ISOLATION AND MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* FROM POULTRY AND POULTRY FARM WORKERS IN KANO, NIGERIA

BY

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Ph. D/ PHARM-SCI/1454/2011-2012

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FACULTY OF PHARMACEUTICAL SCIENCES
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

JUNE, 2016

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGTRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA NIGERIA
IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE AWARD OF (Ph.D) IN PHARMACEUTICAL MICROBIOLOGY

DEPARTMENT OF PHARMACEUTICS AND PHARMACEUTICAL MICROBIOLOGY,
FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

JUNE, 2016

DECLARATION

I hereby declare that the work reported in this thesis was carried out by me under the supervision of Prof. J. A. Onaolapo, Dr B. O. Olayinka, both of the Department of Pharmaceutics and Pharmaceutical Microbiology, and Prof. S.O. Olonitola of the Department of Microbiology, Ahmadu Bello University Zaria, Nigeria. This work is original and has not been submitted elsewhere for a higher degree. The work of other investigators have been properly acknowledged and referred to accordingly.

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_____

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CERTIFICATION

This thesis entitled "Isolation and molecular characterization of methicillin resistant *Staphylococcus aureus* from poultry and poultry farm workers in Kano, Nigeria by Kawu Hauwa Bala meets the regulations governing the award of the degree of Doctor of philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to the entire Muslim Umma. To my Sweetheart and loving children for their love, patience, encouragement and support through this pursuit.

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to my supervisors, Prof. J. A. Onaolapo, Dr. B. O. Olayinka and Prof. O. S. Olonitola for their useful suggestions, guidance and contributions during the course of this work and in the preparation of this thesis.

My sincere thanks to my special colleague Igwe James for his support and encouragement throughout the period of this work. I appreciate my sister and research assistant Rukayya Kawu Bala for her contributions, encouragement and assistance throughout the course of this study.

My gratitude goes to the entire staff of Department of Microbiology, Bayero University Kano, for their assistance, encouragement and provision of a good working environment in the laboratory for my work. I am grateful to you all. I am greatly indebted to all staff of the Department of Pharmaceutics and Pharmaceutical Microbiology Ahmadu BelloUniversity, Zaria for all their assistance and training.

Special thanks to my lovely parents who sacrificed their today for my tomorrow. I owe my husband and children lots of thanks for their love, encouragement and understanding during the course of my study. Thank you all for your support. I love you always.

ABSTRACT

The increasing rate of drug resistance associated with methicillin resistant *Staphylococcus aureus* is not only a problem in the clinical sector but also in livestock disease treatment and management. Methicillin resistant *Staph. aureus* is now a leading cause of staphylococcal infections in human and animals. In view of the present serious problem of resistance to antibiotics from *Staph. aureus*, the present study was undertaken to investigate the incidence of methicillin resistance in *Staph. aureus.* The study was carried out in Kano State, Nigeria to evaluate the incidence of methicillin resistant *Staph aureus* from poultry and poultry farm workers. Cloacae and nostril of 1200 poultry birds selected randomly in 12 farms from the three senatorial zones of Kano State and 60 nostrils of poultry farm workers were screened for the presence of *Staph aureus* using standard microbiological techniques. Antibiotic susceptibility pattern was determined using disc agar diffusion (DAD) method. Vancomycin resistance was determined using vancomycin agar screening method. Molecular studies of 16SrRNA, nuc,mecA and PVL gene were carried out using multiplex PCR, the PCR may permit sufficient sensitivity and specificity for the direct detection of *Staph. aureus.*Twenty two isolates were tested for Panton Valentine Leukocidin (PVL) using PCR. Ninety eight isolates (8.2%) were confirmed and characterized as *Staph aureus*, sixty six of the isolates were from broiler and 32 from layers. Cloacae yielded high number of *Staph aureus* than nostril. The result of antibiotic susceptibility test showed general resistance to β-lactam antibiotics; ampicillin and oxacillin and oxytetracycline at 71.4% each, chloramphenicol (61.2%) and sulfamethaxazole/trimethroprim (51 %). However higher percentage of sensitivity was recorded against vancomycin (74.5 %), Augmentin^{R} and cefoxitin (69.4 %), ciprofloxacin (64.3 %), gentamicin 60.2% and neomycin 54.1%. Thirty percent (30.6 %) of the *Staph aureus* isolates were phenotypically identified as methicillin resistant using cefoxitin 30 µg., and one from poultry farm workers. Determination of multiple antibiotics resistance index showed that 80 (81.6 %) were resistant to 3 or more antibiotics MARI $\geq$ 0.3 and 5.1 % had MARI $<$0.3. Eighty three point three percent of the MRSA (83.3 %) were multidrug resistant. Eighty nine point eight percent (83.3 %) of the total staphylococcal (89.8 %) isolates produced β-lactamase and 19/30(63.3%) phenotypic MRSA were β-lactamase hyper producers. Vancomycin resistance determined using vancomycin screening agar showed that 90% of the MRSA were vancomycin resistant (VRSA). The molecular analysis

of the isolate using PCR showed that all the isolates were *Staph aureus* of 800bp. PCR showed a correlation between phenotype and recovery of MRSA and genotypic detection of mecA gene. The prevalence of mecA mediated methicillin resistance in *Staph aureus* is high as 40.7% of the total MRSA isolate carried mecA gene. The amplified product of *Staph aureus* mecA gene showed a correlation between the staphylococcal penicillin binding protein (PBP2a) .Sixteen (53.3%) were PBP2a positive. Only one isolate from farm worker had mecA gene. Twenty two isolates were tested for Panton Valentine Leukocidin (PVL) but only 14 (63.36%) were positive. Analysis showed that PVL was not associated with truly community acquired as all PVL positive isolates in this study were from poultry. Further analysis showed that 3 of the seven housekeeping genes (*pta,gmk,and yqil*) were present. 35 % expressed Spa typing at variable regions. Multilocus sequence typing of 8 isolates selected showed that the main sequence type (ST398) of livestock associated methicillin resistant *Staph. aureus* (LA-MRSA) was not present in the poultry used in this study. The multidrug resistant nature of most of the isolates, and the high resistance level to especially β-lactam antibiotics is a sign of misuse and overuse of the agents in the environment (poultry farms). These call for rapid and accurate detection of multi drug resistant methicillin resistant *Staph. aureus*,and drawing up of guidelines for the prompt, effective and appropriate use of antibiotic therapy and for control of CA-MRSA and LA-MRSA.

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CHAPTER ONE

1.0 INTRODUCTION

Staphylococcus aureus, a Gram positive coccus is a frequent cause of skin infections, such as boils and pimples. Since the 1970s the usual treatment for these infections has been penicillins and penicillinase resistant antibiotics such as methicillin. Today however, this treatment is likely to fail. In 2003, well over 60% of the *Staph.aureus* strains isolated in hospitals were resistant to this antibiotic (Eugene *et al*., 2009). Methicillin resistance in this bacterial species represents a threat to human health. Originally, methicillin resistant *Staph.aureus* (MRSA) was a nosocomial pathogen, but in the 1990s, MRSA spread into communities worldwide (Lee, 2003). About a third of healthy individuals carry *Staph. aureus* on their skin and nose (Grundmann *et al*., 2010). It is also a common cause of wound and urinary tract infections (Sina *et al*., 2011).

Carriage of *Staph.aureus* Sequence Type (ST) 398 has primarily been reported as occurring among persons in contact with livestock, including swine and cattle (Wulf *et al.*, 2008; Smith *et al*., 2009).This association has given rise to the characterization of this strain as live stock associated (Wulf and Voss, 2008).

Pigs have been shown to be major reservoir of methicillin resistant *Staph. aureus* multilocus sequence type 398 (Leonard and Markey, 2008). It has also shown potential for zoonotic transmission (Huijsdens *et al*., 2006). This clonal complex associated with disease in livestock has also been implicated in human infection (Witte *et al*., 2007). It is known to cause diseases in poultry, feed and companions animals (Person *et al*., 2009; Hunter *et al*., 2011).

Methicillin resistant *Staph.aureus* (MRSA) is becoming increasingly recognized among persons in the community without established risk factors (Kluytman *et al*., 2006;

Faria *et al*., 2005). MRSA in animal disease have not, until now been considered a source of infection to humans,although transmission appears to be primarily between animals, undistinguished isolates have been found in their human contacts, particularly those with occupational exposure (Armand *et al*., 2005; Khanna *et al*., 2008). MRSA CC398 was first detected in 4 pigs and one healthy pig farmer in France (Aubury *et al*., 2004). Clinical infection was described in the daughter of a pig farmer in the Netherlands in 2004 (Voss *et al*., 2005). That study also showed that 23% of pig farmers in a small survey in the same region were seropositive for MRSA CC 398.

Staph.aureus is perhaps the most notorious of all the bacterial pathogens associated with human infection. In 1942 the year penicillin G was introduced, some resistant strains of *Staph. aureus* were found (Kunin, 2000). *Staph. aureus* is the first bug to battle penicillin in 1967 due to its ability to produce β-lactamase. Resistant semisynthetic penicillins in the early 1960s provided temporary respite which ended with the emergence of methicillin resistant *Staph. aureus,* discovered shortly after methicillin became available for clinical use (Miall *et al*., 2001; Fluit *et al*., 2001).

MRSA is of concern not only because of its resistance to methicillin but also because it is generally resistant to many other chemotherapeutic agents (Vidhani *et al*., 2003). Multidrug resistant *Staph.aureus* evolved following acquisition of discrete preformed antimicrobial resistance genes by horizontal gene transfer and resistance determinants generated by chromosomal mutation which poses great challenges in treatment of staphylococcal infections (Jesen and Lyon, 2009).

Methicicillin resistant *Staph.aureus* (MRSA) strain carries a large heterologous mobile genetic element, staphylococcal cassette chromosome (SCC) which includes the central element of methicillin resistance, the mecA gene (Ito *et al*., 1999). Until now, six

SCCmec type (I-VI) have been identified in *Staph. aureus* which are defined by combination of the mec gene complex class with the ccr allotype (Ito *et al*., 2001; Oliveira *et al*., 2006). SCC mec typing has been established as an important addendum to the characterization and identification of MRSA clones and is routinely used in many laboratories. Several strategies have been developed for SCC mec type (Okuma *et al*., 2002) and their broad application has led to the detection of several variants or subtype of the major SCC type (Olivera and de Lancastre 2002; Ito *et al*., 2003). Community acquired methicillin resistant *Staph. aureus* (CA-MRSA) has a characteristic staphylococcal cassette chromosome type IV (SCCmec IV) gene, lacking in non β–lactam determinant and possessing distinct necrotizing toxin, Panton valentine leukocidin (PVL).

Methicillin resistant *Staph.aureus* (MRSA) isolates in livestock have gained particular attention during recent years (Wulf and Voss, 2008). The identification of livestock associated MRSA in food producing animals has raised questions regarding the presence of MRSA in food of animal origin. Several studies were conducted in different parts of the world to screen food of animal origin intended for human consumption for the presence of MRSA and also to identify the MRSA types present (de Boer *et al*., 2009). MRSA are now regarded as a major cause of hospital and community acquired infection worldwide and the problem is exacerbated by the emergence of multidrug resistant MRSA (MDR-MRSA). Such isolates demonstrated a reduced susceptibility to almost all clinically available antibiotics and are often only susceptible to glycopeptides and investigational drugs (Lopez *et al*., 2005).

1.1 STATEMENT OF RESEARCH PROBLEMS

Methicillin resistant *Staph.aureus* is a major cause of hospital and community infections that are becoming increasingly difficult to combat because methicillin resistant *Staph. aureus* resist almost all currently available antibiotics (Blum, 1995). Community acquired MRSA is the major cause of skin and soft tissue infections (SSTIs) that has become increasingly problematic due to its high virulence and the ease with which they spread in the community (Reyes *et al*., 2011). The presence of livestock associated MRSA in farmers constitutes a major threat to public health and health care system (Golding *et al*., 2010). Methicillin resistant *Staph.aureus* prevalence in humans is strongly associated with prevalence in animals and intensity of contact with animals positive for methicillin resistant *Staph. aureus* (Graveland *et al*., 2010). Zoonotic transmission of this clone of MRSA (ST398) has also been documented especially among farm workers and their family (Lee, 2003).

MRSA has been isolated from food of animal origin such as dairy products, beef, chicken (Normonno *et al*., 2007) and although food borne transmission is plausible (Lee, 2003). Methicillin resistant *Staph.aureus* (MRSA) has also been detected in several species and animal derived products (Leonard and Markey, 2008). An emerging subtype of methicillin resitant *Staph.aureus,* a clonal complex CC398, is associated with animals, particularly pigs (Armand *et al*., 2005). MRSA is a significant cause of nosocomial and community morbidity and mortality which over two decades have become a worldwide problem exacerbated by the emergence of multi drug resistant methicillin resistant *Staph. aureus* (Aires *et, al*. 1998).

The leading role of MRSA in these infections is associated with its resistance to most currently available antibiotics resulting in treatment failure (Enright *et al*., 2002). The panton

valentine leukocidin (PVL) encoded by *LukF-LUkS* genes associated with CA-MRSA are responsible for the wide spread of skin and soft tissue infection (David *et al.,* 2007). No antibiotic resistance marker has distinguished a species more than what methicillin resistance has for *Staph. aureus*. Methicillin resistance indicates resistance to all β-lactam antibiotics, including cabarpenems. MRSA isolates are increasingly resistant to multiple non β-lactam antibiotics, with reports of strains not susceptible to vancomycin creating a lot of problems in clinical settings for the successful chemotherapy of infections.

Although the mechanisms, risk factors and other information about emergence of methicillin resistant *Staph. aureus* in animal are rather poorly understood, the close contact between human and various animal species and antimicrobial use in animals presumably facilitate the emergence and spread of MRSA (Wesse *et al*., 2010).

Here in Nigeria, several studies conducted across the country showed that methicillin resistant *Staph. aureus* has been isolated and is a common cause of hospital and community acquired infections with varying prevalence (Sina *et al*., 2011; Adegoke and Komolafe, 2009). Also, nasal carriage of *Staph.aureus* amongst students has also been reported (Abdulhadi *et al.,* 2008).

There have however, been little studies on the prevailing resistance of poultry farm isolates to commonly used antibiotics, especially in the northern part of the country particularly in Kano. Therefore it is the aim of this study to isolate and identify such resistant strains and carryout their molecular characterization.

1.2 JUSTIFICATION OF THE RESEARCH

The occurrence of methicillin resistant *staph.aureus* not only in livestock but also in food of animal origin might represent a relevant issue with regard to human health and food safety for consumption. The MRSA isolated from human skin and soft tissue infections were mainly the MRSA (ST398) seen in cattle and poultry (Dullweber, 2010; Mulders *et al*., 2010).

The emergence and involvement of a distinct clone complex of MRSA (ST 398) associated with livestock in human disease in many countries suspected to have arisen from the increasing use of antibiotics in animal feeds, especially in poultry give a good reason for the study since poultry farming is common in Nigeria.

This study will provide compelling epidemiological and microbiological evidence that persons living with chicken or working on farms will be at increased risk of being colonized or infected with LA-MRSA. It is important to identify the origin of the isolates and their dissemination on the farm, and to evaluate the potential health hazard.

Since MRSA (ST 398) is implicated in human disease and has been reported in poultry in different parts of the world, they may also be present in poultry farms in Nigeria and this study will ascertain the presence and prevalence of MRSA (ST 398) in poultry farms in Kano State, Nigeria. Persons working on farms or living with the chickens are at a high risk of contacting the MRSA and transmitting it to their family and community at large, and may likely cause staphylococcal infection. Poultry farming in rural community is practiced at small scale level, in addition to the large poultry farms scattered all over the country; it is possible that these animals carry methicillin resistant *Staph. aureus* (Olayinka *et al*., 2010).

The presence of methicillin resistant *Staph.aureus* has been reported in Nigeria (Olayinka *et al*., 2010) but there is no report of LA-MRSA of clonal complex ST398 in

human infections in communities and hospital settings in the country. Besides, people working on the farms have little or no knowledge about the MRSA and are at high risk of contacting the bacteria through handling, and cleaning of the farm and this could easily be transferred to community. Thus molecular analysis of the isolates and a comprehensive analysis of their antimicrobial resistance properties would provide relevant information for health workers in the country and plan preventive and therapeutic measures in combating this emerging infection and minimizing risk factors. Transmission of MRSA ST398 from zoonotic reservoir to human could exacerbate presence of MRSA. There is absolute need to study this clonal complex 398 in other food producing animals not only poultry in the country. It is hoped that information from this research work will increase awareness among health care professionals that animals are a possible source of MRSA infection and that the potential for animal to human transmission exists.

1.3 AIM AND OBJECTIVES

AIM: To isolate and molecularly characterize MRSA isolates from poultry and poultry farm workers in Kano State.

Specific Objectives

1. To isolate *Staph. aureus* from chicken nares and cloacae (broilers and layers) and from the farm workers.
2. To screen the isolates for antimicrobial susceptibility using DAD.
3. To identify methicillin resistant isolates using cefoxitin.
4. To use latex agglutination method to determine the presence of PBP2a.
5. To determine those MRSA isolates that harbor mecA gene using PCR method
6. To screen the isolates for staphylococcal cassette chromosome (SCCmec) typing and lukF-lukS gene by multiplex PCR.
7. To determine multilocus sequence typing (MLST) for the clonal complex for confirmation of LA-MRSA.
8. To subject all MRSA isolates to Spa sequence typing.

1.4 RESEARCH HYPOTHESIS

1.4.1 Null Hypothesis (H_0)

Methicillin resistant *Staph.aureus* (MRSA) will not be isolated from both poultry and poultry farm workers in Kano State, Nigeria.

1.4.2 Alternate Hypothesis (Ha)

Methicillin resistant *Staph.aureus* (MRSA) will be isolated from both poultry and poultry farm workers in Kano State, Nigeria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE GENUS *STAPHYLOCOCCUS*

Staphylococci are Gram positive cocci, 0.5 to 1.5 mirometer in diameter, non motile, non-spore forming, facultatively anaerobic. The name *Staphylococcus* is derived from the Greek term "staphyle" meaning a bunch of grapes. This name refers to the fact that the cells of these Gram-positive cocci grow in a pattern resembling a cluster of grapes. However this organism in clinical material may also appear as single strain, pairs or short chains (Humphreys, 1998).

The Scottish surgeon, Sir Alexander Ogston named the organism *Staphylococcus* (Baird, 1990). In 1884, Rosenbach described the two pigmented colony types of *Staphylococcus* and proposed appropriate nomenclature.He called the yellow *Staph. aureus* and the white *Staph.albus* now named *Staph.epidermis* (Todar, 2005). The genus *Staphylococcus* contains about forty species and subspecies today. Only some of them are important as humuan pathogens:

- *Staphylococcus aures*
- *Staphylococcus epidermidis*
- *Staphylococcus hominis*
- *Staphylococcus haemolyticus*
- *Staphylococcus saprophyticus* and others

Taxonomically, the genus *Staphylococcus* is in the bacterial family staphylococcaceae which include three lesser known genera *Gamella*, *Micrococcus* and *Salinicoccus* (Todar, 2005).

Traditionally the genus *Staphylococcus* has been confused with the genus *Micrococcus* with the two genera being used interchangeably until definitive characteristics were established and modern taxonomic evidence has confirmed that there is a distance in their relationship (Richardson, *et al*, 1992). There are consequently three levels of consideration when laboratory identification of an aerobic, catalase positive, coagulase positive, Gram positive, coccus is considered. These are:

1. Differentiation between *Staphylococcus* and *Micrococcus*.
2. Distinction between coagulase positive and coagulase negative species.
3. Speciation of the isolates

The genus *Staphylococcus* can be defined into two sub groups on the basis of its ability to clot blood plasma by enzyme coagulase:

- Coagulase – positive
- Coagulase – negative

Subgroup of coagulase negative species from human *staphylococci* contains only one specie; *Staph. aureus* other coagulase – positive species are animal *staphylococci* e.g *Staph. intermedius*.

Subgroup of coagulase-negative species from human *staphylococci* are rarely pathogenic and considered to be opportunistic pathogens of human (Tormo *et al.*, 2005). They include :

- *Staph. epidermidis*
- *Staph. hominis*
- *Staph. xylosus*
- *Staph.haemolyticus*

- *Staph.saprophyticus*
- *Staph. simulans*
- *Staph. warneri* (Foster, 1996).

Staphylococci are ubiquitous. All persons have coagulase negative *Staphylococci* on their skin and transient colonization of moist skin folds with *Staph. aureus* is common. Colonization of the umbilical stump skin and perineal area of neonates with *Staph.aureus* is common.

Staph.aureus and coagulase negative staphylococci are also found in the oropharynx, gastrointestinal and urogenital tract. Because staphylococci are found on the skin and in the nasopharynx shedding of the bacteria is common and is responsible for many hospital-acquired infections.Both coagulase positive and non-coagulase staphylococcus are involved in human and animal infections (Jensen and Lyon, 2009). Coagulase positive *staphylococci* that include *Staph.hyicus* and *Staph. flexneri* have been implicated in human and animal infection (Tormo *et al*., 2005). They also include the *Staph.intermedius* group (SIG) namely *staph. intermedius,Staph.pseudintermedius*and *Staph. delphini* A and B which are rarely in humans and originally thought of dogs and cats but not found in a variety of other animals (Tormo *et al*., 2005).

2.1.1 Differentiation Of *Staphylococci* From *Micrococci*

Staphylococci are Gram positive, catalase positive cocci that divide in more than one plane to form irregular, three dimensional clusters of cells. They are traditionally differentiated from the morphologically similar members of the genus *Micrococcus* based on

their different behavior under anaerobic growth conditions (Schleifer and Kroppenstedt, 1990).

All *Staphylococci* are oxidase-negative but some *micrococci* are oxidase positive such that a positive oxidase test indicates a coccus other than *Staphylococcus*. A major distinguishing character is the large difference in the guanine plus cytosine (G + C) content of the DNA of the two genera (Baird, 1990). The successful separation of *Staphylococci* from *micrococci* and packet forming aerococci (the sarcinas) brought about established characteristics distinguishing *Staph. aureus* from other *Staphylococcal* species.

The differential effect of two lytic substances; lysozyme and lysostaphin on *Micrococci* and *Staphylococci* respectively is another distinguishing characteristic. The lysosstaphin susceptibility test is considered the most sensitive test that combines ease of use and speed in obtaining results for distinguishing the two genera (Adeleke1998; Gunn *et al*., 1981). The metabolism of *micrococci* is usually strictly respiratory, being obligate aerobes, while the *staphylococci* are facultative anaerobes that grow by aerobic respiration or most often fermentation that yield principally lactic acid (Bannerman and Peacock, 2007).

The results of the oxidation-fermentation (OF) test is a reflection of the ability of staphylococci to grow and produce acid from glucose when grown anaerobically, while the micrococci oxidized the sugar or failed to attack it. It is however been suggested that, separation of *staphylococci* from *micrococci* by this classical test may not be conclusive (Schleifer and Kroppenstedt, 1990).

There are many species of *staphylococci* that are known to produce only small amounts or no acid from glucose under anaerobic conditions. There are also *micrococci* (*micrococcus kristina*e and *m.varians*) that produce small to moderate amount of acid from glucose when grown anaerobically.

2.1.2 Differentiation Of *Staph. aureus* From Other Coagulase Negative *Staphylococci*

Generally, *Staph.aureus* is Gram positive cocci, appearing in clusters and are catalase positive (Cheesbrough, 2002).The two tests often used to distinguish *Staph.aureus* from other *staphylococci* are the coagulase test (Coagulation of human or animal plasma) and the thermostable nuclease test (breakdown of DNA nucleases that survive boiling) (Baird, 1990).

Unlike *Staph.aureus* that is extremely widely distributed, the other coagulase positive *Staphylococcal* species are of animal origin with some being specifically associated with particular animals. *Staph.aureus* from the skin of pigs, poultry and sometimes beef animals (Devrise *et al*., 1978), *Staph. intermedius* carnivorous animals and birds.

2.2 *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus is a Gram positive spherical cells mostly arranged in irregular grape like cluster. Catalase positive, oxidase negative, Vogues Proskaeur positive, ferments glucose to produce acid and gas or acid only, ferments lactose to produce acid and gas or acid only, and ferments mannitol to produce acid. It is coagulase positive and DNAse positive (Lamikanra *et al*., 1985). *Staph.aureus* is a facultative anaerobe growing well under aerobic conditions within 24 hours (Cheesbrough, 2000). It is found in people and frequently colonizing the nares the armpit, the perineum, skin fold and the vagina without giving rise to disease (Rodrigues *et al.*, 2010).

It is a major opportunistic pathogen that is responsible for a wide range of chronic and acute diseases of man and animal (Jesen and Lyon, 2009) and leading cause of hospital and community acquired infections worldwide (Reyes *et al*., 2011).Speciation of staphylococci isolates is essential to distinguish *Staph. aureus* from coagulase negative

staphylococci (CoNS), the production of coagulase an enzyme that enables the organism to clot plasma is a feature peculiar to pathogenic staphylococci and often associated with increased virulence of *Staph. aureus*. This has been used to classify *staphylococcus* species into the two groups; those that produce coagulase – the coagulase positive *staphylococcus* and those that lack the enzyme – the coagulase negative group (Baba *et al*., 2002).

