodstream infections (9.5–39.0%), skin and soft tissue infections (62.8–90.0%), ear, nose and throat infections (16.7–29.0%), and surgical site infections (20.4–32.0%). A few prospective studies have reported a higher incidence rate of *S. aureus* infection in Africa than in industrialized countries. The annual incidence of *S. aureus*

bacteraemia was 3.28 cases per 1,000 hospital admissions (South Africa) with the highest incidence in children aged <5 years (Naidoo *et al*., 2013). For comparison, the annual incidence rates of community-acquired *S. aureus* bloodstream infection in the USA were 2.3 cases per 100 000 person-years for methicillin-sensitive *S. aureus* (MSSA) and 1.5 cases per 100 000 person-years for MRSA (Landrum *et al.,* 2012). There is also evidence that the major MRSA clones in Europe occur majorly in geographical clusters. By implication this indicates that MRSA strains spread through regional health care networks and therefore control efforts should be aimed at interrupting the spread within and between health care institutions in order to achieve success (Grundmann *et al*., 2010)

2.6 Bacterial Drug Resistance

Bacteria are capable of resisting the action of antibiotics through activation of latent mobile genetic elements, mutagenesis of its own DNA and physical exchange of genetic material with another organism (Dwyer *et al.,* 2009). Worldwide emergence of resistant bacteria has been associated with misuse and increasing use of antibiotics in healthcare settings and the lack of specific drugs for treating patients (Buffet-Bataillon *et al.,* 2012).

2.6.1 Causes of Antibiotic Resistance

History has revealed that there are several underlying factors that lead to resistance on an introduction to a new antibiotic, as antibiotic use provides the potential for selection of resistant strains. There are several underlying reasons for this phenomenon:

(1) antimicrobial use is the key driver of resistance and, paradoxically, this selective pressure comes from a combination of overuse in many parts of the world (e.g., for minor infections or in food-producing animals), misuse due to lack of access to appropriate treatment, and failure to complete treatment courses;

(2) inherent microbial characteristics also play a role for example, *S. aureus* resistance to penicillin is highly prevalent, and yet *Streptococcus pyogenes* strains are uniformly susceptible to penicillin, which remains the drug of choice for treating infections caused by this organism (Marchese *et al.,* 2000) and

(3) Societal and technological traits also contribute to the spread of antibiotic resistance due to substantial increases in the availability and ease of travel within and between countries (Lowy, 2003).

2.6.2 Antistaphylococcal Agents and Resistance

2.6.2.1 Beta-Lactam Drugs

The β-lactam drugs are antibiotics which produce a bactericidal effect on microorganisms by inhibiting their membrane-bound enzymes whose functions are to catalyze important stages in the biosynthesis of the organisms' cell wall. The antibiotics achieve this feat by covalently binding to one or more penicillin-sensitive enzymes, termed penicillin-binding proteins (PBPs) (Jensen & Lyon, 2009). Before antibiotics were discovered, infections with *S. aureus* had a mortality rate of close to 80%. When penicillin became available in the 1940s, the medical community thought that death from *S. aureus* infections was a thing of the past. However, within a few years S. *aureus* began to show resistance to penicillin. By 1948, up to 50% of hospital strains were resistant, with the level of resistance rising to 80% by 1957. Resistance is due to the production of a penicillinase (or β-lactamase). More than 90% of staphylococcal isolates now produce β -lactamase, which inactivates β-lactam antibiotics by hydrolysis of their β -lactam ring (Lowy, 2003). *BlaZA* encodes β -lactamase and is part of a transposable element on a plasmid, which often also contains genes for resistance to other antibiotics (e.g., gentamicin and erythromycin) (Lowy, 2003). Other antimicrobials related to penicillin (β-lactam drugs) were then developed: such as methicillin, oxacillin, and

ampicillin. A few years after the development of methicillin, resistant strains were observed (methicillin-resistant *Staphylococcus aureus* – MRSA), and eventually methicillin was removed from the market. Resistance occurs following the chromosomal acquisition of novel DNA, resulting in the production of a new penicillin-binding protein, termed PBP2a, with a low binding affinity for methicillin (Woodford, 2005). These PBPs are actually transpeptidases; enzymes that are involved in the construction of the peptidoglycan portion of the bacterial cell wall. They function to catalyze reactions that allow cross-linking of peptidoglycan subunits as these subunits are incorporated into the cell wall (either for repair of cell wall damage or for constructing new cell wall for cell division). Penicillin will gain entry into the bacterial cell where it will bind to the PBPs and block the ability of the PBPs to function normally (Kernodle, 2000).

Bacteria fought back by acquiring a plasmid that contained a gene (*blaZ*) that encoded a *β*-lactamase enzyme (Olsen *et al*., 2006). All penicillin drugs (and related *β*-lactam drugs) have a *β*-lactam ring at the core of their structure. The *β*-lactamase enzymes (also known as penicillinases) hydrolyze the peptide bond in the *β*-lactam ring, which opens up the ring and makes it impossible for the drug to bind to the PBPs. MRSA strains are considered to be resistant to all the penicillin (and most *β*-lactam) drugs, and since methicillin is no longer produced, oxacillin is used for susceptibility testing. Because of this, the name ORSA (oxacillin-resistant *Staphylococcus aureus*) is sometimes used instead of MRSA, but refers to the same strains. The gene that encodes the PBP2a protein is *mecA* (Ito *et al*., 2001). This gene is acquired through horizontal transfer of a mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*). These genetic elements contain two required components; the *mec* gene complex, and the *ccr* gene complex (which contains site-specific recombinase genes). The SCC*mec* elements have been classified into eight types (I-VIII) based on the structure and combination of *mec* and *ccr* gene complexes present

(Chongtrakool *et al*., 2006). These elements also differ in what other antimicrobial resistance genes are carried on them. Types I, IV, V, VI, and VII generally do not carry other resistance genes. Types II, III, and VIII may contain one or more other resistance genes, such as *ermA* (erythromycin), *aadD* (tobramycin), and *tetK* (tetracycline). These types are also used to help distinguish CA-MRSA and HA-MRSA strains. Most HA-MRSA strains carry SCC*mec* types I, II, III, VI, and VIII; while most CA-MRSA strains carry types IV, with some carrying types V and VII (Hanssen and Ericson, 2006; Malachowa & DeLeo, 2010).

Ampicillin, which is a broader spectrum drug, has not fared well against *S. aureus* isolates. By the 1990s most isolates of *S. aureus* were resistant to ampicillin (Goto *et al.,* 2009). Many more penicillin drugs were developed (such as amoxicillin, piperacillin, and ticarcillin); some were directed at *S. aureus*, and some are broader spectrum and aimed at the gram negative bacteria as well. In the 1970s, compounds with a *β*-lactam structure, but weak antimicrobial activity, were discovered. These compounds were not useful as antimicrobials when used alone, but were found to be *β*-lactamase (an enzyme produced by the microorganisms to fight the *β* lactam drugs) inhibitors. Three of these inhibitors have been used in combination with penicillin drugs; amoxicillin/clavulanic acid, ampicillin/sulbactam, and piperacillin/tazobactam. These combination drugs improved the ability of the penicillin drugs to kill the microorganisms, but did not completely alleviate the resistance problem (Greenwood, 2008). Over the years, more *β*-lactam drugs have been discovered or developed synthetically. These include the cephalosporins, carbapenems, and monobactams. The carbapenem drugs, which are structurally related to the *β*-lactamase inhibitor drugs, are considered to have the best and broadest spectrum of activity against both Gram positive and Gram negative bacteria. As with other *β*-lactam drugs, the cephalosporins and carbapenems have had antimicrobial resistance issues. Most of these drugs should not be used in monotherapy against MRSA strains (Greenwood, 2008, Papp-Wallace *et al.,* 2011). The

monobactams, so named because they have no other ring fused to the *β*-lactam ring structure, have not had much success against Gram positive cocci.

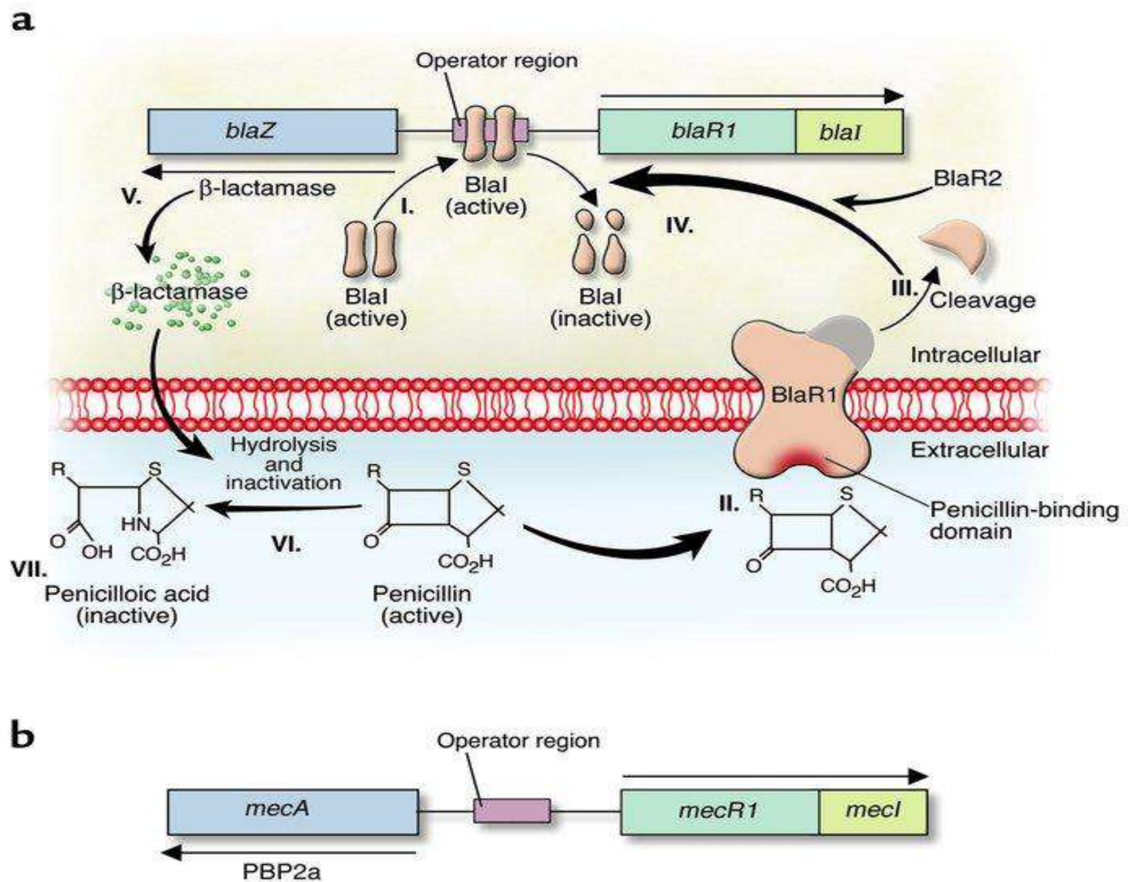

Figure 2.2: Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin. (a). I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR*1-*blaI*. In the absence of penicillin, β-lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV.

Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ*and *blaR*1-*blaI* to commence. V–VII. β-Lactamase, the extracellular enzyme encoded by *blaZ*(V), hydrolyzes the β-lactam ring of penicillin (VI), thereby rendering it inactive (VII).

(b) Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. Exposure of MecR1 to a β-lactam antibiotic induces MecR1 synthesis. MecR1inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and β-lactamase (Lowy, 2003).

2.6.2.2 Macrolide, Lincosamide and Streptogramin

Macrolides, lincosamides and streptogramins, which were first introduced in 1952, constitute a group of antibiotics collectively known as MLS. They effectively block protein synthesis by targeting the 50S ribosomal subunit of bacteria (Schito, 2006).

Following the development of allergies to penicillin antibiotics, these drugs collectively described as MLS provided immediate and suitable alternatives for treating infections caused by *Staphylococcus* species. It didn’t take long for resistance to these groups of antibiotics to develop in some strains of *S. aureus* as resistance genes were already present, and use of these antibiotics exerted a selective pressure.

The resistance to MLS drugs in *S. aureus* is mediated by two different phenotypes. The first is as a result of modification of the ribosome by 23S rRNA methylases, mediated primarily by *ermA, ermB* or *ermC* which are carried by chromosomes or plasmids. The result from the action of these enzymes on the ribosome is a conformational change which lowers the ability of these drugs to bind to the ribosome (Lim *et al.,* 2002). The second resistance type is mediated by msrA and involves the active efflux of the antimicrobial agent by an ATP-dependent pump, thereby maintaining intracellular concentrations below the level required for binding to ribosomes.

2.6.2.3 Aminoglycosides

Aminoglycosides are antibiotics that are important in the therapy of staphylococcal infections. They are synergistically used in combination with either glycopeptides or beta-lactams for treatment of complicated staphylococcal infections. These drugs have been reported to enter bacterial cells by energy-dependent binding to the cell wall and energy-dependent transport across the cytoplasmic membrane, finally binding to one or more

ribosomal sites, thus inhibiting protein synthesis (Ida *et. al.,* 2001). Resistance in staphylococci results from any of three events:

(1) A chromosomal mutation leading to altered aminoglycoside binding to ribosomes;

(2) Ineffective transport of aminoglycosides into the bacterial cell, producing low-level cross-resistance to most aminoglycosides; and, most commonly,

(3) Enzymic modification of aminoglycosides (Lowy, 2003).

In the last case, resistant strains have the aminoglycoside-modifying genes *acc, aph* and *ant,* which code for aminoglycoside acetyltransferases, phosphotransferases and adenyltransferases, respectively. The acetylated, phosphorylated or adenylated aminoglycosides do not bind to ribosomes, and thus do not inhibit protein synthesis (Woodford, 2005).

2.6.2.4 Glycopeptides

Following the spread of MRSA, glycopeptides (usually vancomycin and teicoplanin) became the choice drug for treatment of MRSA infection (Appelbaum, 2006). However, the increased usage of vancomycin to treat MRSA infections led to the emergence of vancomycin-resistant staphylococci (Hiramatsu *et al.,* 2001). The first case of vancomycin resistance among staphylococci occurred in 1987 and was identified in a *Staphylococcus haemolyticus* strain. In 1997, the first report of a vancomycin-intermediate resistant *S. aureus* (VISA) strain was reported from Japan, with reports subsequently following from other countries including France (Chesneau *et al.,* 2000), Scotland (Hood *et al.,* 2000) and two isolates in South Africa (Ferraz *et al.,* 2000). These VISA isolates were all MRSA strains. Complete resistance to vancomycin was reported in Michigan in the United States in 2002 and subsequently in Pennsylvania (CDC, 2002; Tenover *et al.,* 2004).

Identification of two forms of vancomycin resistance has been demonstrated (Walsh & Howe, 2002). The first form involves the VISA strains with a minimum inhibitory concentration of 8 to 16µg/ml (Walsh & Howe, 2002). The reduced susceptibility to vancomycin by *S. aureus* is hypothesized to be as a result of changes in peptidoglycan synthesis (Walsh & Howe, 2002). There is a visible irregularly shaped and thickened cell wall in these VISA strains due to increased amounts of peptidoglycan (Hiramatsu *et al.,* 2001). Evidently, there is a decrease in cross-linking of the peptidoglycan strands resulting in the exposure of more D-alanyl-D-alanine residues (Walsh & Howe, 2002).

The second form of vancomycin resistance involves vancomycin-resistant *S. aureus* (VRSA) with a minimum inhibitory concentration (MIC) of ≥128 µg/ml (Walsh & Howe, 2002). The mechanism is hypothesized to be due to conjugation with vancomycin resistant *Enterococcus faecalis* (VRE) (Showsh *et al.,* 2001). The process of conjugation results in the transfer of the *vanA* operon of the *E. faecalis* bacterium to the MRSA strain (Showsh *et al.,* 2001). The *vanA* gene together with its regulator genes, vanSR, from VRE is carried by a transposon, Tn1546, which is integrated into the plasmid (pLW1043) and conjugatively transferred into *S. aureus* (Hiramatsu *et al.,* 2004). Vancomycin-resistant *S. aureus* is therefore, an MRSA with a pLW1043 carrying the *vanA* gene (Hiramatsu *et al.,* 2004). The pLW1043 also carries other resistance mediating genes against gentamycin, penicillin and trimethoprim (Hiramatsu *et al.,* 2001).

The mechanism of resistance in VRSA is caused by the alteration of the terminal peptide to D- Ala-D-Lac instead of D-Ala-D-Ala (Gonzalez-Zorn & Courvalin, 2003). The D-Ala-D-Lac synthesis occurs with minimal or low concentration of vancomycin (Gonzalez-Zorn & Courvalin, 2003).

2.6.2.5 Fluoroquinolones

Fluoroquinolones are broad spectrum and bactericidal antibiotics. The fluoroquinolone drugs kill bacteria by inhibiting bacterial DNA synthesis (Hooper, 2002). Important examples of the fluoroquinolone group include ciprofloxacin, ofloxacin and norfloxacin. Introduced in the 1980s, fluoroquinolones were initially developed for the treatment of Gram-negative bacteria, such as *Pseudomonas* species with limited activity against Gram-positive bacteria (Hooper, 2002). Over the years, new fluoroquinolones with increased activity against Gram-positive cocci were developed including grepafloxacin, levofloxacin, moxifloxacin, sparfloxacin and trovafloxacin (Hooper, 2002). However, the use of these drugs has been highly regulated because of increased development of resistance by bacteria to this group of drugs (Hooper, 2002).

Fluoroquinolone resistance of *S. aureus* emerged rapidly in US hospitals in 1988 after the introduction of ciprofloxacin with 80% of the infections identified as MRSA. Ciprofloxacin was initially developed for the treatment of Gram- negative and Gram-positive bacteria other than *S. aureus*, thus exposure of *S. aureus* to fluoroquinolones was minimal. *Staphylococcus aureus* resistance to fluoroquinolones is suggested to be as a result of exposure of the bacteria to fluoroquinolones in the mucosal and cutaneous surfaces in the nasal cavity. MacDougall *et al.* (2005) reported a 38% resistance in 616 *S. aureus* strains from 17 US hospitals isolated in 2000. An earlier study had reported an 85% fluoroquinolone-resistance in 1846 MRSA strains isolated from Kuwaiti hospitals between March and October 2005 (Udo *et al.,* 2008).

The DNA gyrase and topoisomerase, which are responsible for DNA replication are the two enzymes targeted by fluoroquinolones. The DNA gyrase alters the supercoiling of the DNA whilst the topoisomerase IV separates DNA strands, which are interlocked to allow separation of the daughter chromosomes into daughter cells. The activity of the

fluoroquinolone drugs differs with the different types of drugs on the level of inhibitory activity against the two enzymes (Takei *et al.,* 2001).

2.6.2.6 Linezolid

Linezolid belongs to the class of antibiotics known as oxazolidinones. It has a broad spectrum of activity against gram-positive organisms, including staphylococci, enterococci and streptococci. Essentially all strains of *S. aureus*, including MRSA, from clinical surveys carried out are inhibited by this agent at or lower than the susceptibility breakpoint of 4µg/mL (Eliopoulos, 2003). Linezolid acts by binding to the 50S ribosomal subunit and prevents the formation of the 70S ribosome complex. Its 100% bioavailability following oral administration makes it an attractive therapeutic option for MRSA infections. Reports also have credited that the rates of linezolid resistance have remained comparatively low and stable as demonstrated by various surveillance programs (Gu *et al.,* 2013; Mendes *et al.,* 2014). The most frequent cause of resistance involves mutations in the bacterial 23S ribosomal subunit, the binding site for linezolid (Mendes *et al.,* 2014). Mutations in the 50S L3 and L4 ribosomal proteins have also been described. A plasmid-mediated acquisition of the *cfr* gene confers a multi-drug resistant phenotype to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (Shore *et al.,* 2010).

2.6.2.7 Daptomycin

Daptomycin, is a drug approved for the treatment of enterococcal and staphylococcal infections, including MSSA and MRSA infections, specifically those implicated in skin and tissue infections, *S aureus* bacteraemia, and right-sided endocarditis. Genetic changes associated with the development of daptomycin resistance can occur during treatment in patients with deep-seated or high bacterial burden infections (Gasch *et al.,* 2014), especially in the *mprF* gene resulting in altered cell membrane charge and daptomycin binding (Yang *et al.,* 2013; Humphries *et al.,* 2013). Co-resistance to vancomycin and daptomycin can result

from mutations in *walKR* and *rpoB* genes, and these mutations can arise during vancomycin treatment failure without daptomycin exposure (Humphries *et al.,* 2013; Bertsche *et al.,* 2013). Other changes targeting teichoic acids, phospholipid genes and cell surface charge have been implicated (Peleg *et al.,* 2012). Interestingly, daptomycin resistance was found in heterogenous vancomycin-intermediate *Staphylococcus aureus* (hVISA) and VISA isolates collected before the introduction of daptomycin in Australia (Kelley *et al.,* 2011), further demonstrating that non-susceptibility can emerge even without daptomycin selection pressure.

2.6.2.8 Ceftaroline

Hetero-resistance to ceftaroline has been reported in laboratory isolates of MRSA, hVISA, VISA, daptomycin-non susceptible S. *aureus* (DNSSA) and linezolid non susceptible *S. aureus* (LNSSA) (Saravolatz *et al.,* 2014). Mutations in PBP2a lead to lower binding affinity, reduced efficacy and higher MICs (Mendes *et al.,* 2012; Alm *et al.,* 2014). A study by Abbott *et al*. (2014) has demonstrated ceftaroline non-susceptibility among multi-drug resistant MRSA clinical isolates, particularly in ST239 MRSA (an endemic hospital MRSA clone).

2.6.2.9 Rifampicin

Although rifampicin is not a new MRSA-active agent, nor is rifampicin resistance a new phenomenon, it is important to appreciate that single mutations in the *rpoB* gene, which are commonly encountered in rifampicin-resistant *S. aureus*, can confer reduced susceptibility to both vancomycin and daptomycin (Cui *et al.,* 2010; Gao *et al.,* 2013). Mutations in *rpoB* have also been associated with reduced susceptibility to host antimicrobial peptides and promote persistent infection (Gao *et al.,* 2013). Using dual therapy with vancomycin or daptomycin plus rifampicin without additional anti-MRSA agents is not recommended.

2.7 Methicillin Resistant *Staphylococcus aureus* (MRSA)

MRSA is any strain of *S. aureus* that has become resistant to beta-lactam antibiotics which include penicillin (methicillin, oxacillin, dicloxacillin etc.) and the cephalosporins. It is a well-recognized nosocomial pathogen that is often implicated in a wide range of infections, the most common being the skin infection, food poisoning, pneumonia, lung abscess, empyema and infective endocarditis. Severe cases of these infections are associated with significant morbidity and mortality especially in those with underlying conditions (Wang *et al*., 2001; Gould *et al*., 2012; Tong *et. al.,* 2020). Mortality from MRSA infections is twice as high as when compared with methicillin susceptible *Staphylococcus aureus* (MSSA) (Moellering 2014). MRSA like most *S. aureus* form yellowish colonies although white variants are also common. The biochemical basis for the identification of MRSA includes the production of catalase, coagulase, DNase and the alternative PBP2A which encodes resistance to methicillin (Bannerman, 2003).

2.7.1 Historical Evolution of Methicillin Resistant *Staphylococcus aureus* (MRSA)

Earlier treatment of *Staphylococcus aureus* infections involved the use of a beta-lactam drug, penicillin (Geddes, 2008). It was after the discovery in 1940 of the active beta-lactam ring enzyme “penicillinase” from *Escherichia coli* that was capable of hydrolyzing penicillin that resistance to this antibiotic was first observed. Some years later in 1944, *S. aureus* was also reported to produce penicillinase (Rayner & Munckhof, 2005). Treatment of *S. aureus* then became difficult due to the ability of the bacterium to rapidly develop multi-drug resistance.

Towards the late 1940s, 50% of *S. aureus* strains obtained from patients were resistant to penicillin in the USA. Greenword *et al.* (2002) reported that 90% of *S. aureus* strains recovered from across different hospitals all over the world were penicillin resistant. Going further from the study of Greenwood *et al.* (2002), it was observed that 90 to 95% of *S.*

aureus strains worldwide are penicillin resistant, with the plasmid encoded penicillinase readily transferable through transduction or conjugation.

A smart replacement for the treatment of *S. aureus* infections following the loss of efficacy of penicillin therapy was methicillin; a semisynthetic penicillin which was introduced in 1960 (Chambers, 2001). In less than one year after the introduction of methicillin as an anti-staphylococcal drug, some methicillin resistant *S. aureus* (MRSA) strains were reported in 1961 (Lowy, 2003). It was the late Professor Patricia Jevons who reported the first human *S. aureus* strain to be methicillin resistant in a UK hospital (Kim, 2009). MRSA isolates were soon obtained from other European countries, and later from Japan, Australia, and the United States (Lowy, 2003).

The dissemination of methicillin resistant *S. aureus* strains from hospital settings was subsequently reported by File (2008). It was the United States who in1968 reported the first major outbreak of MRSA (Palavecino, 2004) while in the 1970s, *S. aureus* strains had become resistant to most penicillinase-stable penicillins. MRSA was initially believed to be of human origin until some MRSA strains were isolated from mastitic cow in the year 1972. Afterwards, reports of MRSA infection associated with different domestic and wild animals have been documented (Rich & Roberts, 2004).

2.7.2 Mechanism of Methicillin Resistance

Development of methicillin resistance arose as a result of the production of an altered penicillin binding protein (PBP2a), which is usually a direct consequence of the acquisition of another penicillin-binding protein (PBP2a) gene, namely *mec*A (Gaze *et al*., 2008). *Mec*A gene is responsible for the synthesis of PBP2a which is a 78KDa protein bound on the cell membrane that catalyze the transpeptidation reaction that is responsible for the cross-linkage of peptidoglycan chains (Fishovitz *et al.,* 2014). PBP2a which has a low affinity for beta-

lactam antibiotics becomes a substitute for other PBPs and enables the organism survive exposure to high concentration of the antibiotics. Thus, resistance to methicillin leads to resistance to all beta-lactam agents.

The difference between PBP2a and other PBPs is that PBP2a's active site does not allow binding of all beta-lactams but allows the transpeptidation reaction to proceed even when other PBPs are inhibited (Lim & Strynadka, 2003).

2.7.2.1 Regulation of PBP2a Expression

The *mecA* gene is carried on the staphylococcal cassette chromosome mec (SCC*mec*), while the regulator genes *mec*R1 and *mec*I are responsible for its expression. The regulator gene *mecR1* is activated by beta-lactam antibiotics and serves as a signal transducer that inactivates the *mecI* repressor gene product. The two genes, *mec*R1 and *mec*I are situated adjacently to *mecA* on the staphylococcal chromosome and their transcption is divergent from *mecA* (Stapleton & Taylor, 2002).

The mecR1 gene is responsible for expression of membrane-bound signal transduction protein (MecR1) while mecI encodes a transcriptional regulator (MecI). The promoter and operator regions are located between *mec*A and *mec*R1. *Mec*R1 and MecI have high protein sequence similar with the proteins, BlaR1 and BlaI, respectively and these are involved in the inducible expression of the staphylococcal β-lactamase gene, blaZ due to the similarities in the operator region, BlaI is able to regulate PBP2a expression. As a result, PBP2a expression can become inducible in the presence of any plasmid carrying the blaZ regulatory genes and this is a common phenomenon among clinical isolates of MRSA (Stapleton & Taylor, 2002).

2.7.2.2 Staphylococcal Cassette Chromosome *Mec* (Scc*mec*)

The gene *mec*A is located in a large chromosomal cassette called staphylococcal cassette chromosome *mec* element (SCC*mec*) which has a size of about 20–60 Kb. The Scc*mec* carries various mobile genetic elements that are integrated in it and more than 80 SCC*mec* elements have been identified in several staphylococci species. SCC*mec* is transferred among Staphylococcal species by horizontal gene transfer and integrates at a specific site known as integration site sequence (ISS). To date, 12 SCC*mec* types have been identified varying between approximately 21Kb to 67 Kb and each type is classified using a Roman numeral based on the unique combination of ccr complex and mec complex (Reichmann and Pinho, 2017). A complete SCCmec structure in *S. aureus* contains a *mec* complex (*mecA*, *mec*RI and mecI), a ccr complex (ccrA, ccrB, ccrC), and a J region (region other than *mec* and *ccr* complexes) (IWG-SCC, 2009).

Among the twelve SCC*mec* types (I–XII) that have been reported so far, five of them SCC*mec* I, II, III IV, and V with sizes 34, 53, 67, 21-24 and 28 kb respectively are globally distributed, while others only distributed in certain countries (Deresinsk, 2005). In general, distinguishing between HA-MRSA and CA-MRSA strains has relied solely on these five types (I-V) according to Deresinski (2005) stating that SCCmec type IV and V are more widely found among CA-MRSA, and the other three types (SCCmec I, II, III) are frequently found among HA-MRSA. An early study by Ito et al. detected only three types of SCCmec structures (SCCmec type I, II, III) isolated from human (Ito *et al*., 2004).

2.7.2.3 *Mec* gene complex.

The mec gene complex is composed of *mecA*, its regulatory genes and associated insertion sequence**.** *Staphylococcus aureus* has different *mec* complexes, which are classified into class A, class B, class C and class D (Ito *et al*., 2004). The *mec* gene complexes are structured as

follows: class A, IS431-*mec*A-*mec*R1-*mec*I; classB, IS431- *mec*A-Delta *mec*R1-IS1271; class C, IS431-*mec*A-Delta *mec*R1-IS431 and class D, IS431-*mec*A-Delta *mec*R1 (Ito *et al.,* 2004). In a study by Katayama *et al.* (2000), strains belonging to class C were found to have an intermediate level of methicillin resistance (MIC 16 to 64 mg/ml) when compared to other classes. Strains found to have neither the IS431 nor the IS127 were classified as class D *mec* strains (Katayama *et al.,* 2000). These four *mec* complexes together with the *ccr* gene complexes classify the different SCCmec types (Ito *et al*., 2001).

2.7.3 Healthcare-associated MRSA (HA-MRSA)

MRSA strains from hospital settings have been increasing gradually in the United States and most other parts of the world. Reports obtained in 2011 surveillance programme in USA indicate that there is a decline in the trend of MRSA infections specific to hospital settings (Raymund *et al.,* 2013). Depending on the sample size and study area, high rates of MRSA (>50%) have been obtained in USA, Asia and Malta, intermediate rates (25-50%) reported in Africa, Europe and China while in some part of Europe, the incidence is relatively lower than 50% (Mejìa *et al.,* 2010). Stafeni *et al.* (2012) computed the prevalence rates of HA-MRSA in some European countries like Ireland, France, and UK and reported a decline in hospital cases. While results obtained in Asia particularly South Korea (77.6%), Vietnam (74.1%), Taiwan (65%) and Hong Kong (56.8%) on HA-MRSA infections is still relatively high. The major lineage implicated in the hospital spread of MRSA between these continents is CC8 (ST239) (Harris *et al.,* 2010). Colonisation increases the chance of infection with MRSA acquired from hospitals (Safdar & Bradley, 2008). High occurrences of hospital colonization usually occur from contact with MRSA colonized patients or contaminated objects. Respiratory infection is a risk factor for the spread of MRSA through aerosols which can lead to serious infections and complications. Generally, HA-MRSA results in dermatitis, septicemias, endocarditis and lung diseases which are mostly seen in immunodepressed

people. Risk factors include hospitalization, dialysis, surgery, and previous history of MRSA infection (Umaru *et al.,* 2011). Individuals with some form of breach on their skin due to wounds or are carrying indwelling catheters as well as others who are immune compromised are at an increased risk of developing infection. Some strains have been described as notorious when it comes to spreading between patients and may spread between hospitals, possibly following the movement of colonized patients or hospital staff from one hospital to another. These strains are known as epidemic MRSA (or E-MRSA). During the 1990s there was a marked increase in infections caused by MRSA in hospitals in the UK due to the emergence and spread of two particular strains of EMRSA known as E-MRSA-15 and E-MRSA-16.

2.7.4 Community-Associated MRSA strains

A community-associated MRSA isolate is one previously associated with clinical specimen but recovered from a patient residing in a surveillance area who had no established risk factors for MRSA infection (Kluytmans-Vandenbergh & Kluytmans, 2006). Attempts at distinguishing between CA-MRSA and HA-MRSA from an epidemiological and clinical point of view led the CDC to describe CA-MRSA infection as any MRSA infection diagnosed for an outpatient within 48h of hospitalization in the absence of hospital associated risk factors including: haemodialysis, surgery, residency in a long-term care facility or hospitalization within the preceding year or the presence of an indwelling catheter at the time of culture (Morrison *et al.,* 2006).

The widely pronounced risk factors associated with acquisition of CA-MRSA include the isolation of MRSA two or more days following admission into a hospital, previous history of hospitalization, dialysis, surgery or residence in a long-term care facility within a year before the MRSA-culture date, presence of a permanent indwelling catheter or percutaneous medical device at the time of laboratory culture (Kluytmans-Vandenbergh & Kluytmans, 2006).

Table 2.1 Prevalence of MRSA Carriage in Some Countries Across the World

Country size	Sample	% Prevalence	Source	Reference
Argentina	591	16	Hospital	Egea *et al.,* 2014
Bangladesh	49	53.1	Hospital specimens	Afroz *et al.,* 2008
Bolivia	585	0.5	Community	Bartoloni *et al* 2013
Cameroon	295	34.6	Hospital staff/patients	Gonsu *et al.,* 2013
Chile	246	80	Hospital	Guzman-Blanco, 2009
Equator	1363	25	Hospital	Guzman-Blanco, 2009
Ethiopia	118	44.1	Hospital	Shibabaw *et al.,* 2013
Indonesia	1502	4.3+	Hospital	Santosaningsih *et al.,* 2014
Kenya	950	7.0	Hospital	Aiken *et al.,* 2014
Sudan	426	69.4	Hospital	Elimam *et al.,* 2014
Nigeria	208	19.2	Hospital	Olowe *et al.,* 2013
North India	6743	46	Hospital	Arora *et al.,* 2010

The etiologies of CA-MRSA are debatable; there are some propositions that CA-MRSA arose as a consequence of horizontal transfer of the methicillin resistance-determinant, *mec*A, into a methicillin-susceptible *S. aureus* strain whereas other studies are of the opinion that CA-MRSA are descendants of hospital isolates (Chambers, 2001). Community-associated MRSA is more virulent than typical HA-MRSA, due to the frequent production of Panton-Valentine leukocidin (*PVL*) toxin (Wannet *et al.,* 2005). Panton-Valentine leukocidin toxin is associated with deep skin infection, soft tissue infection and necrotizing pneumonia and has been identified as a genetic marker for CA-MRSA (Vandenesch *et al*., 2003).

Health-care associated MRSA is associated with bloodstream, urinary and respiratory tract infections whereas CA-MRSA infections are associated with deep skin infection, soft tissue infection and necrotizing pneumonia (File, 2008). The severity of CA-MRSA infections can result in hospitalization and possibly death following the release of the *PVL* toxin (Roberts *et al.,* 2008). Among 100 patients with MRSA infection in a study by Davis *et al.* (2007), SCC*mec* type IV was detected in 71% of the MRSA strains isolated with 54% of them possessing the *pvl* genes.

2.8 Diseases caused by MRSA

Staphylococcal diseases usually develop following either the production of a toxin or through the invasion and destruction of host tissues (Murray *et al.*, 2005). Diseases that arise from staphylococcal toxins include staphylococcal scalded skin syndrome (SSSS), staphylococcal food poisoning and toxic shock syndrome (TSS) (Murray *et al*, 2005). Other staphylococcal diseases include suppurative infections, wound infections and catheter related infections (Murray *et al.,* 2005).

2.8.1 Bacteremia

Staphylococcus aureus remains a common cause of community onset bloodstream infections (Collignon *et al.,* 2005). Staphylococcal bacteremia mortality rate was approximately 20% to 50% between 1992 and 1998 in Belgium (Blot *et al.,* 2002). The increased risk in staphylococcal bacteremia is mostly attributed to catherization and patients with a high nasal carriage (85%) of *S. aureus* in hospital settings. Although MRSA bacteremia incidence has decreased over the past decade, other risk factors for HA-MRSA bacteremia include prior hospitalization within one year, presence of indwelling catheter, human immunodeficiency virus (HIV), previous MRSA infection, malnutrition and undeveloped immune system in infants (Naidoo *et al.,* 2013; Tong *et al*., 2015).

2.8.2 Impetigo

Impetigo is a contagious skin infection caused by *Staphylococcus* sp. or *Streptococcus* sp. It is more common in children than in adults. There are two forms of impetigo; non-bullous impetigo and bullous impetigo. The non-bullous impetigo is more common and can be caused by either bacterium. Here small red papules are observed and will eventually evolve to small blisters and finally scab over with a characteristic honey colored crust. In bullous impetigo, which is only caused by *Staphylococcus* sp., the top skin layer (epidermis) and the lower skin layer (dermis) separate due to a toxin that is produced by *Staphylococcus*, thus leading to the formation of a blister (termed *bulla* in medicine). These blisters which are fragile and contain yellow liquid often break and leave red and raw skin. Finally, a dark crust will develop (Cole & Gazewood, 2007).

2.8.3 Endocarditis

Staphylococcus aureus infections was reported to be the leading cause of infectious endocarditis with an impressive mortality rate despite improved diagnostic and treatment procedures. It is well known to be an aggressive and often fatal infection. Individuals with

increased chances of developing endocarditis include elderly patients, children, prosthetic valve patients, intravenous drug users and hospitalized patients (Valente *et al.,* 2005). Valente *et al.* (2005) recorded a 12% incidence rate of infective endocarditis among children in North Carolina, USA between 1998 and 2001 following complications of *S. aureus* associated bacteremia. Prognosis of *S. aureus* related endocarditis is worsened in patients with HIV infection, as it usually presents as an advanced infective endocarditis. Studies by Fernandez- Guerrero *et al.* (2009) observed that patients with left sided endocarditis developed more complications coupled with higher mortality than those with right sided endocarditis. In a previous study by Fowler *et al.* (2005), *S. aureus* accounted for 25.9% and 54.2% of infective endocarditis in Australia/New Zealand and Brazil, respectively.

2.8.4 Toxic Shock Syndrome (TSS)

Toxic shock syndrome is a rare, severe life-threatening disease caused by *S. aureus* getting into the blood and releasing harmful toxins. The disease is characterized by fever, rash, desquamation, organ dysfunction, and shock (Sharma *et al.,* 2018)

In the 1980's, the disease was frequently observed in women with the onset of menstruation where it reached epidemic proportions and the sudden increase was attributed to the introduction of hyper-absorbable tampons. The prevalence of TSS decreased moderately when the tampons were removed from the market, with 3 to 15 per 100,000 women of menstrual age/year subsequently. Non-menstrual cases have been associated with localized infections, surgery or insect bites. Researchers suggested that cases of non-menstrual toxic shock syndrome have a higher mortality rate compared to cases of menstrual involved toxic shock syndrome (Waldvogel, 2000). These female cases have been associated with caesarean section surgeries and long-term diaphragm use (Waldvogel, 2000). Initial symptoms include diarrhea, fever, myalgias and vomiting (Waldvogel, 2000). Hypovolemic shock develops due

to loss of colloids and fluids (Chuang *et al.,* 2005). A sunburn-like rash develops within a few hours with the involvement of conjunctival inflammation (Waldvogel, 2000).

Diagnosis and treatment of TSS includes identification of the *S. aureus* strain and resistance profiling of the identified strain (White *et al*., 2005). Electrolytes and fluid replacement should be given to the patient as part of the overall therapy (White *et al.,* 2005). An adjuvant treatment approach included agents that can block TSS superantigens, such as intravenous immunoglobulin that contains superantigen neutralizing antibodies (Chuang *et al.,* 2005).

2.8.5 Food poisoning

Staphylococcus aureus is the leading cause of gastroenteritis resulting from the consumption of contaminated food (Le Loir *et al*., 2003). *Staphylococcus aureus* food poisoning is due to the release of toxins in the food during its growth, causing symptoms ranging from abdominal pain to nausea, vomiting and sometimes diarrhea but never diarrhea alone. The onset of *S. aureus* food poisoning is rapid, ranging from 30 min to 8 h after ingestion, with spontaneous remission after 24 hrs.

Staphylococcus aureus enterotoxins (SEs) involved in food poisoning are highly stable and resistant to neutralization by proteolytic enzymes, such as pepsin or trypsin. There are 14 different SE types, which have similar structures (Le Loir *et al*., 2003). *Staphylococcus aureus* enterotoxins are small proteins that are produced in food, soluble in water and are rich in lysine, aspartic acid and glutamic acid (Le Loir *et al*., 2003). These SE's are more heat resistant in food than in laboratory medium.

Various high sugar, protein and salt content foods are involved with *S. aureus* food poisoning including milk and milk products (cheeses and ice creams), sausages, canned meat, salads (potato salads) and sandwich fillings. The foods that are involved in *S. aureus* food poisoning differ from one country to another. The main sources of contamination of these foods are

food-handlers by manual contact, coughing or sneezing since up to 50%-70% of the human populations are *S. aureus* carriers (Solberg, 2000; Le Loir *et al*., 2003). In a study by Gadaga *et al.* (2008), 32% of food handlers were found to be carriers of *S. aureus* in Zimbabwe compared to 6.4% food handlers carrying *E. coli.* Other sources involve contamination from animal origins either by animal carriage or zoonosis (Le Loir *et al*., 2003).

2.8.6 Staphylococcal Scalded Skin Syndrome (SSSS)

Staphylococcal scalded skin syndrome is described as a disease manifested by a bullous exfoliative dermatitis in infants less than 1 month old (Patel & Finlay, 2003). The disease presents occasionally with an onset of general localized erythema and spreads to the entire body in less than two days. The disease is rare in adults and the symptoms are usually followed by an upper respiratory infection or a purulent conjunctivitis and tender flaccid bullae. The bullae expand and burst to expose moist and erythematous skin which gives rise to the scalded appearance (Patel & Finlay 2003). The disease has been attributed to the production of an exotoxin known as epidermolytic toxin (ET) which cleaves desmoglein 1 complex-an important dersmosomal protein (Patel & Finlay, 2003). In a study conducted by Mockenhaupt *et al.* (2005), SSSS accounted for between 0.09 and 0.13 of cases per 1 million patients with a 51% mortality rate in Germany from 2003 to 2004. In the study by Mockenhaupt *et al.* (2005), 11% and 40% was observed in children and adults, respectively. Staphylococcal scalded-skin syndrome has been shown to be due to exfoliative toxins (Yamasaki *et al.,* 2005). The exfoliative toxin genes, *eta* and *etb*, have been detected in 30% and 19% of SSSS presenting patients by polymerase chain reaction (PCR) (Yamasaki *et al.,* 2005).

2.9 Diagnostic Identification of MRSA from Clinical Specimens

Identification of Methicillin-resistant *Staphylococcus aureus* has always relied upon use of various phenotypic and genotypic techniques (Fluit *et al.,* 2001). Identification begins with phenotypic identification of *S. aureus* which involves gram-staining, catalase, coagulase, DNAse, culture on mannitol salt agar or blood agar and sugar fermentation tests (Waldvogel, 2000). Following identification of *S. aureus,* further tests may be carried out on suspicion of MRSA. Susceptibility testing may be employed using any standard method, latex agglutination methods can be employed to detect PBP2a and/or PCR tests performed to detect *mecA* gene (Brown *et al*., 2005). Furthermore, disc diffusion (DD) testing with cefoxitin or oxacillin has been well correlated with the presence of *mecA*-mediated methicillin resistance and sensitivity of 98% and specificity of 100% reported (Velasco *et al.,* 2005). The result obtained from the cefoxitin DD is usually used to report the test as susceptible or resistant as it is considered to be most sensitive and was easier to interpret. On the basis of studies carried out by different authors CLSI now approves the use of cefoxitin DD for detecting methicillin resistance in *Staphylococcus* (CLSI, 2009).

Another test that can be used to detect MRSA is a latex agglutination test based on the reaction of latex particles sensitized with monoclonal antibodies against PBP2a of *S. aureus* and PBP2a extracted from tested colonies. The test is usually performed according to the manufacturer's instructions. The assay is based on the detection of the PBP2a in approximately 20 minutes (Chapin & Musgnug, 2004). Monoclonal antibodies against PBP2a sensitize latex particles of isolated MRSA colonies. The assay is rapid and sensitive when compared to other phenotypic methods such as the standard agar disk diffusion test. Lee *et al.* (2004) reported a 100% sensitivity and specificity for the detection of MRSA using the MRSA-Screen latex agglutination test (Lee *et al.,* 2004). In another study by Cuevas *et al.* (2003), the latex agglutination PBP2a test had a sensitivity of 100% and a specificity of

98% for evaluating 137 MRSA isolates. However, several reviews showed that any test involving the clumping factor may give false positive results (Brown *et al.,* 2005).

2.9.1 Molecular identification and characterization assays of MRSA strains

Due to the limitations of time involved in conventional identification of *S. aureus* isolates, molecular based detection techniques, including conventional PCR and real-time PCR, have been developed for instant characterization of MRSA isolates (Fluit *et al.,* 2001). Molecular techniques are often combined with antimicrobial susceptibility testing methods for the routine diagnostic MRSA detection because of the inability of susceptibility testing alone to confirm the presence of MRSA as a result of fluctuations in the test conditions (Trindade *et al.,* 2003). The identification of MRSA has become simplified following the introduction of polymerase chain reaction (PCR) technique (Van Pelt-Verkuil *et al.,* 2008).

2.9.2 Application of PCR assays for identification of MRSA

Polymerase chain reaction is a process that allows amplification of pre-determined DNA regions (genes) by the use of small target specific DNA sequences referred to as primers (Van Pelt-Verkuil *et al.,* 2008). It is a rapid, powerful and reliable molecular method for MRSA typing and can generate microgram amount of DNA from a few molecules of specific target nucleic acid within a short period of time (Hoffmann *et al.,* 2009). In carrying out the test, two oligonucleotide primers flank and define the target sequence that is to be amplified (Hoffman *et al.,* 2009). These primers hybridize to the opposite strands of the DNA and serve as initiation points for amplification and a thermostable enzyme, DNA Taq polymerase catalyzes this synthesis (Hoffman *et al.,* 2009).

The use of PCR for the detection of the *mecA* gene and SCCmec typing has been described previously by Oliveira and De Lencastre (2002), and used as a reference method for SCCmec typing (Zhang *et al.,* 2005). In the case of MRSA, PCR-based assays detect the *mecA* gene

responsible for mediating methicillin resistance in staphylococci. Other genes such as *femA, femB,* 16S rRNA, *pvl* and *nuc* genes may be detected in MRSA isolates but these genes may be absent in some MRSA strains (Fluit *et al.,* 2001; McClure *et al.,* 2006).

Polymerase chain reaction-based methods have been shown to have shortened the turn-around time (2h to 4h) in identifying MRSA isolates resulting in prompt treatment for MRSA associated infection. However, when PCR method is used to detect multiple genes in *S. aureus* simultaneously, it is called multiplex PCR (M-PCR).

2.9.3 Plasmid Profile Analysis

Plasmid analysis was the first molecular technique used for epidemiological investigation of MRSA and MSSA. The method is relatively easy to perform and interpret but poor in discrimination (Mehndiratta & Bhalla, 2012). In this technique, the isolates are differentiated according to the number and sizes of plasmids carried by an isolate, but its reproducibility suffers due to the existence of plasmids in different molecular forms such as supercoiled, nicked or linear, each of which migrates differently on electrophoresis. The discriminatory power of this method can be improved by restriction endonuclease analysis which exploits the differences in the position and number of restriction sites between two unrelated plasmids of the same size (Mehndiratta & Bhalla, 2012).

The plasmids contain resistant genes against a number of antimicrobial agents, so it has been useful to assess the relatedness of individual clinical isolates of *S. aureus* in the epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance (Shahkarami *et al.,* 2014).

Agbagwa & Jirigwa (2015) determined the antibiotic-resistant pattern and plasmid profile of *S. aureus* obtained from wound swabs and found similar antibiotic resistance pattern, while

different plasmid sizes were observed in the isolates. Jaran also found no direct correlation between the patterns of antibiotic resistance and plasmid profiles in clinical isolates of *S. aureus* in hospitals of Saudi Arabia. This disparity can be due to R-plasmids of different sizes which are also responsible for the presence of multiple resistances.

The technique has not been found to be very useful for the investigation of outbreak of infections because the plasmids can be spontaneously lost or readily acquired and related strains can exhibit different plasmid profiles. Also, certain genes are contained in transposons that can be readily acquired or deleted, while some isolates may also lack plasmids and will not be typeable by this method (Shahkarami *et al.,* 2014; Agbagwa & Jirigwa, 2015).

2.9.4 Random Amplified Polymorphic DNA-PCR

RAPD-PCR is a typing method based on the use of short random primers which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. In other words, random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) is a technique that uses short primers of 10 base pairs (bp) with random sequences of nucleotides to randomly amplify DNA targets producing fragments, which serve as genetic markers. The amplification products are separated by agarose gel electrophoresis to generate a bacterial fingerprint and the banding patterns are used to compare the relatedness of bacterial strains. The method does not require prior special knowledge of specific DNA target sequences (Ranjbar *et al.,* 2013). This makes it a flexible tool with general applicability. RAPD-PCR has the additional advantage of a short turnaround time and requiring a limited amount of bacterial DNA to carry out the analysis. However, the discriminatory power is variable according to number and sequence of arbitrary primers and amplification conditions. The short-fall of this method is that it suffers from problems in low inter-run and inter-laboratory reproducibility and from a lack of consensus

rules for interpretation of pattern differences. The RAPD technique has also been shown to be suitable for routine genotyping of hospital-acquired MRSA (Neslihan and Isil, 2014). However, the technique has been documented to have an inferior discriminatory power when compared with pulsed-field gel electrophoresis (PFGE).

2.9.5 Pulsed-field gel electrophoresis for genotyping of MRSA strains

Pulsed-field gel electrophoresis (PFGE) is considered the "gold standard" of all known molecular typing methods because it has good reproducibility, discriminatory power and typeability but PFGE is sensitive to genetic instability, has limited availability and requires at least 3–4 days to complete a test (Strandén *et al*. 2003; Sabat *et al.* 2006). The technique was developed in 1984 by Schwartz and Cantor (Trindade *et al*., 2003) and is based on the digestion of bacterial DNA with restriction enzymes that recognize specific sites along the chromosome (Trindade *et al*., 2003). The restriction enzyme digestion generates large DNA fragments that cannot be separated by conventional electrophoresis. The electric field is pulsed at different angles across the gel allowing the DNA fragments to separate in order of size. The PFGE system can separate DNA fragments of up to 10 megabase pairs (Kiadó, 2006). The PFGE system consists of a power supply system, with a voltage of up to 750 volts, a switch unit that can alternate current at different directions of a computer system to control the resolution in PFGE and cooler system that regulates the PFGE system since DNA is temperature sensitive (Basim, 2001). Pulsed-field gel electrophoresis has been found to have a high discriminatory power and was illustrated in epidemiologic studies done in Brazil (Trindade *et al*., 2003). A high discriminatory power is an important characteristic of a typing technique defined as the probability that isolates with related and identical phenotypic and genotypic profiles are clonal and part of the same transmission (Trindade *et al*., 2003).

The PFGE technique has been extensively used for MRSA typing compared to other techniques (Trindade *et al*., 2003). However, the limitation of PFGE is the extended time

before the results are available, and in addition, PFGE requires high-cost reagents and specialized equipment including restriction enzymes, specialized power supply and high molecular weight markers (Stranden *et al*., 2003). Though procedural steps in the technique are straight forward, the time needed to complete analysis can take up to a week, which reduces the ability of the laboratory to analyze large numbers of samples (Stranden *et al*., 2003). The DNA fragments are run on an electrophoresis gel with alternating electrical current.

2.9.6 Restriction Endonuclease Analysis (REA) of Chromosomal DNA

In this technique restriction endonuclease enzyme is used to cut DNA at a specific nucleotide recognition sequence. The size and number of restriction fragments generated is dependent on the recognition sequence of the enzyme and the composition of DNA. The resulting fragments are separated based on their size on agarose gel electrophoresis. The patterns are then stained by ethidium bromide and examined under UV light. As a result of differences in their DNA sequences, different strains of the same species can have different REA profiles. All strains can by typed with good reproducibility. The main shortcoming of this method is the generation of large complex number of bands. These bands overlap and make interpretation difficult and lower the discriminatory power (Mehndiratta & Bhalla, 2012).

2.9.7 Staphylococcal cassette chromosome mec (SCCmec) typing

S. aureus becomes methicillin resistant through the acquisition of a mobile genetic element called Staphylococcal Cassette Chromosome mec (SCCmec) that contains *mecA* gene complex and ccr gene complex. Several ccr and *mec* allotypes have been discovered among SCCmec element. There are currently eight types of SCCmec (type I to type VIII) and many sub-types have been distinguished among MRSA strains. However, it is believed that each SCCmec type encodes for resistance to different antibiotics. (Chongtrakool *et al*., 2006; Zhang *et al*.,2009). Differences among these SCCmec types have made the basis for variation

among MRSA strains. Reports indicate that healthcare associated MRSA (HA -MRSA) strains contain majorly type I, type II and type III SCCmec cassettes while CA-MRSA strains contain type IV and type V cassettes, notwithstanding that several variants have also been reported (Arakere *et al.,* 2005).

2.10 Treatment of *S. aureus* infections

The use of antibiotics in the treatment of infection should be dependent on the result of antimicrobial susceptibility testing, although most strains may appear susceptible during testing but becomes resistant when treatment commences (Grema *et al*., 2015). Penicillin is still the main drug of choice for staphylococcal infections as long as the isolate is sensitive to it (Kowalski *et al.,* 2003). However, experts' advice that in area with high prevalence of MRSA, there should be a change in the empirical treatment of these infections from β lactams to antibiotics with activity against MRSA (Kluytmans & Struelen, 2009). In patients with histories of a delayed-type penicillin allergy a cephalosporin, such as cefazolin or cephalothin can be administered as an alternative choice of treatment. Vancomycin, a glycopeptide, is the cornerstone for treating invasive MRSA infections. Alternatively, teicoplanin, which belongs to the same class, is available in some countries. However, these drugs have limited efficacy for deep seated infections, partly because of poor diffusion into tissues such as the bone and lung and because of a gradual decrease in susceptibility of MRSA over recent years. These glycopeptide intermediate resistant strains have mutations that affect the synthesis of the bacterial cell wall and are not easy to detect in microbiology laboratories (Lewis *et al*., 2007). Patients who are intolerable to vancomycin are treated with a fluoroquinolone (ciprofloxacin), lincosamide (clindamycin), tetracycline (minocycline) or trimethoprim-sulfamethoxazole, which is also known as co-trimoxazole. Novel quinolones, such as ciprofloxacin with increased anti-staphylococcal activity are available but their use may become limited due to the rapid development of resistance during therapy.

An evaluation of glycosylated polyacrylate nanoparticles in an earlier study by Abeylath *et al.* (2007) revealed significant *in vitro* activities against methicillin-resistant *S. aureus* and *Bacillus anthracis*. Other recent investigative drugs include, silver nano particles, oleanolic acid from extracted *Salvia officinalis* (Sage leaves) (Horiuchi *et al.,* 2007; Yuan *et al.,* 2008). Two novel antibiotics, neocitreamicins I and II, isolated from a fermentation broth of a *Nocardia* strain have shown to have *in vitro* activity against *S. aureus* and vancomycin-resistant *Enterococcus faecalis* (VRE) (Peoples *et al.,* 2008). Conclusively an accurate empirical therapy against *S. aureus* infections would be an important step towards the reduction of the development of resistance in the different strains.

2.11 Newer Agents with MRSA Activity

Fortunately, there have been several new antimicrobials approved or in development that have activity against multi-resistant Gram-positive pathogens including MRSA. Some of these drugs include;

2.11.1 Ceftaroline

Ceftaroline is a cephalosporin with activity against MRSA because of its affinity for penicillin-binding protein 2a (PBP2a) and has been approved for use in acute bacterial skin and skin structure infections (ABSSSIs) and community-acquired pneumonia (CAP) (Poon *et al.,* 2012).

2.11.2 Tedizolid

A further improvement to the class of oxazolidinone antibiotics is tedizolid (previously referred to as torezolid). It was reported that improved pharmacokinetics from tedizolid facilitates once-daily dosing, and its *in-vitro* potency is up to 16 times that of linezolid (Rybak *et al.,* 2013; Locke *et al.,* 2014). It has been specifically designed to be active against

linezolid non-susceptible (LNS) *S. aureus*, including strains containing the multidrug resistance cfr gene (Locke *et al.,* 2014; Moellering, 2014).

2.11.3 Telavancin

The lipoglycopeptides are semi-synthetic derivatives of glycopeptides. Telavancin is potently bactericidal due to dual mechanisms of action with inhibition of cell wall synthesis and cell membrane depolarization (Saravolatz *et al.,* 2009). It has *in-vitro* activity against MRSA, VISA, DNS and LNS *S. aureus* (Rodvold & McConeghy, 2014; Rybak *et al.,* 2013). Telavancin was approved in Europe and the United States for HAP caused by Gram-positive pathogens including MRSA in which alternative treatments are not suitable, on the basis of the results of investigations carried out by Rubinstein *et al.* (2011).