2.2.1 Culture Characteristics And Laboratory Identification

Colonies on solid media are found regular smooth slightly convex and 2-3mm in diameter after 24h incubation.*Staph. aureus* cells produce cream yellow or orange pigment. Several standard laboratory procedure (Phenotypic and biochemical) have been developed for the identification of *Staph. aureus.* Testing for the presence and types of beta haemolysis on blood agar plates represents a simple and rapid method currently used to differentiate *Staph. aureus* from other staphylococci in primary non selective cultures though with limited sensitivity (Barrow and Feltham,1993). Selective agar like modified Baird parker agar has been used successfully for the detection and identification of *Staph. aureus* and other coagulase positive staphylococci (Kateete *et al*., 2010).

All *staphylococci* grow well in the presence of bile salt and on media with 4-6.5% Nacl (Brooks *et al*., 2007). *Staph. aureus* is isolated by streaking clinical specimen or materials for epidemiological survey onto solid media such as blood agar (BA) tryptisoy agar (TSA) or brain heart infusion agar (BHIA), MacConkey agar (MA) (Cheesbrough 2000). Specimens likely to be contaminated with other microorganisms can be cultured on mannitol salt agar containing 4 - 6.5 % sodium chloride which allows the salts tolerant *staphylococci* to grow (Foster, 1996; Brooks *et al*., 2007).

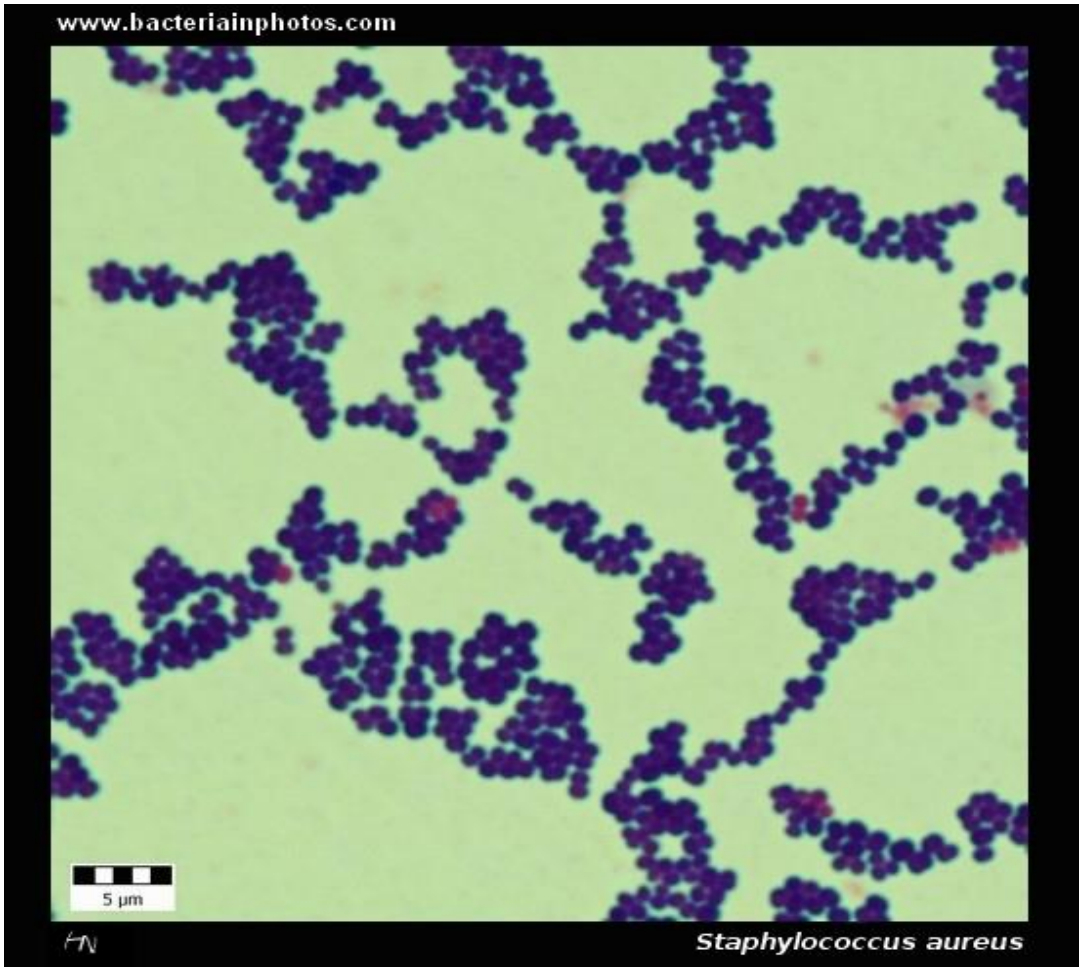


Figure 2:1: Gram stain of *Staph aureus* under oil immersion (X100) objective lens.

The simplest biochemical test that is used to differentiate between *staphylococcus* species and other Gram positive cocci is the catalase test (Isenberg, 1998). Catalase is an enzyme that hydrolysed hydrogen peroxide into water and oxygen during metabolism. It is used to differentiate *Staph. aureus* from *streptococci*; both of which are Gram positive cocci. The production of catalase enzyme by staphylococci is characterized by the production of bubbles or effervescence (Cheesbrough, 2000)

Mannitol fermentation, Protein A, DNAse, phosphate and coagulase productions are commonly used for the phenotypic identification of *Staph. aureus* (Brown *et al*., 2005; Luckzk Kadlubowska *et al*., 2006). Coagulase production has been a useful tool in the identification of *Staph. aureus*. The test utilizes two methods for the identification of coagulase enzyme; the slide agglutination test that detects bound coagulase and the tube agglutination test that detects the free coagulase (Foster, 1996). *Staph.aureus* can be confirmed by testing colonies with tube coagulase clumping factor and latex particles with immunoglobuling for agglutination (Foster, 1996; Kateete *et al*., 2010).

Inspite of these, there is no single phenotypic test including the tube coagulase test that can guarantee reliable results in the identification of *Staph. aureus* (Kateete *et al*., 2010). However, molecular methods have been developed to identify *Staph. aureus* especially methicillin resistance (Brown *et al*., 2005).

2.2.2 Carriage Of *Staph. aureus*

A fundamental biological property of *Staph.aureus* is its ability to asymptomatically colonize both healthy and hospitalized individuals; most often involving the anterior nares (Bien *et al*., 2011; Chambers and Deleo, 2009). After various experiments, three patterns of *Staph. aureus* nasal carriage has been distinguished. Appropriately 20 % of individual are always colonized, carry one type of strain and are called persistent carriers. A large

proportion of the populations, about 60 % are intermitten carriers, and the strains change with varying frequency. While a minority of the population (20 %) called non-carriers almost never carry *Staph. aureus* (Kluytmans *et al*., 1997; Foster, 2004).

Humans are the main source of *staphylococcus* species with diverse clonality and toxigenicity (GomezSanz *et al*., 2013). Humans and animals have persistent, intermitten or transient nasal colonization from where *Staph.aureus* and MRSA can cause infection or be transmitted to other person (Murray *et al*., 2002).*Staph.aureus* nasal carriage of 20-50% exists in the general population (Brooks *et al*., 2007 ; Chambers, 2001). But higher carriage rates have been observed in specific populations; injection drug users, persons with insulin dependent diabetes, patients with dermatological conditions, patients with long term in dwelling intravascular catheters and health care workers (Chambers, 2001). Almost 25% of healthcare workers are stable nasal carriers and 30 % to 50 % of them also carried the bacteria on their hands (Farzana *et al*., 2008). Asymptomatic carriers of *Staph.aureus* are at high risk of infection and are presumed to be an important source of *Staph. aureus* strains that spread among individuals (Chambers and Deleo, 2009). In hospitals, the asymptomatic hosts candisseminate *Staph. aureus* to immune compromised patients (Martins *et al*., 2012).

In infants, colonization of the nasopharynx, perineum or skin (especially if the cutaneous barrier has been disrupted or damaged) occurs and shortly after birth and may recur any time thereafter (Payne *et al*., 1996). Young children have also been known to have higher colonization rates, probably because of their frequent contact with respiratory secretions (Chamber 2001; Adcock *et al*., 1998).

Factors that enhance colonization by *Staph.aureus* are complex and are not fully understood. However, it does appear to involve the host's contact with other carriers,*Staph. aureus* ability to adhere to host cells and evade the immune response; and the antimicrobial

properties of the nasal fluid which allows it to be transmitted among individuals in both healthcare and community settings (Murray *et al*., 2002; Kumar *et al*., 2011). Consequently, a combination of bacterial resistance mechanisms, defective nasal fluid and lack of alternative clearance mechanisms (host associated determinants) results in nasal colonization by *Staph.aureus* (Wertheim *et al*., 2005).

2.2.3 Pathogenesis Of *Staph. aureus*

Staph.aureus is a complex pathogen with numerous classes of virulence factors (Peacock, 2002). The armamentarium of virulence factors of *Staph. aureus* is extensive, with both structural and secreted products playing a role in the pathogenesis of infection. Two noteworthy features of staphylococci are that a virulence factor may have several functions in pathogenesis and that multiple virulence factors may perform the same function. In establishing an infection *Staph.aureus* has numerous surface proteins, called "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) that mediate adherence to host tissues. MSCRAMMs bind molecules such as collagen, fibronectin, and fibrinogen, and different MSCRAMMs may adhere to the same host-tissue components MSCRAMMs appear (Foster and Hook 1998; Menzie 2003; Patti *et al*., 1994; Wetheim *et al*., 2005).

Once *Staph.aureus* adheres to host tissue or prosthetic materials, it is able to grow and persist in various ways. *Staph.aureus* can form biofilms (slime) on host and prosthetic surfaces, enabling it to persist by evading host defenses and antimicrobials (Donlan and Costerton 2005). *Staph.aureus* is able to form small colony variants (SCVs), which may contribute to persistent and recurrent infection. During infection *Staph.aureus* produces numerous enzymes, such as proteases, lipases and elastases that enable it to invade and destroy host tissues and metastasize to other sites. *Staph. aureus* is capable of producing

toxic shock. It does this by interacting with and activating the host immune system and coagulation pathways. Peptidoglycan, lipoteichoic acid, and α-toxin may all play arole (Wang *et al*., 2007; Projan and Novick, 1996; Timmerman *et al*; 1993; Heumann, 1994; Bhakdi and Tranum, 1991).

Staph.aureus is a common human colonizer and pathogen that causes infections ranging from skin and soft tissue. A broad array of virulence factors contribute to *Staph. aureus* pathogenesis and include performing toxins (alpha hemolysin, panton valentine leukocidin), superantigens (enterotoxins, toxic shock syndrome toxin-1), phagocytosis inhibitors (polysaccharide capsulate, protein A), and immune evasion molecules (chemotaxis, inhibitory protein, staphylokinase, aureolysin) (Corrigan *et al*., 2009 ; Bartlette and Hulten, 2010).

Staph.aureus is pathogenic for human as well as for all domestic and free living warm-blooded animals. It causes disease through the production of toxin or through direct invasion and destruction of tissue. Mobile genetic elements (MGEs) as means of transfer of genetic information among and within bacterial species play central role in adaption process (Malachowa and Deleo, 2010).Various host factors predispose individuals to infection, including the loss of the normal skin barrier, the presence of undelying disease such as diabetes or AIDS and defects in neutrophil function (Moran *et al*., 2006; Nabera, 2009; Chambers and Deleo, 2009). The primary mode of transmission of *Staph.aureus* is by direct contact, usually skin to skin contact with a colonized or infected individuals, hands and probably fomites (Shaffer, 2003).

Genome sequencing of *Staph.aureus* strains has helped to identify a number of virulence factors but the key determinants for infection are still unknown (Feng *et al*., 2008).

On entry into a host *Staph.aureus* will persist, escape host defenses and antibacterial agents, or multiply and further disseminate (Bien *et al.,* 2011). Virulent strains of *Staph. aureus* produce large quantities of extracellular proteins involved in pathogenesis, producing a typical staphylococcal lesion involving pus formation in contrast to strains that cause toxic shock syndrome which secrete very small quantities of exoproteins and high levels of toxic shock syndrome toxin – 1 (Vojtor *et al*., 2002).

The colonization process of *Staph.aureus* is mediated by several adhesions comprising proteins covalently anchored to cell peptidoglycans that specifically attach to the plasma or extracellular matrix (ECM) components collectively termed the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs).

Staphylococcal protein A (SPA), a cell wall anchored protein of *Staph.aureus* with capability to interact with several host components is virulence factors in staphylococcal infections (Palmquist *et al*., 2002). The production of staphylococcal protein A is controlled by several global regulators include agr, SarA, SarS, SarT, rot and mgrA, which appear to form a regulatory network in which SarS, an activator of Spa, is a key regulator (Lindsay and Foster, 1999).

Most strains of *Staph. aureus* produce exoproteins, exotoxins and enzymes including nuleases, proteases, lipases, hyaluronidase, and collagnase whose main functions may be to convert local host tissues into nutrient required for bacterial growth. An exception is the fatty acid modifying enzymes (FAME) that may be important in absecess, where it could modify antibacterial lipids and prolong bacterial survival (Foster, 1996).

Panton ValentineLeukocidin (PVL), α-hemolysin, Y-hemolysin, leukocidin, are among cytolytic exotoxins produced by *Staph. aureus*. These cytolytic exotoxins provided inflammation when they insert and oligomerize to form B-barrel fores in the plasma

membrane, causing leakage of the cells content and lysis of the target cell (Ferry *et al.*, 2005).

Alpha hemolysin causes osmotic cytolysis with cytotoxicity to human platelets, endothelial cells, thrombocytes and monocytes (Foster, 1996). PVL is a bi-component cytolysin (luks-lukf) with high affinity to leukocytes, while other bicomponents toxins, Y-hemolysin and leukocidin show cytotoxity to erythroyctes and leukocytes, respectively (Ferry *et al.,* 2005).

Other *staphylococcal* toxins that stimulate proliferation of T-lymphocytes are pyrogenic toxin superantigens (PTSAgs) and the exfoliative toxin (ETA and ETB) involved in staphylococcal scalded skin syndrome (SSSS) (Foster 1996; Wu *et al*., 2010). Other specific proteins associated with *Staph. aureus* that can have profound impact on the innate and adaptive immune system include staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein of *Staph. aureus* (CHIPS), staphylokinase (SAK), extracellular fibrinogen binding protein (EFB), extracellular adherence preotein (Eap and Formyl peptide receptor-like-1 inhibitory protein (FLIPs) (Bien *et al*., 2011).

Infections cause by *Staph. aureus* can be classified into three major groups:

a) Superficial wound infections and absecess.

b) Toxinoses such as food poisoning, scalded skin syndrome and toxic shock syndrome.

c) Systemic and life threatening conditions such as endocarditis, osteomyelitis, pneumonia, brain abscesses, meningitis and bacteremia (Jarrud *et al*., 2002; Nabera, 2009).

2.2.4 Virulence Factors In *Staph. aureus*

Staph.aureus is a pathogen expressing multiple factors that mediate host colonization, invasion of damaged skin and mucous dissemination through the body and evasion of host

defense mechanisms (Chanda *et al*., 2010). The pathogenicity and virulence of *Staph.aureus* infections is associated to various bacterial surface components.Common examples are capsular polysaccharide and protein A), including those recognizing adhesive matrix molecules e.g. clumping factor (CLF) Fibronectin Binding Protein (FBN) and the extracurricular proteins like coagulase, haemolysins, enterotoxins, toxic shock syndrome toxins, exfoliatins toxins and panton valentine leukocidin (Labandeira-Rey *et al*., 2007).

Virulence factors can generally be separated into three; "antigens, enzymes and toxins" based on their function:

- **Antigens e.g; Adhesins:** They are surface attached proteins that allow the bacteria to attach to a wide variety of human tissues. In *Staph.aureus* the adhesion genes which include clf and fnb that encode the fibrinogen and the fibronecting binding proteins respectively. Typical members of the family MSCRAMM are the staphylococcal protein A(SpA), collagen binding protein, clumping factor andFibronectin binding protein (FnBP) A and Fibronectin binding protein (FnBP) B encoded by the finbA and FinbB genes respectively, play prominent roles in *Staph.aureus* colonization and attachment of host tissues or implanted biomaterials (Greene *et al*., 1995).
- **Immune dilators:** are proteins that interfere with host immunity preventing defense against infections.
- **Enzymes:** Are of exoproteins group that convert local host tissue into nutrients required for bacterial growth such as:
- Coagulase – *Staph. aureus* strains possess two forms of coagulase; bound and free. Coagulase bound to the staphylococcal cell wall can directly convert fibrinogen to insoluble fibrin and cause the *staphylococci* to clump. The cell free coagulase

accomplishes the same result by reacting with a globuling plasma factor. The role of coagulase in the pathogenesis of disease is speculative but coagulase may cause the formation of fibrin layer around a staphylococcal abscess thus localizing the infection and protecting the organism from phagocytosis.

- Catalase – All *staphylococci* produce catalase which catalysis the conversion of toxic hydrogen peroxide to water and oxygen. Hydrogen peroxide can accumulate during bacterial metabolism or after phagocytosis.
- Hyaluronidase: This enzme hydrolyzes hyalernic acid. This is the acidic mucopolysaccharides present in the cellular matrix of connective tissue. Hyaluronidase facilitates the spread of *Staph. aureus* in tissues. More than 90 *% of Staph. aureus* strains produce this enzyme.
- Fibrinolysin also called staphylokinase is produced by virtually all strains of *Staph. aureus* and can dissolve fibrin clot. Staphylokinase is distinct from the fibrinolytic enzyme produced by *streptococci*.
- **Toxins:** Are secreted proteins that cause tissue damage and generate pus in abscesses which is believed to facilitate transmission between hosts.This includes
- Enterotoxins are produced by 30 % to 50 % of the *Staph. aureus* strains. Eight serologically distinct enterotoxins (A-E, G-I) have been identified.Enterotoxin A is the most commonly associated with disease. Enterotoxin C and D are found in contaminated milk products and enterotoxin B causes staphylococcal psedoembranous enterocolitis.
- Toxin shock syndrome Toxin-1 (TSST-1 formerly called pyrogenic exotoxin C and enterotoxin F is a heat and proteolysis resistant chromosomally medicated exotoxin. The ability of TSST-1 to penetrate mucosal barriers even though the infection remains

localized in the vagina or at the site of a wound is responsible for the systemic effect to TSS. Death in patient with TSS is due to hypovolemic shock leading to multi-organ failure. Others toxins are exfoliative, α -toxins, β- toxinsα- toxins and PVL

2.3 HISTORY OF ANTIMICROBIAL CHEMOTHERAPY

Antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine. Contrary to the common belief that the exposure to antibiotics is confirmed to the modern "antibiotic era" research has revealed that this is not the case. The traces of tetracycline, for example have been found in humanskeletal remains from ancient Sudanese Nubia dating back to 350-550CE (Nelson *et al*, 2010).

The society of potent antimicrobial agents was one of the greatest combinations of medicine in the 20th century (File, 1999). The history of this development will include both the accidental discovery and deliberate search for both the synthetic chemotherapeutics and antibiotics. Another possibility of exposure to antimicrobials in the pre-antibiotic era could be through the remedies used for millennia in traditional/alternative medicine, in particular in Traditional Chinese Medicine (TCM). The best known example is the discovery of a potent antimalarial drug, qinghaosu (artemisinin), which was extracted in the 1970s from artesmisia plants, used by Chinese herbalists for thousands of years as a remedy for many illnesses (Cui and Su, 2009). Antimicrobial activity seems present in a number of other herbs used in TCM (Wang *et al*., 2007) and the discovery of active component in these ancient remedies may enrich the arsenal of antimicrobials used by the mainstream medicine. Before the early 20th century, treatments for infections were based primarily on medicinal folklore. Mixtures with antimicrobial properties that were used in treatments of infection were described over 2000 years ago, (Lindbald, 2008). Many ancient cultures, including the

ancient Egyptians and ancient Greeks, used specially selected mold and plant materials and extracts to treat infections (Forrest, 1982; Milton, 1989). More recent observations made in the laboratory of antibiosis between microorganisms led to the discovery of natural antibacterial produced by microorganism. Louis Pasteur observed "If we should intervene in the antagonism observed between some bacteria, it would offer perhaps the greatest hopes for the therapeutics" (Boucher *et al*., 2013).

In 1929, Alexander Fleming identified penicillin, the first chemical compound with antibiotic properties. Fleming was working on a culture of disease causing bacteria when he noticed the spores of little green mold in one of his culture plates. He observed that the presence of the mold killed or prevented the growth of the bacteria. He observed the plate was dotted with colonies, save for one area where a blob of mold was growing. The zone immediately around the mold later identified as a rare strain of *Penicillium notatum* was clear, as if the mold had secreted something that inhibited bacterial growth. Fleming found that his mold juice was capable of killing awide range of harmful bacteria such as streptococcus, meningococcus and the diphtheria bacillus. He then set his assistant, Stuart Craddock and Fredrick Ridley, the difficult task of isolating pure penicillin from the mold juice. It proved to be very unstable and they were only able to prepare solutions of crude material to work with. Fleming published his findings in the British Journal of experimental pathology in June 1929, with only a passing reference to penicillin's potential therapeutic benefits. At this stage it looked as if its main application would be in isolating penicillin – insensitive bacteria from penicillin-sensitive bacteria in a mixed culture. This at least was of practical benefit to bacteriologists and kept interest in penicillin going.

2.3.1 Development Of Chemotherapeutic Agents

The first antimicrobial agent in the world was salvarsan, a remedy for syphilis that was synthesized by Ehrlich in 1910. In 1935, sulfonamides were developed by Domagk and other researchers. These drugs were synthetic compound and had limitations in terms of safety and efficacy (Powers, 2004). During the subsequent two decades, new classes of antimicrobial agents were developed one after another, leading to age of antimicrobial chemotherapy. In 1944, streptomycin, an aminogylycoside was obtained from the social bacterium *Streptomyces griseus*. The synthesized antimicrobial agent nalidixic acid, a quinolone antimicrobial drugs, was obtained in 1962 (Hashimoto, 2000; Powers, 2004).

Modern day chemotherapy, refers specifically to the synthesis of drugs with specific biostatic or biocidal effects on the parasite and little or no toxic effects upon the host, is the brain child of the German Jew, Paul Ehrlich (Collard, 1976). He developed the concept of "magic bullet" which would wipe out the parasite from the body of the host following a single dosa sterilisa magma when he tried to apply his knowledge of differential staining of cells to the problem of synthesizing specific chemotherapeutic agents.

The theories of specific receptors and differential affinities led to the development and production of agents such as mepacrine the first synthetic malarial prophylactic agent and prontosil red, the parent compound of the sulphonamides. Prontosil red was synthesized by Gelmo in 1908 but first used in 1935 by Domagk in the treatment of mice infected with β-haemolytic streptococci (Collard, 1976; Russel, 1983). It was subsequently found that, in vivo, prontosil was converted into sulphanilamide. Domagk received a nobel prize in psychology or medicine for his discovery of sulphonamides (Bosch and Rosich, 2008).The chemical structure of prontosil converted to sulphanilamide is shown in figure 2.1.

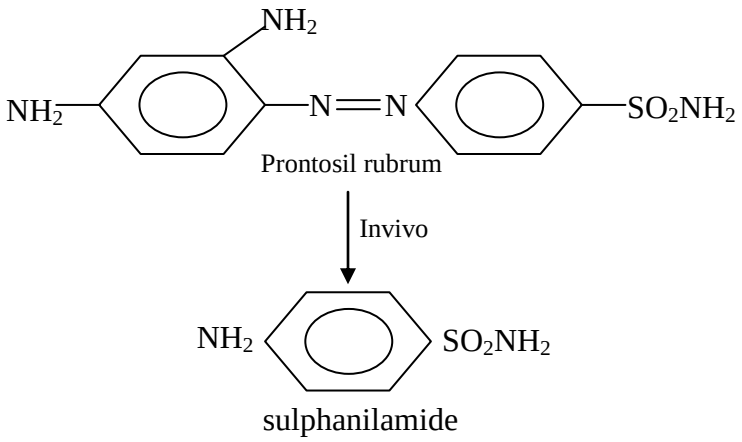


Fig. 2.1: Conversion of prontosil rubrum to sulphanilamide in vivo

Various chemical modifications of the nucleus of sulphanilamide over time have produced compounds with higher antibacterial activity, although this was often accompanied by greater toxicity (Russell, 1983).

This first published account of microbial antagonism is that of William Roberts in the Philosophical transactions of the Royal society in 1874, here he noted that the growth of fungi often suppressed the growth of bacteria and goes on to say "I have repeatedly observed that liquids in which *Penicilium glaucum* was growing luxuriantly could with difficulty be artificially infected with bacteria (Collard, 1976). Two years later, Jon Tyndal published an account of antagonism between a penicillium and various bacteria in liquid cultures and noted that in the struggle between the mold and the bacteria, the mold was usually successful.

In 1938-39, Howard florey, backed by a team of chemists headed for Earnest Chain, paved the way for the first clinical trials (with dramatic results in cases of staphylococcal and streptococcal infections) when they successfully isolated penicillin in 1941. Penicillin was

tested for the first time on a police officer who had a life threatening *Staph. aureus* infection (Nester *et al*., 1998). He improved so dramatically that, within 24 hours, his illness seemed under control.

Unfortunately, the supply of purified penicillin ran out, and the man eventually died of the infection. Later with greater supplies of the drug the experiment was repeated and two deathly ill patients were successful cured.

In 1944 Selman Waksman and group changed the prognosis of tuberculosis with the discovery of streptomycin obtained from soil bacterium *Streptomyces grisues* (Powers, 2004). There after chloramphenicol, tetracyclines, macrolides and glycopeptides were discovered from soil bacteria. This was seen as an ideal complement to penicillin, being active against Gram-negative enterobacteriaeae and the mycobacteria against which penicillin was ineffective.Waksman's success led to a worldwide intensive search for antibiotics producing soil organism. Tyrothrocin (Gramicidin + tyrocidin) was produced by Brevis. Later work led in 1947 to the discovery of polymixin. Thousands of antibacterial compounds were purified, spectrum of activity and toxicity determined leading to the discovery of some other therapeutic compounds often referred to as broad spectrum antibiotic.