Other antimicrobial agents with activity against MRSA that are currently evaluated include: (i) oritavancin, a semisynthetic glycopeptide (Guay, 2004); (ii) tigecycline, a monocycline derivative (Guay, 2004) and (iii) DW286, a fluoroquinolone (Kim *et al.,* 2003). Amongst these three antibiotics, tigecycline has been approved by the Food and Drug administration (FDA) (Stein & Craig, 2006). Newer drugs such as omadacycline, ceftobiprole, oritavancin and dalbavancin look promising on the treatment of MRSA. Other existing drugs like fusidic acid and fosfomycin are currently under investigation for potential use in the treatment of MRSA infections (Burke & Rose, 2014).

2.12 New Combination Therapy

Combinations of vancomycin or daptomycin with β-lactams are being increasingly used to treat serious and invasive MRSA infections. Many *in-vitro* studies have demonstrated synergy with these combinations, even if the β-lactam itself does not possess anti-MRSA activity (Rybak *et al.,* 2013). The proposed mechanism is the ‘seesaw effect’, in which β-lactams thin the cell wall to allow vancomycin to bind to target sites during cell wall

synthesis, or in which β-lactams increase the negative cell surface charge to allow improved daptomycin binding and bactericidal activity (Ortwine *et al.,* 2013). Recent studies have also demonstrated an impact of β-lactams on susceptibility to host immune factors (Sakoulas *et al.,* 2014). Further studies to systematically evaluate the impact of combination therapy are warranted.

2.13 Vaccine development for the prevention of *S. aureus* infections

The wide-spread infections caused by multi-drug- resistant *S. aureus* have demanded priority in the development of an effective therapeutic approach. Although some vaccine candidates have shown protective efficacy in preclinical phase or early clinical phase studies, so far, no vaccine has been approved for human use (Ansari *et al.,* 2019). The first attempt to develop *S. aureus* vaccine was by the use of CP5 and CP8 conjugated to recombinant *P. aeruginosa* exoprotein A. The formula was called Staphvax and was developed by biopharmaceuticals in 2002 though unsuccessful (Ansari *et al*., 2019). Continuous attempts were made by different institutions like University of Chicago and the Absynth biologics which uses clotting factors to produce abscess and membrane protein, respectively (Cheng *et al*., 2010). However, trial on mice did not produce the desired result of abscess development and antibody production (Hu *et al.,* 2003). Primarily, the vaccine development focuses on the driving of antibody response which is able to block the toxins involved in the killing of immune cells as well as helping in the opsonization of bacterial cells. Therefore, several attempts have been made in the development of safe and effective vaccines. However, some vaccine candidates failed to show significant protection and this may be because of over-reliance on the antibody-mediated protective response (O'Brien & McLoughlin, 2019).

Some researchers have however come up with vaccines containing multiple antigens. Recently, two types of vaccines namely, SA3Ag vaccine possessing CP5, CP8, and ClfA and *S. aureus* four- antigen (SA4Ag) vaccine possessing CP5, CP8, ClfA and recombinant P305A

developed from a lipoprotein manganese transporter C (MntC) have been successfully manufactured by researchers, which have shown superior immunogenicity when compared to previous vaccines. The reports have demonstrated that the previous vaccines produced anti-staphylococcal antibodies capable of binding with *S. aureus* leading to their engulfment by phagocytic cells while the multi-antigen vaccines (SA3Ag and SA4Ag) are capable of inducing high level anti-staphylococcal immunoglobulins that lead to the killing of *S. aureus* by increasing the phagocytosis of bacteria and were concluded to be safe with no significant increase in systemic adverse effects or local adverse effects in healthy adults (O'Brien & McLoughlin, 2019). These vaccines are still undergoing clinical trials. Other vaccines currently being researched on include recombinant *pvl* vaccine, combined vaccine containing recombinant *S. aureus* surface protein A((SasA) and the internal heavy chain translocation domain C-fragment of tetanus neurotoxin (TenT-Hc). There are also trials going on in the use of antistaphylococcal monoclonal antibodies for immunization (Ansari *et al.,* 2019)

2.14 Other Management Strategies: (Medicinal Plants)

The demands for antibacterial agents are high as resistance to most antimicrobials continues to soar; consequently, the search for potential drug is beamed towards plant sources. Infections caused by resistant bacteria represent a major public health burden in terms of morbidity, mortality, increased expenditure on patient management and infection control measures (Woodford & Livermore, 2009). Plants which have served as a source of medicinal compounds have continued to play a dominant role in the maintenance of human health since ancient times (Cragg *et al.,* 2012). It is recorded that several plants contain substances that have been used for therapeutic purposes, and which are precursors for the synthesis of drugs. According to the World Health Organization plant extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. Over 50% of

all modern clinical drugs are of natural product origin (Erecevit & Kirbag, 2017). Many hundreds of plants worldwide are used in traditional medicine as treatments for various bacterial infections. Conventional drugs usually provide effective antibiotic therapy for bacterial infections but there is an increasing problem of antibiotic resistance and a continuing need for new solutions. Although natural products are not necessarily safer than synthetic antibiotics, some patients prefer to use herbal medicines (Karen & Edzard, 2003). Some of the reasons for the use of plant products include the high cost of important conventional drugs and/ or inaccessibility to western health care facilities, efficacy, popularity and low cost. Moreover, medicinal plants possess immunomodulatory and antioxidant properties, which stimulate both non-specific and specific immunity leading to antibacterial activities (Okwu *et al.*, 2019). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficacy. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant (Nascimento *et al.,* 2000).

2.14.1 Plant Derived Antimicrobials

Medicinal plants possess antimicrobial compounds which inhibit bacterial growth by different mechanisms than the presently used antimicrobials and may have a significant clinical value in treatment of resistant microbial strains. Extracts isolated from several plants have been reported to have biological activities such as antimicrobial, anti-inflammatory and antioxidant activities (Mehta *et al*., 2001; Parham *et al*., 2020).

Phenolic compounds are one of the most diverse groups of secondary metabolites found in edible plants. They are found in a wide variety of fruits, vegetables, nuts, seeds, stems and

flowers as well as tea, wine, propolis and honey, and represent a common constituent of the human diet. In nature they are involved in plant growth and reproduction, provide resistance from pathogens and predators and protect crops from disease and pre-harvest seed germination (Oluwusulu & Ibrahim, 2006). There are different classes of polyphenols known as tannins, lignins and flavonoids. Each class of polyphenols possesses chemical characteristics that set them apart from one another.

Flavonoids are the most widely occurring polyphenol and are present in almost every form of human consumed vegetation. Dietary flavonoids have attracted interest because they have a variety of beneficial biological properties, which may play an important role in the maintenance of human health. Flavonoids are potent antioxidants, free radical scavengers and metal chelators; they inhibit lipid peroxidation and exhibit various physiological activities including anti-inflammatory, anti-allergic, anti-carcinogenic, anti-hypertensive, anti-arthritic and antimicrobial activities. Consumption of phenol-rich beverages, fruit and vegetables has commonly been associated with reduction of the risk of cardiovascular diseases in epidemiological studies (Whiting, 2001). Flavonoids have been found to be the most abundant polyphenols. The biosynthesis of flavonoids is stimulated by sunlight (ultraviolet radiation), so higher concentrations of flavonoids can typically be found in the outer most layers of fruits and vegetables (i.e., the skins). Flavonoids can be divided into six subclasses according to the degree of oxidation of the oxygen heterocycle: flavones, flavonols, isoflavones, anthocyanins, flavanones and flavonols (catechins and anthocyanidins).

Extraction of polyphenols can be performed using a solvent like water, hot water, methanol, methanol/formic acid, methanol/water/acetic or formic acid etc. Therefore, the total polyphenol amounts detected from the same plant and their corresponding antioxidant and antimicrobial activities may vary widely, depending on external conditions applied. It has been reported that the antimicrobial action of phenolic compounds was related to inactivation

of cellular enzymes, which depends on the rate of penetration of the substance into the cell or caused by membrane permeability changes (Schultz *et al*., 1992).

Terpenoids and essential oils are also plant derived antimicrobials. The oils are secondary metabolites which are highly enriched in compounds based on isopropene structure. They are called terpenes. When compounds contain additional elements usually oxygen, they are termed terpenoids. Terpenes or terpenoids are active against bacteria, viruses and protozoa. The mechanism of action of terpenes is not fully known but it is speculated to involve cellular membrane disruption (Zengin & Baysal, 2014).

Heterocyclic nitrogen compounds are called alkaloids. The mechanism of action of highly aromatic planar quaternary alkaloids is attributed to their ability to intercalate with DNA of microorganisms.

Lectins and polypeptides are often positively charged and contain disulphide bonds. Their mechanism of action may be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Sher, 2004)

2.14.2 *Acalypha wilkesiana*

Acalypha wilkesiana is a shrub commonly known as copperleaf or Jacobs coat that can grow in tropical and sub-tropical climates.

2.14.2.1 Uses

In Southern Nigeria, expressed juice or boiled decoction of the leaves of *A. wilkesiana* is used in traditional health care practice, for the management of gastrointestinal disorders, fungal skin infections, hypertension and diabetes mellitus. The leaf-poultice is used in the treatment of headache, swellings, colds and malaria (Oladunmoye, 2006).

2.14.2.2 Pharmacological Properties

Several lines of evidence point to the fact that members of the genus *Acalypha* are pharmacologically active (Mothana *et al.,* 2008; Canales *et al.,* 2011). Antibacterial and antifungal properties have been observed from *A. indica* L., *A. fruticose* Forssk., *A. communis* Müll. Arg., *A. siamensis* Gagnep., *A. monostachya* Cav., *A. hispida* Burm. f. and *A. wilkesiana* Müll. Arg. (Oladunmoye., 2006; Mothana *et al.,* 2008; Canales *et al.,* 2011). Antioxidant and protective properties were elicited by *A. indica* L., *A. fruticose* Forssk., and *A. racemose* Wall. ex Baill. (Mothana *et al.,* 2008). Extracts of *A. wilkesiana* Müll. Arg., *A. alopecuroidea* Jacq. and *A. australis* L. have displayed anti-cancer properties against different cell lines *in vitro* (Madlener *et al.,* 2010).

Medicinal plant members of the family Euphorbiaceae impart anti-infective and wound healing properties. For instance, a bark extract of *Jatropha curcas*L. (Euphorbiaceae) which is traditionally used to treat diarrhea and dysentery inhibited the growth of *Bacillus subtilis*, *Sarcinalutea* and *Salmonella thyphi* (Gupta *et al.,* 2011). An extract of *Acalypha indica* L. (Euphorbiaceae) inhibited Gram-positive bacteria and traditionally used for bronchitis, pneumonia and asthma (Somchit *et al.,* 2010). *Croton hirtus* L'Hér. and *Euphorbia hirta* L. (Euphorbiaceae) exhibited antibacterial activity against *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* (Wiart *et al.,* 2004).

In vivo studies evidenced the anti-diabetic properties from *A. wilkesiana* Müll. Arg. and *Acalypha indica* L. (Ikewuchi *et al.,* 2011) but the active constituents are yet unknown.

2.14.3 *Alchornea cordifolia* (Euphorbiaceae).

Alchornea cordifolia (Euphorbiaceae) is a medium-sized shrubby tree found along the coastal regions of West Africa. It also occurs from Senegal east to Kenya and Tanzania and south throughout Central Africa to Angola.

2.14.3.1 Uses

The plant is an important crude drug in the indigenous system of medicine for the management of pain, rheumatism, arthritis, pile, toothache and some other inflammatory disease. The leaves are mostly used, but the stem bark, stem pith, leafy stems, root bark, roots and fruits are also used in local medicine.

The leaves or leafy stems, as an infusion or chewed fresh, are taken for their sedative and antispasmodic activities to treat a variety of respiratory problems including sore throat, cough and bronchitis, genital-urinary problems including venereal diseases and female sterility, and intestinal problems including gastric ulcers, diarrhoea, amoebic dysentery and worms. As a purgative, they are also taken as an enema; high doses taken orally are emetic. They are also taken as a blood purifier, as a tonic and to treat anaemia and epilepsy. The leaves are eaten in

West Africa and Congo as an emmenagogue and to facilitate delivery, and in Gabon as an abortifacient. A cold infusion of the dried and crushed leaves acts as a diuretic. Leaf and root decoctions are widely used as mouth wash to treat ulcers of the mouth, toothache and caries, and twigs are chewed for the same purposes. Dried leaves or roots, alone or with tobacco, are smoked to cure cough. The leaves and root bark are externally applied to treat leprosy and as an antidote to snake venom. In Gabon and Congo, a root decoction or maceration is taken to treat amoebic dysentery and diarrhea and used as eye drops to cure conjunctivitis. In Nigeria a decoction of bruised fruit is taken to prevent miscarriage. The sap of the fruit is applied to cure eye problems and skin diseases.

2.14.3.2 Ethno-medicinal Properties of *Alchornea cordifolia*

Much research has been carried out into the antibacterial, antifungal and antiprotozoal properties of *Alchornea cordifolia* as well as its anti-inflammatory activities, with significant positive results. In West Africa pulped root is widely taken to treat venereal diseases. Dried leaves or roots, alone or with tobacco, are smoked to cure cough, they are also taken as a blood purifier, as a tonic and to treat anaemia and epilepsy. Leaf and root decoctions are widely used as mouth wash to treat ulcers of the mouth, toothache and caries, and twigs are chewed for the same purposes. The root and stem bark are used in the treatment of jaundice (Agbor *et al.,* 2004).

The cytotoxicity of the crude extract as reported by Banzouzi *et al.* (2002) is very low. Alcohol extracts from root-bark, stem-bark, leaves, fruits and seeds disrupted mitotic cell division in onion (*Allium cepa* L.) (Ayisi *et al.,* 2003).

Adeshina *et al.* (2010) reported that ethyl acetate extract of *Alchornea cordifolia* leaves possesses antimicrobial activity against the clinical and typed isolates of *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* and *Candida albicans.* A study by

Gatsing *et al* (2010) on the antibacterial activity, bioavailability and acute toxicity evaluation of *Alchornea cordifolia* leaf extract showed remarkable activity against the growth of *P. aeroginousa, E. coli, S. aureus* and *K. pneumonia* with zones ranging from 13mm to 26mm. In the same study an MIC value of 2.5mg/ml, 3.75mg/ml, 5mg/ml and 10mg/ml were for the test organisms respectively. Furthermore, Adeshina *et al.* (2010) carried out studies on the antimicrobial and phytochemical attributes of ethyl acetate extract of *Alchornea cordifolia* against *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* and *Candida albicans.* The result obtained revealed that the extract possesses broad spectrum of activity against the test Gram-negative and Gram-positive bacteria and the fungi/yeast with diameter of zones of inhibition ranging from 10.0-35.0mm. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extract against the test organisms were found to fall between 0.625 - 10 mg/ml.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study sites

The samples were collected from general hospitals located in three different zones in Abia state namely: Umuahia, Aba, and Isiukwuato. Umuahia is the capital of Abia state and boasts of a federal medical center (FMC), which is a tertiary referral hospital. It comprises of several clinics and admission wards. It is the largest referral hospital in the state and the centre for many Microbiology diagnostic works in Umuahia. Aba is a commercial city with trading as the main occupation of majority of the inhabitants. It has a large teaching hospital known as Abia State University Teaching Hospital Aba which caters to many sick patients from the environ. Isiukwuato is the last zone and has a general hospital.

3.1.2 Culture Media

Nutrient agar (NA), Nutrient broth (NB), Mannitol salt agar (MSA), Mueller Hinton agar (MHA), DNAse agar were all from Rapid lab. Ltd, England. All media were prepared according to manufacturer's instruction, sterilized at 121°C for 15 minutes.

3.1.3 Chemical Reagents

Hydrogen peroxide (Jopel Chemical and Allied Product Ltd, Aba, Nigeria), Sodium Chloride, Lugols iodine, crystal violet, Ethanol, starch soluble (BDH Chemicals Ltd., England), Phosphase buffer, dimethyl sulfoxide (DMSO), analar grades of hydrochloric acid, sulphuric acid, methylene blue, acetone (Jopel Chemical and Allied Product Ltd, Aba, Nigeria).

3.1.4 Glassware

Bijou bottles, glass slides, petri dishes, test tubes, measuring cylinder, conical flasks, pipettes.

3.1.5 Equipment

Autoclave (portable), Adelphi Mfg. Co. Ltd., UK., Refrigerator, Centrifuge (model 5417R), weighing Balance, Microcentrifuge Incubator, (Baird and Tatlock Ltd. Essex), Light Microscope (Wild Mill, Switzerland), Hot air oven, (Baird and Tatlock Ltd.). Essex and micropipette (Pipetman), Gilson, France. Thermostated water bath (Gallenhamp). Votex machine (lab-line instruments, inc. USA). PCR Machine; thermocycler, spectrophotometer Nanodrop 1000, inqaba, biotech, south Africa).

3.1.6 Reference bacterial strains

Confirmed clinical isolates of MRSA obtained from Federal Institute of Research Oshodi (FIRO) was used as a positive control in the detection of methicillin resistant *Staphylococcus aureus* (MRSA).

3.1.7 Antibiotics used are:

The following antibiotic discs from Oxoid were used: Cefoxitin (30 µg), Oxacillin (1 µg) Cloxacillin (5 µg), Clindamycin (2 µg), Erythromycin (15 µg), Gentamycin (30 µg), Ciprofloxacin (5 µg), Azithromycin (15 µg), Levoflaxacin (5 µg) , Vancomycin (30 µg), and Ceftazidime (30 µg), Cefuroxime (30 µg), Ceftriaxone (30 µg), Ofloxacine (5 µg), and Augmentin (30 µg).

3.1.8 Polymerase Chain Reaction (PCR) primers

The oligonucleotide primers used in the detection of various genes were obtained from Inquaba, South Africa and are listed in Table 3.1.

3.1.9. Samples types

Samples were obtained from patients' urine, wound, secretions, blood, and swabs. The sections were collected aseptically with sterile swab sticks while urine and blood samples were collected in sterile sample bottles.

Table 3.1: Number and types of samples screened

Site	Umuahia	Aba	Isiukwuato
Wound and abscess	81	68	16
Blood	5	10	-
Urine	93	112	70
Ear swab	20	3	5
Nasal	15	2	8
High vaginal swab	52	46	23
Urethral swab	34	59	28
Total	300	300	150

3.2 Methods.

3.2.1 Isolation of organisms.

The samples collected were inoculated into mannitol salt agar and incubated at 37°C for 24 h for primary isolation of staphylococci. Isolates that produced colonies exhibiting characteristic deep golden yellow coloration were selected.

3.2.2 Pure cultures

The isolates selected were sub cultured on nutrient agar using streak plate method. Pure cultures of the isolates obtained were inoculated on nutrient agar slants, incubated at 37°C for 24 h and stored in refrigerator at 4°C as stock cultures for further tests.

3.3 IDENTIFICATION OF BACTERIAL ISOLATES

Identification of isolates was done by standard microbiological procedures (Adesida *et al*., 2005).

3.3.1 Gram stain

Gram stain classifies bacterial isolates into two main groups namely: Gram-positive and Gram-negative on the basis of differential interactions of Gram's reagent with the varying cell wall components of these two groups of bacteria.

Procedure: A drop of normal saline was placed on a well labelled clean grease-free glass slide using a sterile inoculating loop; a colony of an overnight culture of the bacterial isolate was emulsified with the normal saline to make a thin smear. The smear was air dried and then heat fixed. The slide was flooded with crystal violet (primary stain) for 60 s after which the stain was rinsed from the slide with water. The smear was flooded with Lugol's iodine (mordant) to fix the primary stain. The iodine was rinsed with water after 60 s. The slide was then flooded with a decolourizer (acetone) and rinsed off almost immediately. The counter

stain; safranin was added and left for 60s before being rinsed off. The stained smear was air-dried, and then observed under the microscope using X100 oil immersion objective lens of the microscope. A cluster of purple colonies was indicative of staphylococci.

3.4. BIOCHEMICAL TESTS

3.4.1 Catalase test

Staphylococci will produce catalase, an important virulence factor which degrades the microbicidal H_2O_2 into O_2 and H_2O. This ability differentiates staphylococci from streptococci.

Procedure: A drop of 3% hydrogen peroxide was placed on a clean grease-free glass slide.

About 2 colonies of the bacteria was picked from a culture plate using a sterile wire loop and placed on the hydrogen peroxide; presence of bubbles indicated a positive catalase test.

3.4.2 Coagulase test

Staphylococcus aureus is distinguished from other staphylococci by the production of coagulase an enzyme that clots plasma.

Procedure: About one or two drops of blood plasma was placed on a clean grease-free glass slide and 2 colonies of the organism was picked using a sterile wire loop from a 18 h nutrient agar plate. The colonies were emulsified in the blood plasma and observation of agglutination indicated a positive coagulase test.

3.4.3 Deoxyribonuclease (DNAse) Test

The method as described by Cheesbrough (2000) was used to differentiate *Staphylococcus aureus* (producer of the enzyme deoxyribonuclease) from other *Staphylococcus* spp.

Deoxyribonuclease agar media was prepared and sterilized by autoclaving at 121ºC for 15 minutes. An overnight broth culture of the organisms was spot inoculated onto agar surfaces of the DNAse agar and incubated at 37 ºC for 18hours. At the end of the incubation period, the agar surface was flooded with 1N hydrochloric acid and excess drained off. Development of clearing around the colonies within 5 minutes of flooding with the acid was an indication of the production of the Deoxyribonuclease enzyme by the organisms.

3.4.4 Indole Test

The isolates were inoculated into a bijou bottle containing 3mL of sterile peptone water and incubated at 35-37ºC for 18h. Indole production was tested by adding 5-7drops of Kovac's reagent and examined for a ring of red color in the surface layer within 5 mins which indicates a positive test while the absence of a ring of red layer indicates a negative indole.

3.4.5 Citrate utilization Test

Slopes of Simmon's citrate agar was prepared in bijou bottles according to manufacturer's description. The slopes were then aseptically streaked with a saline suspension of the isolates followed by butt stabbing. Incubation was done at 37ºC for 48h. A bright blue colour is positive test while green is negative.

3.5 Antibiotic Susceptibility Tests

Disk diffusion tests was performed for each of the isolates previously identified as *S. aureus* by following the method recommended by the Clinical Laboratory Standard Institute (CLSI, 2013). Discrete colonies of isolates on nutrient agar plates were emulsified in 3 – 4 ml of sterile physiological saline and the turbidity adjusted to 0.5 McFarland standard (approximately a cell density of 1.5 x 10^8 cfu/ml). Using sterile swab sticks, the surface of Mueller Hinton agar (MHA) in a 90 mm diameter plate was inoculated with the bacterial

suspension. The inoculated plates were allowed to dry for 10 minutes before the antibiotic discs were applied aseptically to the surface of the agar. After 30 minutes of applying the discs, the plates were inverted, and incubated at 35°C.

3.6 Detection of Methicillin Resistance

3.6.1 Cefoxitin disc diffusion

Clinical Laboratory Standards Institute (CLSI) 2009 has recommended Cefoxitin disc diffusion method for the detection of MRSA due to its ability to enhance induction of PBP2a.

A 0.5 Mc Farland standard suspension of the isolate was made and a lawn culture was done on MHA plate. Cefoxitin (30 µg) discs was placed on the plates and incubated at 35°C for 18 hours and the results were interpreted by measuring the zone diameters with the aid of sliding callipers, including the disc diameter. An inhibition zone diameter of ≤21 mm was reported as Methicillin resistant and ≥22 mm was considered as Methicillin susceptible (CLSI, 2013)

3.6.2 Latex Agglutination Test

The MRSA screen test is a latex agglutination test based on the reaction of latex particles sensitized with monoclonal antibodies against PBP2a of *S. aureus* and PBP 2a extracted from tested colonies. The Mastalex MRSA (Mast group limited UK) test kit was used and the test was performed according to the manufacturer's instructions. Briefly isolates were sub-cultured onto nutrient agar and incubated at 37°C for 18 h to obtain fresh growth. To extract PBP 2a from the tested colonies, a loopful of cells was suspended in 4 drops of extraction reagent1.This suspension was placed in a heating block (95°C) for 3min. After allowing the suspension to cool to room temperature (610 min), 1 drop of extraction reagent 2 was added and the mixture was vortexed thoroughly. The suspension was then centrifuged at 3000rpm for 5 min. The actual latex agglutination test was performed with the supernatant; 50 µl of the

supernatant was mixed with 1 drop of sensitized latex. For the negative control, 50 µl of the supernatant was mixed with 1 drop of negative control latex. Mixing for 3 min was performed with a shaker and agglutination was observed under normal lighting condition.

3.7 Determination of inducible and constitutive MLSB resistance

To perform the test, the erythromycin 15µg disk was placed on the surface of a Mueller-Hinton agar plate previously inoculated with the bacterial suspension at a distance of 20 mm (edge to edge) of the clindamycin 2µg disk. The plate was then incubated for 18 hrs. (CLSI, 2019). The test was considered positive when there was flattening of the clindamycin zone adjacent to the erythromycin disc in the shape of the letter D (D effect or zone). Thus, when the inhibition zone of the erythromycin disk is ≤ 13 mm and the clindamycin disk is ≥21 mm, and both portions are circular, the test will be considered negative for iMLSB resistance

3.8 Test for β-lactamase Production

Beta-lactamase production was determined using iodometric method. Suspensions of the isolate was prepared in triplicates by emulsifying bacterial colonies from an overnight NA culture with sterile wire loops in 0.5ml of phosphate buffered saline (PBS) containing 0.06 mg/ml (10,000 units/ml) of penicillin G. As control, cell suspension of the standard typed culture of *S. aureus* was regularly set up. Thereafter, 2 drops of freshly prepared 1 % aqueous starch solution was added to each bacterial suspension and gently shaken. One drop of iodine solution was then added and allowed to stand for 10mins at 28°C. β-lactamase producing organisms change the colour of the reaction mixture from blue-black to colourless within 10 mins.

3.9 Molecular Screening

Molecular characterization of 40 isolates was carried out on the basis of being methicillin resistant phenotypically. This was done in the Molecular Laboratory of Niger Delta University, Amassomma, Bayelsa State. The isolates were analyzed for carriage of *mecA*gene, 16SrRNA, *pvl, blaz* and *ermB* genes. Clonal relatedness was determined among the isolates with *mec A* gene by PCR-RAPD technique. Some of the isolates were randomly selected for sequencing inInqaba Biotechnological, Pretoria South Africa.

3.9.1 DNA extraction (Boiling Method)

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500µl of normal saline and heated at 95^0C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA were transferred to a 1.5ml microcentrifuge tube and stored at -20°C for other downstream reactions.

3.9.2 DNA Quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitres of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

3.9.3 Amplification of *mecA* genes

The *mecA* genes from the isolates were amplified using *mecA* -F (5′CAAGATATGAAGTGGTAAATGGT3′) and *mecA*-R (5′TTTACGACTTGTTGCATACCATC3′) primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30

microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4µM and 50ng of the extracted DNA as template. The PCR conditions was as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 51ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator for a 533bp product size.

3.9.4 Amplification of *ermB* gene

The *ermB* genes from the isolates were amplified using the *ermB* F: 5' GCA TTC ATC ATC AAT CAA AAT G -3' and *ermB* R: 5'-CTA TAA CCT TCT GTG CTT TGC A-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions was as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 56ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator for a 198bp product size.

3.9.5 Amplification of *blaZ* gene

The *blaZ* genes from the isolates were amplified using the *blaZ* F: 5'-TAAGAGATTTGCCTATGCTT-3' and *blaZ* R: 5'-TTAAAGTCTTACCGAAAGCAG-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: The X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions was as follows: Initial

denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 48ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator for a 300 bp product size.

3.9.6 Amplification of *pvl* genes

The *pvl* genes from the isolates were amplified using the staphpVL F GCTGGACAAAACTTCTTGGAATAT3' and staphpVL R5'GATAGGACACCAATAAATTCTGGATTG3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: The X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4µM and 25ng of the extracted DNA as template. The PCR conditions was as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 60ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product will be resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator for a 1100bp product size.

3.9.7 Amplification of the 16SrRNA of the bacterial isolates

The 16SrRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: The X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions was as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The

product was resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue light transilluminator for a 1500bp.

3.9.8 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl, the components included 0.25µl BigDye® terminator v1.1/v3.1, 2.25µl of 5 x BigDye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

3.9.9 Random Amplified Polymorphic DNA for Clonal Relatedness

Random Amplified polymorphic DNA was carried out using the saOLP6: GAGGGAAGAG, saOLP11: ACGATGAGCC and saOLP13: ACCGCCTGCT primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: The X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.2µM and 20ng of the extracted DNA as template. The PCR conditions will be as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 40ºC for 30 seconds; extension, 72ºC for 40seconds for30 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator and clonal relatedness was shown among organisms with the same number of bands.

3.9.10 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary

history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor 1969).

Table 3.1: Sequences and amplified product size of primers used in polymerase chain reaction assay

Genes	Oligonucleotide sequences (5! 3!)	Product size (bp)
mecA	MecA-F (5′-CAAGATATGAAGTGGTAAATGGT-3′) mecA-R (5′-TTTACGACTTGTTGCATACCATC-3′).'	533
Pvl	staphpVL F: GCTGGACAAAACTTCTTGGAATAT-3'staphpvl R5'-GATAGGACACCAATAAATTCTGGATTG-3'	1100
Blaz	blaz F: 5'-TAAGAGATTTGCCTATGCTT-3' blaz R: 5'-TTAAAGTCTTACCGAAAGCAG-3'	300
ermB	ErmB F: 5' GCA TTC ATC ATC AAT CAA AAT G -3' ermB R: 5'-CTA TAA CCT TCT GTG CTT TGC A-3' primers	198

Table 2: Cyclic polymerase chain reaction conditions of different primer set

Genes	Initial Denaturation	No of cycles	Denaturation	Annealing temp	Extention	Final Extention
mecA	95ºC for 5min	35cycles	95ºC for 30s	51ºC for 40s	72ºC for 50s	72ºC for 5min
Pvl	95ºC for 5min	35cycles	95ºC for 30s	60ºC for 40s	72ºC for 50s	72ºC for 5min
blaZ	95ºC for 5min	35cycles	95ºC for 30s	48ºC for 40s	95ºC for 30s	95ºC for 30s
ermB	95ºC for 5min	35cycles	95ºC for 30s	56ºC for 40s	95ºC for 30s	95ºC for 30s

3.10 Plant Antimicrobial Sensitivity Testing

3.10.1 Collection of Plant Materials

The plant leaves were collected from Umudike in Abia state and the selection of the two plants were done on the basis of their wide usage in treatment of microbial infections. The plants were identified by a taxonomist from the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture Umudike.

3.10.2 Extraction of Plant Material

The leaves of the plants were plucked and air-dried at room temperature for several days. The dried leaves were pulverized using a milling machine to obtain fine powder. The active ingredients were extracted by percolation using ethanol and water. Briefly, 50 g of each leaf powder was added to 200ml of ethanol and 200ml of water respectively. The mixture was covered and then allowed to stand for 24hours for extraction. The mixture was then separated by passing through a clean muslin cloth and Whatman's No 1 filter paper, after which the filtrate was evaporated to dryness in oven set at 50°C (Handa *et. al* 2008). The dried crude extracts were stored in the refrigerator (at 4°C) under aseptic conditions for subsequent use.

3.10.3 Antimicrobial Activity of the Plant Extracts against MRSA Isolates

The activities of the plant extracts were determined using agar well diffusion techniques (Nweze & Onyishi, 2010). The ethanolic and water extract of the local herbs were tested against the MRSA isolates. 0.4g of each crude extract was reconstituted in 2ml of Dimethyl sulphoxide (DMSO) to obtain extract concentration of 200mg/ml. This was serially diluted in 2-folds to obtain the following lower extract concentrations: 100, 50, and 25 mg/ml. An 18hour old standardized inoculum (corresponding to 0.5 McFarland turbidity standard) of each test bacterial isolate was inoculated on dried surface of Mueller-Hinton agar by spreading with a sterile cotton-tipped swab to achieve a confluent growth. The inoculated

plates were allowed to dry after which wells were punched on the agar at equidistant positions using a sterile standard 6mm cork borer. Subsequently, 50µl of different concentrations of the extract was separately introduced into the different wells that had been labeled accordingly using a micropipette. Also, 50µl of DMSO as well as gentamycin (antibiotic) was introduced into the well bored in the centre of the plate as negative and positive control respectively. This procedure was repeated in duplicate for all the test organisms, and allowed to stay for 30 min on the bench after which they were incubated for 24hour at 37°C. At the end of incubation, observed zones of inhibition were measured and recorded to the nearest millimeter.

3.10.4 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the extracts was investigated by diluting a given volume of the extract to various concentrations according to Macro-broth dilution technique (Baron and Finegold, 1990). The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration or the highest dilution of an antimicrobial agent that prevents visible growth after 18-24 hours of incubation. It was determined by making dilution of various concentrations (50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78mg/ml) of the ethanol extract in test tubes. 1ml stock solution of the plant extract 100mg/ml was diluted in sterile test tube containing 0.95ml of Mueller Hinton Broth (MHB) to obtain further dilutions. Serial dilution technique was employed by transferring 1ml from the first test tube to the second test tube and from the second to the third. This continued to the seventh test tube from where 1ml was discarded to give concentration of 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78mg/ml. Another test tube was also prepared with MHB (control) of the test tube which was inoculated with standard suspension (50µl) of the test organisms and incubated at 37°C overnight. After incubation, the turbidity in each tube was checked. The tube that contained the lowest concentration which showed no turbidity i.e., a clear view, was observed to be the MIC of the

antimicrobial agent for the organism tested, the lower the MIC, the more susceptible is the test organism.

3.10.5 Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) is the lowest concentration of an antimicrobial agent required to kill a microorganism. This was determined by streaking the content of the tubes used for MIC determination which showed reduced turbidity on freshly prepared nutrient agar plates. The MBC was then identified as the lowest concentration that completely inhibited the growth of the test bacteria.

3.10.6 Rate of Killing of the Organisms by the Extracts

5 ml of 50 mg/ml of the ethanol leaf extracts and 5 ml of the bacterial culture was added together in a sterile test tube and allowed to stand for about 24 hours. At intervals of one hour, 1 ml of the mixture was pour plated on nutrient agar medium and incubated at 37°C for 24 hours, according to the method of Ogundare & Akinyemi (2011). The microbial load was thereafter determined.

3.11 Phytochemical Analysis

The prepared plant extracts were analyzed for the presence of alkaloids, glycosides, saponins, phenolic compounds, tannins, flavonoids (Raman, 2006).

3.11.1 Determination of Saponins

This was done by the double solvent extraction gravimetric method (Harborne, 1993). Five grams (5g) of the sample was mixed with 50ml of 20% aqueous ethanol solution and incubated for 12 hours at a temperature of 55°C with constant agitation. After that, the mixture was filtered through Whatman No 42 grades of filter paper. The residue was re-extracted with 50ml of the ethanol solution for 30 minutes and the extracts weighed together.

The combined extract was reduced to about 40ml by evaporation and then transferred to a separating funnel and equal volume (40ml) of diethyl ether was added to it. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. This aqueous layer was re-extracted with the ether after which its pH was reduced to 5 with dropwise addition of dilute NaOH solution. Saponin in the extract was taken up in successive extraction with 60ml and 30ml portion of normal butanol. The combined extract (ppt) was washed with 5% NaCl solution and evaporated to dryness in a previously weighted evaporating dish. The saponin was then dried in the oven at 60°C (to remove any residual solvent) cooled in a desiccator and re-weighed. The saponin was determined and calculated as a percentage of the original samples.

$$\% \text{ saponin} = \frac{W2 - W1}{W} \times \frac{100}{1}$$

where, W = weight of sample used

W1 = weight of empty evaporation dish

W2 = weight of dish + saponin extract

3.11.2 Determination of Tannins

This was determined by Folin Denis colorimetric method. Five grams (5g) of the powdered sample was put inside a volumetric flask and 50ml of distilled water was dispensed inside the volumetric flask. The mixture was shaken for 30 minutes at room temperature and filtered to obtain the extract. A standard tannic acid solution was prepared, 2ml of the standard solution and equal volume of distilled water were dispersed into a separate 50ml volumetric flasks to serve as a standard and reagent blank respectively. Then 2ml of each of the sample extracts was put in their respective labeled flask. The content of each flask was mixed with 35ml distilled water and 1ml of the Folin Denis reagent was added to each. This was followed by

2.5ml of saturated Na_2CO_3 solution. Therefore, each flask was diluted to the 50ml mark with distilled water and incubated for 90minutes at room temperature. Their absorbance was measured at 760nm in a spectrophotometer with the reagent blank at zero.

The tannin content was calculated as shown below:

$$\% \text{ Tannin} = \frac{100 \times au \times C \times Vt}{W \times as \times Va}$$

Where, W = weight of sample

au = absorbance of test sample

as = absorbance of standard tannin solution

C = Concentration of standard tannin Solution

Vt = Total volume of extract

Va = Volume of extract analysed

3.11.3 Determination of Alkaloids

The alkaline precipitation gravimetric method (Harbone, 1993) was used. A measured weight of the processed sample (5g) was dispersed in 100mls of 10% acetic acid in ethanol solution. The mixture was well shaken and allowed to stand for 4 hours at room temperature being shaken every 30min. At the end of this period, the mixture was filtered through Whatman No 42 grade of filter paper. The filtrate (extract) was concentrated by evaporation; to a quarter of its original volume. The extract was treated with drop-wise addition of concentrated NH_3 solution to precipitate the alkaloid. The dilution was continued until the NH_3 was in excess. The alkaloid precipitate was removed by filtration using weighed what- man No 42 filter paper. After washing with 1% NH_4OH solution, the precipitate in the filter paper was dried at 60^oC and re-weighed after cooling in a desiccator. The Alkaloid content was calculated as shown below:

% alkaloid = W2 – W1 x 100 /Wt of sample

Where W1 = Weight of empty filter paper   W2 = Weight of filter + Alkaloid ppt

3.11.4 Determination of Flavonoids

Flavonoid was determined using the method described by Harborne (1993). A measured weight of the sample (5g) was boiled in 100ml of 2M HCL solution under reflux for 40mins. It was allowed to cool before being filtered. The filtrate was treated with equal volume of ethyl acetate and the mixture was transferred to a separation funnel. The flavonoid extract (contained in the ethyl acetate portion) was received by filtration using weighed filter paper. The weight was obtained after drying in the oven and cooling in desiccators. The weight was expressed as a percentage of the weight analyzed. It was calculated as shown below:

%Flavonoid = W2 – W1 x 100
Wt of sample 1
Where W1 =Weight of filter paper x Flavonoid precipitate W2 = Weight of filter paper alone

3.11.5 Determination of Phenols

This was determined by the Folin – Ciosptean spectrophotometer (AOAC 1990). The total phenol was extracted in 200mg of the sample with 10ml concentrated methanol. The mixture was shaken for 30min at room temperature. The mixture was centrifuged at 500rpm for 15min and the supernatant (extract) was used for the analysis. 1ml portion of the extract from each sample was treated with equal volume of Folin-Ciosptean reagent followed by the addition of 2mls of 2% Na2CO3 solution. Standard phenol solution was prepared and diluted to a desired concentration. 1ml of the standard solution was also treated with the F-D reagent. Blue colouration was measured (absorbance) in a colour meter at 560nm wavelength. Measurement was with a reagent blank at zero. The phenol content was calculated using the formula below:

$$\%phenol = \frac{100}{W} \times \frac{au}{as} \times \frac{cVt}{100\ Va}$$

Where W = Weight of sample

au = absorbance of test sample

as = absorbance of standard phenol sample

c=concentration of standard phenol sample

vt = total extract volume

va = volume of extract analyzed

3.11.6 Determination of Cyanogenic Glycoside

The method of Amadi *et al.* (2004) was adopted for this assay. Each sample was weighed into a 250cm^{3}round bottom flask and 200cm^{3}of distilled water was added to one gram of each powdered sample and allowed to stand for 2hours for autolysis to occur. Full distillation was carried out in a 250cm^{3}conical flask containing 20cm^3 of 2.5% NaOH (sodium hydroxide) in the sample after adding an antifoaming agent (tannic acid). Cyanogenic glycoside (100cm^3), 8cm^3 of 6M NH_4OH (ammonium hydroxide), and 2cm^3 of 5% KI (potassium iodide) were added to the distillate(s), mixed, and titrated with 0.02M $AgNO_3$(silver nitrate) using a microburette against a black background. Turbidity which was continuous indicates the end point. Content of cyanogenic glycoside in the sample was calculated as

$$\text{Cyanogenic glycoside (mg/100 g)} = \frac{\text{Titre Value (cm}^3) \times 1.08 \times \text{exact volume}}{\text{Aliquot volume (cm}^3) \times \text{sample weight (g)}} \times 100$$

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Identification of *Staphylococcus aureus*

A total of 750 samples collected from different clinical samples from three senatorial zones of Abia state; Umuahia, Aba and Isiukwuato were screened for the presence of *S. aureus*. All isolates that were gram positive cocci, coagulase positive, catalase positive, DNAse positive and fermented mannitol were characterized as *S. aureus*. A total of two hundred and sixty-five (265) clinical isolates of *S. aureus* were recovered from the various clinical specimens of patients submitted during the study. The results obtained show that the highest prevalence of *S. aureus* occurred in Aba 39.7%, Umuahia was 37.3% while the lowest was in Isiukwuato with a prevalence of 22.7% (Fig 4.1).

4.1.2 Prevalence of *S. aureus* among age and gender

Out of the 265 *S aureus* isolates, 126(47.5%) were recovered from males, while 139(52.5%) were from females, 13 (4.9%) were from the age group of 0–11 years, 28 (10.6%) from the age group of 11–20 years and 59 (22.3%) from the age group of 21-30, 58(21.9%) from 31-40, 56(21.1%) from 41-50, 28(10.6%) from 51-60 and 23(8.7%) from >60 years. The statistical analysis showed that there was no significant association between prevalence and age in the overall prevalence (P >0.05). There was also no significant association between prevalence gender and (P >0.05). The number of female patients was slightly higher than males but not significantly different. The summary is on Table 4.1

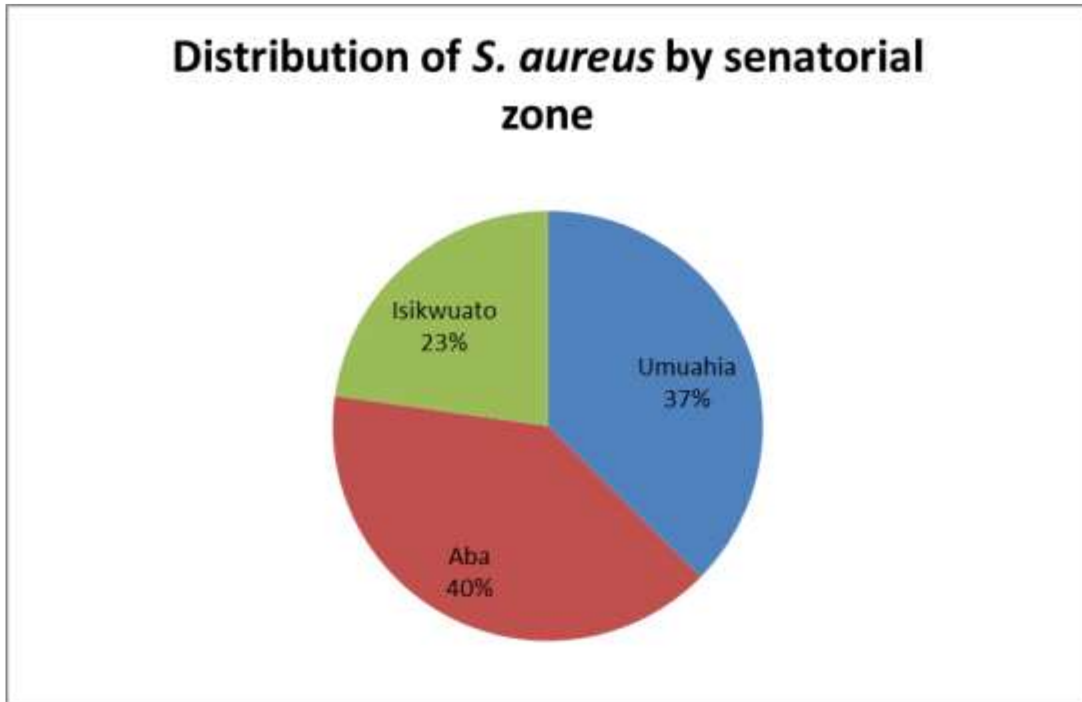

Fig 4.1: Distribution of *S. aureus* by senatorial zone

Table 4.1: Prevalence of *S. aureus* among various age groups and genders

Age group	Umuahia			Aba			Isikwuato			Overall		
	Male(%)	Female(%)	Total(%)	Male%	Female(%)	Total(%)	Male(%)	Female(%)	Total(%)	Male	Female	Total(%)
<11	4(44)	5(56)	9(8.0)	1(25)	3(75)	4(3.4)	-	-	-	5	8	13(4.9)
11-20	7(70)	3(30)	10(8.9)	6(37.5)	10(62.5)	16(13.4)	1(50)	1(50)	2 (5.9)	14	14	28(10.6)
21-30	4(21.1)	15(79)	19(17)	15(57.7)	11(42.3)	26(21.8)	5(35.7)	9(64.3)	14(41.2)	24	35	59(22.3)
31-40	11(44)	14(56)	25(22.3)	12(41.4)	17(58.6)	29(24.4)	2(50)	2(50)	4 (11.8)	25	33	58(21.9)
41-50	12(52.2)	11(47.8)	23(20.5)	14(56)	11(44)	25(21)	5(62.5)	3(37.5)	8 (23.5)	31	25	56(21.1)
51-60	8(57)	6(50)	12(10.71)	6(54.5)	5(45.5)	11(9.2)	2(66.7)	1(33.3)	3 (8.8)	16	12	28(10.6)
>60	6(50)	6(50)	12(100)	4(50)	4(50)	8(6.7)	1(33.3)	2(66.7)	3 (8.8)	11	12	23(8.7)
Total	52(46.4)	60(53.6)	112(100)	58(48.7)	61(51.3)	119(100)	16(47.1)	18(52.9)	34 (100)	126	139	265(100)

4.1.3 Prevalence of *S. aureus* among clinical sample types

An overall prevalence of *S aureus* recovered from clinical sample in the three zones was as follows; a total of 113 (42.6%) of the *S. aureus* isolates were recovered from samples of urine, 62 (23.4%) from wound swabs, 43(16.2%) from high vaginal swabs (HVS), 33(12.5%) from urethral swab, 6(2.3%) from ear swabs etc. However, the prevalence among the zones varied as shown in Table 4.2

4.1.4 Antibiotic susceptibility profile of *S aureus* isolates

The anti-microbial susceptibility profile of 265 *S. aureus* strains from clinical samples obtained from the 3 zones are shown in Fig. 4.2, 4.3, and 4.4. The rate of resistance was highest for ceftazidine among the isolates from the three zones (100% for Aba, Umuahia and Isikwuato). The antibiotic susceptibility test of the *S. aureus* isolates from Aba showed that the isolates were generally resistant to most of the antibiotics used with resistance to augmentin being the highest (75%) followed by cloxacillin 77% and azithromycin 73%. In Umuahia highest resistance occurred against augmentin 76%, ceftriaxone 71% and cloxacillin 69.9% while Isikwuato had cloxacillin 82.4%, ceftriaxone 70.6% and augmentin 61.8%. The overall antibiogram for the three zones showed the resistance pattern to be as follows; all 265 (100%) of the isolates were resistant to ceftazidine, 203 (76.6%) to cloxacillin, and 195 (73.6%) to augmentin (Table 4. 3). However, there was 100% susceptibility to vancomycin among the isolates from the three zones.

Comparing resistance among the clinical samples, most of the isolates were highly resistant to augmentin, cloxacillin and ceftriaxone, with the isolates from HVS being resistant to augmentin with resistance as high as 86% (Table 4.4).

Table 4.2: Distribution of Staphylococcal Isolates Obtained From Different Clinical Samples

Sample Type	Umuahia			Aba			Isiukwuato		
	No. of Samples	No of *S. aureus*(%)	NSI (%)	No of Samples	No. of *S. aureus* (%)	NSI (%)	No. Of Samples	No. Of *S. aureus* (%)	NSI (%)
Wound and abscess	81	31(38.3)	50(61.7)	68	28(41.2)	40(58.8)	16	3(18.8)	13(81.2)
Blood culture	5	1(20)	4(80)	10	3(30)	7(70)	-	-	-
Urine	93	40(43)	53(57)	112	51(45.5)	61(54.5)	70	22(31.4)	48(68.6)
Ear swab	20	6(30)	14(70)	3	-	3	5	-	5(100)
Nasal	15	3(20)	12(80)	2	-	2	8	1(12.5)	7(87.5)
High vaginal swab	52	19(36.5)	33(63.5)	46	20(43.5)	26(56.5)	23	4(17.4)	19(82.6)
Urethral swab	34	12(35.3)	22(64.7)	59	17(28.8)	42(71.2)	28	4 (14.3)	24(85.7)
Total	300	112(37.3)	188(62.7)	300	119(39.7)	181(60.3)	150	34(22.7)	116(77.3)

NSI: Non-Staphylococcal Isolates

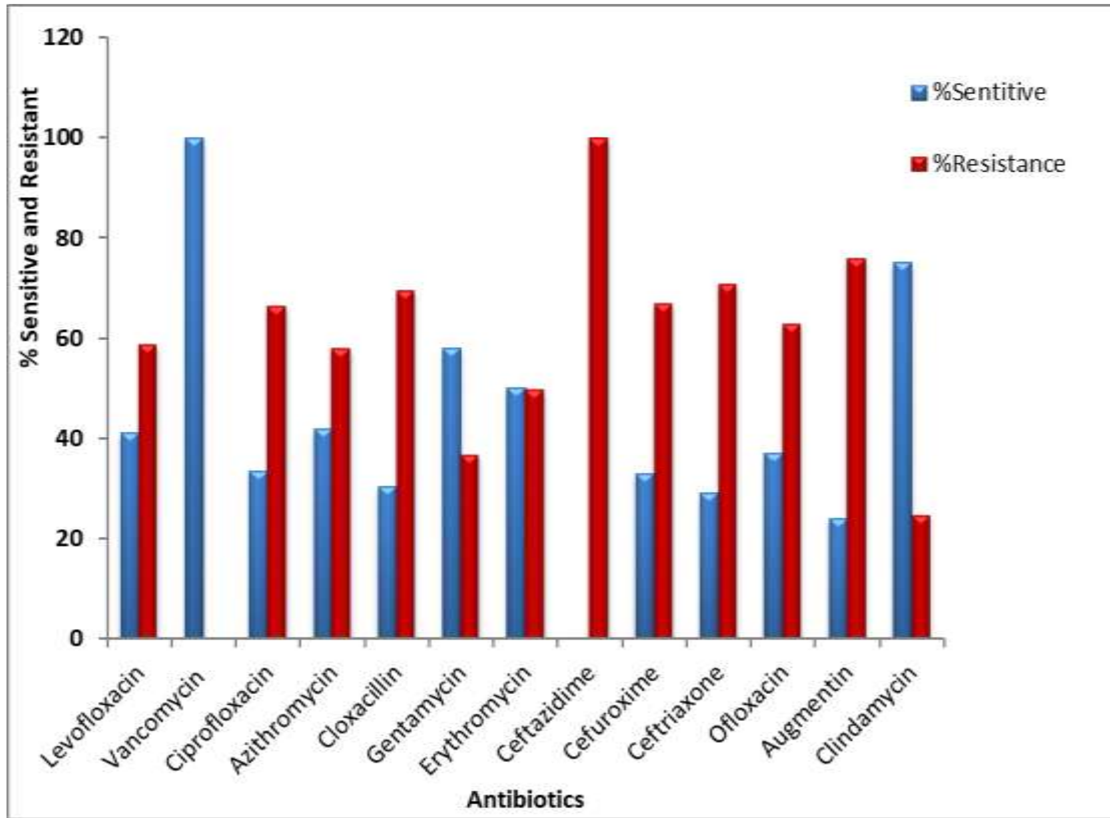

Fig 4.2: Antibiotic susceptibility profile of *S. aureus* isolates from Umuahia

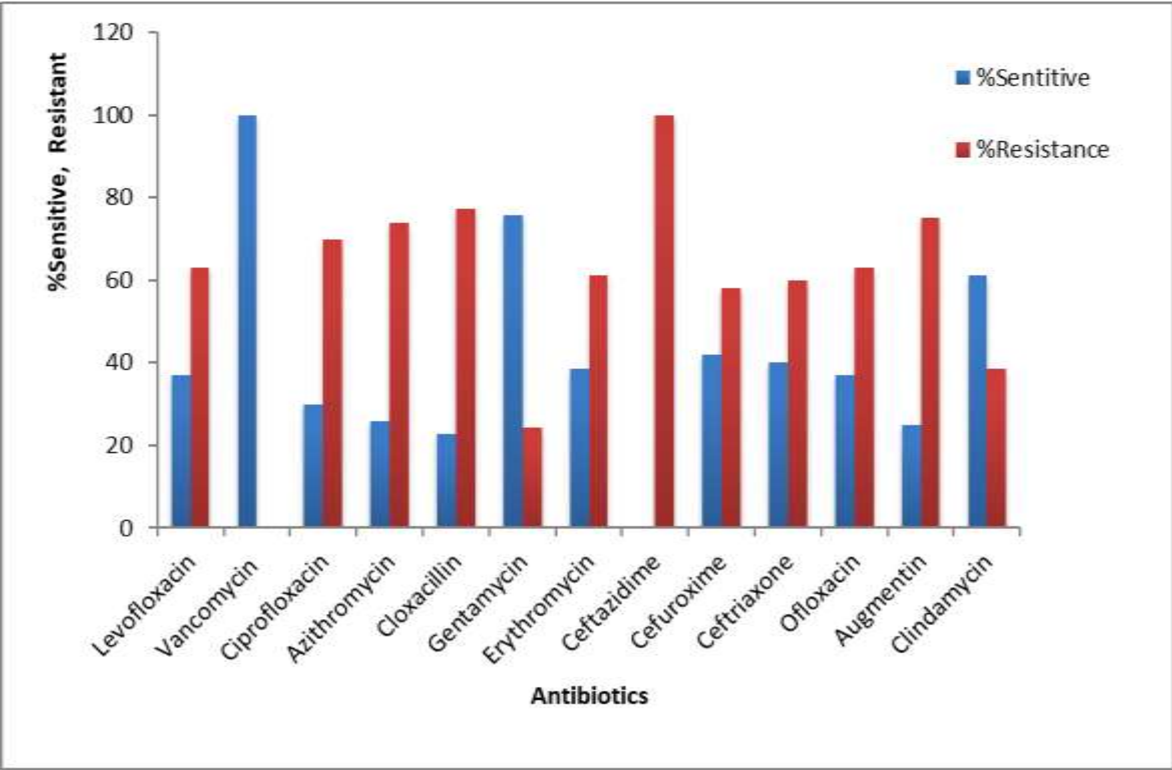

Fig 4.3: Antibiotic susceptibility profile of *S. aureus* isolates from Aba

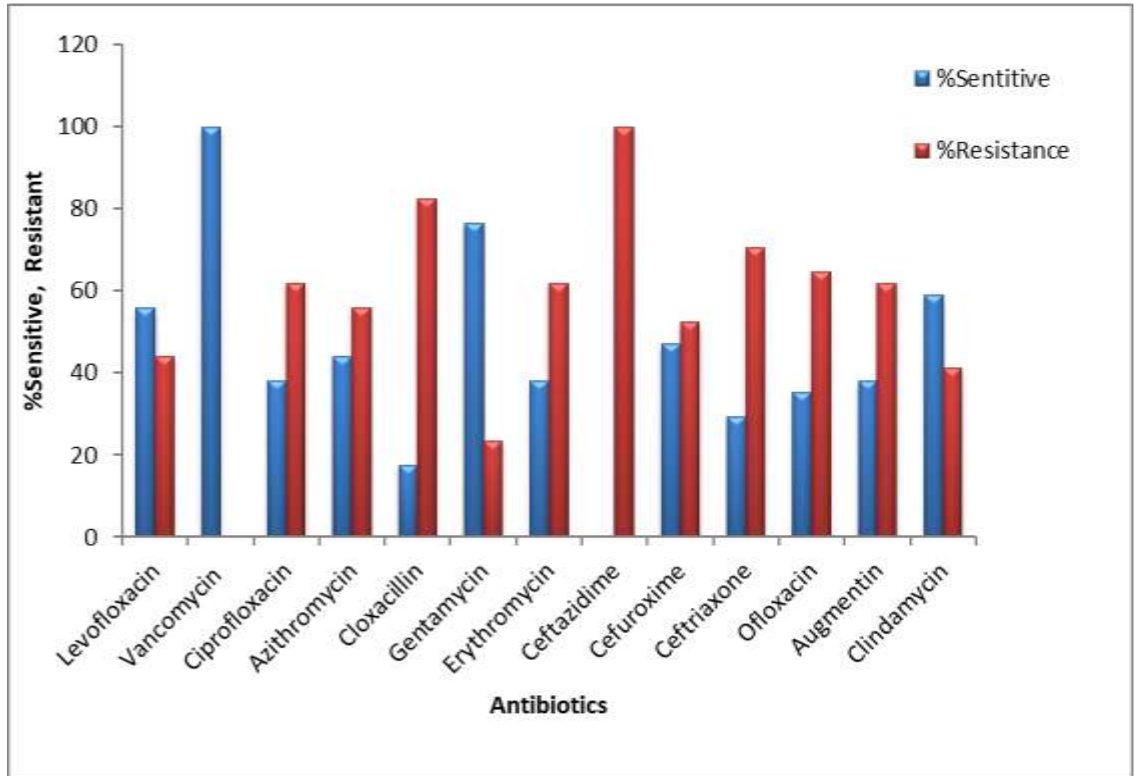

Fig 4.4: Antibiotic susceptibility profile of *S. aureus* isolates from Isiukwuato

Table 4.3: Antibiotic susceptibility profile of *S. aureus* isolates from the 3 zones

Antibiotic	Sensitive No. (%)	Resistance No. (%)
Vancomycin	265(100)	0(0)
Gentamycin	181(68.3)	84(31.7)
Clindamycin	177(66.8)	88(33.2)
Erythromycin	115(43.4)	150(56.6)
Levofloxacin	108(40.8)	157(59.2)
Cefuroxine	103(38.9)	162(61.1)
Ofloxacine	98(37.0)	167(63.0)
Azithromycin	93(35.1)	172(64.9)
Ceftriaxone	90(34.0)	175(66.0)
Ciprofloxacine	87(32.8)	178(67.2)
Augmentin	70(26.4)	195(73.6)
Cloxacillin	62(23.4)	203(76.6)
Ceftazidine	0(0)	265(100)

Table 4.4: Comparison of *S. aureus* Resistance from Various Sample Types.