A large number of antibiotics have been introduced in clinical practice. They differ markedly in physical, chemical and pharmacological properties, antibacterial and mechanism of action.

2.4 ANTIBIOTICS: DEFINITION

The term antibiosis meaning against life, was introduced by the French bacteriologist Jean Paul Vuillemin as a descriptive name of the phenomenon exhibited by the early antibacterial drug (Calderon and Sabundayo, 2007).

Antibiosis was first described in 1877 in bacteria when Louis Pasteur and Robert Koch observed that an airborne bacillus could inhibit the growth of *Bacillus anthracis* (Landsberg 1949). These drugs were later renamed antibiotics by Selman Waksman, an American Microbiologist, in 1942 (Calderon and Sabundayo, 2007; Waksman, 1947).

The term antibiotic was first used in 1942 by Selman Waksman and his collaborators in journal articles to describe any substance produced by a microorganism that is antagonistic to the growth of the other microorganisms in dilution (Waksman, 1947). Antibiotics are a type of antimicrobials used in the treatment and prevention of bacterial infection. They may either kill or inhibit the growth of bacteria. Several antibiotics are also effective against fungi, protozoans and some are toxic to humans and animals. Antibiotics are not effective against viruses such as the common cold or influenza (Goodman *et al,* 1941). Any substance of natural, synthetic or semisynthetic origin which at low concentrations kills or inhibits the growth of microorganisms but causes little or no host damage (WHO, 2000). Today, however, with increased knowledge of causative agents of various infectious diseases antibiotic(s) has come to denote broader range of antimicrobial compounds, including antifungal and other compounds.

2.4.1 Sources Of Antibiotics

There are mainly three classes of antibiotics:

1. Those obtained from natural sources i.e β-lactam antibiotic (such as penicillins, cephalosporins) or protein synthesis inhibitors (such as aminoglycosides, macrolides, tetracyclines, chloramphenicol, and polypeptides); most antibiotics in current use have been produced from streptomyces species.
2. Synthetic Antibiotics: obtained from chemical synthesis example, chloramphenicol, sulphanamides, quinolones and the oxazolidones.

3. Semi Synthetic antibiotic: This means that part of the molecules is produced by a fermentation process and the product is then further modified by a chemical process. Many penicillins e.g. ampicillin (figure 2.3c), amoxicillin, Methicillin (figure 2.3b) piperacillin (figure2.3d) and ticarcillin, cephalosporins and the carbapenems are produced in this way (Russel, 1998; Von Nussbaum *et al*., 2006).

2.4.2 The Beta-Lactam Antibiotics

The β-lactam antibiotics form a large biochemical class of agents that have greatly revolutionized antimicrobial chemotherapy. β - lactam antibiotics are agents that contain a β-lactam ring in their molecular structure(figure 2.2).

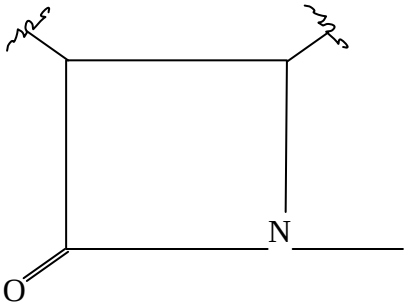
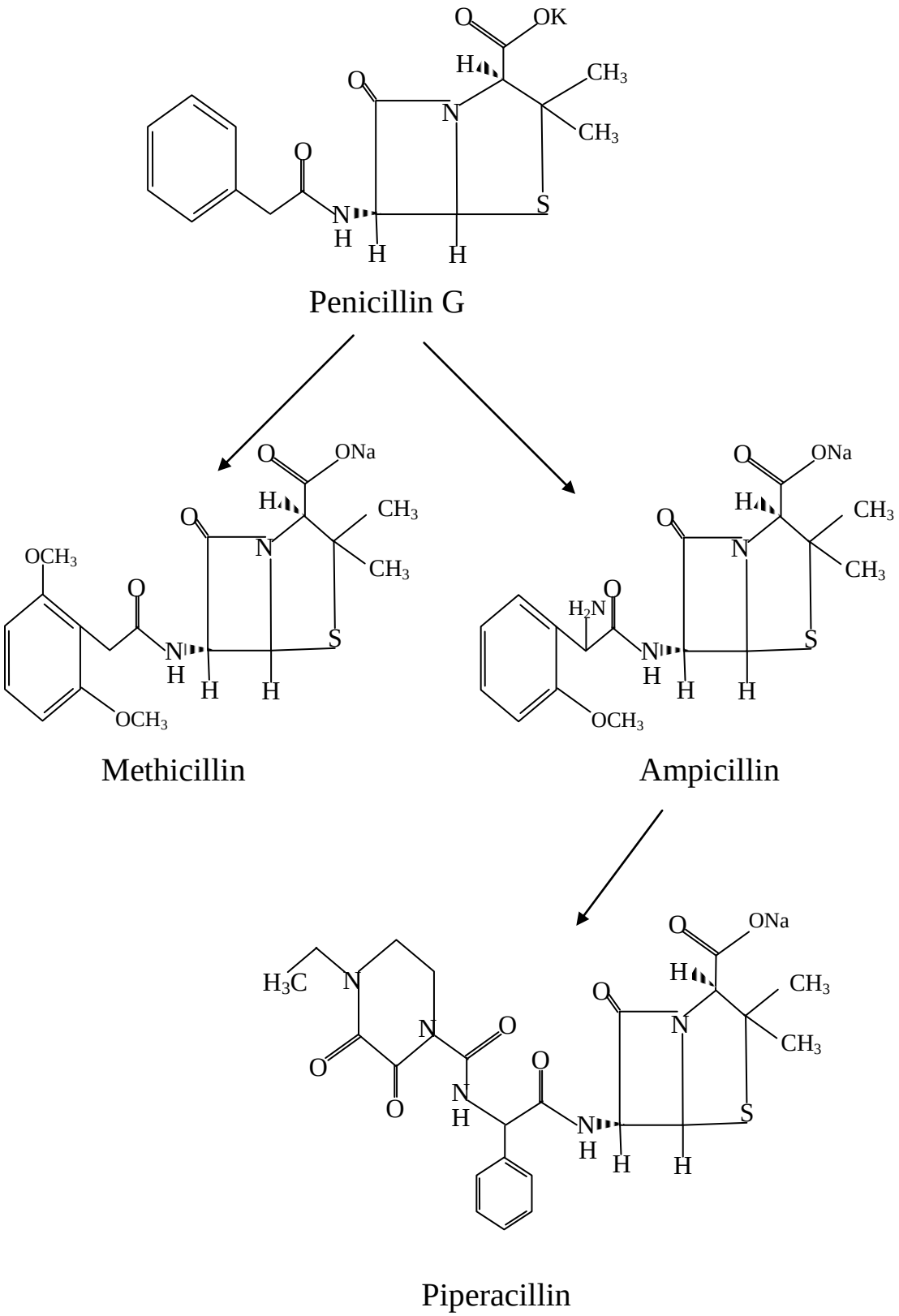


Figure 2.2 Structure of the β-lactam ring

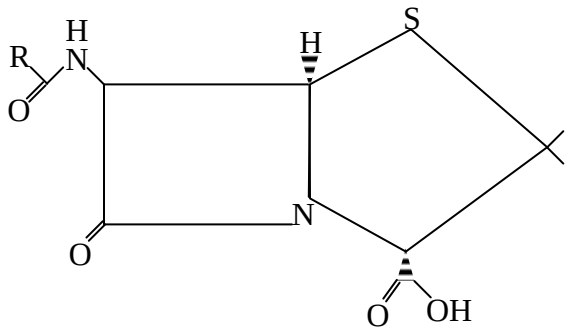
The β-lactam antibiotics include penicillins (fig. 2.4A) and penicillin derivatives; penams (fig. 2.5A), cephalosporins (fig. 2.4B), cephems (fig.2.5E), carbacephem (fig.2.5H) monobactams (fig 2.5G) oxapenam (fig.2.5C), oxacephem (fig.2.5I) and carbapenems (fig.2.5F) (Holten and Onusko, 2000).

The β- lactam antibiotics are useful and are frequently prescribed antimicrobial agents with similar structure and mechanism of action (Petri, 2006). Up until 2003, when measured by sales more than half of all commerciailly available antibiotics in use were β-lactam compounds (Elander, 2003).



Figuure 2.3: Evolution of antibiotics

(A)



(B)

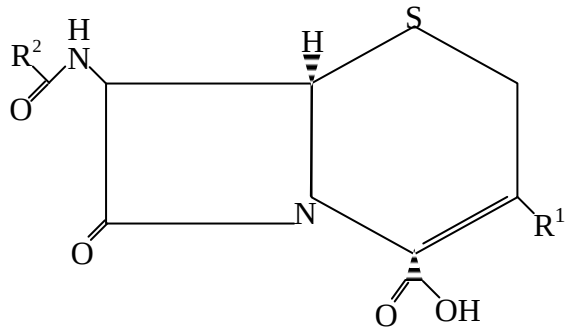


Figure 2.4: Core structure of (A) Penicillins (B) Cephalosporins

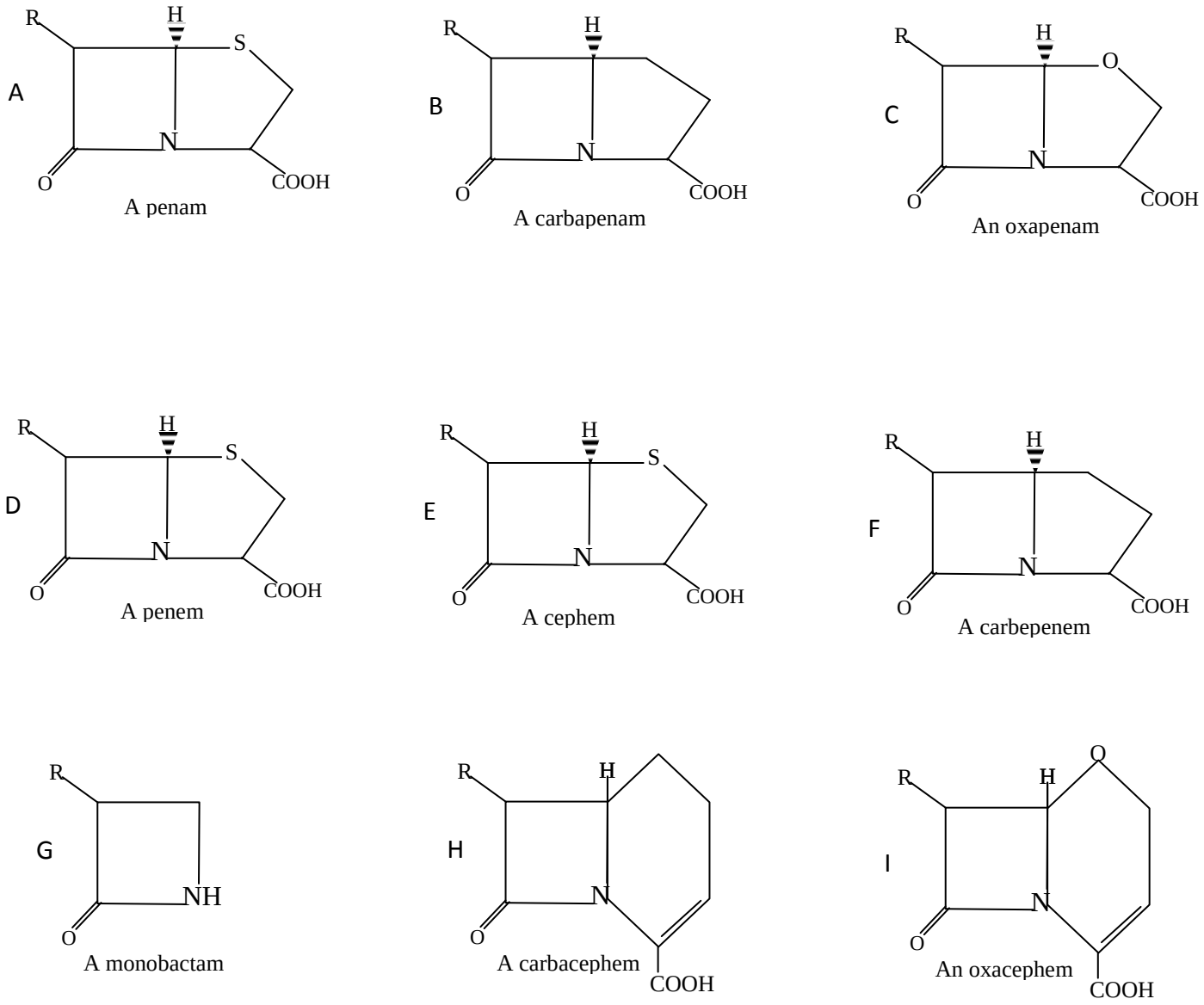


Fig. 2.5: Examples of some beta lactam antibiotics

2.4.2.1 *Mechanism of action of the penicillins and cephalosporins*

Knowledge of the mechanism of action of β-lactam antibiotics has been incomplete, but numerous researchers have supplied information that allows understanding of the basic antibacterial phenomenon (Ghuysen, 1991; Bayles, 2000). The cell wall of bacteria has been reported to contain heteropolymeric component of peptidoglycan which provides rigid mechanical stability by virtue of its highly cross-linked lattice work structure. The last step of peptidoglycan synthesis of the bacterial cell wall has been reported to be inhibited by the β-lactam antibiotics (Petri, 2006b).

Although inhibition of the transpeptidase enzyme has been ascribed as the major active site, there are other reported additional target sites for penicillins and cephalosporins, called penicillin binding proteins (PBPs). All bacteria havebeen reported to possessseveral PBPs entities; for example, *Staph. aureus* have been documented to have four PBPs. The PBPs affinity for different β-lactam antibiotics varies, though the interactions eventually become covalent. Inhibition of the transpeptidase enzyme has been reported to cause spheroplast formation and subsequent rapid lysis of the bacteria cells. However, inhibition of the activities of other PBPs has been reported to delay cell lysis (PBP 2) or to enhance the production of long, filamentous forms of the bacterium (PBP 3). The lethality of penicillin for bacteria appears to involve both lytic and nonlytic mechanisms. Penicillin's disruption of the balance between PBP-mediated peptidoglycan assembly and murein hydrolase activity results in autolysis. Non-lytic killing by penicillin may involve porin-like proteins in the bacterial membrane that collapse the membrane potential (Bayles, 2000).

The first-generation cephalosporins, such as **cephalothin and cefazolin**, have been reported to have good antibacterial activity against Gram-positive bacteria and relatively

modest activity against Gram-negative microorganisms. Most Gram-positive cocci are susceptible with the exception of *enterococci*, methicillin-resistant *Staph. aureus*, and *Staph. epidermidis*. Most oral cavity anaerobes are shown to be sensitive, except *Bacillus* fragilis group. *Moraxella catarrhalis, Escherichia coli, Klebsiella pneumoniae*, and *Proteus mirabilis* are reported to be susceptible to first generation cephalosporins (Petri, 2006b). The second-generation cephalosporins are documented to have increased activity against Gram-negative microorganisms. Second-generation cephalosporins such as **cefoxitin, cefotetan, and cefmetazole** have been reported to be active against the *B. fragilis* group.

Third-generation cephalosporins are generally reported to be less active than first-generation cephalosporins against Gram-positive cocci, but much more active against the Enterobacteriaceae, including β-lactamase-producing strains. Third-generation cephalosporins such as **ceftazidime and cefoperazone** are reported to be active against *Pseudomonas aeruginosa* but less active against Gram-positive cocci.Fourth-generation cephalosporins, such as **cefepime**, are reported to have an extended spectrum of activity and increased stability from hydrolysis by plasmid and chromosomally mediated β-lactamases. Fourth-generation cephalosporins are reported to be particularly useful for the empirical treatment of serious infections in hospitalized patients when Gram-positive microorganisms, Enterobacteriaceae, and Pseudomonas are the etiologies (Petri, 2006a).

2.4.3 Aminoglycosides

The aminoglycoside antibiotics include gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin, and neomycin. In contrast to most inhibitors of microbial protein synthesis, which are bacteriostatic, the aminoglycosides are reported to be bactericidal in

action. Mutations affecting proteins in the bacterial ribosome, the target for these antibiotics, can confer marked resistance to aminoglycoside action. However, most commonly reported bacterial resistance are acquisition of plasmids or transposon-encoding genes for aminoglycoside-metabolizing enzymes or impaired transport of aminoglycoside into the bacterial cell (Chambers, 2006).

Mechanism of action of aminoglycosides has been known to disrupt the normal cycle of ribosomal function by interfering, at least in part, with the initiation of protein synthesis, leading to the accumulation of abnormal initiation complexes (Pantosti *et al*., 2007). Aminoglycosides are known to also cause misreading of the mRNA template and incorporation of incorrect amino acids into the growing polypeptide chains. Aminoglycosides vary in their capacity to cause misreading presumably owing to differences in their affinities for specific ribosomal proteins. Although there appears to be a strong correlation between bactericidal activity and the ability to induce misreading (Hummel and Bock, 1989), it remains to be established that this is the primary mechanism of aminoglycoside-induced cell death (Chambers, 2006).

2.4.4 Tetracyclines

Tetracycline is a semi-synthetic derivative of chlortetracycline. Tetracyclines are close congeners of polycyclic naphthacenecarboxamide. They have activity against Ricketttsia, Gram- positive and Gram-negative bacteria, aerobes, anaerobes, and Chlamydia. Because of these activities, they are reffered to as “broad spectrum” antibiotics (Chambers, 2006). Tetracyclines are bacteriostatic antibiotics. Tetracyclines are known to inhibit bacterial protein synthesis by binding to the 30S bacterial ribosome and preventing access of aminoacyl tRNA to the acceptor (A) site on the mRNA-ribosome complex. These

antibiotics have been reported to enter Gram-negative bacteria by passive diffusion through the hydrophilic channels formed by the porin proteins of the outer cell membrane and by active transport via an energy-dependent system that pumps all tetracyclines across the cytoplasmic membrane. Entry of these drugs into Gram-positive bacteria has been reported to require metabolic energy, but the details are not well understood. (Chambers, 2006).

2.4.5 Trimethoprim-Sulfamethoxazole

The antibacterial spectrum of trimethoprim has been reported to be similar to that of sulfamethoxazole, although the former drug usually is 20 to 100 times more potent than the latter. Most Gram-negative and Gram-positive microorganisms are sensitive to trimethoprim, but resistance has been reported to develop when the drug is used alone. *Pseudomonas aeruginosa, Bacteroides fragilis, and enterococci* usually are reported to be resistant to trimethoprim. There is a significant variation reported in the susceptibility of Enterobacteriaceae to trimethoprim in different geographical locations because of the spread of resistance mediated by plasmids and transposons (Petri, 2006a).

The antimicrobial activity of the combination of trimethoprim and sulfamethoxazole has been reported to result from its actions on two steps of the enzymatic pathway for the synthesis of tetrahydrofolic acid. Sulfonamide inhibits the incorporation of para-aminobenzoic acid (PABA) into folic acid, and trimethoprim prevents the reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is essential for one-carbon transfer reactions, e.g., the synthesis of thymidylate from deoxyuridylate. Selective toxicity for microorganisms is achieved in two ways. Mammalian cells use preformed folates from the diet and do not synthesize the compound. Furthermore, trimethoprim is a highly selective inhibitor of dihydrofolatereductase of lower organisms:

About 100,000 times more drug is required to inhibit human reductase than the bacterial enzyme. This relative selectivity is vital because this enzymatic function is essential to all species (Petri, 2006 a).

2.4.6 Quinolones

The first quinolone, nalidixic acid, was isolated as a by-product of the synthesis of chloroquine. (Petri, 2006a). It has been available for the treatment of urinary tract infections for many years. The introduction of fluorinated 4-quinolones, such as **ciprofloxacin , moxifloxacin ,** and **gatifloxacin** represents a particularly important therapeutic advance because these agents have broad antimicrobial activity and are effective after oral administration for the treatment of a wide variety of infectious diseases. Relatively few side effects are known to accompany the use of these fluoroquinolones, and microbial resistance to their action does not develop rapidly (Andriole, 1993; Hooper, 2000). Rare and potentially fatal side effects, however, have resulted in the withdrawal from the market of temafloxacin (immune hemolytic anemia), trovafloxacin (hepatotoxicity), grepafloxacin (cardiotoxicity), and clinafloxacin (phototoxicity). In all these cases, the side effects were so infrequent as to be missed by prerelease clinical trials and detected only by post marketing surveillance (Sheehan and Chew, 2003).

The quinolone antibiotics has been reported to target bacterial DNA gyrase and topoisomerase IV (Drlica and Zhao, 1997). For many Gram-positive bacteria (such as *Staph. aureus*), topoisomerase IV is the primary activity inhibited by the quinolones. In contrast, for many Gram-negative bacteria, DNA gyrase is the primary quinolone target (Alovero *et al.*, 2000; Hooper, 2000). The individual strands of double-helical DNA must be separated to permit DNA replication or transcription. However, anything that separates the strands

results in "overwinding" or excessive positive supercoiling of the DNA in front of the point of separation. To combat this mechanical obstacle, the bacterial enzyme DNA gyrase is responsible for the continuous introduction of negative supercoils into DNA. This is an ATP-dependent reaction requiring that both strands of the DNA be cut to permit passage of a segment of DNA through the break; the break then is resealed (Petri, 2006a).

The drugs inhibit gyrase-mediated DNA supercoiling at concentrations that correlate well with those required to inhibit bacterial growth (0.1 to 10 µg/ml). Mutations of thegene that encodes the A subunit polypeptide can confer resistance to these drugs (Hooper, 2000). Topoisomerase IV also is composed of four subunits encoded by the parC and parE genes in *E. coli* (Drlica and Zhao, 1997; Hooper, 2000). Topoisomerase IV separates interlinked (catenated) daughter DNA molecules that are the product of DNA replication. Eukaryotic cellsdonot contain DNA gyrase (Petri, 2006a). However, they do contain a conceptually and mechanistically similar type II DNA topoisomerase that removes positive supercoils from eukaryotic DNA to prevent its tangling during replication. This enzyme has been reported as the target for some antineoplastic agents (Goodman and Gilman, 2006). Quinolones inhibit eukaryotic type II topoisomerase only at much higher concentrations (100 to 1000 µg/ml) (Mitscher and Ma, 2003).

The fluoroquinolones are reported as potent bactericidal agents against *E. coli* and various species of *Salmonella, Shigella, Enterobacter, Campylobacter, and Neisseria* (Eliopoulos and Eliopoulos, 1993). Resistance to quinolones has been reported to develop during therapy via mutations in the bacterial chromosomal genes encoding DNA gyrase or topoisomerase IV or by active transport of the drug out of the bacteria (Oethinger *et al.,* 2000). No quinolone-modifying or -inactivating activities have been identified in

bacteria (Gold and Moellering, 1996). Resistance has increased after the introduction of fluoroquinolones, especially in Pseudomonas and Staphylococci (Pegues *et al*., 1998; Peterson *et al*., 1998). Increasing fluoroquinolone resistance also is being observed in *C. jejuni, Salmonella, N.gonorrhoeae,* and *S. pneumoniae* (Centers for Disease Control and Prevention, 1994; Thornsberry *et al*., 1997; Molbak *et al*., 1999; Smith, 1999). Fluoroquinolones are potent broad-spectrum antimicrobial agents that are increasingly used to treat *E. coli* infection. Despite initial optimism, resistance to these antibiotics has increased significantly since their introduction into medicine and agriculture in the late 1980's and early 1990's. Consequently, the mechanism of resistance in fluoroquinolone-resistant bacteria has been the subject of intense research and, in recent years, dramatic advances have been made in understanding of these mechanisms. The primary target of fluoroquinolone action documented is DNA gyrase and topoisomerase IV. The 2 enzymes are tetramers, composed of 2 A subunits and 2 B subunits, encoded by the gyrA and gyrB genes for DNA gyrase (topoisomerase II) and parC and parE genes for topoisomerase IV. The mechanism of resistance to fluoroquinolones has been mainly linked to mutations in the quinolone-resistance-determining region (QRDR) of gyrA and parC genes and, less frequently, in the gyrB and pares genes.

2.5 BACTERIAL DRUG RESISTANCE

Bacterial resistance to antibiotics has emerged as global problem involving all known classes of both natural products and synthetic compound. Its emergence is a set back and increases the challenges posed by infectious diseases (Dantas and Sammer, 2012). Antimicrobial resistance is recognized when the microorganism multiply at an antibiotic concentration which can be achieved in the tissues (Simon *et al.,* 1993). The microbial cells

now survive and are able to thrive in hitherto macrobiotic or microbiotic concentration of the chemotherapeutic agent. Resistance to antimicrobials has been described as a natural phenomenon (WHO, 2001).

Resistant bacteria have always been around; they existed long before humans began to use antibiotics therapeutically. What is new in the world of resistance is how quickly new resistant strains arise. The widespread use and misuse of antibiotics contribute to the problem (Saga and Yamaguchi, 2009). The mortality of patients with *Staph.aureus* bacteremia in the pre antibiotic era exceeded 80% and over 70% developed metastatic infections (Skinner and Keefer, 1941). Antibiotic resistance in an antibiotic free environment was a rare event (Ayliffe, 1997). According to Davies and Davies (2010), development of generations of antibiotic – resistant microbes and their distribution in microbial population are the result of many years of incessant selection pressure from human applications of antibiotics through under use, over use and misuse.