Antibiotic	Wound(n=62) No. (%)	Blood (n=4) No. (%)	Urine(n=113) No. (%)	Ear (n=6) No. (%)	Nasal (n=4) No. (%)	HVS (n=43) No. (%)	U/S (n=33) No. (%)
Levofloxacin	37(59.7)	1 (25)	71(62.8)	2(33.3)	2(50)	24(55.8)	18(54.5)
Vancomycin	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Ciprofloxacin	41 (66.1)	1 (25)	65(57.5)	1(16.7)	2(50)	25(58.1)	20(60.6)
Azithromycin	28 (45.2)	1 (25)	73(64.6)	2(33.3)	2(50)	25(58.1)	12(36.4)
Cloxacillin	41 (66.6)	2 (50)	89(78.8)	4(66.7)	2(50)	33(76.7)	26(78.8)
Gentamicin	17 (27.4)	0(0)	29(25.7)	3(50)	1(25)	12(27.9)	8(24.2)
Erythromycin	34 (54.8)	1(25)	70(61.9)	3(50)	3(75)	21(48.8)	19(57.6)
Ceftazidime	63 (100)	4(100)	113(100)	6(100)	4(100)	43(100)	33(100)
Cefuroxime	33 (53.2)	2(50)	65(57.5)	4(66.7)	3(75)	24(55.8)	26(78.8)
Ceftriaxone	35 (56.5)	2(50)	65(57.5)	5(83.3)	3(75)	28(65.1)	23(69.7)
Ofloxacin	41 (66.1)	3(75)	74(65.5)	1(16.7)	2(50)	26(60.5)	15(45.5)
Augmentin	47 (75.8)	3(75)	76(67.3)	5(83.3)	2(50)	37(86.0)	24(72.7)
Clindamycin	17 (27.4)	0(0)	33(29.2)	2(33.3)	1(25)	14(32.6)	8(24.2)

Key; HVS=high vaginal swab, U/S=urethral swab

4.1.5 Phenotypic Detection of Methicillin Resistance

A total of 265 (35.3%) of *S. aureus* isolates were screened phenotypically using cefoxitin and oxacillin discs for methicillin resistance. 164(61.9%) of these were resistant to 30mg of cefoxitin (zone <-21mm) while a total of 162 (61.1%) were resistant to oxacillin (zone <-10mm). The percentage rate of detection of MRSA from cefoxitin and oxacillin discs was comparable at 61.9% and 61.1% respectively. This is represented in table 4.5.

4.1.6 Prevalence of MRSA and MSSA from the 3 Zones

The prevalence of MRSA among the various clinical samples from the 3 zones showed that the highest occurrence was 68.1% in Aba, 61.6% in Umuahia and 41.2% in Isikwuato as shown in table 4.6.

4.1.7 Antibiogram of MRSA and MSSA among the 3 Zones

The antimicrobial susceptibility profile of MRSA isolates from clinical samples obtained from the 3 zones showed that the rate of resistance was highest for ceftazidine among the isolates from the three zones (100% for Aba, Umuahia and Isikwuato). The antibiotic susceptibility test of the MRSA isolates from the zones showed that the isolates were generally resistant to most of the antibiotics used with the resistance to beta lactams and quinolones being the highest while resistance was significantly lower among the MSSA with beta lactams and macrolides being the highest. It is interesting to note that resistance to gentamycin was least among the MSSA from the three zones (Umuahia 11.6%, Aba 2.6% and Isikwuato 5.0%). However, there was 100% susceptibility to vancomycin among the isolates from the three zones. This is shown in table 4.7

Table 4.5: Phenotypic detection of MRSA using Oxacillin and Cefoxitin discs

Zone	Resistant		Sensitive	
	Oxacillin (%)	Cefoxitin (%)	Oxacillin (%)	Cefoxitin (%)
Umuahia	65 (58)	69(61.6)	47(42)	43(38.4)
Aba	79 (66.4)	81(68.1)	40(33.6)	38(31.9)
Isikwuato	18(52.9)	14(41.2)	16(47.1)	20(58.8)
Total	162(61.1)	164(61.9)	103(38.9)	101(38.1)

Table 4.6: Prevalence of MRSA and MSSA from clinical samples from the 3 zones

Sample type	Umuahia *S. aureus*	MRSA No. (%)	MSSA No. (%)	Aba *S. aureus*	MRSA No. (%)	MSSA No. (%)	Isikwuato *S. aureus*	MRSA No. (%)	MSSA No. (%)
Wound and abscess	31	17(54.8)	14(45.2)	28	18(64.3)	10(35.7)	3	3(100)	-
Blood culture	1	0	1(100)	3	1(33.3)	2(66.7)	-	-	-
Urine	40	24(60)	16(40)	51	36(70.6)	15(29.4)	22	5(22.7)	17(77.3)
Ear swab	6	4(66.6)	2(33.3)	-	-	-	-	-	-
Nasal	3	2(66.6)	1(33.3)	-	-	-	1	1(100)	
Hvs	19	13(68.4)	6(31.6)	20	15(75)	5(25)	4	3(75)	1(25)
Urethral swab	12	9(75)	3(25)	17	11(64.7)	6(35.3)	4	2(50)	2(50)
Total	112	69(61.6)	43(38.4)	119	81(68.1)	38 (31.9)	34	14 (41.2)	20 (58.8)

Table 4.7: Antibiotic Resistance profile of MRSA and MSSA among the zones

Antibiotics	Umuahia			Aba			Isikwuato		
	MRSA resistant No. (%)	MSSA resistant No. (%)	Total	MRSA resistant No. (%)	MSSA resistant No. (%)	Total	MRSA resistant No.(%)	MSSA resistant No. (%)	Total
Levofloxacine	55(79.7)	12(29.9)	67	61(75.3)	14(36.8)	75	11(78.6)	4(20.0)	15
Vancomycin	0(0)	0(0)	0	0(0)	0(0)	0	0(0)	0(0)	0
Ciprofloxacine	57(82.6)	12(29.9)	69	67(82.7)	12(31.6)	79	11(78.6)	8(40.0)	19
Azithromycin	52(75.4)	6(4.0)	58	58(71.6)	21(55.3)	79	8(57.1)	8(40.0)	16
Cloxacillin	66(95.7)	12(29.9)	78	77(95.1)	20(52.6)	97	13(92.9)	15(75.0)	28
Gentamycin	36(52.2)	5(11.6)	41	22(27.2)	1(2.6)	23	7(50.0)	1(5.0)	8
Erythromycin	40(58.0)	16(37.2)	56	54(66.7)	19(50)	73	7(50.0)	14(70.0)	21
Ceftazidine	69(100)	43(100)	112	81(100)	38(100)	119	14(100)	20(100)	34
Cefuroxime	64(92.8)	11(25.6)	75	54(66.7)	15(39.5)	69	9(64.3)	9(45.0)	18
Ceftriaxone	67(97.1)	13(30.2)	80	57(70.4)	14(36.8)	71	12(85.7)	12(60)	24
Ofloxacine	59(85.5)	11(25.6)	70	58(71.6)	17(44.7)	75	13(92.9)	9(45.0)	22
Augmentin	64(92.8)	21(48.8)	85	69(85.2)	20(52.6)	89	13(92.9)	8(40)	21
Clindamycin	21(30.4)	7(16.3)	28	31(38.3)	15(39.5)	46	5(35.7)	9(45.0)	14

4.1.8 Comparison of Antimicrobial Susceptibility Profiles of MRSA and MSSA

All the 165 MRSA isolates were sensitive to vancomycin, 120(73.2%) to clindamycin, and 92 (56.1%) to gentamycin. For MSSA counterparts it was observed that the highest susceptibility was against gentamycin (88.1%), levofloxacine 72.3%. All the MRSA isolates were resistant to cefoxitin (100%), ceftazidine (100%), 157 (95.7%) to cloxacillin, 146 (89.0%) to augmentin and 136 (82.9%) to ceftriaxone. Lower level of resistance was observed among MSSA isolates; augmentin 48.5%, azithromycin 47.5% and cloxacillin 46.5%. There was a significant association between resistance among MRSA and MSSA (p=0.00) (Table 4.8)

4.1.9 Resistance Profile of MRSA from Clinical Sites to Various Antibiotics

Comparing the antibiotic susceptibility pattern of the MRSA isolates from the different clinical samples, it was observed that susceptibility was generally highest with the antibiotic clindamycin; 9.1%, 21.6%, 30.3% in urethral swab, wound and urine samples respectively while the highest resistance was observed among the drugs augmentin (95.5%, 91.9% and 75.8 in urethral swab, wound and urine respectively) and cloxacillin (100%,83.8%, and 92.4% in urethral swab, wound and urine respectively). The summary of percentage resistance is shown in Table 4.9.

Multiple-drug resistance among MRSA isolates

High rates of multidrug resistance were observed among the MRSA isolates with 115 (70.1%) being designated as such having expressed resistance to four or more classes of the antibiotics tested, also no isolate was fully susceptible to all the tested antibiotics. 18 of the MRSA isolates were resistant to more than 6 classes of the antibiotics tested (Table 4.10).

Table 4.8: Antibiotic sensitivity profile of MSSA and MRSA isolates

Antibiotics	MSSA (N=101)		MRSA (164)		TOTAL (265)	
	S	R	S	R	S	R
Levofloxacine	73(72.3)	28(27.7)	35(21.3)	129(78.7)	108(40.8)	157(59.2)
Vancomycin	101(100)	0(0)	164(100)	0(0)	265(100)	0(0)
Ciprofloxacine	61(60.4)	40(39.6)	26(15.9)	138(84.1)	87(32.8)	178(67.2)
Azithromycin	53(52.5)	48(47.5)	40(24.4)	124(75.6)	93(35.1)	172(64.9)
Cloxacillin	55(54.5)	46(46.5)	7(4.3)	157(95.7)	62(23.4)	203(76.6)
Gentamycin	89(88.1)	12(11.9)	92(56.1)	72(43.9)	181(68.3)	84(31.7)
Erythromycin	54(53.5)	47(46.5)	61(37.2)	103(61.6)	115(43.4)	150(56.6)
Cefoxitin	101(100)	0 (0.0)	0(0.0)	164(100)	101(38.1)	164 (61.9)
Ceftazidine	0(0)	101(100)	0(0)	164(100)	0(0)	265(100)
Cefuroxime	66(65.3)	35(34.7)	37(22.6)	127(77.4)	103(38.9)	162(61.1)
Ceftriaxone	62(61.4)	39(38.6)	28(17.1)	136(82.9)	90(34.0)	175(66.0)
Ofloxacine	64(63.4)	37(36.6)	34(20.7)	130(79.3)	98(37.0)	167(63.0)
Augmentin	52(51.5)	49(48.5)	18(11.0)	146(89.0)	70(26.4)	195(73.6)
Clindamycin	57(56.4)	44(43.6)	120(73.2)	44(26.8)	177(66.8)	88(33.2)

Table 4.9: Resistance Pattern of MRSA from Clinical Sites to Various Antibiotics

Antibiotic	Wound (n=37)%	Blood (n=1)%	Urine (n=66)%	Ear (n=4)%	Nasal (n=3)%	HVS (n=31)%	U/S (n=22)%
Levofloxacin	32(86.5)	1(100)	55(83.3)	2(50.0)	2(66.7)	22(71.0)	14(63.6)
Vancomycin	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Ciprofloxacin	28(75.5)	1(100)	58(87.9)	1(25)	2(66.7)	24(77.4)	16(72.7)
Azithromycin	18(48.6)	1(100)	46(69.7)	2(50.0)	2(66.7)	23(74.2)	14(63.6)
Cloxacillin	31(83.8)	1(100)	61(92.4)	3(75)	2(66.7)	30(96.8)	22(100)
Gentamicin	17(45.9)	0(0)	25(37.9)	3(75)	1(33.3)	11(35.5)	7(31.8)
Erythromycin	23(62.2)	1(100)	43(65.2)	3(75)	3(100)	20(64.5)	12(54.5)
Ceftazidime	37(100)	1(100)	66(100)	4(100)	3(100)	31(100)	22(100)
Cefuroxime	27(73.0)	1(100)	50(75.8)	3(75)	3(100)	23(74.2)	20(90.9)
Ceftriaxone	29(78.4)	1(100)	52(78.8)	4(100)	3(100)	25(80.6)	19(86.4)
Ofloxacin	32(86.5)	1(100)	48(72.7)	1(25)	2(66.7)	24(77.4)	16(72.7)
Augmentin	34(91.9)	1(100)	50(75.8)	4(100)	2(66.7)	27(87.1)	21(95.5)
Clindamycin	8(21.6)	0(0)	20(30.3)	2(50.0)	1(33.3)	12(38.7)	2(9.1)

Table 4.10: Prevalence of multiple-drug resistance among MRSA isolates.

Parameter	Frequency of multi-drug resistance	
	Number	**(%)**
Fully sensitive	-	-
Resistant to 1 class	3	1.8
Resistant to 2 classes	14	8.5
Resistant to 3 classes	32	19.5
Resistant to 4 classes	64	39.0
Resistant to 5 classes	33	20.1
Resistant to 6 classes	18	11.0
Resistant to 7 classes	-	-

4.1.10 Prevalence of Inducible Clindamycin Resistance among Isolates.

Out of the 265 *S. aureus* isolates, 150(56.6 %) of them were erythromycin resistant. These isolates when subjected to D test, 73 (27.5 %) isolates showed resistance to erythromycin and clindamycin indicating constitutive MLSB phenotype. Out of the 177 isolates that showed clindamycin sensitivity, 32 (12.1 %) isolates showed positive D test indicating inducible MLSB phenotype, while 51 (19.2 %) showed true sensitivity to clindamycin (D test negative indicating MS phenotype) Table 4.12.

In MRSA, 57(34.8) % had the susceptible phenotype (E-S, CL-S). Constitutive MLSB phenotype was 45(27.4 %) and the inducible MLSB phenotype was 29(17.7 %), while in methicillin sensitive Staphylococcal isolates (MSSA), the constitutive MLSB phenotype was 28(27.7%) and the inducible MLSB phenotype was 3(3.0 %). The E-S and CL-S phenotype predominated over the inducible resistance phenotype and constitutive resistance phenotype among MRSA and MSSA isolates. The percentage of inducible and MS resistance was higher amongst MRSA isolates when compared with MSSA isolates. There was significant association between MRSA and inducible clindamycin resistance (iMLSB) among staphylococci isolates (X2 =**,** 6.9892 **P**=0.008) Table 4.11.

The result obtained from the three zones showed that constitutive MLSB phenotype among MRSA was highest in Isikwuato (35.7%) and lowest in Aba (27.7%) while the inducible MLSB phenotype was highest in Aba (21.0%) and lowest in Isikwuato (14.3%). The results obtained for inducible MLSB phenotype and negative ms phenotype was observed to be same in Umuahia (14.5%) and Isikwuato (14.3) Table 4.11.

Table 4.11: D test among methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) among the zones

Susceptibility pattern phenotype	Umuahia *S. aureus*	MRSA No. (%)	MSSA No. (%)	Aba *S. aureus*	MRSA No. (%)	MSSA No. (%)	Isikwuato *S. aureus*	MRSA No. (%)	MSSA No. (%)
ERY-S,CL-S	53(47.3)	27(39.1)	26(60.5)	45(37.8)	25(30.9)	20(52.6)	11(32.4)	5(35.7)	6(30.0)
ERY-R,CL-R (constitutive mls_b)	28(25)	22(31.9)	6(14.0)	33(27.7)	18(22.2)	15(39.5)	12(35.3)	5(35.7)	7(35.0)
ERY-R,CL-S (D-test positive/mls_b)	11(9.8)	10(14.5)	1(2.3)	18(15.1)	17(21.0)	1(2.6)	3(8.8)	2(14.3)	1(5.0)
ERY-R,CL-S (D-test negative ms)	20(17.9)	10(14.5)	10(23.3)	23(19.3)	21(25.9)	2(5.3)	8(23.5)	2(14.3)	6(30)
Total	112(100)	69(61.6)	43(38.4)	119	81(68.1)	38(31.9)	34(100)	14(41.2)	20(58.8)

Key: Ery –erythromycin, Cl –clindamycin, S – sensitive, R –resistant, Imls$_b$- inducible mls$_b$phenotype, Ms - ms phenotype, Constitutive mls$_b$ =constitutive mls$_b$ phenotype

Table 4.12: Overall phenotypic pattern of inducible clindamycin resistance among MRSA and MSSA

Susceptibility pattern (phenotype)	MRSA No (%)	MSSA No (%)	Total (%)
ERY-S, CL-S	57(34.8)	52(51.5)	109(41.1)
ERY-R, CL-R (constitutive mls_b)	45(27.4)	28(27.7)	73(27.5)
ERY-R, CL-S, D-test positive($Imls_b$)	29 (17.7)	3(3.0)	32(12.1)
ERY-R, CL-S (D-test negative ms)	33(20.1)	18(17.8)	51(19.2)
Total	164(61.9)	101(38.1)	265

Key: Ery –erythromycin, Cl –clindamycin, S – sensitive, R –resistant, inducible $MLS_{B=}$ phenotype=inducible resistance to clindamycin, MS - MS phenotype, Constitutive MLS_B =constitutive resistance to clindamycin.

4.1.11 Beta lactamase production among *S. aureus* isolates

The detection of β-lactamase production was carried out among the methicillin resistant *S. aureus* isolates. 164 isolates were tested and only 109 (66.5%) were positive, while 55(33.5%) were negative (Fig 4.5), the highest level of resistance was observed against all the β-lactam antibiotics

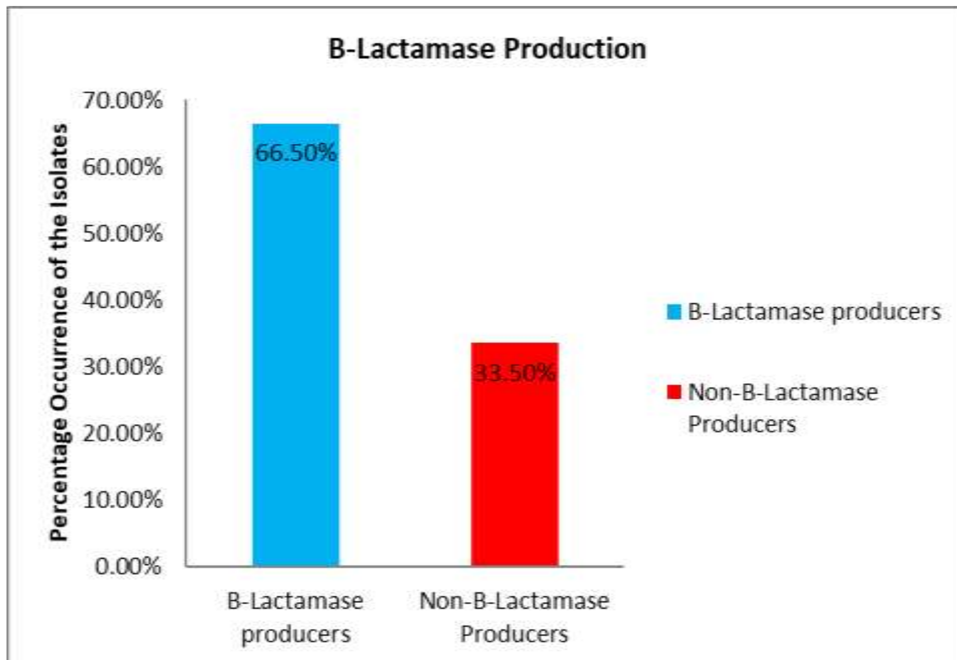

Fig 4.5; β-lactamase production among the MRSA isolates.

4.1.12 Amplification of 16SrRNA

The 16SrRNA was amplified and detected among 13 isolates which were selected randomly and they showed amplification at 1500 bp. This showed that all isolates were *S. aureus* as 16SrRNA gene is generally accepted as a genius specie specific marker for detecting *S. aureus*. However, 2 isolates were not amplified. The result is shown in Fig 4.6

4.1.13 Phylogenesis Obtained from 16SrRNA Sequencing

The obtained 16SrRNA sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16SrRNA of the isolates A13, A25, A12, A14, A7, A2 and A5 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Staphylococcus* sp and revealed a close relatedness to *Staphylococcus aureus* than other *Staphylococcus* sp (Fig4.7).

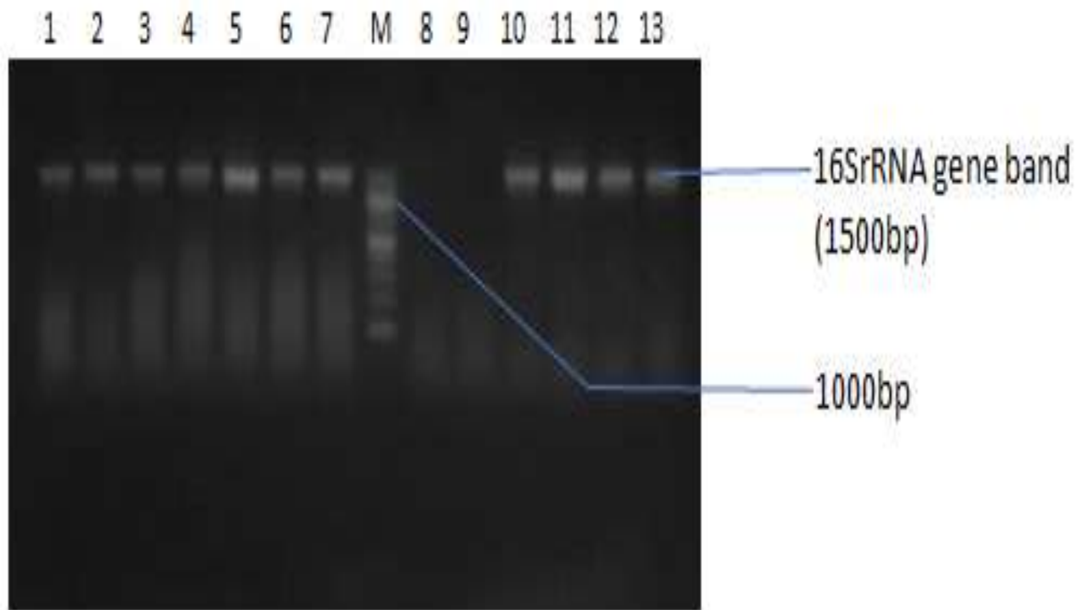

Fig 4.6: Agarose gel electrophoresis showing the 16rRNA gene of the bacterial isolates. Lane M represents the 100bp molecular ladder.

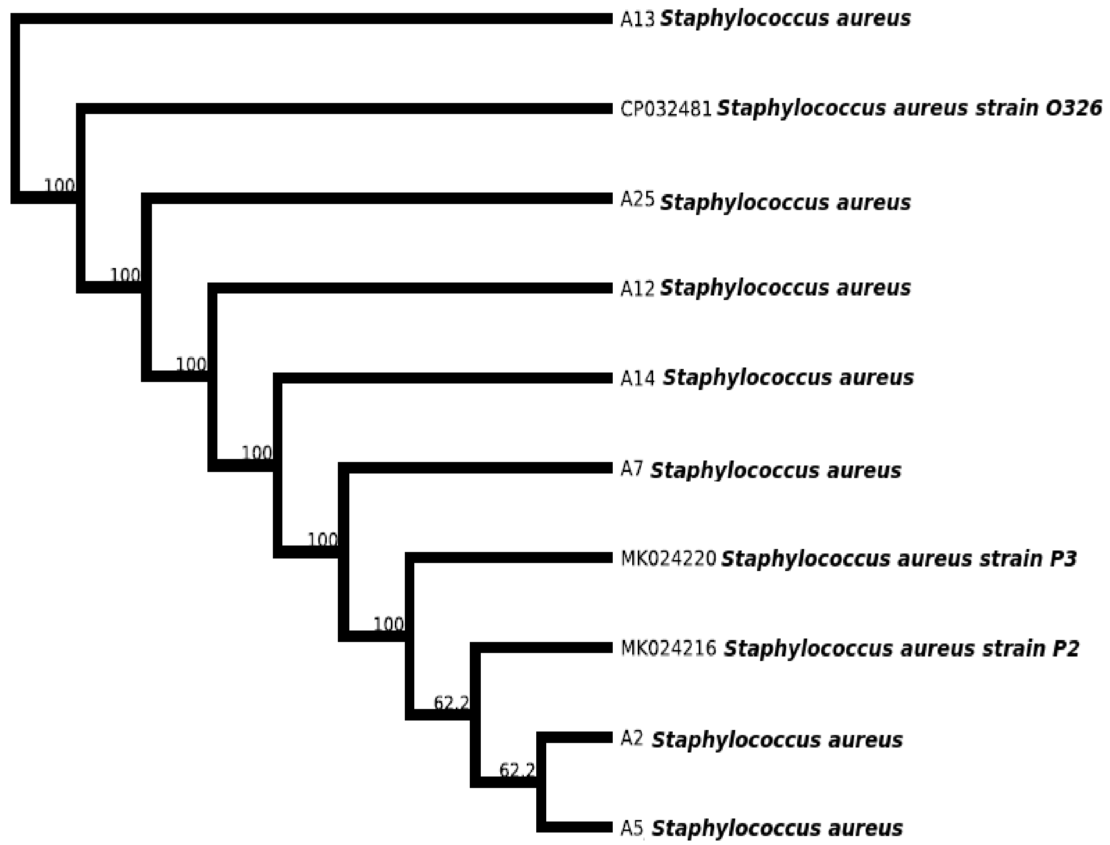

Fig 4.7: Phylogenetic tree showing the relationship between the query 16SrRNA nucleotide of 7 *S. aureus* strains.

4.1.14 PCR Amplification of Antibiotic Resistance Genes

4.1.14.1 Amplification of *mecA* Gene

Out of the 40 cefoxitin resistant isolates tested, 12 (30%) possessed the *mec A* gene and the size of the amplicon corresponded to 533bp. The result is shown in Fig 4.8

4.1.14.2 Amplification of *blaZ* gene

*BlaZ*gene codes for the presence of βeta lactamase. A total of 7(17.5%) of the isolates tested positive to the *blaZ* gene. The amplified band was seen at 300bp as seen in Fig 4.9.

4.1.14.3 *ermB* gene amplification

The presence of *erm B* gene which codes for inducible macrolide resistance was observed in 8 (20%) of the isolates. The amplicon size was seen at 198bp as shown in Fig 4.10.

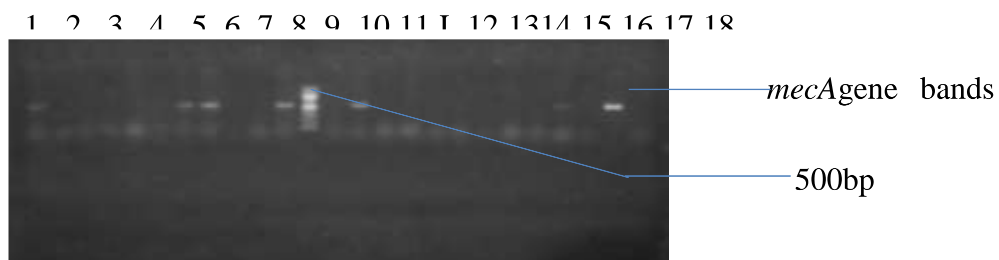

Fig 4.8: Agarose gel electrophoresis of the Mec A gene of the *S. aureus* isolates. Lane 1,7,811,13,20,23 showing *mecA* at 533bp. Lane L represents the 100bp molecular ladder

Fig 4.9: Agarose gel electrophoresis showing the amplified *blaZ* gene bands. Lanes 1,6,8,10,17 and 20 showing the amplified bands at 300bp while lane L represents the 100bp molecular ladder.

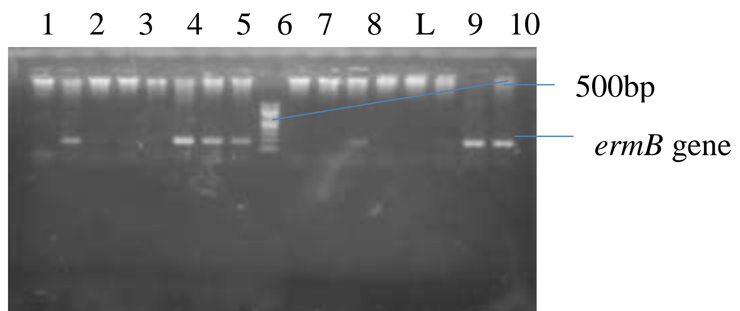

Fig 4.10: Agarose gel electrophoresis of the *erm*B gene of the *S. aureus* isolates. Lane 2,6-8, 11 15, 16 showing the *ermB* gene band at 198bp Lane L represents the 100bp molecular ladder.

4.1.15 Virulence gene profiling

4.1.15.1 Amplification of *pvl* gene

All 40 isolates were amplified for the detection of the virulence determinant; Panton Valentine Leukocidin (*pvl*) gene. *pvl* is associated with tissue necrosis and leucocyte destruction. Until recently genes coding for *pvl* were infrequently encountered being observed in <5% of *S. aureus* isolation worldwide. The amplicon size was at 1100bp. A total of 4 (10%) isolates possessed the *pvl* gene. A closer look at these 4 isolates of MRSA that harboured the *pvl* genes in this study showed the isolates were from wound specimen (n=1), HVS (n=1) and from urine specimen (n=2). This is shown in Fig 4.11.

4.1.16 Gene Distribution among the MRSA Isolates.

The prevalence of genes among the MRSA isolates is shown in fig 4.12, while the gene cluster was represented in table 4.13, the highest cluster was found in isolates with *mecA* and *blaz* genes 7.5%, while no isolate possessed all 4 genes. 2 isolates had the combinations of the 3 genes.

4.1.17 Occurrence of Genes Among the Clinical Samples

The prevalence of the genes among the clinical samples is shown in Fig 4.13, with the highest prevalence occurring among the wound samples and none from ear swabs.

4.1.18 RAPD Analysis

RAPD-PCR was used to determine the genetic relationship of 12 MRSA strains from clinical samples. Dendrogram analysis of RAPD-PCR amplification patterns of the obtained isolates with the chosen primer generated 3 distinct but related RAPD clusters. Genetic fingerprinting and phylogenetic diversity between different MRSA strains were determined

by converting RAPD data into a Jaccard similarity matrix and analysed by UPGMA to produce a phylogenetic tree. These genotypes were assigned RAPD types A to C (Figure 4.14).

4.1.19 Molecular size of obtained RAPD Amplicons

The RAPD profiles consisted of two to four amplicons ranging from 170 to 1000 base pairs in length. Apart from two isolates, i.e., strain 5 and 6, all other isolates generated some conserved bands that could be detected in the patterns from all isolates. According to our results, RAPD types A with 4 isolates (40%), type B with 4 1solates too (40%) were the most frequently encountered, followed by RAPD type C with 2 isolates (20%) as the third (Table 4.14).

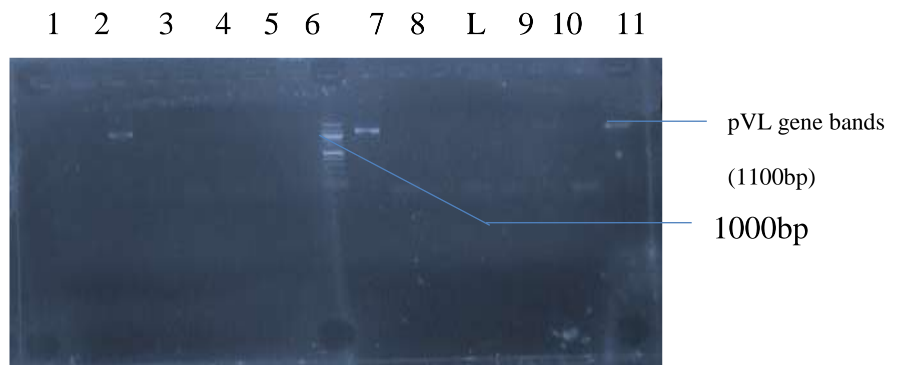

Fig 4.11: Agarose gel electrophoresis of the pVL gene of the *S. aureus* isolates. Lane 3, 9 and 16 showing the pVL gene band at 1100bp. Lane L represents the 100bp molecular ladder.

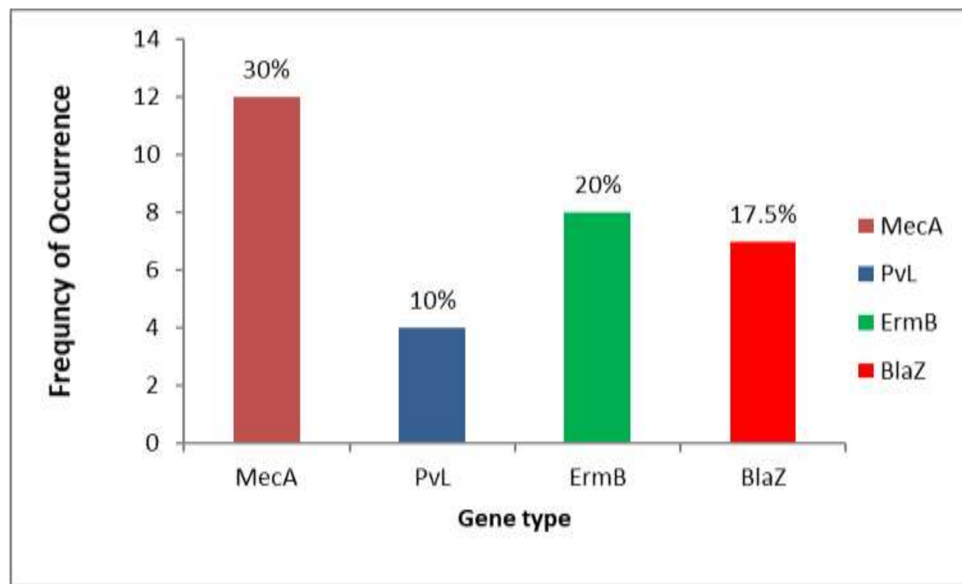

Fig 4.12: Gene distribution among the MRSA isolates

Table 4.13: Pattern of Gene Cluster among the Isolates.

Gene Cluster	**No (%) Occurrence**
MecA+Blaz	3 (7.5)
MecA+ErmB	2 (5.0)
MecA+Pvl	2 (5.0)
Pvl+ErmB	1 (2.5)
Pvl+Blaz	1 (2.5)
ErmB +Blaz	2 (5.0)
MecA+Pvl +Blaz	1 (2.5)
MecA+Pvl +ErmB	1 (2.5)
MecA+ ErmB +Blaz	0 (0.0)
Pvl + ErmB +Blaz	0 (0.0)

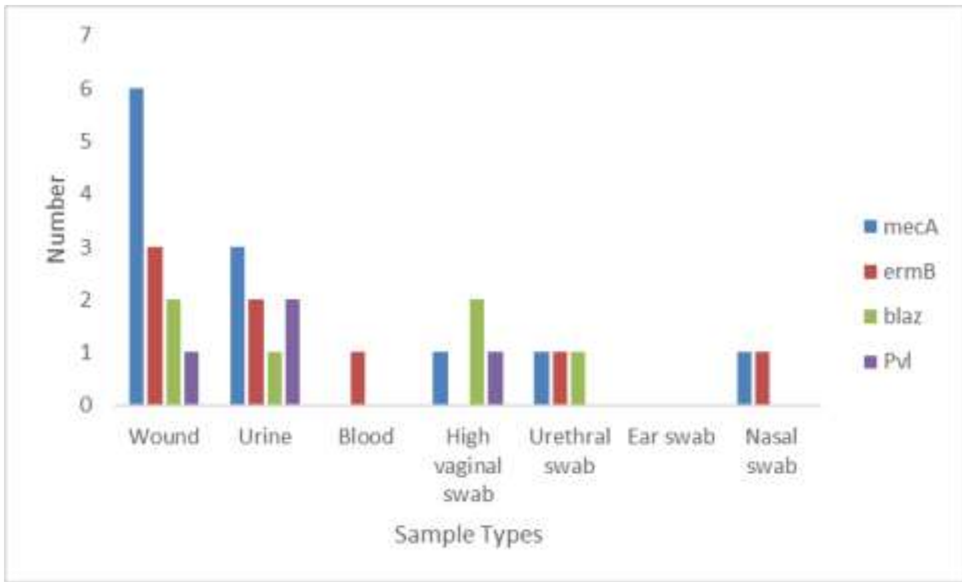

Fig 4.13: Prevalence of genes among the clinical samples.

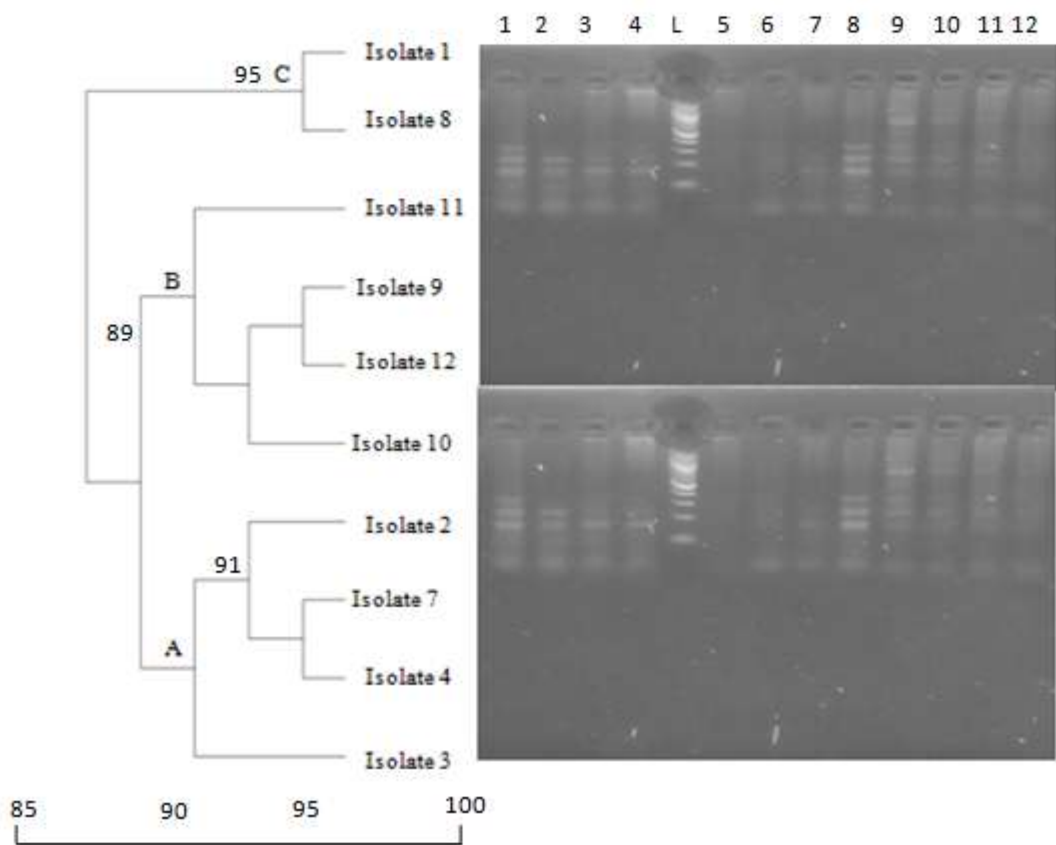

Fig 4.14: Dendogram of genetic relationship of the isolates (1-12) revealing three clusters (A, B and C) between ten strains of methicillin-resistant *Staphylococcus aureus* obtained with saOLP13 RAPD primers.

Table 4.14: Molecular Size of RAPD Amplicon (bp)

RAPD Type	No Of Bands	100	170	220	300	500	700	900	1000	No Of Isolates	Isolate Designation
A	2		1	1						4	2,3,4,7
B	4		1	1	1				1	4	9,10,11,12
C	3		1	1	1					2	1,8

4.1.20 Antibacterial Activities of the Plant Extracts

Two plant spp- *Alchornea cordifolia* and *Acalypha wilkesiana* were investigated to evaluate their antibacterial activity against 9 isolates of MRSA obtained from clinical samples using agar well diffusion method. The results obtained from both water and ethanol crude extracts of *A. cordifolia*., and *A. wilkesiana* showed good activity for the ethanol extracts while there was no activity for the water extracts on *A. wilkesiana* as shown on Tables 4.16 and 4.17 respectively. The largest diameter of zone of inhibition for the two plants against the isolates was 21.0 mm at 200mg/ml while the least activity was 9.0mm at 50mg/ml. The result was comparable to the standard antibiotic gentamycin used as control

4.1.21 MIC and MBC Obtained from the Two Plants

The MIC and MBC of the plants were carried out to determine their bacteriostatic and bactericidal properties. The concentration of the plant extracts is shown in Table 4.18, the inhibitory effect of *Alchornea cordifolia* extract started at 12.5mg/ml

4.1.22 The Rate of Killing of the Extracts on the Strains

The results of rate of killing of the organisms in Fig 4.15 and Fig 4.16 shows a gradual reduction in the number of colonies from 0hour- 24hours in the test isolates.

4.1.23 Quantitative Phytochemical Composition of the Two Plants

Phytochemical screening of crude extracts of *Alchornea cordifolia* and *Acalypha wilkesiana* revealed the presence of flavonoids and phenolic compounds as shown in Table 4.20

Table 4.16: Antimicrobial effect of the ethanol extracts of *Alchornea cordifolia and Alcalypha* against MRSA

Isolates	Alchornea cordifolia Concentrations (mg/ml)			Alcalypha*wilkesiana* Concentrations (mg/ml)			Gentamycin
	200	**100**	**50**	**200**	**100**	**50**	
MRSA1	15.0±0.01	0.0±0.00	0.0±0.00	16.0±0.00	0.0±0.00	0.0±0.01	24.0±0.02
MRSA2	19.0±0.01	16.0±0.01	0.0±0.00	21.0±0.01	14.0±0.00	10.0±0.01	29.0±0.01
MRSA3	15.0±0.02	15.0±0.00	0.0±0.00	17.0±0.02	0.0±0.00	0.0±0.03	31.0±0.01
MRSA4	16.0±0.01	12.0±0.01	0.0±0.00	18.0±0.02	11.0±0.01	0.0±0.01	29.0±0.003
MRSA5	20.0±0.01	16.0±0.03	11.0±0.03	19.0±0.01	16.0±0.01	10.0±0.01	25.0±0.01
MRSA6	15.0±0.02	0.0±0.00	0.0±0.00	15.0±0.03	0.0±0.00	0.0±0.03	32.0±0.02
MRSA7	16.0±0.04	14.0±0.01	9.0±0.00	18.0±0.00	15.0±0.02	0.0±0.02	31.0±0.01
MRSA8	15.0±0.01	0.0±0.00	0.0±0.00	15.0±0.01	0.0±0.00	0.0±0.01	31.0±0.01
MRSA9	21.0±0.03	17.0±0.01	0.0±0.00	21.0±0.01	15.0±0.01	10.0±0.01	32.0±0.02

Values are the mean ± standard deviation of two replication of each parameter.

Table 4.17: Antimicrobial effect of the Aqueous extracts of *Alchornea cordifolia and Alcalypha wilkesiana* against MRSA

Isolates	*Alchornea cordifolia* Concentrations (mg/ml)			*Alcalypha* Concentrations (mg/ml)			Gentamycin
	200	100	50	200	100	50	
MRSA1	8.0±0.01	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	25.0±0.01
MRSA2	9.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	25.0±0.01
MRSA3	9.0±0.01	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	25.0±0.01
MRSA4	8.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	23.0±0.00
MRSA5	9.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	23.0±0.01
MRSA6	8.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	23.0±0.00
MRSA7	9.0±0.01	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	23.0±0.02
MRSA8	9.0±0.01	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	24.0±0.03
MRSA9	8.0±0.01	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	27.0±0.02

Values are the mean ± standard deviation of two replication of each parameter.

Table 4.18: MIC and MBC of Ethanol extract of *Alchornea cordifolia* against Methicillin Resistant *Staphylococcus aureus*

Plant	Isolate ID	Concentration (mg/ml) 100	50	25	12.5	6.25	3.12	1.56	MIC	MBC
Alchornea	MRSA1	-	-	+	+	+	+	+	25	50
cordifolia	MRSA2	-	-	-	+	+	+	+	12.5	25
	MRSA3	-	-	-	+	+	+	+	12.5	25
	MRSA4	-	-	+	+	+	+	+	25	50
	MRSA5	-	-	-	+	+	+	+	12.5	25
	MRSA6	-	-	+	+	+	+	+	25	50
	MRSA7	-	-	+	+	+	+	+	25	50
	MRSA8	-	-	+	+	+	+	+	25	50
	MRSA9	-	-	-	+	+	+	+	12.5	25

KEY

+ = Growth of the organism in broth medium indicated by turbidity of the medium after overnight incubation.

_ = No growth of the organism in broth medium indicated by clarity of the medium after overnight incubation.

Table 4.19: MIC and MBC of Ethanol extract of *Alcalypha wilkesiana* against methicillin resistant *Staphylococcus aureus*

Plant	**Isolate ID**	**Concentration (mg/ml)** 100	50	25	12.5	6.25	3.12	1.56	**MIC**	**MBC**
Alcalypha	MRSA1	-	+	+	+	+	+	+	50	100
	MRSA2	-	-	+	+	+	+	+	25	50
	MRSA3	-	-	+	+	+	+	+	25	50
	MRSA4	-	+	+	+	+	+	+	50	100
	MRSA5	-	-	+	+	+	+	+	25	50
	MRSA6	-	-	+	+	+	+	+	25	50
	MRSA7	-	-	+	+	+	+	+	25	50
	MRSA8	-	-	+	+	+	+	+	25	50
	MRSA9	-	-	+	+	+	+	+	25	25

KEY

+ = Growth of the organism in broth medium indicated by turbidity of the medium after overnight incubation.

_ = No growth of the organism in broth medium indicated by clarity of the medium after overnight incubation.

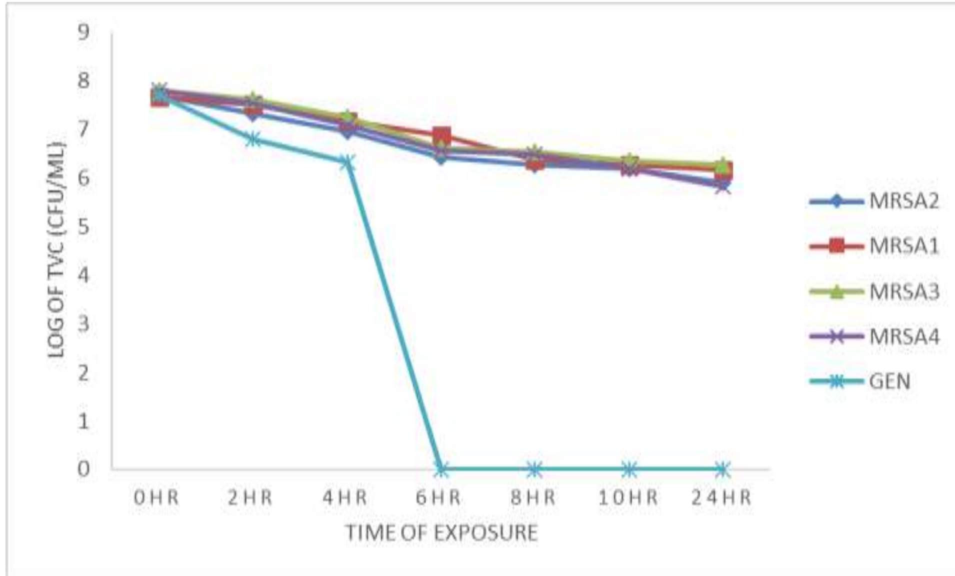

Fig 4.15: Rate of Kill of the Isolates by *Alchornea cordifolia*

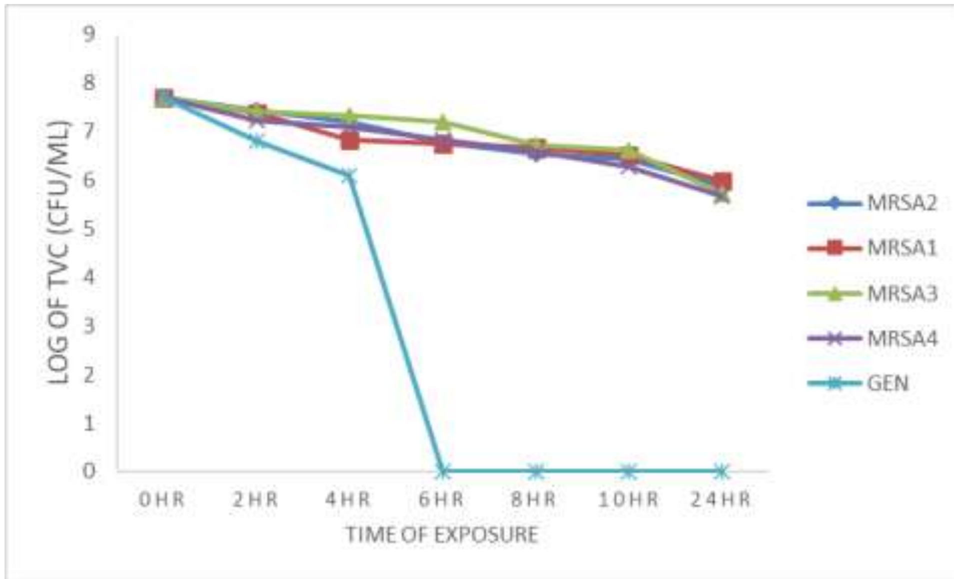

Fig 4.16: Rate of Kill of the Isolates by *Alcalypha wilkesiana*

Table 4.20: Quantitative Phytochemical Composition of the Plants

Phytochemicals	*Alchornea cordifolia*	*Alcalypha wilkesiana*
Alkaloid (%)	1.85 ± 0.01^{b}	2.64 ± 0.02^{a}
Tannin (%)	0.70 ± 0.02^{e}	0.64 ± 0.02^{c}
Saponin (%)	4.13 ± 0.01^{a}	0.24 ± 0.01^{d}
Flavonoid (%)	1.08 ± 0.01^{c}	1.84 ± 0.01^{b}
Phenol (%)	1.03 ± 0.02^{cd}	0.26 ± 0.01^{d}
Glycosides (%)	1.05 ± 0.05^{c}	0.09 ± 0.00^{e}

Values are the mean ± standard deviation of two replication of each parameter. Values with different superscript down a column are significantly different from each other

4.2 DISCUSSION

This study presents the comparative analysis of phenotypes, antibiogram, molecular characteristics of S*taphylococcus aureus* isolates from clinical samples in Abia State. In Nigeria, *S. aureus* causes significant epidemiologic and therapeutic challenges as deduced from many literatures while identification and antibiotic susceptibility testing of *S. aureus* isolates have been based on phenotypic methods, there is generally paucity of data on the characterization of these isolates using molecular methods (Esan *et al.,* 2009). Over the past 20 years, the incidences of both community-acquired (CA) and hospital-acquired (HA) *S. aureus* infections have increased, while antibiotic treatment is increasingly affected by the spread of new strains that are resistant to multiple antibiotics, including methicillin (Ghebremedhin *et al*., 2009).

4.2.1. Prevalence of *S aureus* and MRSA

Staphylococcus. aureus is a well-known nosocomial pathogen with an alarmingly increasing level of developing resistance to many available antimicrobial agents (Akerele *et al.,* 2015). The overall prevalence of *S. aureus* from clinical samples obtained in this study was 35.3%. This is in line with other studies; 24.5%, and 48% obtained in other parts of Nigeria (Nsofor *et al*.,2016; Uwaezuoke & Aririatu 2004). This pattern of prevalence may be as a result of the level of *S. aureus* infection in the study locality. The high incidence of *S. aureus* observed among the clinical samples shows the versatility of this organism amongst other bacteria, which makes it the most endemic pathogen in clinical settings (Nsofor *et al*.,2016).

Though MRSA does not show a predilection for any particular age or sex, no age is exempt from these infections (Osinupebi *et al.*, 2018). In the present study, it was found that highest

rate of MRSA was observed in age group of 31-40 years showing a prevalence rate of 25.6% while age group <11 had the least prevalence of MRSA with 1.8%.

The overall MRSA prevalence of 61.9% of *S. aureus* isolates in this study may be considered high although it falls within the range determined in previous reports from some other parts of the country. Onemu & Ophori (2013) recorded 79% from Benin city, and a study amongst Nigerian women recorded 71.2% (Onanuga *et al*., 2005). Some centers however have reported lower rates of 26.9%, 47.8% from Abuja and Oshogho respectively (Olowe *et al* 2007; Abdullahi & Iregbu 2018). Different studies from other countries have recorded variations of MRSA prevalence, 23.6% in Australia to over 61% in Taiwan and Singapore more than 70% in Japan and Hongkong and 72% in Eritrea (Tebelay & Adane, 2016; Garoy *et al.,* 2019). Differences in the length of study period, number of study sites, sample size, sample type, economic level of the accessed regions and laboratory procedures employed are attributed as factors that may cause variations in the prevalence rate of MRSA (Tebelay & Adane, 2016; Ghia *et al*., 2020).

4.2.2 Antibiotic resistance in *S. aureus* strains

Antimicrobial resistance has been observed to be a paramount threat in the twenty first century. *S. aureus* has always been a stumbling block for antimicrobial chemotherapy and methicillin resistance of *S. aureus* remains a major problem. Some years back, cloxacillin was highly recommended in treatment of staphylococcal infections in view of the excellent in vitro sensitivity results. This could be seen from the reports obtained at Owerri, Nigeria (Uwaezuoke & Aririatu, 2004) with 85.4% sensitivity. However, these results are at variance with current trends in MRSA susceptibility to cloxacillin as could be seen from the results obtained in the present study and others conducted from various parts of the country

(Iroha *et al*, 2012; Joshua & Ronke 2015; Ejikeugwu *et al*., 2018). The high level of resistance could be associated with earlier exposure of these antibiotics to isolates which may have enhanced development of resistance.

Resistance to ceftazidime, cefuroxime and ceftriaxone (which are second generation cephalosporins), among the isolates were 100%, 77.4% and 82.9% respectively. These drugs were very effective against *S. aureus* years ago but resistance is on the increase probably because it is readily available with many cheap brands in the market. The Japanese experience cites the introduction of 2nd and 3rd generation cephalosporins in the early 1980s as playing a significant part in the emergence and spread of MRSA in Tokyo hospitals. The steady increase of MRSA in Italy, Europe and Britain has also been attributed to the use of cephalosporins (Dancer, 2001). Resistance to beta lactam drugs including cephalosporins, augmentin and oxacillin used in this study is not surprising. This is consistent with the observation that clinical staphylococcal isolates are resistant to a large number of commonly prescribed antimicrobial agents and to beta lactams in particular (Olukoya *et al*., 2005). It is believed that more than 80% of staphylococcal isolates produce penicillinase regardless of the clinical setting (Lowy 2003; Pantosti *et al.,* 2007).

High resistance of isolated MRSA strains to fluoroquinolone was observed; ciprofloxacin 133(84.1%), levofloxacin 129(78.7%) and ofloxacine 130(79.3%) while there was lower level of resistance among the MSSA counterparts at 27.7%, 39.6% and 36.6% respectively. The commencement of resistance to flouroquinolones may be connected with the increasing availability of cheaper generation of the agents within our locality leading to increased spontaneous mutations that are present in large bacterial populations and which contain chromosomal mutations that alter the target proteins and/or increase the level of efflux

pump expression (Hooper, 2002; Rogues *et al.,* 2007). This is consistent with studies from other parts of the world (Clement *et al*., 2009; Maj puneet *et al*., 2015; Kot *et al.,* 2020). Previous studies also have implicated fluoroquinolones as being culpable to MRSA acquisition even though the mechanisms responsible for this action have not been fully elucidated. It further revealed fluoroquinolone use as being significantly associated with MRSA but not MSSA acquisition (Clotilde *et al*., 2014).