The introduction of penicillin in the early 1940s dramatically improved the prognosis of patients with staphylococcal infection. However, as early as 1942, penicillin – resistant *staphylococci* were recognized, first in hospitals and subsequently in the community (Ramelkamp and Maxon, 1944). By the late 1960s, more than 80% of both community and hospital acquired staphylococcal isolates were resistant to penicillin. This pattern of resistance, first emerging in hospitals and then spreading to the community, is now a well-established pattern that recurs with each new wave of antimicrobial resistance (Chambers, 2001). Today 80% of staphylococcus strains do not respond to penicillins (Appelbaum, 2008). Increasing resistance to antibiotics is a consequence of selective pressure but the actual incidence of resistance varies between different bacteria species (Jawetz *et al*., 2007).

Antibiotic resistant pathogens constitute an important and growing threat to the general public and it occurs when a microbe acquires a gene (Muto *et al*., 2003) or undergoes changes (Jesen and Lyon, 2009) which allow the microbe to inactivate the antibiotic or otherwise nullify its antimicrobial activity. This may occur as a result of spontaneous genetic mutation or involve acquisition of mobile genetic element such as plasmids, transposons, integrons, or gene cassettes (Roberts, 2012). Antibiotic resistant organism has resulted in prolonged suffering serious infections and higher risk of death than with antibiotic susceptible strains of the same species. The consequence is an increasing cost of medical care, prolonged hospital stay and associated morbidity and mortality (Muto *et al*., 2003). Acquired resistant in pathogens with introduction of new drugs is a common feature of antibiotic use. Antibiotic resistances had been regarded as a problem of human and animal pathogens following the observation of a correlation between the introduction of new antibiotics and the emergence of resistance in the clinics in previous susceptible population of bacteria (Wright, 2012).The changed prognosis of tuberculosis as a result of the discovery in 1944 of streptomycin; a specific antibacterial therapy which often produced a cure within months and could be given if need be on an out-patient basis suffered a blow within a year. This is because patients ceased to respond to treatment and relapsed as a result of their infecting organism developing resistance to streptomycin (Collard, 1976).

The introduction of sulfonamides in 1936 for the treatment of gonorrhea produced remarkable results, with practically all strains of gonococci being susceptible to the chemotherapeutic agent. Unfortunately, most strains were resistant to sulfonamide six years later (Jawetz *et al.,* 1974). Also, before 1962, meningococci were uniformly susceptible to sulfonamides. The subsequent emergence of sulfonamide resistant meningococcal and its spread rendered sulfonamides useless in the prevention and treatment of a meningococcal

infections (Franklin, 1987; Jawetz *et al*., 1974).Incidence of occurrence of resistance to either a single chemotherapeutic agent or many agents at the same time (multiple-drug resistance) has been reported in many pathogenic microorganisms (Ribner, 1987; Oduyebo *et al*., 1997; Pellegrino *et al*., 2002). Today, antimicrobial resistance is considered a serious ever-increasing problem (Masterton, 2000) and one of the greatest current challenge to the effective treatment of infections with every indication that will become an even greater public health challenge in future (Karlousky and Sahm, 2002).

2.5.1 Origin of Drug Resistance

Antimicrobial resistance encountered in clinical practice settings could be inherent or acquired (Franklin, 1987) or could be non-genetic or genetic (Jawetz *et al.,* 1974). This means resistance may either be a characteristic associated with the entire species or it may emerge in strains of the microorganisms normally susceptible to the antimicrobial agent.

2.5.1.1 *Non genetic or inherent*

Some microorganisms are known to be naturally or innately resistant to certain groups of chemotherapeutic agents (Franklin, 1987). Such innately resistant microorganisms lack the target for a specific antimicrobial agent (Nester *et al*., 1998).Here the inherent properties of the bacterium are responsible for preventing antibiotic action (Godfrey and Bryan, 1984). Microorganisms may lose the specific target structure for a drug for several generations and thus be resistant e.g. penicillin susceptible organism may change to L-forms (protoplasts) during resistant to cell wall inhibitor pump e.g. penicillins, cephalosporins and may remain so for several generations in this form as “persisters” when these organisms revert to their bacterial parent forms by resuming cell wall production, they will then become fully susceptible to penicillin again (Jawetz, 1992; Nester *et al*., 1998).

Gram negative bacteria as a group are known to be resistant to a number of antimicrobial agents that are very effective against the Gram positive organisms, and the vice versa. The inherent resistance of Gram positive bacteria is associated to some drugs thus preventing the attainment of an inhibitory concentration within the cell (Franklin, 1987; Lowbury and Ayliffe, 1974) or preventing the antibiotic from reaching their intracellular target (Rang and Dale, 1994). This type of natural resistance led Ehrlich Shiga to wrongly conclude in 1902 that atoxyl (Sodium arsenilate) was ineffective in the treatment of mouse trypanosomiasis. They had used a strain to trypanosome that was naturally resistant to arsenicals (Collard, 1976).Active replication of bacteria is usually required for most antibacterial drug actions; non-multiplying (metabolically inactive) microorganism may be phenotypically resistant to drugs. However, their offspring are fully susceptible e.g. mycobacteria often survive in tissues for many years after infection yet are restrained by the host's defenses and do not multiply such "persisting" organisms are resistant to treatment and cannot be eradicated by drugs (Rang and Dale, 1994; Jawetz *et al*., 1974).

2.5.2 Genetic Origin

The emergence of most drug resistant microorganism has been attributed to genetic changes and subsequent selection processes by antimicrobial drugs. The rapid multiplication rate and large sizes of bacterial populations confer on such population a remarkable ability to respond to changes in their growth environment. The introduction of a new drug initially marks a victory on the causative organisms that are not inherently resistant to the drug, such that high success rate is reported in therapy (Franklin, 1992). However, reports of treatment failures are soon encountered after months or years of continuoususe of the new drug, as a result of the emergence and spread of resistant strains of the implicated organism (Gold and Moellering Jr., 1996). The acquired ability of the microorganism to thrive in the presence of

the new drugs is a reflection of the genetic composition of the resistant strains which gradually replaced the sensitive strains as a result of the antibiotics selective pressure (Simon *et al*., 1993).

The genetic information in a cell can be altered by many factors including; a change in the nucleotide sequence of DNA (mutation), transfer of sets of genes from other cells and of certain genes (transposable elements) from one location to another in the genome of the same cell (Nester *et al*., 1998). Genetic mechanism may be chromosomal or extra chromosomal.

2.5.2.1*Chromosomal Resistance*

Chromosome mediated resistance occurs by spontaneous mutation in a locus that control susceptibility for the drug. The antimicrobial drug serves as a selecting mechanism to suppress susceptible organism and favour the growth of drug resistant mutation of the cell structure. The properties of a cell depend largely on its proteins, which include both enzymes and proteins that form part of the cell structures such as the proteins in the cytoplasmic membrane and the ribosome (Nester *et al*., 1998). The substitution of even one amino acid for another (change in genetic information) in any given protein may cause the protein to be non functional, thereby changing the properties of the cell.Spontaneous mutation is not a frequent cause of the clinical drug resistance in a given patient, but occurs with high frequency to rifampicin. Mutation can result in the loss of PBPs, making such mutants resistant to β-lactam drugs. An example is resistance of *Mycobacterium tuberculosis* to rifampicin is caused by mutation in RNA polymerase and that to isoniazid by mutation in catalase.

2.5.2.2 *Extra Chromosomal*

Bacteria may also acquire extra ch romosomal genetic elements called plasmids. A plasmid is a circular body of double stranded DNA which is separate from the chromosome and carries genes that encode various tracts such as virulence and antimicrobial resistance (Akkina and Johnson, 1999). Plasmid mediated drug resistance is more common than that of chromosome; R factors (drug resistance plasmid) are a class of plasmid that carried genes for resistance to antimicrobial drugs. A single plasmid can carry genes that code for resistance to several drugs (multi drug resistance MDR) such as streptomycin, chloramphenicol, tetracycline and sulphonamides. Plasmid genes control the formation of enzyme capable of destroying the antimicrobial drugs. E.g. β-lactamases destroy β-lactam ring of penicillins and cepholosporins.

There are two types of plasmids based on their ability to transfer from one bacterium to another. The self-transmissible termed conjugative plasmids can transfer to other bacteria via sex pili while the non-conjugative termed transducible plasmids are not capable of transfer.

Table 2.1: Various transfer elements involved in the spread of resistance genes and their characteristics

Element	Characteristic	Roles in spread of resistant genes
Self-transmissible Plasmid	Circular, autonomously replicating, element; carries genes for conjugal DNA transfer	Transfer of resistance genes, mobilization of other elements that carry resistance genes
Conjugative transposon	Integrated element that can excise to form non-replicating circular transfer intermediate; carries genes for conjugal DNA transfer	Same as self-transmissible plasmid
Mobilizable Plasmid	Circular, autonomously replicating element; carries genes that allow it to use conjugal apparatus provided by a self-transmissble plasmid	Transfer resistance genes
NBV	Integrated elements that cannot excise or transfer themselves, can be triggered to excise and transfer by conjugative transposons; transfer intermediate is a non-replicating circle carrying a gene that allows the NBV to take advantage of the conjugal transfer apparatus of a conjugative transposon	Transfer of resistance genes
Transposon	Can move from one DNA segment to another within the same cell	Can carry resistance genes from chromosome to plasmid and vice versa
Genetic Cassette	Circular, nonreplicating DNA segment containing only open reading frames; integrates into integrons	Carries resistance genes
Integron	Integratd DNA segment that contains an integrase, a promoter, and an integration site for gene cassettes	Forms cluster of resistance genes, all under the control of the integron promoter

The table above outlined the various gene transfer elements and the characteristics of the different elements involved in the spread of resistance genes (Salyers and Amabile-Ceuvas, 1997).

2.5.2.3 *Transfer of genetic information*

Another mechanism responsible for the acquisition of antibiotic resistance is the transfer of genetic information. Chromosomal or plasmid DNA containing resistance genes can be transferred from bacteria to bacteria of the same or different species and genus (Top *et al*., 2000; White and McDermott, 2011). The intercellular transfer genetic materials may occur by conjugation, transduction and transformation.

Conjugation: This is the most common and important of these mechanisms for the transfer of antimicrobial resistance. In most cases of conjugation the transferable DNA is plasmid, but chromosomal DNA may also be transferred. Conjugation involves cell to cell contact during which chromosomal or plasmid DNA is transferred from one bacterium to another (Stearns and Hoekstra, 2005). The ability to conjugate is encoded in conjugative plasmids; these are plasmids that contain transfer genes which code for the production, by the host bacterium of surface tubules of proteins that connect the two cells "sex pili", the conjugative plasmid then passes from one bacterium to the other which is usually of the same species. Some plasmids can cross the species barriers. Many R plasmids are conjugative. Non conjugative plasmids can make use of sex pili if they co-exist in the "donor" cell with conjugative plasmids (Campbell and Reece, 2002).

Transduction: is a process by which plasmid DNA is enclosed in a bacterial virus (or phage) and transferred to another bacterium of the same species (Stearns and Hoekstra, 2005). Transfer of gene for β-lactamase production is mediated by bacteriophage. Transduction is a relatively ineffective means of transfer of genetic materials but there is evidence that it is clinically important in the transmission of resistance genes between strains of staphylococci and between strains of streptococci.

Transformation: It is a natural occurrence and is a direct uptake of donor DNA by recipient cells. The bacterium takes up naked DNA from its environment and incorporates it into its genome. It is possible only when the incoming DNA comes from a cell belonging to the same strain as the host bacterium or one that is very closely related.

2.6 BIOCHEMICAL MECHANISM OF RESISTANCE

Resistance in bacteria arises through a process from low level to high level except a plasmid is acquired which already contains genes for full blown resistance (Levy, 1998). Bacterial resistance to a particular antibiotic or related series of drugs usually depends on a single isolate leading to higher levels of resistance (Hawkey, 1998).

The alteration in genetic composition of the bacteria as a result of either mutational changes or acquisition of any of the resistance gene transfer elements are expressed through various biochemical mechanisms that protect the bacteria from killing effects of antimicrobial agents.

2.6.1 Enzymatic Inactivation Of The Antibiotic

Enzyme inactivation of the antimicrobial agent remains the predominant biochemical method of resistance to a wide variety of antibiotic structural types (Anon, 2001). Resistance by enzyme inactivation involves the hyper production of an enzyme that inactivates the antibiotic by an enzymatic cleavage or chemical modification such that they no longer interact with the target site or are no longer taken up by the organism (Powell, 2000). The most common example of the type of resistance is that mediated by β-lactamase which are widely distributed in both Gram positive and Gram negative species (Hawkey, 2000).The antibiotics concerned are β-lactam ring of penicillins and cephalosporins. The four membered, strained lactam ring is the chemically activated functional moiety in the β-

lactams that acylate and irreversibly modify the cell wall crosslinking PBPs (Walsh, 2000; Yoneyama and Katsumata, 2006). Hydrolytic deactivation opens the β-lactam ring producing a product that is nonfunctional as a PBP pseudo substrate and useless as an antibiotic. This enzymatic weapon is secreted into the periplasmic space by the β-lactamase producing strains before reaching the site of action, the PBPs in the cytoplasmis membrane. It has been estimated that a single β-lactamase molecule can hydrolyse 10^3 penicilling molecules per second *(Walsh, 2000).*

Staphylococci are the principal bacteria producing β-lartamase and the genes which code for the enzymes are on plasmids that are transferred by transduction. The penicillinases increasing in occurrences from only above 5 % of *Staph.aureus* isolates when benzyl penicillin was introduced, to 80 to 90 % of isolates, both *Staph. aureus* and the coagulase negative staphylococci through plasmid transfer and strain selection (Livermore, 1995). However, plasmids encoding staphylococcal penicillinase have spread to *Enterococcus faecalis* isolates (Murray, 2000).

In this bacterium the enzymes is inducible, its synthesis is at a very low level in the absence of the drug. The enzyme may diffuse through the envelope and inactivate antibiotic molecules in the surrounding medium. Gram negative organisms can also produce β-lactamases, which are a significant factor in their resistance to the semisynthetic broad spectrum β-lactam antibiotics. Here the enzyme may be determined by either chromosomal genes or by plasmid genes. The enzymes are produced constitutively (i.e. they are synthesized even when the substrate is absent and remain attached to sites in the cell wall preventing access of drug to the membrane associated target site, they do not inactivate the drug in the surrounding medium. Many of these β-lactamases are encoded by transposons some of which may also carry resistance determinants to several other antibiotics.

Other antibiotic classes, such as the aminoglycosides which do not possess such hydrolytically labile groups (like the β-lactam ring) can still be neutralized by deactivating the periphery of the aminoglycosides with three types of chemical substituents, thus interrupting the binding of the RNA targets in the ribosome. Inactivation of aminoglycosides may be brought about by three different classes of enzyme by phosphorylation, adenylation and acetylation. The bacterial enzyme transfer an acetyl group (derived from acetyl coenzyme A) an adenylyl group or a phosphate group (both derived from ATP). The aminoglycoside modifying enzymes are therefore broadly classified as N-acetyl transferases (AACs), O-adenyl transferases (ANTs) or O-phosphotransferases (APHs), and together comprise over 50 different enzymes which are further divided into subtypes according to the position on the drug at which the modification occurs (Smith and Baker, 2002).

Many Grams-positive pathogens possess a usually bi-functional aminoglycoside modifying enzyme (Davies, 1994). The best known O-adenyltransferase is ANT (4^1) la from *Staph. aureus* which confers resistance to a variety of aminoglycosides (Dessen *et al.*, 2001).

2.6.2 Alteration Of The Target Site

The protein on the 30s subunit of the ribosome, which is the binding site for aminogylcosides, may be altered as a result of a chromosomal mutation. A plasmid mediated alteration of the binding site protein on the 50s subunit underlies resistance to erythromycin. Many of the antibiotics inactivate specific enzyme in the microorganism or the ribosome as in the case of a large number of protein synthesis inhibitors. The antibiotic would need to bind to the target receptor molecule in the bacteria. By altering the target receptor molecule, the antimicrobial is unable to bind and therefore does not have any effect.

Apart from the mutation leading to a target protein being unable to bind the antibiotic, in some situation the target receptor retains its function even after formation of the

complex (Powell, 2000). Example of such mechanisms abound. The ribosome of staphylococci can become insensitive to erythromycin following specific enzymatic modifications of rRNA (Davies, 1994). This sequence-specific methylation of the 23S molecule is one of the best known mechanisms for developing macrolide and resistance and it is catalyzed by methyltransferase enzymes Erm (erythromycin resistance methylase) that does not impair protein synthesis. The Erm mechanism is said to be the main route in drug-resistant clinical isolates of *Staph. aureus* and is present in organisms which originally produce erythromycin; *Streptomyces erythreus* as a self-immunity mechanism (Walsh 2000; Dessen *et al*., 2001).

Macrolide-lincomycin resistance in clinical isolates of *staphylococci* and *streptococci* is believed to have resulted from a biochemicalmodification (methylation) in the 50S ribosomal subunit RNA thus reducing binding. Rifampicin resistance results from inactivation of a single target and requires a single aminoacid change in the subunit of the DNA-directed RNA polymerase (Spratt, 1994).

Unlike resistance genes which are shared between bacterial species, rifampicin resistance can result from a single mutation and is a good example of how mutation that confer antibiotic resistance are conserved (Powell, 2000). Resistance to flouroquinolones is frequently associated with mutations in the "quinolone resistance-determining region" (QRDR) of the A subunit of DNA gyrase, the target molecule (Jenkins, 1996).

Some *staphylococci* carry an altered penicillin binding protein (PBP2a) which is coded for by a mutated chromosomal gene; it has much lower affinity for penicillins and it confers intrinsic resistance (Powell, 2000). The resistance is believed to occur as a result of the acquisition of a chromocomal gene (mecA) encoding the new target enzyme penicillin binding protein 2a (PBP2a) that has much lower affinity for the antibiotic. PBP2a mediates

clinically relevant resistance to all β-lactam antibiotics and more specifically, methaicillin resistance in *Staph. aureus*.

Altered PBPs are the cause of resistance in some strains of *Streptococcus pneumonia* to penicillin G. Under susceptible conditions, vancomycin prevents cross liking of peptidoglycan by binding to D-ala-D-Ala dipeptide of the muramylpetide. Most Gram positive bacteria acquire vancomycin resistance by changing D-Ala-D-Ala to D-Ala-D-lactate, which vancomycin does not bind to (Bugg *et al*., 1991), the enzymes responsible are VanA, VanB and VanD on the other hand, two of the enzymes VanC and VanE replace the terminal D-ala with D-ser, resulting in low level vancomycin resistance (Park *et al*., 1997; Yoneyama and Katsumata, 2006).

2.6.3 Decreased Concentration Of Drug At The Target Site

Decreasing the accessibility of the antimicrobial agent to the target site can be achieved by either altering entry of the antimicrobial into the cell (decreased uptake or influx) or increasing the removal of the antimicrobial from the cell (increased efflux) (Yoneyama and Katsumatga, 2006). A third possibility is that the antimicrobial can be altered in its passage across the microbial membrane such that it cannot bind its target (Powell, 2000). Efflux pumps are membrane proteins that have the function of detoxifying cells by expelling noxious molecules (Pantosti *et al.,* 2007). In *Staph.aureus*, several specific efflux pumps have been associated with resistance to antibiotics, such as tetracycline (Tet K), (Tet L) and macrolides (mef A), (Msr A) (Poole, 2007).

In general, specific efflux pumps can be found either in the chromosome or in plasmids, while multidrug efflux pumps are mainly located in the chromosome (Sofia *et al*., 2013). Tetracycline resistance is believed to be as a result of decreased in the levels of drug

accumulation caused by decreased uptake and increased efflux of the drug molecule. This resistance is usually plasmid mediated in both Gram positive and Gram negative bacteria.

Tetracycline efflux was discovered in the early 1980s tetK serves as an example for an efflux-mediated tetracycline resistance. Under normal conditions, the efflux gene tetk, is not expressed, due to a suppressor that is bound to promoter region. However, in the presence of tetracycline, it binds to the repressor, relieves the suppression, and causes transcription and translation of the efflux pump, thereby leading to tetracycline resistance (Krzysztof *et al*., 2000). Plasmid containing tetracycline resistance markers are known to move among members of the enterobacteriaceae and have been observed between *Staph.aureus, Staph. epidermidis, Streptococcus pneumonia* and *S. faecalis.*Bacteria that acquired the ability to actively pump antibiotic out across the cytomplasmic membrane, the drug is actively pumped out faster than it can diffuse in, so the concentration of the antibiotic remains too low to be effective. The mechanism referred to as active efflux, which may be drug specific or multidrug efflux pump is believed to play an increasingly important role in the resistance to many different antibiotics, including macrolides, quinolones, β-lactams and chloramphenicol (Kaye *et al*., 2000; Levy, 1992).

The ability of resistant organism to alter the compound in their periplasmic space by bacterial enzymes that acetylate, phosphorylate or a denylate aminoglycosides has been attributed to resistance in aminoglycoside (Kaye *et al*., 2000). The enzymatic alteration of the compound leads to binding to the bacterial ribosomes and poor intake into the cell. Research has shown that the genes coding for aminoglycoside altering enzymes are often found on transposons that have been identified in members of the enterobacteriaceae, and *Pseudomonas aeruginosa, Streptococcus pneumonia* and gram positive species such as *Staph. aureus, S. faecalis,* and *S. pyogenes* (Kaye *et al.*, 2000).

2.6.4 Failure To Metabolize The Drugs

Whenthe activity of a drug depends on its conversion by the bacteria to the active form; mutants that do not metabolize the drug will be resistant (Pratt and Taylor, 1990). *Bacteroides fragilis* that do not metabolize nitro-imidazole metronidazole to the active metabolite are resistant to the drug (Powell, 2000).

2.6.5 Metabolic Bypass

An important example of this type of resistance is found in the thymidine requiring streptococci that are not inhibited by trimethoprim and sulphonamides due to the production of adequate concentration of thymidine nucleotides by an alternative pathway (Powell, 2000).

2.7 ANTIBIOTIC RESISTANCE IN *STAPH. AUREUS*

Resistance of *Staph.aureus* to antibiotics was presumed to have occurred in four waves, the last being the emergence of the community acquired methicillin resistant *Staph. aureus*(Lowy, 2003). Prior to the use of antibiotics to treat *Staph.aureus* infections, resistant strains outside of hospital setting were rarely encountered (Ayliffe, 1997).

After World War II and following widespread use of antibiotics penicillin resistant *Staph.aureus* prevalence increased. Resistance to penicillin is associated with production of penicillinase, a predominantly extracellular enzyme that hydrolyses the β-lactam carrying antibiotics encoded by blaZ gene controlled by two adjacent regulatory genes, the antirepressor bla RI and the repressor blaI (Lowy, 2003). A great majority of staphylococcal isolates now produce penicillinase, regardless of the setting. The gene for β-lactamase is part of a transposable element located on a large plasmid often with additional antimicrobial

resistance genes (genes for gentamycin and erythromycin) and spread of penicillin resistance primarily occurs by spread of resistance strains (Lowy, 2003).

The prognosis of patient with staphylococcal infections was dramatically improved by the introduction of penincillin in the early 1940s. However, penicillin resistant *staphylococci* emerged shortly after the introduction of the antibiotic, first in the hospitals and subsequently in the community. This pattern of resistance, which first emerged in the hospitals, then spread to the community is known as well-established pattern that recurs with each new wave of antimicrobial resistance (Chambers, 2001).

The introduction of methicillin in 1959 led to a drop in the prevalence of penicillin resistant *Staph. aureus* (Haley *et al.,* 1982). However, in less than a year of its introduction methicillin resistant *Staph.aureus* was identified (Chambers, 2001; Deleo *et al*., 2009). In addition to use of antibiotics in humans, antibiotic use in animals for therapeutics, for food production, and disease prevention has also promoted antibiotic resistance in human (Ndi and Barton, 2012). Under doses of antibiotics to food producing animals can result in bacterial resistance in livestock, elevating the potential for resistant bacterial strains to cross species boundaries, especially livestock imported from countries where antibiotic use is indiscriminate (Smith, 2005).

What makes *Staph.aureus* a dangerous pathogen is the combination of antibiotic resistance and high virulence . The relative ease with which *Staph. aureus* exchanges genetic material encoding antibiotic resistance and virulence determinants amongst strains and other species such as *Staph.epidemidis*suggest an emerging hyper virulent, multidrug-resistant superbug (Davies and Davies, 2010).

As a "superbug" *Staph.aureus* has higher morbidity and mortality due to multiple mutations encoding it with high levels of resistance to different antibiotic classes specifically

recommended for their treatment. The complexity and sophistication of the staphylococcal genome has enabled it to change and adapt to varying circumstances such as exposure to a new antibiotic, adherence to a clinical device and transfer from an animal to human host (Humphreys, 2012).

Staph.aureus is unlikely to show a change in sensitivity to a drug administered for a single short course, unless the mutation rate of resistance to that drug is very high, like in the case of streptomycin (aminoglycoside) and erythromycin (macrolide) (Garrod *et al*., 1973). Generally, *Staph.aureus* is less sensitive to erythromycin than are pneurmococci or haemolytic streptococci and rapid development of resistance has been observed, especially of staphylococci in vitro (Russell, 2004). It was noticed in vivo that resistance is usually not a serious clinical problem with short course of treatment with erythromycin but resistance is more likely to develop with prolonged use.