The MRSA isolates in this study exhibited excellent susceptibility to vancomycin, and the finding is in line with results from previous studies in Nigeria other parts of the world (Kesah *et al*., 2003; Taiwo *et al*., 2004; Fayomi *et al.,* 2011; Firoozeh *et. al.,* 2020) however, there are some reports of the emergence of vancomycin-resistant *S. aureus* in some centres in Nigeria (Taiwo *et al*.,2011; Onolitola *et al*., 2007). The display of excellent susceptibility of these isolates to vancomycin, is good for therapeutic purposes. This drug is not commonly in use in many hospitals and so does not contribute significantly to selective pressure. It is also not readily available across the counter (Abdullahi & Iregbu, 2018)

The high level of multiple drug resistance shown by the MRSA isolates obtained in this study is of a great concern. Majority of the MRSA isolates showed resistance to more than 4 antibiotic classes, indicating the presence of strong selective pressures from antibiotics used in the study area. One of the cities used in this study is a commercialized city with most inhabitants on high level of self-medication.

4.2.3 Inducible clindamycin resistance and detection of *ermB* genes

Emergence of MRSA has resulted in therapeutic alternatives to treat staphylococcal infections. The macrolides (erythromycin and clarithromycin), lincosamides (clindamycin

and lincomycin) and streptogramin B (quinupristin-dalfopristin), which corresponds to the macrolide-lincosamide-streptogramin B (MLSB) group serve as useful options. Clindamycin is a preferred choice due to its excellent pharmacokinetic properties and is indicated in the treatment of soft-tissue and skin infections caused by *Staphylococcus* species (Drinkovic *et al*., 2001; Nwokah & Abbey, 2016). Resistance to MLSB antibiotics most commonly results from acquisition of erythromycin resistant methylase genes (*erm* gene) which encode enzymes that methylate the 23SrRNA. There is a risk of treatment failures when clindamycin or any non-inducer macrolide is used in the treatment of infections caused by staphylococcal strains carrying inducible erm gene and so it is important to routinely carry out a D Test to detect resistance (Drinkovic *et al*., 2001).

The present study revealed that out of 265 isolated *S. aureus* tested for inducible clindamycin resistance, 32 (12.1%) were positive (D-test positive). This is comparable with a study conducted in Nigeria where 11.2% of their isolates were D-test positive (Nwokah & Abbey, 2016) and Bangalore, India (9.15%) (Sasirekha *et al*., 2014), though higher results have been observed in other places (Parasa *et al*., 2011). This difference or variability could be attributed to difference in geographical location, methicillin susceptibility of the *S. aureus* isolates and age group of the study subjects (Mohanansoundaram, 2011).

The overall prevalence of inducible clindamycin resistance among MRSA isolates was 29 (17.7%), whereas among MSSA, only 3 (3.0%) isolates showed inducible clindamycin resistance. This is similar to result obtained by Okojokwu *et al* (2018) in Jos while some other investigators have reported a higher incidence of iMLSB resistance, others have indicated a lower incidence (Bottega *et al*., 2014; Kumurya, 2015; Nwokah & Abbey,

2016). In reality, incidence of the MLSB phenotype of *S. aureus* depends on the patient population studied, the geographical region, the hospital characteristics and methicillin susceptibility (MRSA or MSSA) (Sasirekha *et al*., 2014)

Also, in the present study, 20.1 % of erythromycin-resistant MRSA isolates showed true clindamycin susceptibility (MS phenotype) and this implies that patients with infections caused by such isolates can be treated with clindamycin without emergence of resistance during therapy. From these findings, it is imperative that phenotypic differentiation of truly clindamycin sensitive from false clindamycin-sensitive staphylococci (iMLSb) be carried out to avoid treatment failure especially for staphylococci isolates from hospital environments.

Identifying MLS resistance phenotype is very important, because iMLSb phenotype under intensive antibiotic selective pressure converts into cMLSb phenotype and may lead to treatment failure in patients with serious staphylococcal infection (Zachariah *et al*., 2016). Erythromycin resistance can be caused by several mechanisms, the predominant form being target modification mediated by one or more *erm* genes encoding a 23S rRNA methylase. Target site modification is mediated by the presence of *erm* genes; *ermA, ermB* and *ermC*. In our study, the greatest number of MRSA isolates showed sensitivity to both macrolide and lincosamide and the most frequent phenotype of resistance was cMLSb, while iMLSb phenotype was the least MLS resistance phenotype among MRSA. There are various reports of prevalence among the erm gene from various countries but paucity of data from Nigeria. Findings by David *et al*. (2018) among Nigerian patients listed *ermB, ermC, msrA* and *msrB* genes as the genes responsible for erythromycin resistance. In Tunisia the most abundant genes were *ermA* and *ermB* (Zmantar, *et al*., 2011) while *ermC* is most prevalent in Brazil

(Pereira *et al*., 2016). In our report, the prevalence of cMLSB phenotype was highest and this motivated our search for the presence of the *ermB* gene, as according to Weiming *et al* (2019), *ermB* is found more in constitutive phenotypes. In this research, there was a significant relationship between phenotypic and genotypic resistance to erythromycin as 5 out of 8 isolates positive for *ermB* gene showed the iMLSB phenotype while only 3 out of the 8 isolates were erythromycin resistant, caused by other mechanisms other than the presence of *ermB* genes. However, in a number of reported studies, no correlation between the presence of genes and resistance phenotypes was observed (Zmantar *et al*., 2011). The high prevalence of *ermB* gene agrees with the findings of Jarajreh *et al*. (2016) who detected high frequency of *ermB* in both HA-MRSA and CA-MRSA but is in contrast with the study in Brazil by Coutinho *et al.* (2010) who reported low frequency of the *ermB* gene,

4.2.4 Phenotypic and molecular detection of MRSA

The accurate detection of β-lactam and *mecA*-mediated resistance in *S. aureus* is essential for the treatment of overt infections and the implementation of infection control practices Phenotypic detection of MRSA using cefoxitin disc diffusion gave an MRSA prevalence of 164(61.9%) with 65(39.6%) being from urine samples, 3(23.1%), wound swabs, 31(18.9%), high vaginal swabs, 22(13.4%), urethral swabs, 4(2.4%), ear swabs, 3(1.8%), nasal swabs and 1(0.6%) from blood samples. In comparison with other reported phenotypic MRSA isolates from different parts of the country Nigeria: Fayomi (2009) reported a prevalence rate of 31% of MRSA among in-patients at a tertiary health facility in Ido-Ekiti. Azeez-Akande *et al*. (2008) reported a MRSA prevalence rate of 37.5% from clinical specimens at University of Calabar Teaching Hospital and from Ilorin, Onanuga *et al*. (2005) reported a higher rate of 71.1% from urine of healthy women in Abuja while Ikeh & Yakeu (2006)

reported an alarming 92.6% MRSA out of the *S. aureus* isolated from bacteria flora on the hands of nursing service workers in Jos University Teaching Hospital. The prevalence of phenotypic methicillin resistance was 61.6%, 68.1% and 41.2% from Umuahia, Aba and Isiukwuato zones respectively. A moderate 43% prevalence was also reported in Jos by Ikeh (2003).

The gold standard for detection of MRSA is the detection of *mecA* gene by polymerase chain reaction. Expression of *mecA* gene yields an altered penicillin binding protein, PBP2a which has a reduced affinity for β- lactam antibiotic binding. In this study 12(30%) out of 40 *S. aureus* isolates were confirmed as methicillin resistant *S. aureus* by the detection of *mecA* gene. Similarly, there are reported cases of detection of *mecA* gene in MRSA isolates from other parts of this country, Nigeria which include the following: In research in Benin City, Nigeria, 4 isolates representing 11% were confirmed to carry *mecA* gene according to molecular technique (Obasuyi, 2013). Another research by Clement *et al.,* (2009) confirmed only one MRSA isolate from health care institutions from Ekiti and Ondo states. In another research carried out by Shittu *et al.,* (2011), two MRSA isolates with *mecA* gene were detected in Ile-Ife, one from Lagos and two from Ibadan (all-in South-Western Nigeria). In the same study, five MRSA isolates with *mecA* gene were detected in Maiduguri (North Eastern Nigeria). Okon *et al*., (2013) reported the detection of 12.5% MRSA from clinical specimens from six tertiary hospitals in North Eastern Nigeria. However, Olowe *et al.,* (2013) reported a higher prevalence of 19.2% MRSA from clinical isolates in Medical Microbiology Laboratory of University Teaching Hospital, Ado-Ekiti.

In the present study out of 40 isolates which were phenotypically MRSA only 12(30%) possessed the *mecA* gene while the rest were *mecA* negative and the question may be what

may be responsible for the resistance of the *mecA* negative isolates. Resistance in MRSA is mediated by PBP2a encoded by chromosomal *mecA*. PBP2a increases resistance to all β-lactam antibiotics including penicillin, cephalosporins, cephamycins and carbapenems by decreasing affinity for binding these antibiotics. In the prototype MRSA strains, the *mecA* gene is only expressed following induction by exposure to the drug. It is under the control of MecIR regulatory proteins which are homologous to the BlaIR proteins that regulate *BlaZ* expression (McKinney *et al.,* 2001; Peacock & Paterson 2015; Fisher & Mobashery 2016). The mechanism of resistance of these *mecA* negative strains may result from the production of modified PBPs 1 and 2 with reduced affinities for β lactamase, production of a new β lactamase, over production of PBP4 or increased β lactamase production (Khorvash *et al*., 2008). Finding of *mecA* gene is the major evidence for the detection of MRSA isolate. This statement was approved by many researchers all over the world e.g.: in Sudan (Maimona *et al*., 2014), Egypt (Hafez *et al.,* 2009), Saudi Arabia (Meshref & Omer, 2011), Japan (Hotta *et al*., 2000), India (Mehndiratta *et al*., 2009).

It is important to note that absolute dependence on *mecA* gene as the defining standard in determining the resistance of *S. aureus* to methicillin became the subject of distrust by many researchers. However, the findings in the project by Mogahid *et al., (*2015) suggested low burden of the *mecA* gene (90.2%); this may open the door to search for other intrinsic factors that may compete with *mecA* gene in producing resistance phenomenon in regions with high prevalence of MRSA. On the other hand, the absence of *mecA* gene within resistant staphylococcal isolates was listed worldwide (Hawraa *et al*., 2014). Additionally, moderate methicillin resistance was observed in isolates that lacked the *mecA* gene mutations. Also, a previous study in Nigeria reported the complete absence of five major

SCCmec types and *mecA* genes including the gene product of PBP2a in isolates which were phenotypically MRSA suggesting a probability of hyperproduction of β- lactamase as a cause of the phenomenon (Olayinka *et al*., 2009: Nwaogaraku *et al*., 2019). Moreover, recently Ba *et al.,* (2014) mentioned specific alterations in different amino acids present in protein binding proteins cascade (PBPs1, 2, and3) which may be the basis of resistance. These alterations were found to include three amino acid substitutions which were identical and were present in PBPs 1, 2, and 3. Moreover, the same amino acid was found to have two other different substitutions in PBP1. Both the identical and different amino acid substitutions were observed in isolates from different multilocus types (Ba *et al*., 2014). These findings provided clear evidence that there are mechanisms other than the presence of *mecA* gene responsible for β-lactam resistance of MRSA and that molecular method alone is not enough for confirmed characterization of MRSA isolates, a point that should be under consideration by regional and reference laboratories (AlSaadi *et. al*., 2020)

In addition, there was a report by van Griethysen *et al*., (2005) about the loss of *mecA* gene during storage of 36/250 (14.4%) confirmed MRSA strains at -80°C with the MicroBank system (Pro-Lab Diagnostics, Canada). Also, in a study on loss of the *mecA* gene during storage of methicillin-resistant *Staphylococcus aureus* isolates in North Western Nigeria by Kumurya (2013), it was reported that *mecA* gene was lost in 95.0% of 100 MRSA isolates after 2 years of storage at −80°C with the Micro bank system (Pro-lab Diagnostics, Austin, Texas).

Considering the time interval between the preliminary characterization, storing and sub-culturing over a considerable period of time before the final molecular characterization, and

the inconsistent power supply in this environment it is therefore not impossible that some *mecA* containing isolates might have lost the gene on prolonged storage.

There was a high level of the resistance among the isolates to the β- lactam antibiotics tested. This is not surprising and is consistent with the observation that clinical staphylococcal isolates are resistant to an increasing number of commonly prescribed antimicrobial agents and to β- lactams in particular (Olukoya *et al.,* 2005). It is now believed that greater than 80% of staphylococcal isolates produce penicillinase regardless of the clinical setting (Pantosti *et al*., 2007). In this study, phenotypic test for β- lactamase production showed that 64% produced β-latamase which is the enzyme that hydrolyses the amide bond of the β-lactam ring resulting in an inactive compound. *BlaZ* gene, the gene coding for β – lactamase was detected in 7/40 (17.5%) of the *S. aureus* isolates. Many of these β-lactamases are encoded by transposons, some of which may also carry resistance determinants to several other antibiotics including quaternary ammonium compounds, dyes (acriflavine and ethidium bromide) or heavy metals (lead, mercury and cadmium) (Pantosti *et al.,* 2007). Methicillin-resistant staphylococci are resistant to all other penicillins, carbapenems, cephems and β-lactam/β-lactamase inhibitor combinations (CLSI, 2006). It is therefore advisable that these antibiotics should be discouraged for treating of methicillin-resistant *Staphylococcus* infections. The antimicrobial susceptibility patterns showed that majority of the MRSA isolates were resistant to 4 classes of antibiotics including flouroquinolones, penicillin/β-lactamase inhibitor combinations, macrolide, and cephalosporins.

The necessity in phenotypic and genotypic tests for discovering resistance in clinically relevant staphylococci has become clearer with the occurrence of strains having borderline minimum inhibitory concentrations of antibiotics.

4.2.5 Prevalence of Panton valentine leukocidin (PVL) genes among the MRSA isolates

The Panton Valentine leukocidin genes code for the production of cytotoxin associated with furunculosis, severe necrotizing haemorrhagic pneumonia, necrotizing fasciitis and other lesions that involve the skin and mucosa (Yu *et al*., 2013) The gene encoding *pvl* is more often found in CA-MRSA strains carrying SCCmec-IV (Salgado & Farr, 2004) and less frequently in HA-MRSA. The reason being that it is believed that HA-MRSA have sacrificed their virulence for high level of resistance to β-lactams (Rudkin *et al*., 2012) Since the *pvl* gene is known to be associated with virulence, (Vandenesch *et al*., 2003) determining the presence of the *pvl* gene in MRSA strains might be important to early and proper therapy for serious MRSA infections (Salliot *et al.,* 2006).

The prevalence of *pvl* toxin genes in this study was 10% and is considered to be low and the isolates were from wound samples, HVS and urine samples. The prevalence of *pvl* toxin gene in this study is in line with work of Orji *et al* (2016) who reported a prevalence of 10.7% pvl gene among *S. aureus* of nosocomial origin in a local hospital in Nigeria. Similar result was also obtained by Sahar *et al* (2013), studies from Algeria and Tunisia reported higher *pvl* prevalence while investigations from South Africa and Zambia reported the lowest prevalence (Mulemba *et al*., 2017). *PVL* -positive MRSA is more frequently reported with SSTIs, and community-associated clones. This diversity of *pvl* gene carriage prevalence among various MRSA strains around the world might be explained by the strong association between *pvl* gene carriage with certain *mecA* gene subtype (Types IV & V) and

the distinct geographical distribution of *mecA* subtypes as proposed by various recent studies (David & Daum, 2010).

Inter strain variability in the in-vitro production of *pvl* has been reported (Hamilton *et al*., 2007). In-vitro, *pvl* production by *S. aureus* strains is highly variable, suggesting important differences in transcriptional and/or translational control of gene expression. Researching *pvl* and its relationship to diseases caused by *S. aureus* gives room for a better insight into the pathogenicity of the diseases as experimental data obtained have supported the implication of *pvl* in the severity of diseases such as skin infection, pneumonia, bone and joint infections (Badiou *et al*., 2010). Consequently, an understanding of the additional environmental and host factors that influence *pvl* gene expression in vivo may be needed. The occurrence of *pvl* toxin gene in pathogenic *S. aureus* portends serious clinical implications – since most of these organisms are known to be multidrug resistant in nature and could present a significant challenge in disease management and infection control in resource-limited countries such as Nigeria.

4.2.6 PCR-RAPD

The RAPD amplification was implemented on 12 MRSA strains using three randomly selected oligonucleotide primers sOLP6, sOLP11 and sOLP13. Molecular typing methods have been evaluated not only for their ability to discriminate among strains for epidemiologic purposes but also for their potential of having a taxonomic value (Mobasherizadeh *et al*., 2016). Previous studies have shown that *S. aureus* is a polymorphic species and has a clonal population structure (Feil *et al*., 2003). Several molecular methods have been used for epidemiological surveillance of MRSA isolates in order to track the distribution, infection source and transmission routes. These methods include multilocus

sequence typing (MLST) (Enright *et al*., 2000), pulsed-field gel electrophoresis (PFGE) and SCC mec typing. Most of these methods suffer from the disadvantages that they require high expertise, complicated laboratory settings and complex procedures to clearly differentiate the various MRSA. Among all these methods, PFGE has been shown to be a gold standard as an accurate and reliable method (Strandén *et al*. 2003). However, it is also very tedious and time- consuming, compared with ease and speed of performance of PCR-RAPD techniques (Sabat *et al.* 2006). In the present study we endeavored to find the molecular variation of MRSA isolates using a rather simple and cost-effective molecular typing method, namely, Random Amplified Polymorphic DNA (RAPD) analysis. Compared with other typing methods for *S. aureus* strains such as PFGE, this procedure generates greater polymorphism, is technically friendly and faster, and requires no radioactive materials (Reinoso *et al.,* 2004).

Our results indicated that RAPD fingerprinting can classify isolates of MRSA into clusters through which the relationship of strains can be evaluated. In our investigation the RAPD application in HA-MRSA genotype analysis resulted in 3 RAPD clusters and incidentally 3 groups also for the 12 MRSA isolates. Of 12 HA-MRSA isolates, 8 (66.7%) grouped into two clusters, i.e., group A and B, and 2 isolates (16.7%) classified into a small cluster termed group C, while two failed to be amplified. The RAPD group A and B that encompassed 80% of all amplified isolates prevailed. Group A comprised of isolates which originated from Umuahia while group B from Aba. The dendogram indicated that the strains isolated from the same hospital were closely related and this means that these strains might have evoived from one clone. Remarkably the small group C had an isolate from Isiukwuato and Umuahia showing band similarities. The reason for this situation could be that patients

admitted from other healthcare settings might have introduced these strains from other institutions in the other town. The dendogram showed that the highest percentage of similarity was 95% among the strains.

Similar observations have been reported in earlier studies carried out by Neela *et al*. (2005) who discovered that two major groups with three clusters each in one group as revealed by the dendogram generated from the RAPD analysis of different *S. aureus* strains showed that the strains isolated from the same hospital were all genetically related and mostly in the same cluster. Nikbakht *et al.* (2008) found that MRSA strains isolated from two different hospitals having similar RAPD pattern suggested about the route of MRSA transmission as being from cross infection between the hospitals. Although RAPD assay is a widely used method for genetic fingerprinting, there is no known specific primer for discrimination. The primers sometimes are insufficient to differentiate the genetic differences among related and unrelated strains (Neslihan & Isil, 2014). While considering the clones spreading in the state, it could be estimated that there were three main different clones. From this finding it can be inferred that group A and B are the major prevalent strains circulating in Abia State. The genetic diversity observed in the clusters may also suggest possible and frequent occurrence of mutants among the strains. Whether these strains have a particular feature that facilitates their colonization and adaptation capacity within the tested population is a question that remains to be answered by further studies including more advance molecular typing assay such as multilocus sequence typing (MLST).

4.2.7 Plant Antimicrobial and Phytochemistry

The results of the antibacterial activity of the ethanolic and hot water extracts of the leaves of *Alchornea cordifolia* and *Acalypha wilkesiana* on the methicillin resistant *Staphylococcus aureus* strains showed that the growth of the microorganisms used for the test was inhibited at extract concentrations of 50mg/ml to 200mg/ml. The inhibition zone ranged from 9.0mm to as high as 21.0mm as reported. The organisms were susceptible to the ethanol extract of *Alchornea cordifolia* at 200mg/ml concentration with zone sizes ranging between 15.0mm to 21.0mm while at a similar concentration for *Acalypha wilkesiana* for the organisms, a diameter zone of inhibition in the range of 15.0mm to 21.0mm was obtained. The ethanol extracts of both plants had the highest inhibitory effect on all the pathogens used for the study, while the aqueous extracts showed the least inhibitory effect. The ability of the extracts of the leaves of these plants to inhibit the growth of these pathogens confers medicinal value on them. The aqueous extract of *Acalypha wilkesiana* however didn't show any evidence of activity against the isolates at all the concentrations tested. The result obtained in this study is comparable to those recorded in similar studies by Ojiako (2014), Abalaka *et al.* (2012) and Vinoth *et al.,* (2012).

Antimicrobial activity of the ethanol extracts of these medicinal plants were observed to be concentration-dependent and the activity varied with concentration against the tested pathogens as shown and minimum inhibitory concentration (MIC) as shown on tables 4.15 and 4.16. The methanolic extract of *Acalypha wilkesiana* showed maximum zone of inhibition against one of the methicillin resistant *S. aureus* (21mm) at 200mg/ml. The diameter of zone of inhibition reduced to 15.0mm as the concentration dropped to 100mg/ml and 10.0mm at 50mg/ml. A similar trend was observed for the other test isolates on both

plants. The zone sizes recorded in this investigation were comparable to similar studies carried out by Patel *et al.,* (2014); Vinoth *et al.,* (2012).

A study by Gatsing *et al.* (2010) on the antibacterial activity, bioavailability and acute toxicity evaluation of *Alchornea cordifolia* leaf extract showed remarkable activity against the growth of *S. aureus* with zones ranging from 13mm to 26mm. In the same study an MIC value of 5mg/ml was obtained against the test organisms respectively. Also, the study of Adeshina *et al.* (2010), on phytochemical and antimicrobial studies of the ethyl acetate extract of *Alchornea cordifolia* against *Staphylococcus aureus* showed that the extract possesses broad spectrum of activity against the test Gram-negative and Gram-positive bacteria and the fungi/yeast with diameter of zones of inhibition ranging from 10.0 - 35.0 mm. The commercial antibiotic gentamycin was observed to be more effective in inhibiting the test organisms. Doughari *et al*. (2007) reported that the state in which an antimicrobial agent is administered affects the effectiveness of such agent, and that antibiotics being in a refined state may record higher antimicrobial activity than crude extracts. However, the antibacterial activities of the crude leaf extracts could be enhanced when purified just as standard antibiotics, leading to higher zones of inhibition on the test organisms and may serve as a substitute to the commercially available antibiotics to which bacteria are developing resistance. For *Alchornea cordifolia* aqueous extracts, maximum zone of inhibition (9.0mm) was observed against the isolates at concentrations of 200mg/ml, while at lower concentration, no noticeable activity was observed. This observation is in line with earlier studies of Ramanathan *et al*. (2013) and Onawunmi *et al.* (2006).

The minimum inhibitory concentration (MIC) of the *Alchornea cordifolia* ranged between 12.5 and 50mg/ml while *Acalypha wilkesiana* had minimum inhibitory concentrations in the

range of 25 to 100mg/ml. This low MIC values showed a strong antibacterial effect on the test organisms. It is note-worthy that MBC values obtained for the extracts against the pathogens were higher than MIC, showing that the extracts are bacteriostatic at lower concentrations and bactericidal at higher concentrations. This suggests that these plant extracts, when used traditionally as antimicrobials inhibit bacterial growth without necessarily killing the bacteria and since most of the traditional preparations lack specific concentrations, this may be the reason for the use of large quantities of the extracts by traditional medical practitioners for their patients' therapy (Akinyemi *et al.,* 2006).

The result of the rate of killing of the organism shows a gradual reduction in the total viable count of bacteria from 1hr to 24hrs in all the test isolates. There was an observable reduction in the number of organisms present at each hour but no cidal effect occurred except with the control gentamycin. This shows that the extracts are bacteriostatic at 50mg/ml concentration.

Phytochemical screening of the selected plant materials shows that the study plants contained alkaloids, glycosides, flavonoids, tannins, phenol and saponin but to varying degrees. These compounds have been shown to be active against potentially significant pathogens (Owolabi *et al*., 2007). Quantitatively, the percentage yields of phytochemical content of the leaves of the *Alchornea cordifolia* plants were as follows: alkaloids (1.85%), flavonoids (1.08%), Glycosides (1.05%), saponins (4.13%), and tannins (0.70%). There was a significant difference in the quantity of saponin over the other phytochemicals in the *Alchornea cordifolia* extract. However, no significant difference existed between the quantity of glycosides and flavonoids ($p>0.05$). The result shows that the leaves of these plants have appreciable amount of these phytochemicals, hence their medicinal value.

Acalypha wilkesiana, had the highest amount of alkaloids (2.64%) when compared with those of *Alchornea cordifolia* (1.85%). The saponins content of *Alchornea cordifolia* was however higher (4.13%), while that of *Acalypha wilkesiana* had the lowest amount of saponins (0.24%). *Alchornea cordifolia*, however had the highest tannin content (0.70%), against that of *Acalypha wilkesiana* (0.64%). The pharmaceutical and therapeutic potentials of plants and their products are as a result of the presence of these phytochemicals in them (Edeoga *et al*., 2005; Bishnu *et al*., 2009).

The findings of this study conform with earlier studies that have reported the presence of tannins, flavonoids, saponins and phenols from extracts of *Alchornea cordifolia* (Abere *et al.*, 2007; Edeoga *et al*., 2005) as well as Oladunmoye (2006) for *Acalypha wilkesiana*. In a study by Ameh *et al.* (2011), tannins, saponins and anthraquinone glycosides were detected in the aerial parts of *Alchornea cordifolia*.

According to Cushnie and Lamb (2005), both alkaloids and flavonoids have antimicrobial activities. Phytoconstituents such as saponins and phenolics compounds have also been reported to inhibit bacterial growth. These secondary metabolites exert antimicrobial activity through different mechanisms. Tannins form irreversible complexes with proline rich protein, resulting in the inhibition of cell protein synthesis and the flavonoids complex with extracellular- soluble proteins and with bacterial cell wall proteins while the lipophilic flavonoids exert antimicrobial activity by disrupting microbial cells membranes (Olowusulu & Ibrahim, 2006).

The susceptibility of the isolates to the study plant extracts implies that chemical compounds in the extracts can be further developed to fight against these resistant microorganisms (Uttu *et al*., 2015).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

There is an alarming level of multiple drug resistance shown by the MRSA isolates obtained in this study and this is a cause for great concern. Majority of the MRSA isolates showed resistance to more than 4 antibiotic classes, indicating the presence of strong selective pressure from antibiotics used in the study area. Therefore, it calls for a review of antibiotic regimen and the need to look for alternative treatment therapy to avoid treatment failure. Knowledge about the nature and number of MRSA clones that are disseminating is required to implement any strategies to control the transmission of MRSA, either within hospitals or the community. The knowledge about the evolution of MSRA could be increased through investigations of historic strain collections to find the molecular events that have led to the origin of MRSA clones, both in healthcare facilities and in the community.

Although clindamycin is an option for treatment of infections caused by MRSA, the iMLSB phenotype may limit the effectiveness of this drug. Therefore, it is essential that the D-test is carried out in our clinical laboratories to minimize medication errors and ineffective treatments. On the whole, the inducible clindamycin resistant isolates obtained in our study was 17.7%. If D-test was not performed, those isolates would have been classified as being clindamycin susceptible thereby leading to likely therapeutic failure.

RAPD-PCR can be successfully utilized to find out the distribution and epidemiological relationship of MRSA, and also monitor the inter or intra- spread of MRSA strains. It could also be implemented in controlling the sources and routes of transmission, tracking the

spread of strains within hospital, and between the hospitals, and especially preventing the nosocomial infections caused by the MRSA, provided that the technique is implemented under careful reproducibility condition and in particular duplication of PCR runs to obtain valid results.

5.2 Recommendation

This study has further established that *S. aureus* is a major causative agent for nosocomial infections; therefore, it is recommended that proper infection control measure be put in place in our hospitals from primary to tertiary institutions. There is also need to carry out routine D-Test when clindamycin is to be used in therapy to forestall treatment failure.

Now that *mecA* gene mediated MRSA has been detected in this part of the country, there is need for the various health institutions and the government to develop means of combating its spread either in the hospital or within the community before it becomes a major health problem in Nigeria. Part of the measures to be taken should include:

1. Hospital workers are to be screened regularly for MRSA.

3. Adjust antibiotics based on results of culture and sensitivity testing.

4. Monitor response to therapy.

5. Patient education: Provide education on drug uses and abuses.

There is the need for our research institutes to be properly funded and the provision of standard laboratories made available in different parts of the country for detailed molecular study on typing and classification of MRSA for epidemiological control.