2.7.1 Multidrug Resistant *Staph. aureus*

The problems posed by the increasing spread of multidrug resistant *Staph. aureus* (MDR-SA) in clinical setting is compounded by spectacular adaptive capacity of this pathogen resulting in the emergence and worldwide spread of lineage that acquired resistance to the majority of available antimicrobial agents, narrowing choice of therapy to a few antibacterial agents, among them the glycopeptide antibioticvancomycin, which has become the mainstay of therapy worldwide (Mwanji *et al*., 2007).

The environment and especially freshwater, constitute ambulance for the evolution and rise of new resistance that provides ‘environmental-hot spots’ for antibiotics and other pollutants from different sources, environmental species with intrinsic antibiotic resistance mechanism and bacteria from different antibiotic sources to interact to acquire resistance

determinants which may involve phages and integrons and introducing same into clinics (Lupo, *et al*., 2012).

The 1950s were marked by an increased prevalence of both virulent and multiple-antibiotic resistant *Staph. aureus*. Since, the 1960s, however, there has been an improvement in this position through the interaction of several agents that were less subject to less of activity through the emergence of resistant variants (Garrod *et al*., 1973). The most important of these agents are the semisynthetic penicillinase stable penicillins (Methicillin, cloxacillin oxacillin and nafcillin) and the cephalosporins (cephalothin, cephaloridine and cephalexin) (Al-Masaudi *et al.,* 1991).

A series of new antibiotics; streptomycin, the tetracycline chloramphenicol, erythromycin and novobiocin, effectiveagainst *Staph. aureus* were introduced during the period of increasing prevalence of penicillin-resistant penicillinase producing *Staph. aureus* (Lowbury and Ayliffe, 1974). The increased use of these agents in the treatment of infection causes by the penicillin-resistant staphylococci was soon followed by the emergence of strains resistant to these antibiotics (Shanson, 1981) after periods of time that varied with the type of antibiotics and with the amount of time that was used.

Resistance to erythromycin developed rapidly, with resistant strains spreading fast in hospital where the antibiotic was used. Resistance was also readily produced to novobiocin but it was not often seen because the antibiotic was little used (Garrod *et al*., 1973; Lowry, 2003). *Staph.aureus* is predominant in most developed countries where they occur as multidrug resistant pathogen (Enright *et al.,* 2000). Multidrug resistant bacteria such as MRSA are endemic in healthcare environment and serves as a potential source for outbreaks in these settings (Henderson, 2006). MRSA is said to be multidrug resistant because of its non-susceptibility to at least one antimicrobial agent in three or more categories and whose

resistance to oxacillin or cefoxitin predicts non-susceptibility to all categories of β-lactam antimicrobials with the exception of the anti MRSA cephalosporins (Magiroakos *et al*., 2012).

The improved medicare due to antibiotic discovery in the 20th century has been eclipsed by the upsurge of antibiotic resistance in hospitals, community and the environment attributed to extraordinary genetic versatility of microbes that have capitalized anthropogenic activities to utilize every sources of resistance genes and horizontal gene transfer mechanism to develop multiple mechanisms of resistance for each and every antibiotic introduced into clinical and agricultural practice (Davies and Davies, 2010). Antimicrobial resistant in *Staph.aureus* is a major public health threat, compounded by emergence of strains resistance to vancomycin and daptomycin; both last line antimicrobials (Howden *et al*., 2011).

The huge prescription of antibiotics and their uncontrolled extensive usage has resulted in the spread of antibiotic resistance in microorganisms (Lamikanra *et al*., 1985; Davies and Davies, 2010). Antibiotic use creates conditions that allow bacteria with mechanism for resistance to compete better. These mechanisms arise not only by mutation of microbial genes which code for antibiotic uptake into cells or the binding sites for antibiotics, but by horizontal spread or longitudinal transfer of resistance determinants (Kayser, 1993) and has accounted in part to antibiotic resistance and virulence among staphylococcus (Chan *et al*., 2011).

2.7.2 Staphylococcal Cassette Chromosome

Staphylococcal cassette chromosomes (SCCs) are relatively large fragments of DNA that always insert into the orfX gene on the *Staph. aureus* chromosome and can encode antibiotic resistance and/or virulence determinants (Malachowa and Deleo, 2010). It is a

mobile genetic element that carries the central determinant for broad spectrum beta-lactam resistance encoded by the MecA gene. The emergence of methicillin resistant staphylococcal lineage is due to the acquisition and insertion of the SCC mec element into the chromosome of susceptible strains.

The SCC mec types differ from one another by the number of genes in their architecture (Hiramatsu *et al.,* 2001). Some SCCmec are carriers of resistance genes that are determinants of multiple antibacterial drugs which include β-lactam antibiotics, macrolides, lincosamides, streptogramins, aminoglycosides and tetracycline and bacterial cell acquiring such SCCmec, acquires a multiple resistance phenotype (Ito *et al.,* 2004).

SCCmec type I, IV, V and VI encode resistance to β-lactam antibiotics only, while SCCmec type II and III carry multi-resistant genes some on plasmids and transposons (Deurenberg, *et al*., 2007). The initial reservoir of SCCmec is not very clear but is suspected to have arisen from coagulase-negative staphylococcal species (Gordon and Lowy, 2008).

SCCmec may have evolved from a primordial mobile element, staphylococcal cassette chromosome (SCC) into which the mec complex was inserted and it is likely that SCCmec serves as the carrier of the mecA gene moving across staphylococcal species, since mecA genes in other staphylococcal species have never been found without the accompaniment of SCC mec like structure.

The SCC mec element type has been defined by the use of the combination of ccr allotype and mec class. In MRSA strains, several types of SCCmec elements have been reported and these SCCmec elements have been further classified by differences in regions other than – ccr and mec, which are designated junkyard (j) regions. These constitute non essential component of the cassette, but some cases these regions carry additional antibiotic resistance determinants (Zhang *et al*., 2005; Milherico *et al*., 2007).

The J regions comprise three parts; J1 (the region between ccr and the right junction chromosome, J2 region from the mec complex to the left extremity orfX (Chongtrakool *et al.,* 2006). Structurally, the SCCmec elements typically share four characteristics; first, they carry the mec gene complex consisting of the methicillin resistance determinant mecA and its regulatory gene system (mec1 and mec R1 or Dmec1) and insertion sequence, IS.

Second they carry the ccr gene complex, consisting of the ccr genes (ccrA and ccrB, or ccrC in type VSCC mec) that encodes recombinases responsible for the mobility (Insertion and excision) of SCCmec element and its surrounding sequence (Mitheirico *et al.*, 2007; Pantosti *et al*., 2007).

Third they have characteristic directly repeated nucleotide sequences and inverted complementary sequences at both ends (Kondo *et al*., 2007).

Lastly, they integrate into the 3 ^{1} end of an open reading frame (ORF), orfX (Chongratakool *et al.*, 2006).

Eleven SCCmec elements are reported to date SSC mec I to XI (Ito *et al*., 2012; Li *et al*., 2011). Among these, SCCmec type I-V are the most commonly reported (Shore *et al*., 2005; Olivera and de Lencastre, 2002).Three types of SCC mec (I, II and III) are carried mostly by healthcare- associated MRSA strains throughout the world (Enright *et al*. 2002; Sola et al., 2006). While type IV and V are widely disseminated among community acquired MRSA infections (Ito *et al*., 2004); Vandenesh *et al*., 2003; Sola *et al*., 2006).The SCC mec IV and V allotypes are smaller thanthe other SCCmec element I, II and III, more genetically mobile being readily transmissible between staphylococci and does not at present carry additional antimicrobial resistance genes (Ma *et al*., 2002; Lowy 2003; Ito *et al*., 2004).

2.8 METHICILLIN RESISTANCE *STAPH. AUREUS*

2.8.1 History And Epidemiology

Methicillin, introduced in 1961 was first of the semisynthetic penicillinase-resistance penicillins. Its introduction was rapidly followed by reports of methicillin resistant isolates (Jevons, 1961). For clinicians, the spread of these methicillin resistant strains has been a critical one. The therapeutic outcome of infections that result from methicillin resistant *Staph.aureus* (MRSA) is worse than the outcome of those the result from methicillin sensitive strains (Cosgrove *et al*., 2003). First reported in British hospital, MRSA clones rapidly spread across international borders. Once identified in a new setting, these unique MRSA clones rapidly spread, often becoming the resident clones and accounting for an increasing percentage of nosocomial infections (Panlilo *et al*., 1992; Couto *et al*., 1995).

Like the penicillin-resistant strains, the MRSA isolates also frequently carried resistance gene to other antimicrobial agents (Lyon *et al*., 1984). The spread of MRSA is reminiscent of the emergence of penicillin resistance in the 1940s. First detected in hospitals in the 1960s, methicillin, resistance is now increasingly recognized in the community (Chamber, 2001). While many of these infections occurred in patients with some antecedent hospital experience, recently there have been an increasing number of subjects with no prior hospital exposure. These community based infections have been reported in the patient from both rural and urban settings (Moreno *et al*., 1995; Groom *et al*., 2001). The prevalence of MRSA reported differs widely among different countries and regions of the world (Lowy, 1998; Kesah *et al*., 2003; Akpaka *et al*., 2006).

The evolutionary origins of MRSA are poorly understood and there is no consensus on the nomenclature, the number of major MRSA clones, or the relatedness of clones described from different countries (Enright *et al*., 2002). The over 3,000 MRSA isolates from

different regions of the world characterized, as reported by de Sousa and de Lancastre, (2004), confirms the existence of only a few epidemic clones worldwide, namely the Iberia, Brazilian, Hungarian, New York/Japan and Paediatric clones. This suggests that acquisition of the staphylococcal cassette chromosome SCC mec, the mobile genetic elements harboring the mecA gene that confer methicillin resistance, has been a rare event (Brurec *et al*., 2011). The evolutionary changes of MRSA have resulted in its continuing threat to public health. The ever increasing burden of MRSA infections in hospitals has led to the increased use of vancomycin, the last remaining antibiotic to which MRSA strains were reliably susceptible (Chambers and Deleo, 2009).

2.8.2 Evolution Of MRSA

The understanding of the evolution of MRSA has been aided immensely by the development of molecular methods that provide characterization of both the strain phylogeny (evolutionary history) and the methicillin resistance determinants (Grundmann *et al*., 2006a). Resolution of MRSA strain on the basis of its sequence at seven housekeeping genes (Enright *et al*., 2000; Feil *et al*., 2003) and by whole genome typing methods, including amplified fragment length polymorphism (AFLP) which documents nucleotide sequence variation, insertions and deletions across genome (Melles *et al*., 2006) have provided consistent molecular epidemiological evidence that supports the view that the evolution of MRSA and of *Staph. aureus* as a species is predominantly clonal (Enright *et al*., 2002; Grundmann *et al*, 2006).

However, horizontal transfer of DNA from other strains or species has been documented and plays an important part in the resistance acquisition in *Staph. aureus* (Ito *et al*., 2003; Robinson and Enright, 2004). This horizontal transfer is brought about mainly by the insertion sequences (IS), transposons, prophages and other incompletely understood

events (Grundmann, *et al.*, 2006). It is believed that, MRSA strains have emerged from the introduction of a large mobile genetic element, staphylococcal cassette chromosome mec (SCC mec) into a prevalent methicillin susceptible *Staph. aureus* strain that continue to flourish (Enright *et al*., 2002; Foster, 2004).

2.8.3 Mechanism Of Methicillin Resistance

Methicillin resistance requires the presence of the chromosomally localized mecA gene (Chamber, 1997; Kernodle, 2000). mecA is responsible for synthesis of penicillin binding protein 2a (PBP2a also called $PBP2^{1}$) a 78KDa protein (Lowy, 2003). PBPs are membrane-bound enzymes that catalyzed the transpeptidation reaction that is necessary for cross-linkage of peptidogylycan chains (Ghuysen, 1994). Their activity is similar to that of serine proteases, from which they appear to have evolved.

PBP2a substitutes for the other PBPs and, because of its low affinity for all β-lactam antibiotics, enable staphylococci to survive exposure to high concentrations of the agents. Thus resistance to methicillin confers resistance to all β-lactam agents recent studies determined the crystal structure of a soluble derivative of PBP2a.

PBP2a differs from other PBPs in that its active site blocks binding of all β-lactams but allows the transpeptidation reaction to proceed (Lim and Strynadka, 2002). Phynotypic expression of methicillin resistance is variable and each MRSA strain has a characteristic problem of the proportion of bacterial cells that grow at specific concentrations of methicillin (Thomasz *et al.*, 1989). Expression of resistance in some MRSA strains is regulated by homogues of the regulatory genes for blaz. These genes, mecI and mecRI, regulate the mecA response to β-lactam antibiotics in a fashion similar to that of the regulation of blaZ by the genes blaR1 and blaI upon exposure to penicillin.

Rosato *et al.,* have recently found that either mecI or blaI must be functional in all MRSA, and they suggest that this may be a protective mechanism preventing over production of a toxin protein. An additional series of genes, the fem genes (factor essential for essential to methicillin resistance, play a role in crosslinking peptidoglycan strands and also contribute to the heterogeneity of expression of methicillin resistance (Berger, 1994).

Most clinical isolates exhibit the heterogeneous pattern of resistance under routine culture condition. However, heterogeneous strains can appear homogenous (i.e. 1 % or more of the cell grow at 50mg of methicillin per ml) under certain culture conditions, such as growth in hypertonic culture medium supplemented with Nacl or sucrose or incubation of 30^0C (Sabath and Wallace, 1974; Chambers 1997). Addition of EDTA (PH 5.2) or incubation at 37 ^{0}C to 43 ^{0}C favours a heterogeneous pattern and may entirely suppress resistance and its detection. The changes in expression of methicillin resistance with different culture conditions are known to be transient and entirely phenotypic. Methicillin resistance express is seen in the borderline (or low level) methicillin-resistant strains of *Staph.aureus.* Such borderline methicillin resistance (BORSA) strains are characterized by methicillin MICs equal or just above the susceptibility break point (e.g. Oxacillin MICs of 4.8mg/ml) (Chambers, 1997., Brown *et al*., 2005). Borderline strains can be divided into two categories on the basis of whether mecA gene is present, BORSA strains that contain mecA are extremely heterogeneous methicillin resistant strains that produce PBP2a. These strains have a small resistant sub-population of cells that can growth at high drug concentration (Chambers, 1997).

The second category of BORSA is those that do not contain mecA gene. These can be differentiated phenotypically from extremely heterogeneous mecA-positve BORSA strains in that highly resistant clones do not occur in the population of cells (Chambers,

1997). The hypothesis is that BORSA in mecA negative strains is either as a result of modification of normal PBP genes or overproduction of staphylococcal β-lactamase (Chambers *et al*., 1989; Thomsz *et al*., 1989).

2.8.4.1 ***Altered penicillin Binding Protein (PBP2a)***

MRSA differ generally from methicillin-sensitive *Staph. aureus* isolates by the presence, in the chromosome of large stretch of foreign DNA (40-60kb), referred to as the mec element, the presence of the mecA gene that encodes the 76 KDa penicillin binding protein, PBP2a (also referred to as $PBP2^{1}$). The mecA gene has been proposed to originate from *Staph.sciuri* (Wu *et al*., 2001). Althought the mechanism of gene acquisition from this specie is not known, two genes, ccrA and ccrB, present on the mec element from one isolate, have been shown to code for recombinase proteins that are capableof excising and integrating the mec element into the chromosome (Katayama *et al*., 2000). In common with other PBPs, PBP2a has the common structure motifs that are associated with penicillin binding yet its affinity of β-lactam antibiotics is greatly reduce. Consequently, at the therapeutic levels of methicillin that would inhibit the transpeptidational activities of other PBPs, PBP2a remains active ensuring the crosslinking of the glycan in peptidoglycan. PBP2a is not able to completely compensate for the other PBPs since cells grown in the presence of methicillin exhibit a marked reduction in the degree of crosslinking. However, the limited degree of crosslinking is enough to ensure survival of the cell.

2.8.4.2 ***Regulation of PBP2a expression***

Adjacent to mecA on the staphylococcal chromosome are two genes, mecR1 and mecI, that are co-transcribed divergently from mecA. The mecR1 gene encodes a membrane-bound signal transduction protein (MecR1) while mecI encodes a transcriptional regulator (MecI). Between mecA and mecR1 are the promoters for these genes and an operator region

that encompasses the -10 sequence of mecR1 (Sharma *et al*., 1998). MecR1 and MecI have high protein sequence homology with the proteins, B1aR1 and B1aI, respectively are involved in the inducible expression of the plasmid-mediated staphylococcal β-lactamase gene, blaZ. The arrangement of the genes coding for B1aR1 and BlaI resembles the mecA system suggesting that mecA may have acquired the regulatory genes of the blaZ system sometime in the past (Song *et al.*, 1987). The operator regions are similar enough to allow BlaI to regular PBP2a expression (Gregory *et al*., 1997). Consequently, the presence of a plasmid carrying the blaZ regulatory genes can render PBP2a expression inducible under the control of BlaR1 and BlaI, a situation that commonly occurs in clinical isolates of MRSA (Hackbarth and Chambers, 1993).

The nature of the signaling system of inducible β-lactamese expression has been elucidated (Zhang *et al*., 2001). BlaI, a DNA binding protein, binds to the operator region as a homodimer and represses RNA transcription from both blaZ and BlaR1-blal. Consequently, in the absence of aβ-lactam antibiotic, β-lactamase is expressed at low levels. Bla1, present in the cytoplasmic membrane, detects the presence of the β-lactam by means of an extracellular penicillin-binding domain and transmits the signal via a second intercellular zinc metalloprotease signaling domain. Binding of β-lactam to BlaR1 stimulates the autocatalytic conversion of the intracellular zinc metalloprotease domain of BlaR1 from an inactive proenzyme to an active protease (Zhang *et al*., 2001). The activated form of BlaR1 is thought to directly or indirectly cleave Blal resulting in fragments that are incapable of forming dimers and binding DNA (Gregory *et al.*, 1997). Without Blal bound to the operator site, transcription of both blaZ and blalR1-blal can commence and β-lactam resistance can be conferred through β-lactamase synthesis.

An additional gene product BlaR2, also regulates β-lactamse synthesis, although the role of this protein has not been elucidated. Whether there are other proteins involved in the signaling system also remains to be determined. Unlike β-lactmase synthesis, expression of PBP2a is not strongly inducible in isolates carrying the normal regulatory genes (mecA and mecR1-mecI) and induction is much slower (15 minutes for β-lactmase expression compared to up to 48 hours of PBP2a synthesis). This is because MecI is a tight regulator of mecA transcription (Kuwahara-Arai *et al*., 1996) and most isolates, referred to as pre-MRSA, are methicillin-sensitive despite carrying the mecA gene. However, selective pressure through antibiotic usage has promoted *Staph. aureus* isolates that have mutations or deletions in mecI or the mecA promoter/operator region giving rise to an inactive repressor and constitutive PBP2a expression (Kobayashi, *et al*., 1998).

2.9 DETECTION OF METHICILLIN RESISTANT *STAPH. AUREUS*

Recently, the British society for antimicrobial chemotherapy, the hospital infection society, and the Infection Control Nurses Association published guidelines for the laboratory diagnosis and susceptibility testing of MRSA (Brown *et al*., 2005). Routine identification of *Staph.aureus* should be performed via tube coagulase tests or latex agglutination tests; routine use of molecular test for identification is not currently practical.

However, molecular testing may be useful when there is a high index of suspicion for MRSA. Susceptibility testing may be preformed through any standard recognized method, and latex methods to detect PBP2a and/or PCR tests to detect mecA gene can provide confirmation of equivocal results (Brown *et al*., 2005).

Disc diffusion (DD) testing with cefoxitin has been well correlated with the presence of mecA – mediated oxacillin, resistance in *Staph. aureus* and has excellent sensitivity (98

%) and specificity (100 %).Swenson *et al*., (2007) identified cefoxitin DD breakpoint of 19mm (resistant) and 20mm (susceptible) for *Staph. aureus*. These authors also reported that the cefoxitin DD test gave results equivalent to oxacillin both microdilution tests and oxacillin DD tests but was easier to interpret and did not require transmitted light for identification of resistance. On the basis of this study, the Clinical Laboratory Standards Institute (CLSI) now recommends the use of the cefoxitin DD test for detecting methicillin resistane in *Staph.aureus,* and the method has been validated in an international collection of staphylococci from the sentry antimicrobial surveillance programs (Pottumarthy *et al*., 2005).

Two novel molecular detection method are worthy of mention, although they are not ready for routine clinical use. The 3-dimensional microarray system described by Nagaoka *et al,* (2005) has sensitivity 10-fold greater than that of standard PCR methods and provides a high level of data productivity. It is a rapid, specific, and easy test for the simultaneous detection of resistance to levofloxicing and the presence of the mecA gene in *Staph. aureus*. Zhang *et al.,* (2005) reported the development of a novel multiplex PCR assay that both characterizes and subtypes SCC mec in MRSA (e.g., type's I-V and subtypes IVa-IVd) using sets of SCCmec type-and-subtype unique and specific primers.

This method has demonstrated excellent (100 %) sensitivity and specify in characterizing 54 known strains of MRSA with various SCCmec types and subtypes. This tool may be useful in understanding the epidemiology and clonal relatedness of various MRSA isolates.

2.10 HOSPITAL ACQUIRED METHICILLIN RESISTANT *Staph. aureus* (HA-MRSA)

Hospital acquired (HA-MRSA) is typically defined as MRSA isolated from in patients that had been MRSA-negative at the beginning of hospitalization or MRSA isolates from in patients 48hours or more after being hospitalized (Tacconelli *et al*., 1998; Brumfitt and Hamilton 1989; Vandenesch *et al*., 2003). Patient to patient transmission of MRSA within healthcare settings primarily occurs via carriage on the hand of healthcare workers (Henderson, 2006), only people with direct exposure to healthcare setting such as hospitalization, outpatient, visit, nursing home admission, antibiotic exposure, chronic illness, surgery or indwelling catheters or in close contact with people with these risk factors were at risk of HA-MRSA infection (Foster, 2004; Zetola *et al.*, 2005). Colonization increases risk of infection and nasal carriage is one of the important sources of staphylococcal nosocomial infection among hospital personnel (Farzana *et al*., 2008).

Methicillin resistance *Staph.aureus* nosocomial infections were originally detected in large territory hospitals and in intensive care units, where colonized and infected patients as well as colonized healthcare workers were a significant source of cross infection. Presently however; MRSA is one of the most common pathogen in hospitals of all sizes with increasing prevalence (de Sousa and de Lencastre, 23004). HA-MRSA has increased during the past decade due to a number of factors including an increased number immune compromised and elderly patients; an increase in the number of invasive procedure, e.g. advanced surgical operation and life support treatment and failure in infection control measure such as hand washing prior to patient contact and removal of non-essential catheters.

Methicillin resistant *Staph. aureus* (MRSA) remains an important pathogen in clinical setting due to development of multiple drug resistant strains, which makes it increasingly difficult to treat, and accounting for its leading role in nosocomial infections globally (Grundmann *et al.,* 2002; Enright *et al.,* 2002). While only few clonal types have been identified to be responsible for most of hospital acquired infections worldwide and disseminating across border (Gordon and Lowy, 2008). The hospital epidemiology of MRSA has changed in the past few years due to the encroachment of community associated MRSA (CA-MRSA) strains into health care settings (Valsesia *et al*., 2010).

2.11 COMMUNITY-ASSOCIATED METHICILLIN RESISTANT *Staph. aureus* (CA-MRSA)

Community associated MRSA infections (CA-MRSA) are MRSA infections in healthy people who have not been hospitalized or had a medical procedure (such as dialysis or surgery) within the past year and who presented no other established risk factors for MRSA infections. CA-MRSA strains were reported from Western Australia from indigenous Australian patients in the 1990s, who had not been previously exposed to the healthcare system (Coombs *et al*., 2005; Udo *et al*., 1993). Numerous reports of MRSA causing infection in patients lacking traditional risk factors for infection with MRSA have since been published, many of which have affected children, young adults and some have been associated with substantial morbidity (Harold *et al*., 1998; Hussain *et al.,* 2000; Abi Hanna *et al*., 2000).

According to Centre for Disease Control (CDC, 2005), Fey *et al*., (2003) and David *et al*., (2007), community acquired methicillin resistant *Staph. aureus* (CA-MRSA) infections do not have typical MRSA risk factors such as recent hospitalization, kidney dialysis, surgery, haemodialysis, indwelling cathethers, long term residence in a health care

facility or intravenous drug use. Clinically, CA-MRSA differs from HA-MRSA by its specific involvement in skin and soft tissue infections, occurrence in diverse and often healthy populations especially children without clear risk factors, difference in antimicrobial susceptibility patterns, genetic markers and its frequent association with PVL genes (Foster, 2004; David and Daum, 2010; Hunter *et al*., 2011).

The origin of CA-MRSA is not clear. It was thought to have emanated only from patients discharged from hospitals into community (de Sousa and de Lencastre, 2004). But the discovery of MRSA strains with distinct genetic and diverse properties indicates that MRSA strains may also have originated from community (Charlebios *et al.,* 2004) causing skin and soft tissue infection in people, mostly children, (Hunter *et al.,* 2011). Furthermore, it appears that MRSA transmission from the hospital setting to the community and new acquisitions of SCCmec DNA in susceptible backgrounds may account for its emergence.CA-MRSA is susceptible to numerous antibiotic to which hospital strains are routinely resistant. Two molecular markers that are not found in typical hospital MRSA strains are strongly associated with CA-MRSA regardless of geographical origin: specific cassette elements encoding mecA and genes encoding PVL (Chambers and Deleo, 2009). This new and distinct strain of *Staph.aureus* with high virulence is associated with high morbidity and mortality in the community (Baba *et al*., 2002; Nebera, 2009; Hunter *et al*., 2011).