5.3 Contributions to Knowledge

1. This study has described the antibiotic resistance profile of *S. aureus* strains obtained from clinical samples within Abia State of Nigeria. This information will be helpful in establishing effective infection control measures in health care settings in Nigeria.
2. Information on antibiotic susceptibility pattern could be used as genetic markers to investigate outbreaks of *S. aureus* infections and also means of educating the populace on dangers of drug abuse
3. This is the first time of detecting Methicillin resistant *S. aureus* (MRSA) molecularly from clinical samples in the state, to the best of our knowledge.
4. This study provides an overview of the genotypes of *S. aureus* clones circulating in health-care settings in Abia State of Nigeria.
5. The data provided on MRSA strains can serve as a baseline for future MRSA surveillance and to study the evolution of MRSA clonal types.
6. The data can also be used in designing treatment options for MRSA in the different zones and also the need to inculcate the different treatment options for other places as well.

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Appendix 1

Mcfarland Standard

1% v/v solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of distilled water. 1% w/ v solution of barium chloride was prepared by distilling 0.5g of dehydrated barium chloride in 50ml of distilled water.0.05ml of barium chloride and 9.95ml of sulphuric acid was measured to make approximately a cell density of 1.5x108 cfu/ml (Cheesbrough, 2002)

Appendix 2

Overall age related prevalence

Age group	Total Isolated	Umuahia	Aba	Isikwuato	P - Value
<11	13	9(8.0)	4(3.4)	0	0.52
11-20	28	10(8.9)	16(13.4)	2 (5.9)	
21-30	59	19(17)	26(21.8)	14(41.2)	
31-40	58	25(22.3)	29(24.4)	4 (11.8)	
41-50	56	23(20.5)	25(21)	8 (23.5)	
51-60	28	14(12.5)	11(9.2)	3 (8.8)	
>60	23	12(10.7)	8(6.7)	3 (8.8)	
	265	112(100)	119(100)	34 (100)	

Umuahia Age related

Age group	Total Isolated	Age Distribution	P - Value
<11	112	9(8.0)	0.04
11-20	112	10(8.9)	
21-30	112	19(17)	
31-40	112	25(22.3)	
41-50	112	23(20.5)	
51-60	112	14(12.5)	
>60	112	12(10.7)	

Aba Age related

Age group	Total Isolated	Age Distribution	P - Value
<11	119	4(3.4)	0.00
11-20	119	16(13.4)	
21-30	119	26(21.8)	
31-40	119	29(24.4)	
41-50	119	25(21)	
51-60	119	11(9.2)	
>60	119	8(6.7)	

Isikwuato Age related

Age group	Total Isolated	Age Distribution	P - Value
<11	34	0	0.001
11-20	34	2 (5.9)	
21-30	34	14(41.2)	
31-40	34	4 (11.8)	
41-50	34	8 (23.5)	
51-60	34	3 (8.8)	
>60	34	3 (8.8)	

Overall Gender related prevalence

Gender	Total Isolated	Umuahia	Aba	Isikwuato	P - Value
Male	126	52(46.4)	58(48.7)	16(47.1)	0.99
Female	139	60(53.6)	61(51.3)	18(52.9)	
Total	265	112(100)	119(100)	34 (100)	

Umuahia Gender Related

Gender	Total Isolated	Gender Isolate	P - Value
Male	112	52(46.4)	0.54
Female	112	60(53.6)	

Aba Gender related

Gender	Total Isolated	Gender Isolate	P - Value
Male	119	58(48.7)	0.82
Female	119	61(51.3)	

Isikwuato Gender Related

Gender	Total Isolated	Gender Isolate	P - Value
Male	34	16(47.1)	0.78
Female	34	18(52.9)	

Appendix 3

INTERPRETATIVE CHART FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antibiotics	Disc potency	Diameter of zone of growth inhibition (mm)		
		Susceptibility	Intermediate	Resistance
Vancomycin	30 µg	.≥ 2	-	≤ 2
Ciprofloxacin	10 µg	≥ 21	16-20	≤15
Azithromycin	15 µg	≥ 18	14-17	≤13
Gentamicin	30 µg	≥ 15	13.14	≤12
Erythromycin	15 µg	≥23	14-22	`≤ 13
levofloxacine	5 µg	≥19	16-18	≤15
Ceftriaxone	30 µg	≥21	14-20	<13
Augmentin	30 µg	≥20	--	≤19
Clindamycin	2 µg	≥21	15-20	≤14
Cefoxitin	30 µg	≥22	-	≤21
Qxacillin	1 µg	≥13	10-12	≤10

Clinical and Laboratory Standards Institute (2017). Performance standards for antimicrobial susceptibility testing: 22nd informational supplement. M100 - S22, Wayne, PA

SEQUENCES OBTAINED	QUERY LENGTH	% SIMILARITY	GeneBank No.	BLAST ID
AGCGGGAGGCTWAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCT AACACTTAGCACTTCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT TTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGC CTTCGCCACTGGTGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGG AATTCCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCC ACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCG CTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGG CTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGATGT GCACAGTTACTTACACATATGTTCTTCCCTAATAACAGAGTTTTACGATCCG AAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGC GGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTT CCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCCTTGGTA AGCCGTTACCTTACCAACTAGCTAATGCAGCGCGGATCCATCTATAAGTGA CGCAAGACCGTCTTTCACTTTTGAACCATGCGGTTCAAAATATTATCCGGT ATTAGCTCCGGTTTCCCGAAGTTATCCCAGTCTTATRGGTRGGTTATCCACG TGTTACTCACCCGTCCGCCGCTAACATCAGAGAAGCAAGCTTCTCGTCCGT TCGCTCGACTTGCATGTATKAGGCACGCCGCCAGCGTTCATCCCTGAGCCR TGATTCAAACTA	891	98	KY007579	*Staphylococcus aureus*

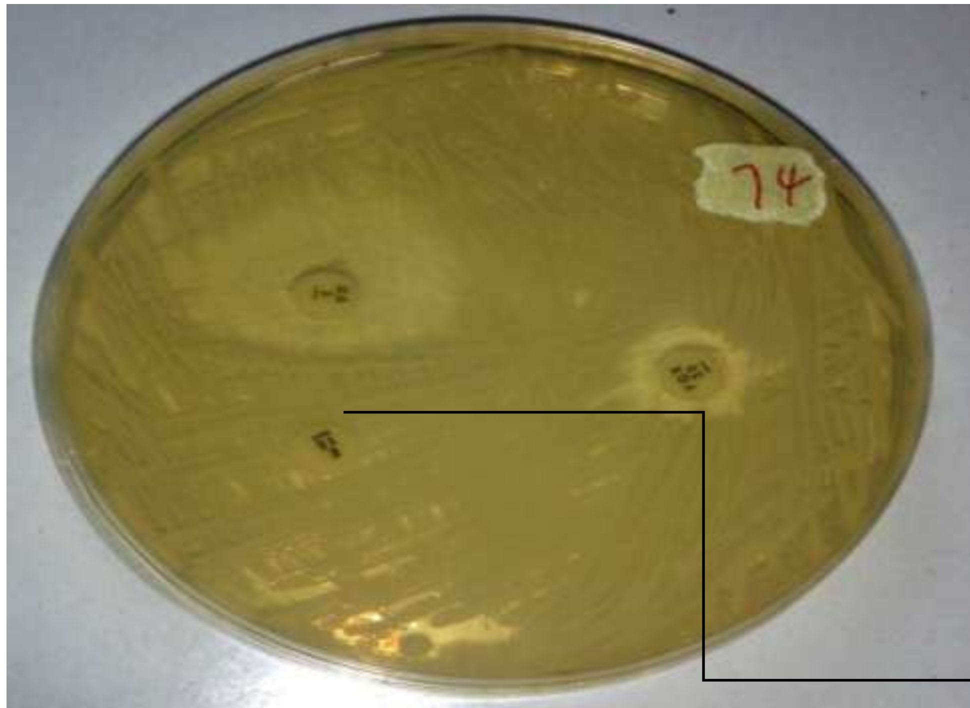
SEQUENCES OBTAINED	QUERY LENGTH	% SIMILARITY	GeneBank No.	BLAST ID
AAAATGGGGGTTAAKCTTGCAGCACTAAGGGGGGAAACCCCCTAACACTT AGCCTTATCGTTTACGGCGTGGACTACCAGGGKATCTAATCCTGTTTGATCC CCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCC ACTGGGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATTCCAC TTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGA GCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTTACGC CCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGTGCTGGCA CGTAGTTAGCCGKGGCTTTCTGATTAGTACCGTCAAGACGGCATAGTTACT TACACGTATGTTCTTCCCTAA	439	97	MH429612	*Staphylococcus aureus*

SEQUENCES OBTAINED	QUERY LENGTH	% SIMILARITY	GeneBank No.	BLAST ID
TCGATTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAAC ACTTAGCACTATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTG ATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTC GCCACTGGTGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATT CCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGG TTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTT ACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGC TGGCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGATGTGCAC AGTTACTTACACATATGTTCTTCCCTAATAACAGAGTTTTACGATCCGAGAC CTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAG ATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTG TGGCCGATCACCCTCTCAGGTCGGCTATGATCTTTGCCTTGGKAAGCCGTT ACCTTTACCAACTAGCTAATGCAGCGCGGATCCATCTATAAGTGACGAAGA CCCGTCTTTCACTTTTGAACCATGCGGTTCAAAATATATCCCGGATTAGCTC CGGTTTCCCCAAAT	752	98	HM559234	*Staphylococcus aureus* strain DMH 4

SEQUENCES OBTAINED	QUERY LENGTH	% SIMILARITY	GeneBank No.	BLAST ID
TCGATCCTTTTGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACAC TTAGCATATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATC CCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGC CACTGGTGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATTCC ACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTT GAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTTAC GCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTG GCACGTAGTTAGCCGTGGCTTTCTGATTAGGTACCGTCAAGATGTGCACAG TTACTTACACATATGTTCTTCCCTAATAACAGAGTTTTACGATCCGAAGACC TTCATCACTCACGCGGCGTTGCTCCGTCRGGCTTTCGCCCATTGCGGAAGAT TCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCMAGTGT GGCCGATCACCCTCTTCAGGTCGGCTATGCATCTTGCCTTGGTAAGCCGTTA CCTTTACCAACTAGCTAATGCAGCGCGGATCCATCTATAAGTGACAGCAGA CCGTCTTTCACTTTTGAACCATGCGGWTCAAAATATTATCCGGTATTAGCT CCGGGTTTCCCGAGTTTATCCCAGTCTTATAGGTAGGCTATCCACGTGTTAC TCACCCTCGCCGCTAACATCAGAGAAGCAGCTTCTCCCGTTGCTCGACTTG CATGATAGGCACGCCGCCAGCTTCATCCTTGAGCCATGACACTCAA	899	97	CP032481	*Staphylococcus aureus* strain O326

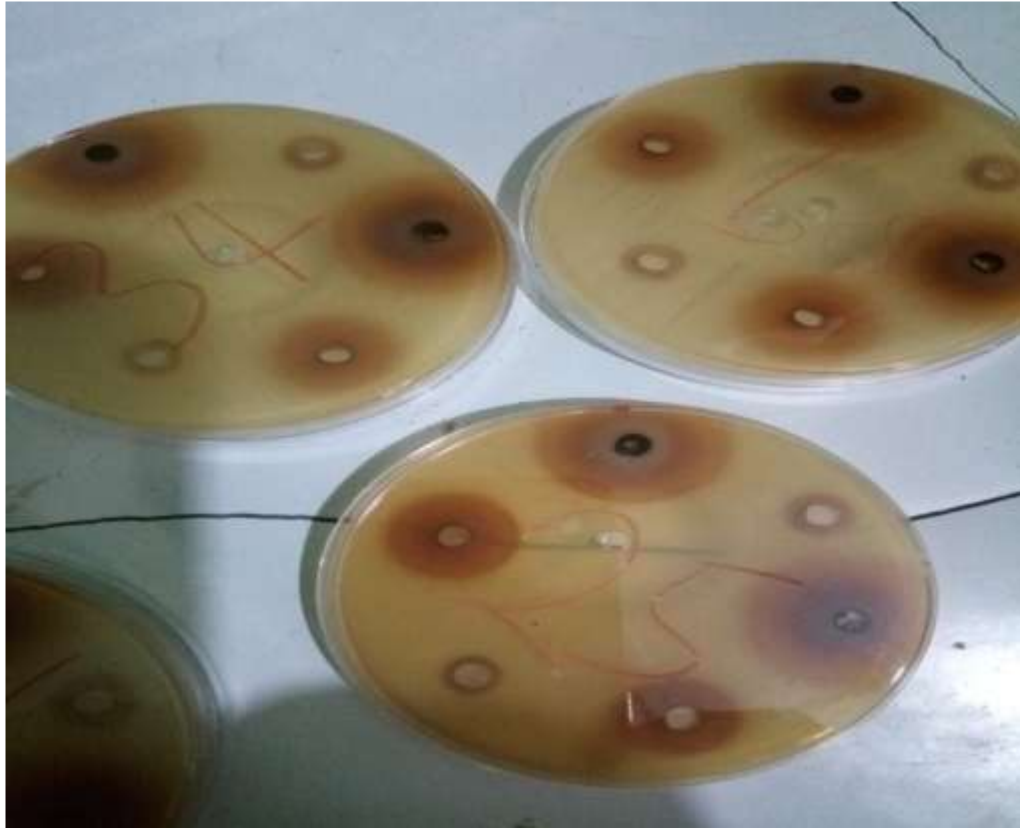
SEQUENCES OBTAINED	QUERY LENGTH	% SIMILARITY	GeneBank No.	BLAST ID
TATTGGCGTTTAACTTCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGC ACTTCGTTTACGGCGGGACTACCAGGGTATCTAATCCTGTTTGATCCCCAC GCTTTCGCACATCACGWCAGTTACGGACCAGAAAGTCGCCTTCGCCACTGG TGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATTCCACTTTC CTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCG TGGGCTTTCACATCAACTTAAAAAACCGCCTACGCGCGCTTTACGCCCAAT AATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTA GTTACCGWGGYTTTCTGATTAGTACC	359	96	FJ957637	*Staphylococcus sp*

Plate of *Staphylococcus aureus* on Mannitol salt Agar



D- Test positive plate

Plates showing an isolate resistant to multiple drugs



Plates showing antibacterial activity of the plant extracts against Methicillin Resistant *Staphylococcus aureus*

Plots Report

Test type: Nucleic Acid

29-Sep-18 4:40 PM

Exit

Report Name

Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1	Default	29-Sep-18	3:59 PM	54.95	1.099	0.570	1.93	0.52	50.00	230	2.126	0.035
2	Default	29-Sep-18	3:59 PM	55.37	1.107	0.548	2.02	0.46	50.00	230	2.382	0.074
3	Default	29-Sep-18	4:00 PM	49.67	0.993	0.615	1.61	0.45	50.00	230	2.203	0.171
4	Default	29-Sep-18	4:00 PM	36.70	0.734	0.368	2.00	0.36	50.00	230	2.028	0.064
5	Default	29-Sep-18	4:00 PM	51.34	1.027	0.693	1.48	0.53	50.00	230	1.919	0.070
6	Default	29-Sep-18	4:01 PM	145.82	2.916	1.640	1.78	0.94	50.00	230	3.113	0.112
7	Default	29-Sep-18	4:01 PM	16.40	0.328	0.118	2.77	1.86	50.00	230	0.177	1.850
8	Default	29-Sep-18	4:02 PM	125.20	2.504	1.280	1.96	1.58	50.00	230	1.588	0.060
9	Default	29-Sep-18	4:02 PM	79.75	1.595	0.806	1.98	1.05	50.00	230	1.521	-0.136
10	Default	29-Sep-18	4:02 PM	67.91	1.358	0.727	1.87	1.32	50.00	230	1.031	0.073
1	Default	29-Sep-18	4:03 PM	68.98	1.380	0.737	1.87	1.29	50.00	230	1.073	0.053
12	Default	29-Sep-18	4:04 PM	47.61	0.952	0.594	1.60	1.22	50.00	230	0.783	0.092
13	Default	29-Sep-18	4:04 PM	50.16	1.003	0.499	2.01	1.23	50.00	230	0.815	0.129
14	Default	29-Sep-18	4:04 PM	42.84	0.857	0.488	1.76	1.19	50.00	230	0.721	0.097
15	Default	29-Sep-18	4:05 PM	53.02	1.060	0.562	1.89	1.31	50.00	230	0.810	0.053
16	Default	29-Sep-18	4:05 PM	74.64	1.493	0.801	1.86	1.27	50.00	230	1.173	0.079
17	Default	29-Sep-18	4:06 PM	66.63	1.333	0.732	1.82	1.30	50.00	230	1.024	0.203
18	Default	29-Sep-18	4:06 PM	58.31	1.166	0.652	1.79	1.29	50.00	230	0.903	0.098
19	Default	29-Sep-18	4:06 PM	273.83	5.477	2.647	2.07	2.04	50.00	230	2.689	0.179
20	Default	29-Sep-18	4:07 PM	50.21	1.004	0.548	1.83	1.38	50.00	230	0.727	0.064
21	Default	29-Sep-18	4:07 PM	44.27	0.885	0.432	2.05	1.42	50.00	230	0.623	0.026
22	Default	29-Sep-18	4:08 PM	66.33	1.327	0.792	1.67	1.24	50.00	230	1.073	0.233
23	Default	29-Sep-18	4:08 PM	43.29	0.866	0.526	1.64	1.19	50.00	230	0.726	0.063
24	Default	29-Sep-18	4:08 PM	62.58	1.252	0.666	1.88	1.28	50.00	230	0.974	0.095
25	Default	29-Sep-18	4:09 PM	32.16	0.643	0.361	1.78	0.77	50.00	230	0.835	0.125

Fig ..: Nanodrop results

Plots | Report

Test type: Nucleic Acid

29-Sep-18 4:40 PM

Exit

Report Name

Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
19	Default	29-Sep-18	4:06 PM	273.83	5.477	2.647	2.07	2.04	50.00	230	2.689	0.179
20	Default	29-Sep-18	4:07 PM	50.21	1.004	0.548	1.83	1.38	50.00	230	0.727	0.064
21	Default	29-Sep-18	4:07 PM	44.27	0.885	0.432	2.05	1.42	50.00	230	0.623	0.026
22	Default	29-Sep-18	4:08 PM	66.33	1.327	0.792	1.67	1.24	50.00	230	1.073	0.233
23	Default	29-Sep-18	4:08 PM	43.29	0.866	0.526	1.64	1.19	50.00	230	0.726	0.063
24	Default	29-Sep-18	4:08 PM	62.58	1.252	0.666	1.88	1.28	50.00	230	0.974	0.095
25	Default	29-Sep-18	4:09 PM	32.16	0.643	0.361	1.78	0.77	50.00	230	0.835	0.125
26	Default	29-Sep-18	4:10 PM	180.31	3.606	1.937	1.86	1.23	50.00	230	2.924	-0.231
27	Default	29-Sep-18	4:10 PM	46.03	0.921	0.659	1.40	0.83	50.00	230	1.105	0.072
28	Default	29-Sep-18	4:11 PM	16.36	0.327	0.238	1.38	0.70	50.00	230	0.468	0.014
29	Default	29-Sep-18	4:11 PM	38.85	0.777	0.381	2.04	1.14	50.00	230	0.684	-0.061
30	Default	29-Sep-18	4:12 PM	50.90	1.018	0.577	1.77	1.10	50.00	230	0.923	0.022
31	Default	29-Sep-18	4:12 PM	30.02	0.600	0.431	1.39	0.98	50.00	230	0.610	0.030
32	Default	29-Sep-18	4:12 PM	140.25	2.805	1.339	2.10	1.60	50.00	230	1.755	0.089
33	Default	29-Sep-18	4:13 PM	270.88	5.418	2.758	1.96	1.60	50.00	230	3.389	-0.050
34	Default	29-Sep-18	4:13 PM	86.91	1.738	1.028	1.69	1.26	50.00	230	1.376	0.090
35	Default	29-Sep-18	4:14 PM	47.15	0.943	0.559	1.69	1.09	50.00	230	0.863	0.045
36	Default	29-Sep-18	4:14 PM	327.07	6.541	3.224	2.03	1.52	50.00	230	4.309	0.317
37	Default	29-Sep-18	4:14 PM	103.07	2.061	1.141	1.81	1.27	50.00	230	1.623	0.129
38	Default	29-Sep-18	4:15 PM	195.08	3.902	1.991	1.96	1.42	50.00	230	2.740	0.227
39	Default	29-Sep-18	4:15 PM	50.94	1.019	0.612	1.66	1.15	50.00	230	0.887	0.137
40	Default	29-Sep-18	4:16 PM	24.47	0.489	0.312	1.57	0.89	50.00	230	0.548	0.032
WT	Default	29-Sep-18	4:16 PM	2.66	0.053	0.025	2.09	0.24	50.00	230	0.220	-0.006
WT	Default	29-Sep-18	4:17 PM	2.81	0.056	0.047	1.20	0.34	50.00	230	0.165	0.032
WT	Default	29-Sep-18	4:17 PM	2.73	0.055	0.052	1.04	0.26	50.00	230	0.214	0.003

Nanodrop results

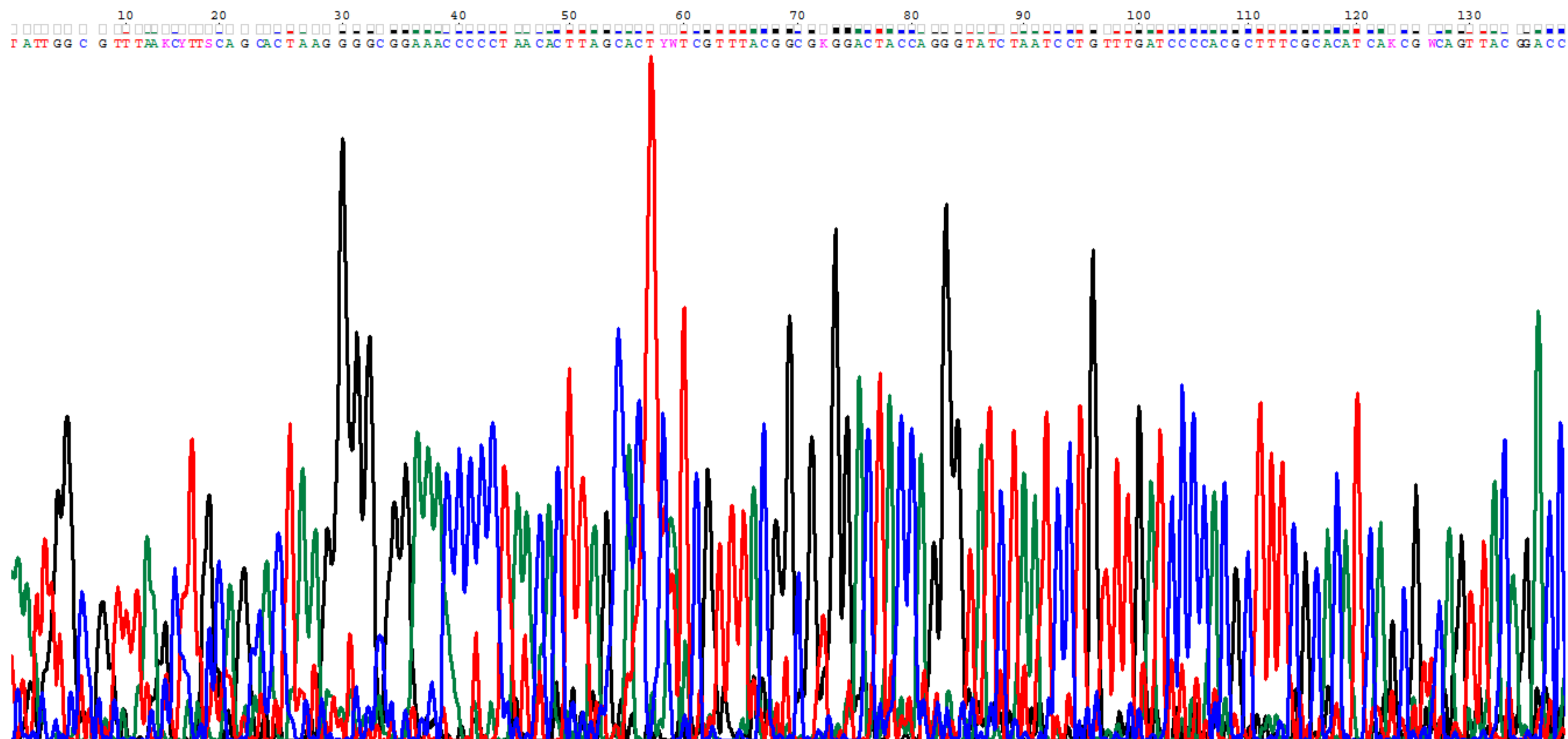


Fig..: Chromatogram of the sequences of *Staphylococcus*sp with Gene bank Number FJ957637

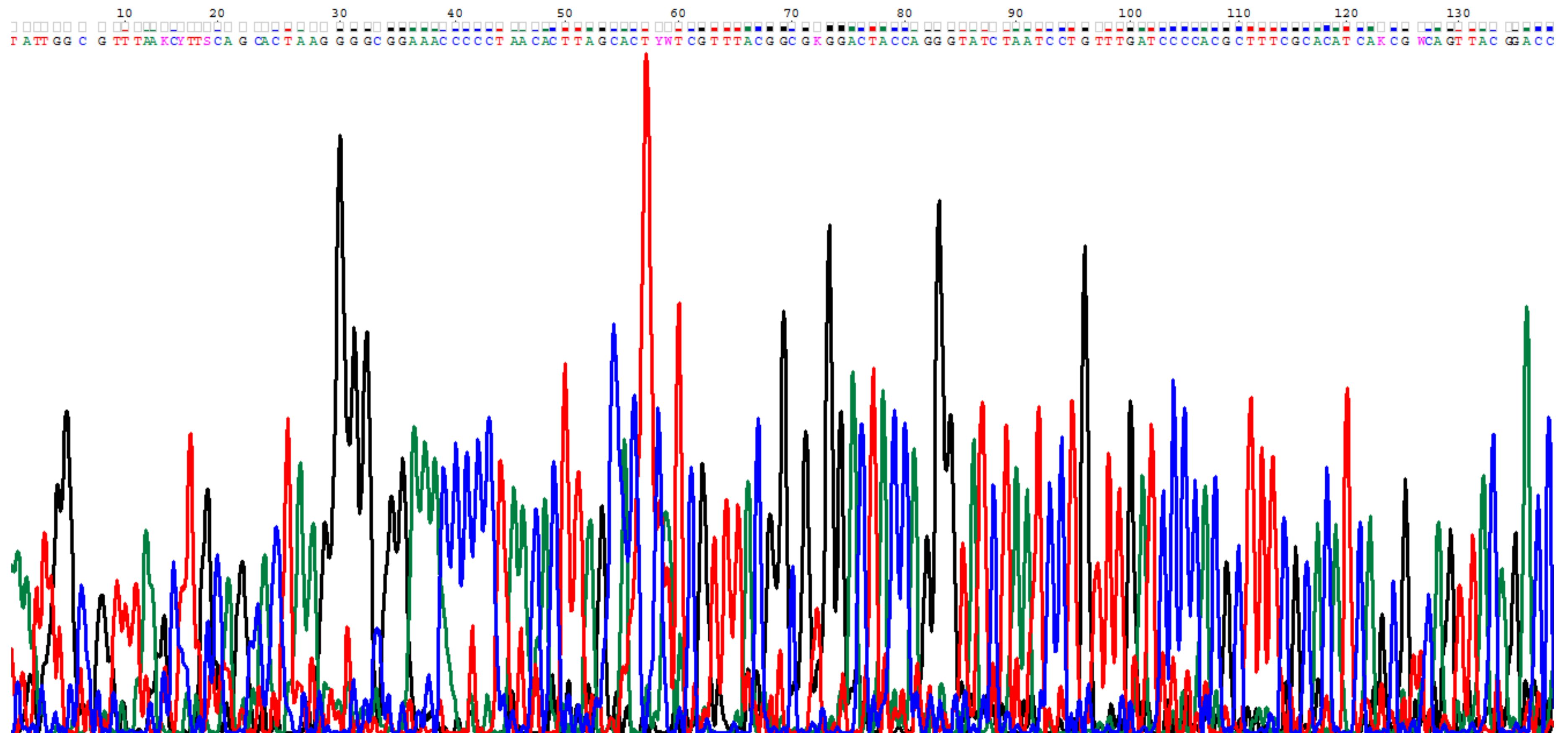


Fig..: Chromatogram of the sequences of *Staphylococcus aureus* with Gene bank Number MH429612