Panton-Valentine leukocidin (PVL) and its genes (lukS-lukF) which elicit tissue necrosis are the most well-known community acquired methicillin resistant *Staph. aureus* virulence factor and though present in most cases of CA-MRSA infection worldwide (Lina *et al.,* 1999; Zetola *et al.,* 2005; Elston and Barlow, 2009), it may not be essential for the evolution of other community-associated strains of MRSA, including ST8; USA 300 and

ST59; USA 1000 (AnDiep *et al*., 2006). Its occurrence in urban regions, overcrowding and young children has become a global problems (Moran *et al*., 2006; David *et al.,* 2007; Ghebhremedhin *et al*., 2009; Nabera, 2009).

CA-MRSA is spread in the same way as MRSA infection, mainly through person-to-person contact or contact with contaminated items such as towel, clothing or athletic equipment. Bacteria that exist normally on the skin cause CA-MRSA and so it is possible to infect a pre-existing cut not protected by a dressing or other bandage. While HA-MRSA strain types are rarely transmitted in the community, CA-MRSA strains are not transmitted in healthcare facilities, making it difficult to differentiate both on the basis of their epidemiology (Otter and French, 2011). Genetic characterization using multilocus sequence typing (MLST), spa typing of the polymorphic region X of *Staph.aureus* protein A and SCCmec has provided the best way to differentiate CA-MRSA from HA-MRSA (Crombs *et al.*, 2012).

The CA-MRSA strains differ from HA-MRSA several ways:

- The CA-MRSA strains were more susceptible to antibiotic classes other than β-lactam antibiotics (Harold *et al.,* 1998).
- The geneotype of the CA-MRSA strains were not the same as isolates from local hospitals (Vandenesch *et al*., 2003).
- The CA-MRSA strains mainly carried a different methicillin resistance determinants mec (Okuma *et al*., 2002).

Recent genomic sequence of CA-MRSA isolates shows that they most often contain a novel variant of the methicillin resistance locus SCC mec IV (Baba *et al*., 2002; Vandensech

et al., 2003) and unlike other SCCmec elements, type IV SCCmec and type 1 SCCmec do not code by additional resistance determinants in CA-MRSA (Vandenesch *et al*., 2003).

- The CA-MRSA strains encode a putative virulence factor called Panton valentine Leukocidin (PVL). PVL is a two component cytolytic toxin with high affinity for human leukocytes (Foster, 2004). The PVL locus is carried on a bacteriophage and is associated with primary cutaneous infections, especially furuncles, and with severe necrotizing community-acquired pneumonia (Vandenesch *et al*., 2003; Gillet *et al*., 2002).

2.12 LIVESTOCK-ASSOCIATED METHICILLIN RESISTANT *Staph. aureus*

Staph.aureus is a gram-positive bacterium that colonizes a variety of animal species (Weese *et al*., 2010). *Staph.aureus* infections in animals are most commonly reported as the cause of mastitis in dairy-producing animals (including cattle and goats) and "bumble foot" in chickens (McNamee and Smyth, 2000), as well as being identified as a pathogen of farmed rabbits (Viana *et al*., 2007). Most reports characterizing animal associated *Staph. aureus* have demonstrated that strains affecting animals are distinct from those affecting humans, suggesting that there are host of specific lineages which only rarely cross species boundaries (Shepheard *et al*., 2013).

In the early part of the 21st century, a novel pig-associated strain of MRSA was identified; sequence type 398 (ST398) and related strains (collectively grouped into clonal complex 398 or (C398) (Fluit, 2012). CC398 was first identified in pigs, and swine workers but has also been found in other animals (including cattle, poultry, and dogs and as well as humans) in a number of countries in Europe, Asia, and North and South America as well as Australia. The discovery of this strain led tothe addition of livestock associated MRSA (LA-MRSA) to the lexicon, to complement hospital-associated (HA) and community-associated

(CA) strains. However while CC398 strains have been found in livestock across the globe, the epidemiology of livestock associated *Staph. aureus* has been found to differ in to other geographical areas. There is also a report of high prevalence of methicillin susceptible *Staph. aureus* (MSSA) of clonal complex (CC398) among pigs in Europe (Hasman *et al*., 2010), studies provide strong evidence that (C398) originated in humans as MSSA and then spread to livestock where it acquired resistance to methicillin and tetracycline and with the jump from human to animals came the decreased capacity for human colonization, transmission, and virulence (Price *et al*., 2012).

The first case of livestock methicillin resistant *Staph.aureus* strains ST398 emerging in healthy poultry in Europe were reported in Belgium in 2008 (Nemati *et al*., 2008). Spatype E1456 of ST398 has been reported in poultry and more specifically in broiler chickens in Belgium that are different from spa types found in other animals species but it is not clear whether this spa type is peculiar to chicken (Persons *et al*., 2009). In the Netherlands, Mulder *et al*., (2010) reported both ST398 and ST9 in broiler chickens and slaughter house personnel. While Hasman *et al.,* (2010) found no ST398 in clinical isolates from poultry in the Netherlands. Other studies in addition have reported ST5 as dominant strain in poultry rather than ST398 (Lowder, *et al*., 2009).

Since its discovery in the early 2000, MRSA of clonal complex 398 (CC398) has becomes a rapidly emerging cause of human infections, most often associated with livestock exposure (Price *et al*., 2012). Livestock associated strains may evolve on farms because of the use of antibiotics in animals husbandry. These may be used as feed additives for growth promotion in industrial livestock and poultry (Silbergeld, *et al*., 2007), for preventing of disease within a herd, or for treatment of an existing disease outbreak. Agricultural-use antibiotics include many classes that are relevant for human health, including tetracyclines,

macrolides, penicillins, and sulfonamides among others. Antimicrobial resistance generated during animal husbandry, may then be spread to the general human population in a number of different manners; contact with contaminated meat products (via handling or ingestion), occupational exposure (farmers, meat parkers, butchers), and potential secondary spread into the large community from those who are occupationally exposed, entry into land transmission via hospitals or other healthcare facilities, or spread through environmental routes including air, water or manure in areas in proximity to live animal farms or crop farms where manure has been used as a fertilizer (Silbergeld *et al.*,2008). Apart from CC398/ST398 with (to11, to34, t108, t567, t1451, t2011, t2510) live stock indicator spa types, other LA-MRSA clonal lineages have also been detected in animals in Europe and elsewhere (Kock *et al.*, 2013). These include CC9/ST5 with t002 as the livestock indicator spa type; CC97/ST97 with t3992 as the livestock indicator spa type. All these clonal and sequence types have been reported in Germany in different animal species according to European baseline study as cited by Kock *et al.*, (2013).

Initially, MRSA ST398 isolates did not cause frequent invasive disease in humans, which may be due to the absence of several common virulence factors, however, the enhanced ability of SO385 a MRSA ST398 isolate from human endocarditis to acquire mobile elements suggests a possible rapid acquisition of determinants which contribute to virulence in human infections in this species (Schijffelen *et al.*, 2010).

Antibiotics such as bacitracin, chlortetracycline, erythromycin, penicillin and flouroquinolos licensed to treat diseases in man and animals are used in the treatment and control of bacterial diseases in poultry (Kilonzo Nthenge *et al*., 2008) and may account for the emergence of antibiotic resistance in poultry (Nemati *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 THE STUDY AREA

The research was conducted in Kano State, Northern Nigeria.The state has three senotorial zones thus; Kano central, Kano North and Kano South. The state has two distinct seasons; the wet (rainy) season and the dry season. The rainy season is usually between March and October. As with any other business in the state, poultry farming is a big business and is practiced throughout the state. There are many poultry farms all over the state. Apart from the commercial poultry farming, the traditional chicken raising system is practiced in the rural communities where chicken serve as pets as well as a source of meat.. Four selected poultry farms from each of the three zones were screened for the presence of MRSA. The three zones are selected purposely because: Kano central has the largest number of local government and the big business is mostly there. North and south constitute most of the rural areas where local chicken farming is mostly practice. Also some of the largest poultry farms are located far from the city.

3.1.1 The Study Population

The study population consisted of:

- Broilers
- Layers
- Poultry farm workers

Four hundred birds were sampled from each zone, that is, one hundred samples for each local government (four local governments from each zone). Six hundred were from the birds' nares, and six hundred from the cloacae. Total number of birds sampled was one thousand two hundred (1200). Total number of people sampled was sixty (60).

3.2 MATERIALS

3.2.1 Culture Media

Nutrient agar (NA), Nutrient broth (NB), Mannitol salt agar (MSA), Mueller Hinton agar (MHA), Brain Heat infusion agar (BHI), Brian Heart infusion broth (BHI) and Baird Parker modified agar,were all from Oxoid Ltd, England.

All media were prepared according to manufacturer's instruction, sterilized at 121^0C for 15 minutes and stored at 4^0C until required.

3.2.2 Chemical Reagents

Hydrogen peroxide (Jopel Chemical and Allied Product Ltd, Aba, Nigeria, Sodium chloride (Fisher Scientific Company New Jersey, USA) Lugols, Iodine, crystal violet, Ethanol, carbol fuschin, starch soluble (BDH Chemicals Ltd., England), Phosphase buffer PH 8, Bromothymol blue, 0.2M Monosodium phosphate, 0.2M Dibasic sodium phosphase, Benzyl penicillin potassium salt, IM hydrochloric acid all from BDH Chemical Ltd. England. Deoxyribonucleic acid (Sigma Chemicals Company St. Louis M.O. USA).

3.2.3 Antibiotic Disc

The following antibiotic discs from Oxoid Ltd. UK were used;

Sulphamethoxazole/trimetophrim (25µg), Vancomycin (5µg), Erythromycin (15µg), Neomycin (30µg), Chloramphenicol (10µg), Oxacillin (1µg), Gentamicin (10µg) Ampicillin(10µg), Oxytetracycline(30µg). Antibiotics were selected on the basis of been commonly used in the selected poultry farms.

3.2.4 Plasma

Undiluted, pooled human plasma was collected from the blood bank of Muhammadu Abdullahi Wase specialist hospital Kano, and stored in freezer until required.

3.2.5 Equipment

Autoclave (portable), Adelphi Mfg. Co. Ltd., UK., Refrigirator, Centrifuge, Weighing Balance, Microcentrifuge Ependorff, 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 (Techne TC-312, Cambridge, United Kingdom)

3.3 METHODS

3.3.1 Sample Collection Areas

- Kano Central
- Kano North
- Kano South

3.3.2 Sample Collection

Three different samples were collected: Nasal and Cloacae swabs of chicks and Nasal swab of workers. Following aseptic technique, samples were collected using a sterile cotton swabs and were taken to laboratory for bacteriological analysis in an ice pack. 400 samples were collected from each senatorial zone, making a total of 1200 samples; 60 samples were from poultry farm workers.

3.3.3 Preliminary Identification of Organism

Samples were inoculated into sterile Brain heart infusion broth and incubated at 37^0 C for 24 h. The overnight culture was then subcultured on the surface of sterile nutrient agar (NA) by streaking and incubation at 37^0 C for 18-24 hours. Cultural characteristics of the resulting colonies were noted.

3.3.4 Isolation and Purification of *Staph.aureus*

3.3.4.1 *Simple Staining*

Simple staining of suspected *Staphylococci* colonies were carried out as described by (Cheesbrough, 2002). Using sterile wire loop, smears were made from discrete colonies with desirable growth characteristics on clean glass slides. After fixing, slides were stained with methylene blue solution. Stained slides were observed under microscope.

Colonies, whose smear appeared as cocci in characteristics clusters were picked into nutrient broth (NB), incubated overnight at 37^0 C.

3.3.4.2 *Gram Staining*

Gram staining was carried out using the method described by (Cheesbrough 2000). A smear of the culture of the test organism was made evenly on a clean glass slide and heat-fixed. The smear was stained with crystal violate, fixed with Lugol's iodine and decolorized rapidly with acetone-alcohol, after which it was counter-stained with dilute carbol fuchsin solution. The stained slides were microscopically examined under the oil immersion objective. Sample colonies that appeared as purple/violet cocci, predominantly in cluster were selected. Sample colonies were inoculated into sterile NB and incubated at 37^0 C for 18 hrs. Each sample was sub-cultured in duplicate onto slants and incubated at 37^0 C for 18 hrs. Slants were kept at 4^0 C until required.

3.3.4.3 *Growth on Selective Media*

A loopful of overnight NB culture of the isolates that were presumptively identified as probable *staphylococci* (based on simple and Gram staining characteristics) were streaked on previously prepared Mannitol salt Agar (MSA) plates. Plates were incubated at 37^0 C for 24 hrs. Isolates that produced colonies exhibiting characteristics deep golden yellow coloration were selected and sub-cultural into NB, overnight culture were subculturedon to NA slants, after overnight incubation at 37^0 C, slant were stored in refrigerator until required.

3.3.5 Biochemical And Confirmatory Tests

3.3.5.1 *Catalase Test*

The ability of the isolates to produce an enzyme catalase was demonstrated by the addition of 1ml of 3 % w/v hydrogen peroxide solution to 24 hr culture of the isolate. Rapid evolutions of gas bubbles indicated the breakdown of hydrogen peroxide into oxygen and water by catalase peroxidase enzyme present a positive result.

$$2H_2O_2 \rightarrow H_2O + O_2$$

3.3.5.2 *Coagulase Test*

The tube coagulase method was carried out as described by Cowan (1985) and Cheesbrough (2000). A positive coagulase test differentiates *Staph. aureus* (which produce free coagulase and converts fibrinogen to fibrin by activating a coagulase-reacting factor present in plasma) from the coagulase-negative *staphylococci* species. Pooled, EDTA-anticoagulated human plasma was diluted 1 in 10 with sterile normal saline. Aliquots of 0.5 ml were pipetted into sterile test tubes into which 0.1ml of 24 hrs NB culture of the isolate was added, mixed and incubated in water bath thermostated at 37^0 C for 4 hrs. At intervals of 30 minutes, the test tubes were observed for clotting of tube contents or fibrin clot in tubes. Two different test tubes were set up for positive control test (containing 24 hrs culture of a known *Staph. aureus*) and negative control (containing only sterile NB). Incubation period may be extended for 24 hr to take care of delayed reactions since *Staph. aureus* is not prone to spontaneous clumping of plasma which usually accompanies the utilization of citrate present in the anticoagulant.

3.3.5.3 *Deoxyribonuclease (DNAse) test*

Deoxyribonucleic acid agar media (containing 0.2 % W/V of deoxyribonucleic acid) was prepared by incorporating appropriate amount of deoxyribonucleic acid into nutrient agar media and sterilized by autoclaving at 121^0 C for 15 minutes. A DNase agar plate was divided into four and the underside was marked with the number of the test organism. An overnight broth culture of the test organism was spot inoculated onto the surface of the plate and incubated at 37^0 C for 24 hrs. The surface of the overnight plate was covered with 1 mol/1 hydrochloric acid solution and excess decanted.

Clear zone around the colonies within 5 minutes of adding the acid indicated the production of the enzymes Deoxyribonuclease by organism (Cheesbrough, 2002).

3.3.6 *Staph. Agglutination Test*

Staphytect plus latex slide agglutination test (Oxoid Ltd England) was used for differentiation of *Staph. aureus* by detection of clumping factor, protein A, and certain polysaccharides found in *Staph. aureus* from those staphylococci that do not possess these properties.

Procedure

The latex reagents were brought to room temperature, and the reagent was mixed by shaking vigorously.Any latex from the dropper pipette was expelled from complete mixing. One drop of test latex was dispensed onto one of the circles on the reaction card and one drop of control latex was dropped onto another circle. Using inoculating wire loop, equivalent of 5 average sized suspected staphylococcal colonies (equivalent to 2-3 mm diameter of growth) were picked from the culture plate and smeared onto a circle and mixed in the control latex reagent. The circle was covered by spreading and the loop was discarded

appropriately. Using a separate loop the same procedure was repeated in the test latex reagent. The card was picked up and rocked for up to 20 seconds and agglutination was observed under normal light condition.

3.3.7 Microgen Staph ID Test

The test was carried out according to the manufacturer's instructions. A single colony from an 18-24 hrs culture was emulsified in the suspending medium supplied in the kit, and mixed thoroughly. Back of the adhesive tape sealing the microwell test strip(s) was carefully peeled. Using a sterile Pasteur pipette, 3-4 drops (approximately 100 ul) of the bacteria suspension were added to each well of the strip(s). After inoculation, 10^{th} and 11^{th} wells were overlaid with 3-4 drops of mineral oil. The top of the microwell test strips was sealed with the adhesive tape removed earlier and incubated at $35\text{-}37^{0}$ C. The Microwell test strips were read after 18-24hours incubation.

Adhesive tape was removed and all positive reactions were recorded with the aid of the colour chart substrate reference table (appendix 4a and 4b). One drop of PYR reagent was added to well 12 and read after 10 minutes. Formation of a very deep pink/red colour indicates positive results. Nitrate reduction test was performed on well 9 after reading and recording the β-glucoronidase reaction, one drop of nitrateA reagent and one drop of nitrateB reagent was added to the well and read after 60 seconds. The development of a red colour indicated that nitrate has been reduced to nitrite. All reports were recorded on the form provided (appendix 5). Using the software provided specie was assigned to each bacterial isolate tested.

3.3.8 Test for β-lactamase Production

3.3.8.1 *Starch solution*

Suspensions of the isolate were prepared in triplicates by emulsifying bacterial colonies from an overnight NA culture with sterile wire loops in 0.5ml of phosphase 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 *Staph aureus* was regularly set up. Thereafter, 2 drops of freshly prepared 1 % aqueous starch solution were added to each bacterial suspension and gently shaken. One drop of iodine solution was then added and allowed to stand for 10 minutes at room temperature. β-lactamase producing organisms change the colour of the reaction mixture from blue-black to colourless within 10 minutes.

3.3.8.2 *Nitrocefin test (β-lactamase hyper producers)*

Oxoid identification stick can be used to detect beta lactamase produced. Nitrocefin is a cephalosporin developed by Glaxo Research Ltd. This compound exhibits a rapid distinctive colour change from yellow to red as the amide bond in the beta lactam ring is hydrolyzed by a beta lactamase.

Procedure

The containers were removed from refrigerator and allowed to reach room temperature. Well separated representative colonies from the primary isolation medium were selected. One stick (colour coded black) was removed from the container, and holding the coloured end, the colonies were touched with the impregnated end of the stick and the stick was rotated picking a small mass of cells. Because the reaction require moisture, the inoculated tip of the stick was placed in the moisture condensate on the lid, in the case where condensate is not available in the inverted plate, one drop of sterile distilled water was added

to moisten the tip of the stick. The reagent-impregnated top of the stick, was examine for up to 5 minutes, and if negative, the stick was re-examined after 15 minutes because some staphylococci may take up to 1 hour before the reaction shows a colour change. In the presence of β-lactamase, impregnated tip of stick changes to a pink-red colour. No colour changewill be observed in organism that does not produce β-lactamase. To ensure correct reading, the colour of the stick was compared to an unused stick.

3.3.9 Antibiotic Susceptibility Test

Antibiotic susceptibility testing was carried out to obtain the susceptibility pattern of *Staph. aureus* isolated from the poultry farms and the poultry farm workers. The isolates were tested against a panel of twelve antibiotics.

The antibiotics susceptibility pattern was determined using the Kirby-Bauer (CLSI, 2008) modified disc agar diffusion (DAD) technique, (Cheesbrough, 2002). Discrete colonies on NA plate were emulsified in 3 ml of normal saline and the turbidity was adjusted to 0.5 McFarland. Using sterile swab sticks, the surface of MHA in 90 mm- diameter plate was inoculated with the bacterial suspension by streaking the surface of the agar in three directions, rotating the plate approximately to 60^0 to ensure even distribution. The inoculated plates were allowed to dry for 10 minutes after which the antibiotic discs were placed on the surface of the agar. The plates were left at room temperature for the pre-diffusion inverted and incubated aerobically at 37^0 C for 16-18 hrs.

The diameter of the zones of growth inhibition were measured to the nearest millimeter and isolates classified as; sensitive, intermediate or resistant based on CLSI interpretative chart zone size (CLSI, 2012).

3.3.10 Determination of Methicillin (Oxacillin) Resistance Using Cefoxitin Disc

CLSI recommends using cefoxitin instead of oxacillin when using disk diffusion method to determine methicillin resistance in *Staph.aureus* (CLSI, 2007). Cefoxitin results are easier to interpret and are thus more sensitive and enhance induction of PBP2a for the detection of mecA mediated resistance than oxacillin results. The gene is located on the staphylococcal chromosome cassette mec and encodes penicillin binding protein (PBP2a) (BergerBachi and Rohrer 2002).

A direct colony suspension of each *Staph.aureus* isolate was prepared equivalent to 0.5 McFarland standards and plated on Mueller Hinton agar surfaces). Cefoxitin 30µg disc was placed on the plate. Plates were incubated at 36^0C for 24h. The zones of inhibition were measured and compared to that of CLSI interpretative chart (CLSI, 2007). Inhibition zones diameter of 21mm was reported as methicillin resistant and $\geq$22mm was considered as methicillin sensitive.

3.3.11 Determination of Multiple Antibiotic Resistance (MAR) Index

The MAR index was determined for each isolate by dividing the number of antibiotics to which the isolate is resistant by the total number of antibiotic tested (Paul et al., 1997).

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which isolate is resistant}}{\text{Total number of antibiotics tested}}$$

3.3.12 Screening For Vancomycin Resistance

Brain-heart infusion (BHI) agar supplemented with 6 µg/ml vancomycin, (BHI-V6) was prepared, sterilized and poured into 90 mm petridish. Plates were inoculated with standard

inoculum prepared from overnight culture and incubated at 35^0 C for 24-48 hrs. Plates were examined for the presence of growth after 24 hrs.

3.3.14 Test For Penicillin-Binding Protein (PBP2a)

Detection of PBP2a was carried out using latex agglutination kit (Oxoid Ltd, England) and performed according to the manufacturer's instructions. The sample colonies were from overnight Baird Parker agar culture. The test is rapid in detecting PBP2a in isolates of Staphylococcus, as an aid in identifying methicillin resistant *Staph.aureus* (MRSA) and coagulase negative staphylococci.

PBP2a Extraction

Four drops of extraction reagent were added into microcentrifuge tube containing approximately 1.5 x 10^9 (3-5µl) cells. This was achieved by using a sterile 5 µl loop to remove sufficient growth to fill the internal diameter of the loop and suspended into microcentrifuge tube.

Tubes were vortexed occasionally if clumps were present until a turbid suspension was visible. Tubes were placed into boiling water and heated for three minutes. The tubes were removed and allowed to cool to room temperature. One drop of extraction reagent 2 was added into the tube and shaken. The tube was centrifuged at 1,500 x g for 5 minutes (i.e 3000 rpm at 15 cm rotation radius) and the supernatant was used for the test.

Latex Agglutination Procedure

For each supernatant to be tested, one circle of test card for testing with test latex and another for testing with control latex were labeled. The latex reagents were mixed by inversion, and one drop of test latex and control latex were added to each labeled circle. 50 µl of the supernatant was added to test and control circle respectively. The latex and the

supernatant in each circle were thoroughly mixed with a mixing stick. The card was rocked for up to three minutes and agglutination was observed under normal lighting condition.

3.4 MOLECULAR ANALYSIS

Molecular characterization of 30 isolates selected on the basis of their being methicillin resistant phenotypically was carried out at International Institute of Trophical Africa (IITA) Ibadan Oyo state. The isolates were analyzed for carriage of mecA gene the genetic determinant of methicillin resistance, 16SrRNA, and the nuc gene. Spa typing and sequence types for the seven housekeeping gene were also determined.

3.4.1 DNA Extraction

The procedure was carried out according to the manufacturer`s instructions. An overnight Lauryl and Buretti (LB) culture of each *Staph.aureus* isolate was centrifuged at 10,000xg for 1min and the supernatant discarded into a disinfecting Jar. Fifty to hundred (50-100) µg of the *Staph aureus* cells that have been resuspended in 200µl of water was added to a ZR bashing bead lysis tube, 750µl lysis solution was also added to the tube. ZR bashing bead lysis tube was centrifuged in a microcentrifuge at 10,000xg for 1 minute. Up to 400 µl supernatant was transferred to a zymo-spin IV spin filter in a collection tube and centrifuged at 7,000 rpm for 1minute, 1200 µl of bacterial DNA binding buffer was added to the filtrate in the collection tube from above. Eight hundred (800) µl of the above mixture was transferred to a zymo-spin 11C column in a collection tube and centrifuged at 10000xg for 1minute. The flow through was discarded and step repeated.Two hundred (200) µl DNA pre-wash buffer was added to the zymo-spin 11C colmn in a new collection tube and centrifuged at 10,000xg for 1minute, 500 µl bacterial DNA wash buffer was also added to the tube and centrifuged again. The zymo-spin 11C column was transferred to a clean 1.5 ml

microcentrifuge tube and 100 µl DNA elution buffer was added diretly to the column matrix, centrifuged at 10,000xg for 30 seconds to elute the DNA.

3.4.2 Polymerase Chain Reaction For Detection of 16SrRNA, Nuc Gene And MecA Gene.

A master mix was prepared in an eppendorf tube and the total volume was determined by the number of samples that were analysed. The total volume of the reactants per reaction was 10 µl. Multiplied by the number of samples to give the total volume of the master mix.The master mix was vortexed to mix thoroughly, this was then aliquated using repeater pipette into the PCR tubes numbered to correspond with the samples.The DNA template of 2.0 µl for each sample was then added to the corresponding tubes. The PCR tubes were then vortexed to mix well.The primer sequence and the predicted sizes used in the multiplex PCR was shown in table 3.1.

The temperatures and cycling times were optimized for each new DNA template target and primers to achieve maximum yield and specificity. PCR process was started with an initial denaturation step at 40^0 C for 5 minutes. This was followed by three steps cycling: denaturation at 94^0 C for 30 seconds, annealing at temperature of 56^0 C, and extension step for one minute at 72^0 C. The total numbers of cycles were 35 and then the final extension time was 7 minutes at a temperature of 72^0C.Holding temperature is at 10^0 C for (∞) infinity.After amplification of the gene, the PCR products were subjected to electrophoresis and viewed in the illumination system. The amplicon were loaded on 1.5% agarose gel. The first well was loaded with the ladder (1KB plus ladder from invitrogen) followed by the PCR products in that order. The expected base pair of amplicon is around 1500bp.

Preparation for electrophoresis;

Tri-acetic acid – EDTA buffer (TAE) gel was prepared. The volume of the TAE and the weight of the agarose used were dependent on the number of wells or gene product. 30 wells were used and so 3g agarose was added to 200 ml× 1TAE and warmed to dissolve in a microwave for 2min, it was removed from the microwave oven and allowed to stand for a few minutes. 8µl ethidium bromide (Et Br) was added to stain the DNA product.

After the addition of the ethidium bromide to the viscous agarose and shaken to mix, it was poured into the gel mould with 30well comb and allowed to stand for 30minutes to harden. The comb was carefully pulled out of the gel. The clamp of the mould was removed and the gel was transferred into the electrophoretic tank.The tank was filled with 1xTAE buffer to the maximum limit and the PCR products were loaded into the wells. The gel was run at 120V for 1hour. Following electrophoresis the PCR product was viewed and the picture of the bands was taken. The primers used are shown in table 3.1

Table 3.1 Primers for MecA, nuc and 16SrRNA

Gene	Primer sequence	Expected amplicon size (bp)
Mec forward	GGGATCATAGCGTCATTATTC	500
reverse	AACGATTGTGACACGATAGCC	
Nuc forward	TCAGCAAATGCATCAAACAG	200
reverse	CGTAAATGCACTTGCTTCAAG	
16SrRNA forward	GTGCCAGCAGCCGCGGTAA	800
reverse	AGACCCGGAACGTATTCAC	

3.4.3 Detection of PVL (luks-lukf) Gene by PCR

The multiplex PCR carried out and the primers used were described and design by Lina *et al*., (1999b). Mastermix was prepared according to the number of templates. The mastermix was vortexed to mix well and dispensed in to PCR tube and 2.0 µl of each DNA template was added t o the corresponding PCR tube. 0.5 µl primer was added (forward and reverse). PCR tubes were vortexed to mix and the PCR product was loaded on an agarose gel and electrophoresed as described in 3.4.2.The primers used are shown in table 3.2

Table 3.2: Primers for Panton Valentine Leukocidin

Gene		Primer sequence	Expcted amp size
PVL	Forward	ATCATTAGGTAAAATGTCTGGACATGATCCA	
	Reversed	GCATCAASTGTATTGGATAGCAAAAGC	451bp

Lina *et al*., (1999b).

3.4.4 Spa Typing:

Amplification of the spa gene X region was performed as described previously (Shopsin *et al*., 1999)nd amplicons were then sequenced by using 3130x1 genetic analyser (Applied Biosystem). Spa typing was determined with the ridom spa server (Harmsen *et al.,* 2003).The primer used is shown in table 3.3

Table 3.3: Primers fo r spa typing

Gene	Primer sequence	Expected amplicon size
Spa 2F	GAACAACGTAACGGCTTCATCC	
Spa 1514R	CAGCAGTAGTGCCGTTTGCCT	250-637bp

(Shopsin *et al*., 1999)

Spa products were purified before sequencing. Two vol. (20µl) of absolute ethanol was added to the PCR product and incubated at room temperature for 15minutes. This was spinned down at 1000rpm for another 15minutes and the supernatant decented.It was spinned down at 10000rpm for 15minutes. Two (2) vol (20µl) of 70% ethanol was added, and the supernatant decented and air dried. About 10µl of ultrapure water was added and the amplicon was checked on 1.5% agarose. The purified PCR products were then sequenced by using 3130x1 genetic analyser (applied biosystem). Spa typing was determined with the ridom Spa server (Harmsen *et al*., 2003).

3.4.5 Multilocus Sequence Typing (MLST)

MLST was performed according to protocol described by Enright *et al.*, (2000). It was carried out to detect the seven house keeping gene. *Arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqil*. A master mix was prepared for each of the seven housekeeping gene.The total volume of the master mix was determined by the number of reaction of the DNA templates. Finally the primers (forward and reverse) and the template DNA were added and agitated to mix and then loaded in the thermocycler. The amplification and gel electrophoresis were carried out as described in 3.4.2. They were viewed in the GelDoc illumination system.

The PCR products were purified as described in 3.4.4 before sequenceing. After sequencing the results were analysed using bioedit soft were (http://www.mlst.net.)(Halin *et al*., 2007).Primers used are shown in table 3.4.

Table 3.4 Primers used for Multilocus

Gene	primer sequence	expected amplicon size
arcC-Forward	TTG ATT CAC CAG CGC GTA TTG TC	
arcC-Reverse	AGG TAT CTG CTT CAA TCA GCG	456bp
AroE-Forward	ATC GGA AAT CCT ATT TCA CAT TC	
AroE-Reverse	GGT GTT GTA TTA ATA ACG ATA TC	456bp
glpF Forward	CTA GGA ACT GCA ATC TTA ATC	
glpF Reverse	TGG TAA AAT CGC ATG TCC AAT TC	465bp
gmk,Forward	ATC GTT TTA TCG GGA CCA TC	
gmk,Reverse	TCA TTA ACT ACA ACG TAA TCG TA	429bp
pta,Forward	GTT AAA ATC GTA TTA CCT GAA GG	
pta,Reverse	GAC CCT TTT GTT GAA AAG CTT AA	474bp
tpi Forward	TCG TTC ATT CTG AAC GTC GTG AA	
tpi Reverse	TTT GCA CCT TCT AAC AAT TGT AC	402bp
yqiL Forward	CAG CAT ACA GGA CAC CTA TTG GC	
yqiL Reverse	CGT TGA GGA ATC GAT ACT GGA AC	516bp

(Enright *et al.*, 2000).

3.4.6 Staphylococcal Cassette Chromosome (SCCmec) Typing.

PCR was carried out to detect mecA gene and to determine the type of staphylococcal cassette chromosome. It was carried out as described previously by Ghaznavi Rad *et al.*, (2010). Primers used were for type 1 to type V SCC mec as shown in table 3.5. Mastermix was prepared to include the mecA and the SCC mec types 1 -V primers according to the number of DNA templates. The master mix was vortexed to mix and aliquated in volumes of 48 µl PCR tubes. The template DNA was added, vortexed to mix and then loaded in the thermocycler. The optimization process was carried out as described in 3.4.2.

The amplified DNA product was run on a gel in which 3 grams of agarose (1.5%) was gradually heated to dissolve in 200 ml of TAE buffer and 8ul of ethidium bromide was added to stain the DNA. The PCR products were loaded on the gel and run on electrophoretic machine at 120 V for 1.5 hrs. Bands were viewed using transilluminator.

Table 3.5: Primers for SCCmec typing

Gene	primer sequence	expected amplicon size
Type I Forward	GCTTTAAAGAGTGTCGTTACAGG	
Type I Reverse	GTTCTCTCATAGTATGACGTCC	613bp
Type II Forward	GATTACTTCAGAACCAGGTCAT	
Type II Reverse	TAAACTGTGTCACACGATCCAT	287bp
Type III Forward	CATTTGTGAAACACAGTACG	
Type III Reverse	GTTATTGAGACTCCTAAAGC	243bp
Type IVa Forward	GCCTTATTCGAAGAAACCG	
Type IVa Reverse	CTACTCTTCTGAAAAGCGTCG	776bp
Type IVb Forward	AGTACATTTTATCTTTGCGA	
Type IVb Reverse	AGTCATCTTCAATATGGAGAAAGTA	1000bp
Type IVc Forward	TCTATTCAATCGTTCTCGTATT	
Type IVc Reverse	TCGTTGTCATTTAATTCTGAACT	677bp
Type IVd Forward	AATTCACCCGTACCTGAGAA	
Type IVd Reverse	AGAATGTGGTTATAAGATAGCTA	1242bp
Type IVh Forward	TTCCTCGTTTTTTCTGAACG	
Type IVh Reverse	CAAACACTGATATTGTGTCG	663bp
Type V Forward	GAACATTGTTACTTAATGAGCG	
Type V Reverse	TGAAAGTTGTACCCTTGACACC	325bp
mecA Forward	TCCAGATTACAACTTCACCAGG	
mecA Reverse	CCACTTCATATCTTGTAACG	162bp
Sa442 Forward	AATCTTTGTCGGTACACGATATTCTTCACG	
Sa442 Reverse	CGTAATGAGATTTCAGTAGATAATACAACA	108bp

(Ghaznavi Rad *et al.*, 2010)

3.5 STATISTICAL ANALYSIS

Data was entered in micrsoft excel 2007 and then transferred to SPSS for analysis. Comparism between proportions were made using one way ANOVA for three means.Differences showing a critical value lessthan F value confidence level 0.05 or 0.01 were considered significant .

CHAPTER FOUR

4.0 RESULT

4.1 SAMPLE POPULATION

A total of 1260 samples collected were screened for the presence of *Staph. aureus*. Samples came from three senatorial zones: Kano central, Kano north and Kano south. The number of samples collected and the site as shown in table 4.1.

Table 4.1: Number of farms and birds screened

Source	Bird N=1200		Farm workers N=60
	Cloacae	Nostril	
Farm 1	50	50	5
Farm 2	50	50	5
Farm 3	50	50	5
Farm 4	50	50	5
Farm 5	50	50	5
Farm 6	50	50	5
Farm 7	50	50	5
Farm 8	50	50	5
Farm 9	50	50	5
Farm 10	50	50	5
Farm 11	50	50	5
Farm 12	50	50	5
Total	600	600	60

Keys:

Farm 1 – 4 Kano central

Farm 5 – 8 Kano North

Farm 9 – 12 Kano South

N = Number of Birds .There were 300 layers and 300 broilers.

4.2 BIOCHEMICAL AND CONFIRMATORY TEST

Isolates that were Gram positive cocci, catalase positive, deoxyribonuclease positive and coagulase positive were presumptively characterized as *Staph. aureus.* (Table 4.2 - 4.4).

Table 4.2: *Staph aureus* from Kano Central Zone

Source of Isolate	Number of samples	Gram+ve staining	*Staph aureus* isolates by Catalase +ve	DNase +ve	Coagulase +ve
Nasal Swab	200	175	175	168	120
Cloacae Swab	200	170	170	163	160
NasalSwab of Workers	20	11	11	9	8
Total	420	356	356	340	288

Table 4.3: *Staph aureus* from Kano North Zone

Source of Isolate	Number of samples	Gram staining +ve	*Staph aureus* isolates by Catalase +ve	DNase +ve	Coagulase +ve
Nasal Swab	200	160	160	145	100
Cloacae Swab	200	120	120	80	50
Nasal Swab of Workers	20	15	15	10	5
Total	420	295	295	235	155

Table 4.4 *Staph aureus* from Kano South Zone

Source of Isolate	Number of samples	Gram staining +ve	*Staph aureus* isolates by Catalase +ve	DNase +ve	Coagulase +ve
Nasal Swab	200	190	190	175	90
Cloacae Swab	200	150	130	115	80
Nasal Swab of Workers	20	10	10	9	9
Total	420	350	330	299	179

4.2.1 **Screening of Isolates Using Staph.Agglutination Test**

Agglutination test was carried out to further identify the isolates. Six hundred and twenty two (622) coagulase positive isolates were subjected to this test. Two hundred and ninety (290) were positive i.e. 46.6%.

4.2.2 **Identification of Isolates Using Microgen Staph ID**

Isolates that were positive by agglutination test (290) were further screened and identified using microgen staph ID. Only 98 (33.8 %) of the isolates showed to be *Staph. aureus*, the rest were other species of *Staphylococcus*. Result is shown in Table 4.5.

Table 4.5: Number of *Staph.aureus* identified by agglutination test and microgen Staph ID

Source	Coagulase positive	Agglutination positive	Agglutination negative	Microgen positive
Kano central	288	101	187	35
Kano North	155	88	67	39
Kano South	179	101	78	24
Total	622	290	332	98

4.3 DISTRIBUTION OF *STAPH. AUREUS* ACCORDING TO SENATORIAL ZONES

Percentage distribution of *Staph.aureus* in the three geopolitical zones is presented in the figure below. Kano Central 35.7 %, Kano North 39.8 %, Kano South 24.5 % (n=98).

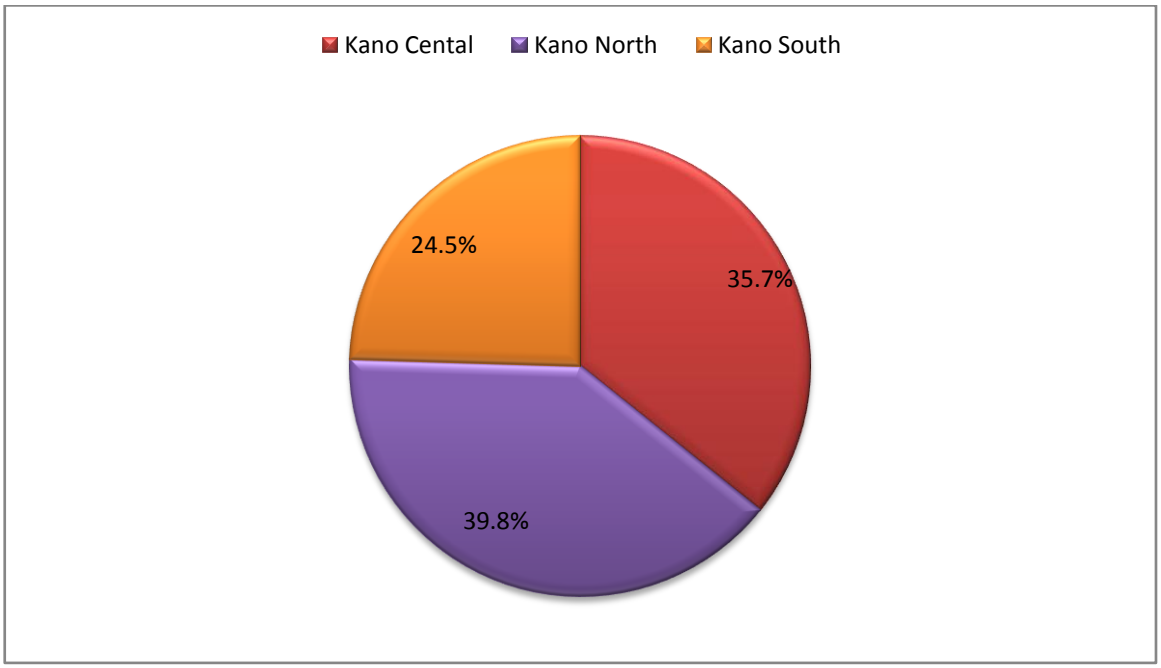


Fig. 4.1 Graphical Presentation of Staphylococcus isolate by geopolitical zones

4.4 RECOVERY OF *STAPH. AUREUS* FROM BROILERS AND LAYERS

Staph.aureus was isolated from both broilers and layers cloacae and nostril. The number of isolates varied from cloacae and nostril and from zone to zone.

Analysis showed that more *Staph. aureus* were recovered from broilers than layers (Table 4.6 and figure 4.2).

Table 4.6 *Staph.aureus* recovered from broilers and layers

Sources	**No. of isolates (%)**			
	Broilers	**%**	**Layers**	**%**
Kano Central	25	37.9	10	31.3
Kano North	27	40.9	12	37.5
Kano South	14	21.2	10	31.3
Total	66		32	

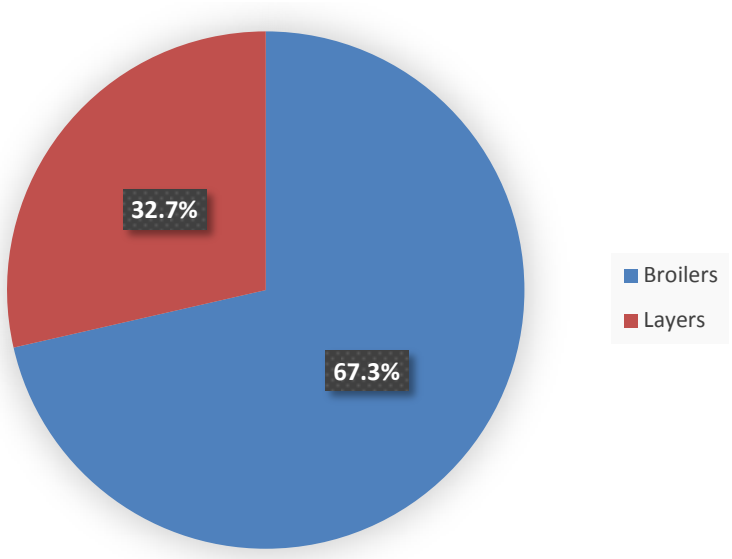


Fig. 4.2: Percentage of *Staph.aureus* recovered from broilers and layers

The *Staph. aureus* isolated from the cloacae, nostril of the birds and the nostril of the farm workers are shown in tables 4.7,4.8 and figure 4.3.

Table 4.7: Comparism of isolates from cloacae, nostril of birds and workers nostril.

Source	No. of *Staph.aureus*	*Staph. aureus* (%)
Cloacae	56	57.1
Nostril	37	37.8
Worker's nostril	5	5.1
Total	98	100

4.5 *STAPH. AUREUS* ISOLATED FROM POULTRY FARM WORKERS

The number of isolates from consented poultry form workers was 5, and the percentage of poultry farm workers that harbored *Staph. aureus* from the twelve farms was 8.3 % (N=60) (Table 4.8).

Table 4.8: Number of farm workers screened and the *Staph.aureus* recovered.

Source	**Nasal swab of workers**	***Staph.aureus recovered***
Farm 1	5	0
Farm 2	5	1
Farm 3	5	0
Farm 4	5	0
Farm 5	5	1
Farm 6	5	1
Farm 7	5	0
Farm 8	5	0
Farm 9	5	0
Farm 10	5	0
Farm 11	5	1
Farm 12	5	1
Total	60	5

4.6 ANTIBIOTIC SUSCEPTIBILITY PROFILE

The number of isolates resistant to each of the antibiotics is shown in table 4.10. Out of the 98 *Staph. aureus* isolates recovered from the twelve farms, high percentage of resistance is seen against oxytetracycline 71.4 % followed by oxacillin and ampicillin (71.4 %) each, chloramphenicol (61.2 %) and erythromycin (55 %). Only 30.6 % were resistance to cefoxitin. Vancomycin showed high activity against 74.5 % of the isolates, augmentin 69.4 %, ciprofloxacin 64.3 % and gentamicin 60.2 % (table 4.10). One isolate was found to be resistant to all the twelve antibiotics, and one isolate was sensitive to all the antibiotics.

Comparing the three sample sites i.e cloacae, nostril and nostril of the workers, higher resistance was seen in cloacae isolates against ampicillin, oxytetracycline and chloramphenicol. Isolates from bird's nostril were susceptible to vancomycin (87 %), gentamicin (80 %) and ciprofloxacin (77 %) while isolates from farm workers were 100 % susceptible to ciprofloxacin, and 86 % to vancomycin. The resistance of the isolates from broilers, layers and farm workers was compared as shown in Table 4.11.

The interpretative chart for the susceptibility test is as shown in appendix 3.

Table 4.9: Antibiotic, potency, zone diameter and the class of antibiotics used in the study.

Antibiotics	Potency	Resistant zone diameter (mm)	Class of antibiotic
Ampicillin	10µg	≤28	β-lactam
Augmentin	30µg	≤19	β--lactam+ β-lactamase inhibitor
Chloramphenicol	30µg	≤12	Miscelleneous
Ciprofloxacin	5µg	≤15	Flouroquinolones
Cefoxitin	30µg	≤21	β-lactam
Erythromycin	15µg	≤13	Macrolides
Gentamicin	10µg	≤12	Aminoglycoside
Neomycin	30µg	≤12	Aminoglycoside
Oxacillin	1µg	≤10	β-lactam
Oxytetracycline	30µg	≤14	Tetracyclines
Trimethomprim/sulfamethaxazole	25µg	≤10	Folate inhibitor
Vancomycin	30µg	≤14	Glycopeptide

Table 4.10: Antibiotic resistance/sensitivity profile of the *Staph.aureus* isolates

Antibiotics	**Potency(µg)**	**No. of *Staph.areus (%)***	
		Resistant	**Sensitive**
Ampicillin	10	70 (71.4)	28 (28.6)
Augmentin	30	30 (30.6)	68 (69.4)
Chloramphenicol	30	60 (61.2)	38 (38.8)
Ciprofloxacin	5	35 (25.7)	63 (64.3)
Cefoxitin	30	30 (30.6)	68 (69.4)
Erythromycin	15	54 (55)	44 (45)
Gentamicin	10	39 (39.8)	59 (60.2)
Neomycin	30	45 (45.9)	53 (54.1)
Oxacillin	1	70 (71.4)	28 (28.6)
Oxytetracycline	30	70 (71.4)	28 (28.6)
Trimethomprim/sulfamethaxazole	25	50 (51)	48 (49)
Vancomycin	30	25 (25.5)	73 (74.5)

Table 4.11: Comparison of Antibiotic resistance of *Staph.aureus* isolates from broilers, layers and poultry farm workers

Antibiotics	**Poultry (%)**		
	Broilers	**Layers**	**Poultryfarm Workers (%)**
Ampicillin	40 (60.6)	30 (93.8)	0 (0)
Augmentin	16 (24.2)	14 (43.8)	0 (0)
Chloramphenicol	35 (53.0)	25 (78.1)	0 (0)
Ciprofloxacin	15 (22.7)	20 (62.5)	0 (0)
Cefoxitin	25 (37.9)	4 (12.5)	1 (20)
Erythromycin	35 (53.0)	18 (56.3)	1 (20)
Gentamicin	11 (16.7)	28 (87.5)	0 (0)
Neomycin	30 (45.5)	13 (40.6)	2 (40)
Oxacillin	36 (54.5)	30 (93.8)	4 (80)
Oxytetracycline	38 (57.6)	32(100)	0 (0)
Vancomycin	10 (15.2)	15 (46.9)	0 (0)
Trimethomprim/sulfamethaxazole	30 (45.5)	19 (59.4)	1 (20)

4.7 IDENTIFICATION OF HETERO RESISTANT STRAINS

The presence of sub population with lesser susceptibility within larger population of fully antimicrobial susceptible microorganisms (heteroresistance) has been recognized. Some isolates were found to exhibit hetero resistance phenotypically to some of the antibiotics used in this study. Highest level hetero-resistance was observed against ciprofloxacin (19.6 %), gentamicin (17.6 %), vancomycin (15.7 %) erythromycin and neomycin (13.7 %).The percentage of such isolates is shown in Table 4.12.

Table 4.12: Percentage of *Staph.aureus* isolates showing hetero resistance.

Antibiotics	**Hetero resistant isolates**	
	Number	**Percentage (%)**
Ciprofloxacin	10	19.6
Chloramphenicol	5	9.8
Erythromycin	7	13.7
Gentamicin	9	17.6
Neomycin	7	13.7
Oxacillin	5	9.8
Vancomycin	8	15.7

4.8 TEST FOR β – LACTAMASE PRODUCTION

The result of β lactamase production test showed that 88(89.8 %) produced β -lactamase. Ten were non β lactamase producers.The result is presented in figure 4.3.

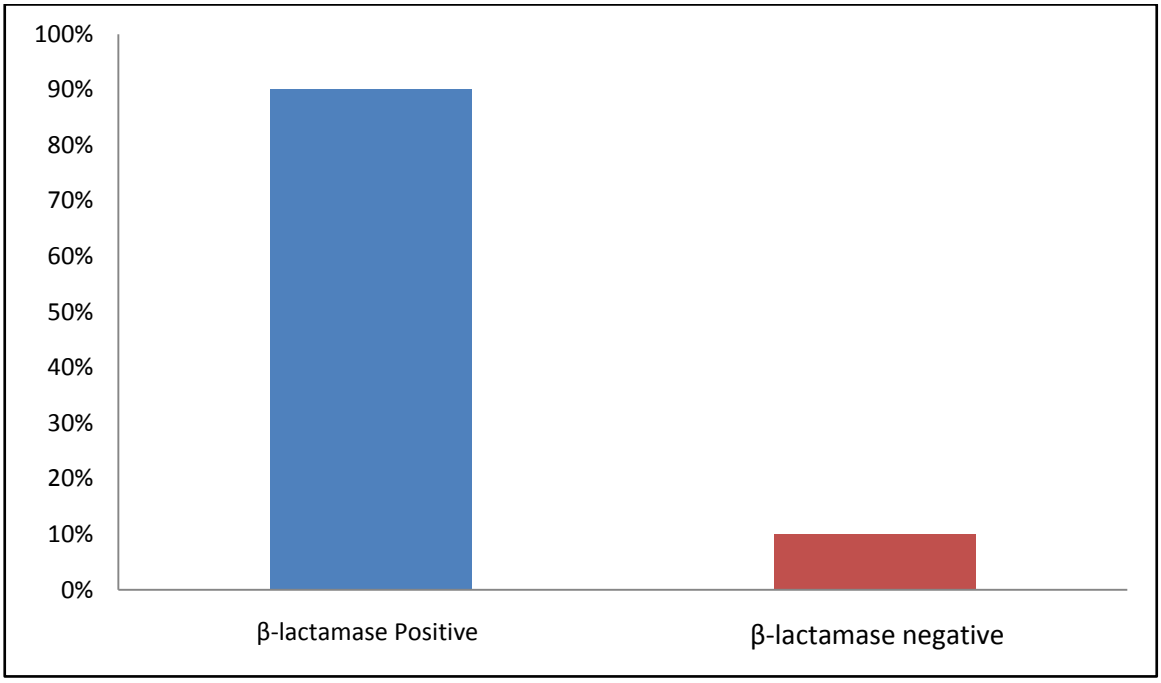


Figure 4.3: Percentage of β-lactamase production

4.9 DETERMINATION OF PHENOTYPIC METHICILLIN RESISTANCE USING CEFOXITIN

A total of 30 (30.6 %) of the 98 *Staph.aureus* isolates were resistant to cefoxitin 30µg (zone ≤ 21mm).This shows that 30.6 % were methicillin resistant *Staph.aureus* (MRSA) phenotipycally

4.10 DETECTION OF VANCOMYCIN RESISTANCE

Out of 30 methicillin resistant isolates, 27(90 %) grew on brain heart infusion agar supplemented with 6µg vancomycin, that is, they were vancomycin resistant (VRSA).

4.11 DETECTION OF PENICILLIN BINDING PROTEIN (PBP2A)

The presence of mecA gene product PBP2a which is responsible for methicillin resistance was determined in isolates that showed phenotypic resistance to cefoxitin. Thirty isolates were tested, 16 (53.3 %) methicillin resistant *Staph. aureus* were PBP2a positive and 14 (46.7 %) were negative.

4.12 DETERMINATION OF β-LACTAMASE HYPER PRODUCTION

The detection of β-lactamase hyper production was carried in methicillin resistant *Staph.aureus* isolates. Thirty isolates were tested and only 19 (63.3 %) were positive. Table 4.13 shows the antibiotic resistance pattern of β-lactamase producing and hyper β-lactamase producing *Staph. aureus*. The highest level of resistance was observed against all the β-lactam antibiotics: amplicillin 73 %, oxacillin 63.3 %, amoxicillin- calvulanic acid 46.73%. Oxytetracycline also had a higher resistance level 73.3 %, chloramphenicol 66.7 %, erythromycin 56.7 %, sulfamethazole/trimethoprim and vancomycin 50% each, ciprofloxacin

46.7% and gentamicin 33.3%.It was observed that 96.7% of the isolates were multidrug resistant being resistant to three or more antibiotic classes (Table 4.13)

4.13 MULTIDRUG RESISTANCE (MDR) AND MULTIPLE ANTIBIOTIC RESISTANT INDEX

Multidrug resistance which is the non-susceptibility to at least one agent in three or more antimicrobial categories of all the 30 phenotypic MRSA isolates was determined.MDR is shown in Table 4.13.

Table 4.13: Antibiotic resistance pattern of methicillin resistant, β-lactmase producing and hyper producing *Staph. aureus.*

Isolate	β-lactamase Producers	β-lactamase hyperproduce	Resistance pattern											Classification
127C	+	+	CIP	OT	SXT	VAN	E	OX	N	C	FOX			MDR
93C	+	+	AMP	OT	AMC	FOX	VAN	E	OX	N	C			MDR
12C	+	_	AMP	OT	FOX	SXT	VAN	C						MDR
63C	+	_	CIP	AMP	OT	FOX	E	OX	N					MDR
139C	+	_	CIP	AMC	OT	FOX	SXT	OX	N	C	E			MDR
65N	+	+	AMP	AMC	OT	FOX	SXT	VAN	E	N	C			MDR
86C	+	_	AMP	CIP	CN	FOX	SXT	VAN						MDR
67N	+	+	CIP	AMP	OT	FOX	SXT	VAN	OX	N	C			MDR
80C	+	_	CN	SXT	E	OX	C	FOX						MDR
62N	+	+	CN	AMP	AMC	FOX	E	C	N					MDR
14C	+	_	CN	CIP	AMP	FOX	OT	SXT	N	C	E	C	OX	MDR
136C	+	+	AMP	AMC	OT	FOX	SXT	VAN						MDR
69C	+		CN	AMP	AMC	OT	N	OX	FOX	OX				MDR
38C	+	_	AMC	CIP	OT	E	OX	C	N	FOX				MDR
73N	+	+	CN	CIP	AMP	AMC	SXT	VAN	FOX	E	OX	N	C	MDR
12N	+	+	AMP	AMP	FOX	VAN	OX	E	N	C				MDR
9C	+	_	AMP	OT	SXT	E	OX	N						MDR
64N	+	_	CIP	AMP	OT	FOX	N		C					MDR
83N	+	_	AMP	AMC	OT	FOX	VAN	E	C					MDR
25C	+	_	CIP	AMP	AMC	OT	E	OX	C	N				MDR
54C	+	_	AMP	AMC	OT	FOX	OX	N	C					MDR
75N	+	_	CN	CIP	AMP	OT	SXT	E	OX					MDR
112N	+	_	CN	OT	FOX	N	C							MDR
27C	+	_	FOX	SXT	VAN	E	N	C	OX					MDR
94N	+	+	AMC	OT	SXT	VAN	E	C	N	OX				MDR
39N	+	+	CIP	FOX										XDR
52C	+	_	CN	CIP	AMP	OT	SXT	VAN	E	OX	C	N		MDR
7N	+	_	CIP	AMP	AMC	FOX	VAN	E	OX	N	C			MDR
3C	+	_	CIP	AMP	OT	SXT	N	FOX						MDR
91N	+	+	CN	CIP	OT	VAN	Ox	N	C	E				MDR

Figure 4.4 shows the percentage antibiotic resistance pattern of β - lactamase positive phenotypic MRSA.

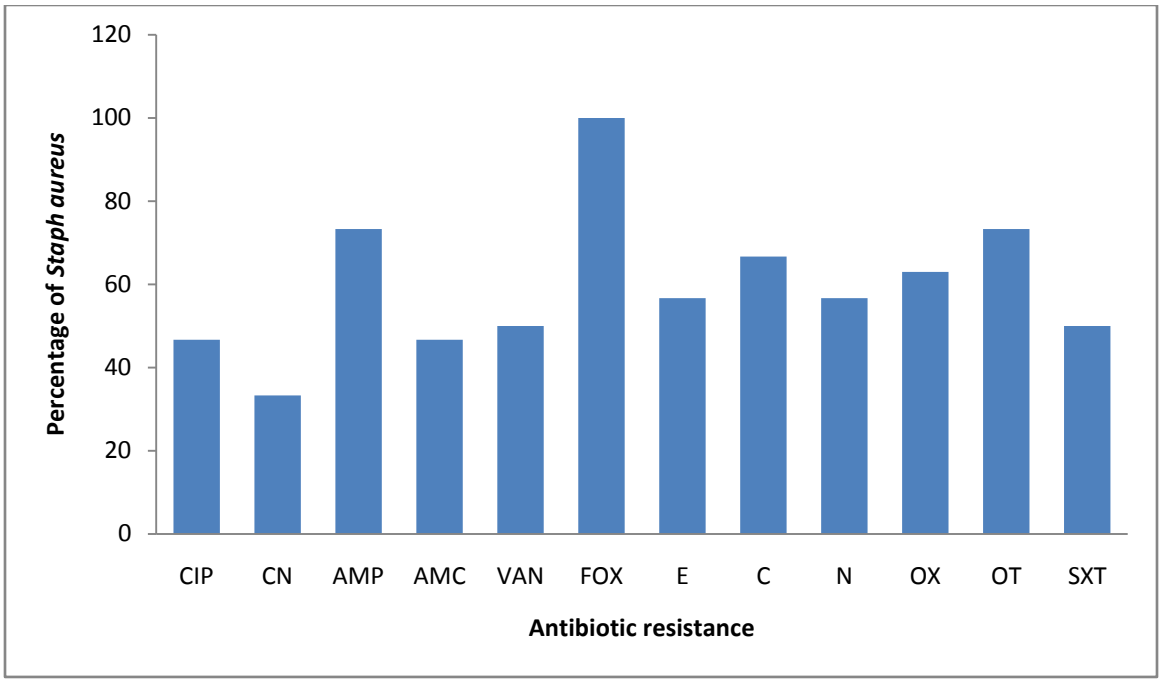


Figure 4.4: Antibiotic resistance pattern of β-lactamase positive methicillin resistant *Staph aureus*

CIP = Ciprofloxacin 5µg

CN = Gentamicin 10µg

Amp= Amplicillin 10µg

Amc= Amoxicillin-clavulanic acid 30µg

Van= Vancomycin 30µg

Fox= Cefoxitin 30ug

E=Erythromycin 15 µg

C=Chloramphemicol 30µg

N=Neomycin 30µg

OT = Oxytetracycline 30 µg

OX= Oxacillin1µ g

SXT=Sulfamethaxazole/trimethoprim25µg

The multiple antibiotic resistant indexes (MARI) for each of the 98 isolates were determined as:

$$\text{MARI} = \frac{\text{Number of antibiotics to which isolate is resistant}}{\text{Total number of antibiotics used}}$$

MARI showed that 80(81.6 %) isolates were resistant to three or more antibiotics. MARI $\geq$ 0.3 indicated that the isolates originated from an environment where antibiotics were frequently used. One isolate showed 100 % resistance to the twelve antibiotics tested. All the isolates were consistently resistant to oxytetracycline, ampicillin and oxacillin (Table 4.14)

Table 4.14 Antibiotic resistant indices of *Staph. aureus* isolates

No. of antibiotic to which resistant	Resistant isolates	MAR index	% of Staph to MARI
1	1	0.1	1.0
2	4	0.2	4.1
3	13	0.25	13.3
4	9	0.3	9.2
5	9	0.4	9.2
6	15	0.5	15.3
7	18	0.6	18.4
8	13	0.7	13.3
9	7	0.75	7.1
10	6	0.8	6.1
11	2	0.9	2.0
12	1	1.0	1.0

4.14 MOLECULAR ANALYSIS

4.14.1: Genomic DNA of *Staph. aureus* Isolates

The result of the DNA bands of the *Staph.aureus* isolates on 1.5 % Agarose gel electrophoresis is shown in plate 4.1

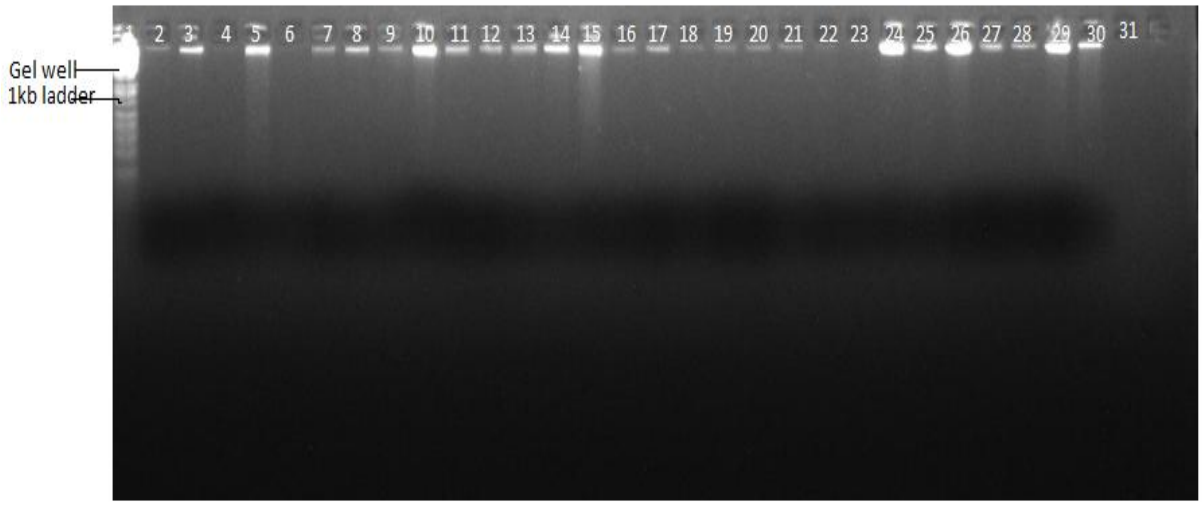


Plate 4 .1: Genomic extracted DNA of *Staph aureus* on 1.5 % agarose gel

Lane 1: gel well / 1kb ladder

Lane 2 – 14: *Staph. aureus* isolates from cloacae of the birds

Lane 15 – 29: *Staph. aureus* isolates from nasal swab of birds

Lane 30– 31 : *Staph. aureus* isolates from farm workers nostril

4.14.2 Detection Of 16SrRNA, And Nuc Gene

The 16SrRNA and the nuc gene were amplified and detected. Twenty five isolates were selected, only 21 showed the amplification of 800bp16SrRNA. This showed that all the isolate were *Staph. aureus* as 16SrRNA and nuc the thermostable nuclease gene were generally accepted as a genus species specific marker for detecting *Staph. aureus.* The multiplex PCR showed the amplication of 16srRNA 800bp. The result is shown in plate 4.2.

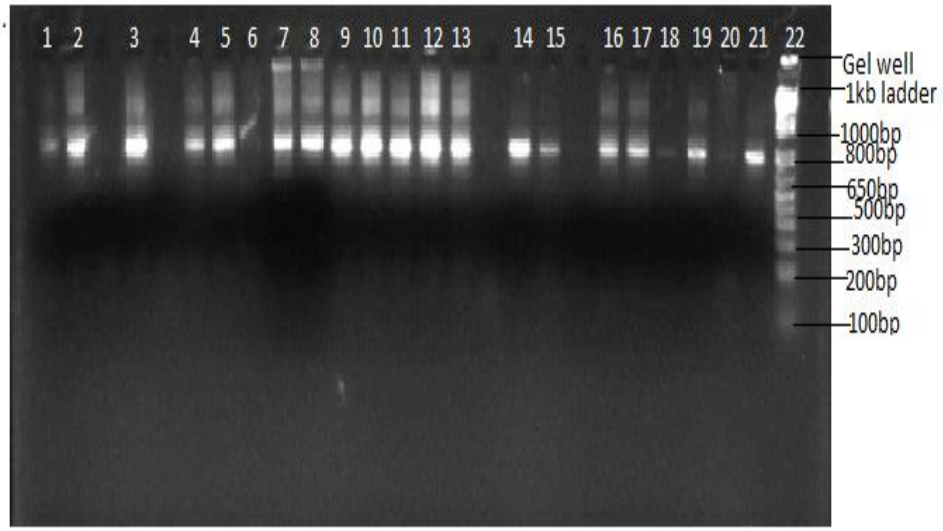


Plate 4.2: PCR product for 16SrRNA of *Staph. aureus* at 800bp .

Clear band confirmed that, all the isolates were *Staph.aureus.*

Lane 1-13: *Staph.aureus* isolates from cloacae

Lane 14-20: *Staph.aureus* isolates from nostril of the poultry.

Lane 21: *Staph.aureus* isolates from poultry farm workers nostril.

Lane 22: 1kb ladder

4.14.3: Detection Of MecA Gene

Out of the 22 cefoxitin resistant isolates tested 6 of the isolates possess mecA gene and the size of the amplicon correspond to 162 bp. Seven possess mecA gene and size of the amplicon correspond to 500 bp as represented by the mass ruler DNA ladder. Altogether the result indicated that 13 isolates (59.0 %) amplified mecA gene coding for methicillin resistant clear bands confirmed that all the 15 isolates were MRSA.The result is shown in plate 4.3.

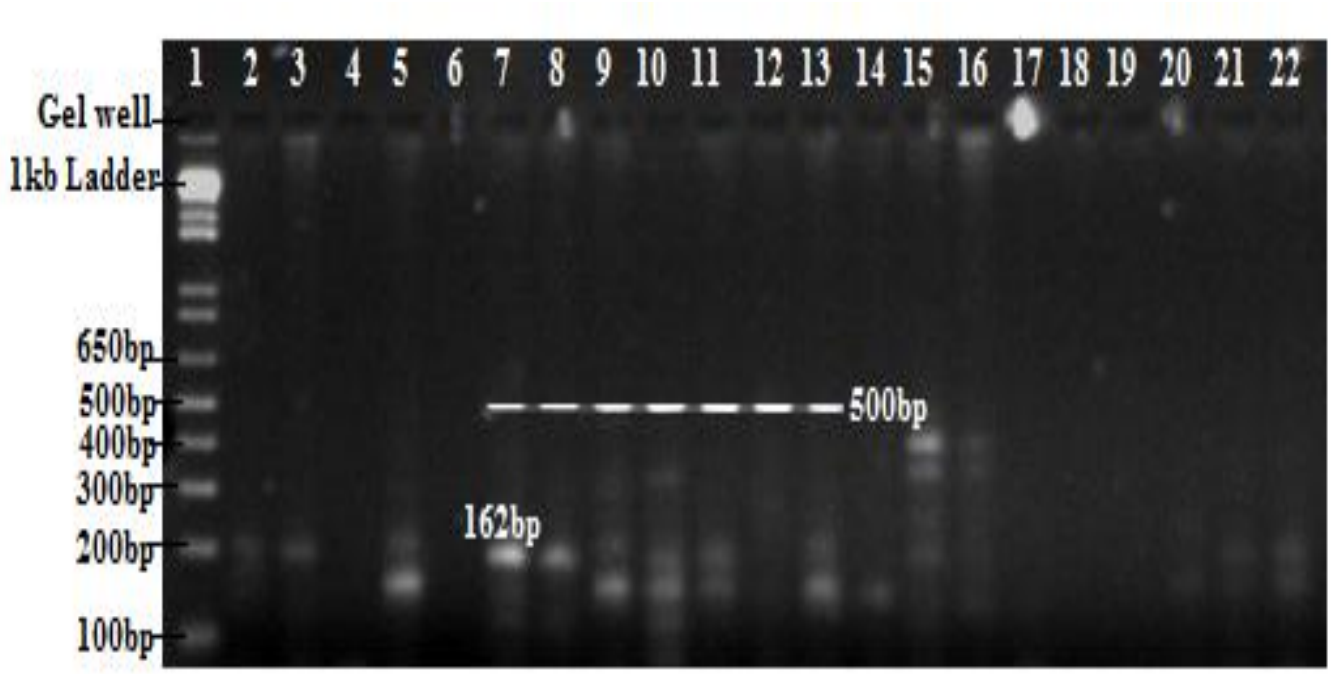


Plate 4.3 Multiplex PCR product for detection of mecA gene in *Staph. aureus*

Lane 1. 1kb ladder

Lane 2, 3 and 5: *Staph. aureus* from cloacae mecA positive

Lane 7- 13: *Staph. aureus* from cloacae mecA positive

Lane 4: *Staph. aureus* from cloacae mecA negative

Lane17 and 19: *Staph. aureus* from bird's nostril mecA negative

Lane 14: *Staph.aureus* from nostril mecA negative

Lane 20, 21:*Staph.aureus* from nostril mecA positive

Lane22: *Staph.aureus* from nostril of farm worker mecA positive.

4.14.4: Detection Of Nuc And Yqil One Of The Seven Housekeeping Genes

The result of the detection of nuc and one of the house keeping genes is shown in plate 4.4.

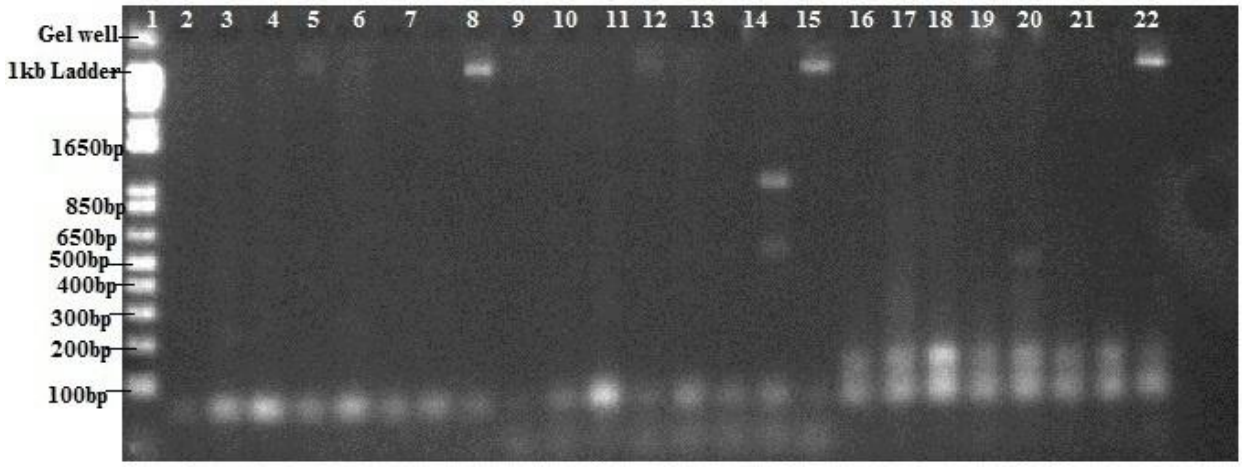


Plate 4.4: PCR product for nuc and yqil genes

PCR product applied 200bp on lane 16-23for nuc

Lane 1: gel well

Lane 2 – 13: *Staph.aureus* from cloacae

Lane 14– 21: *Staph.aureus* from bird's nostril

Lane 22: *Staph.aureus* from workers nostril

Lane 16-21 *Staph.aureus* from bird's nostril nuc gene positive

Lane 8: *Staph.aureus* from bird's nostril *yqil* positive

Lane 15: *Staph.aureus* from bird's nostril *yqil* positive

Lane 22: *Staph.aureus* from worker's nostril *yqil* positive

4.14.5 Spa Typing Of *Staph. aureus*

Sixteen of the isolates were sequenced for spa typing. The size of the amplicon for the spa typing is shown in plate 4.5. Lane one is the gel well and the ladder.Genes of seven (43.8 %) isolates were amplified and showed variation amongst the isolates and the amplicon size which ranges from 250 bp to 637 bp. Ten per cent of spa positive isolates were multidrug resistant isolates (Plate 4.5).

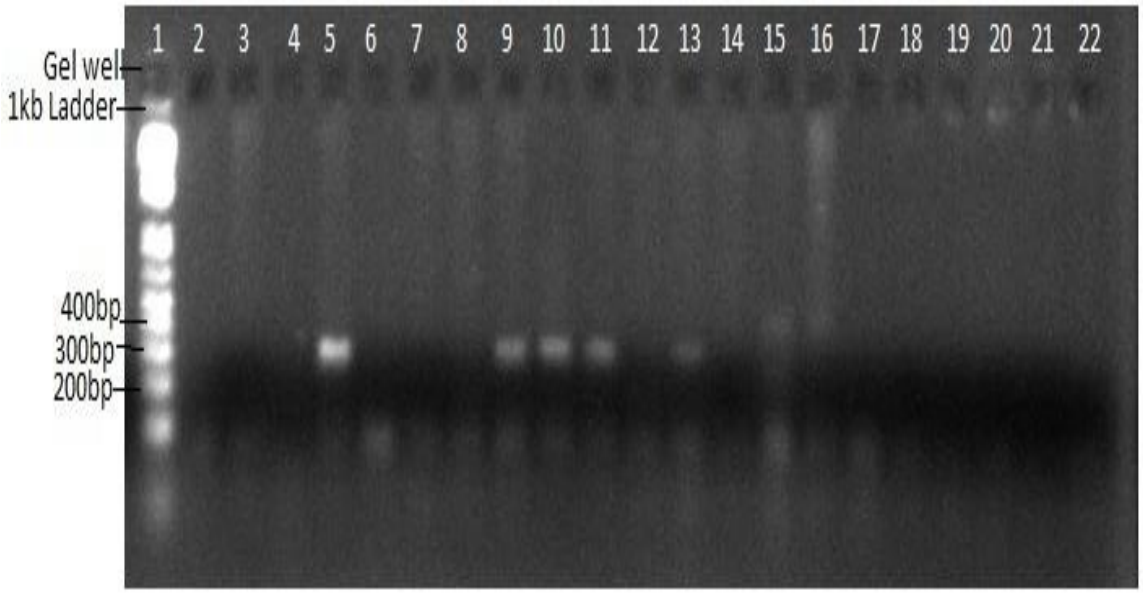


Plate 4.5: Spa typing of *Staph. aureus* on 1.5 % agarose gel electrophoresis

Lane 1: gel well ladder

Lane 2 -13 : Poultry MRSA from cloacae.

Lane 14-21: Poultry MRSA from nostril.

Lane 22: Poultry MRSA from workers nostril. Spa negative

Lane 5: Poultry MRSA from cloacae. Spa positive

Lane 9-11: MRSA from cloacae. Spa positive

Lane 13:*Staph. aureus* from cloacae Spa positive

Lane15 and16: MRSA from nostril Spa positive

4.14.6 PVL Detection

All the isolates were tested for the presence of panton valentine leukocidin (PVL) luks-lukf genes. PVL gene is a virulence determinant in *Staph aureus* and is associated with tissue necrosis and leukocyte destruction. Until recently, genes coding PVL were infrequently encountered, being noted in <5 % of *Staph. aureus* isolates worldwide. Genes from the fourteen isolates (66.7 %) were amplified, (Plate 4.6).

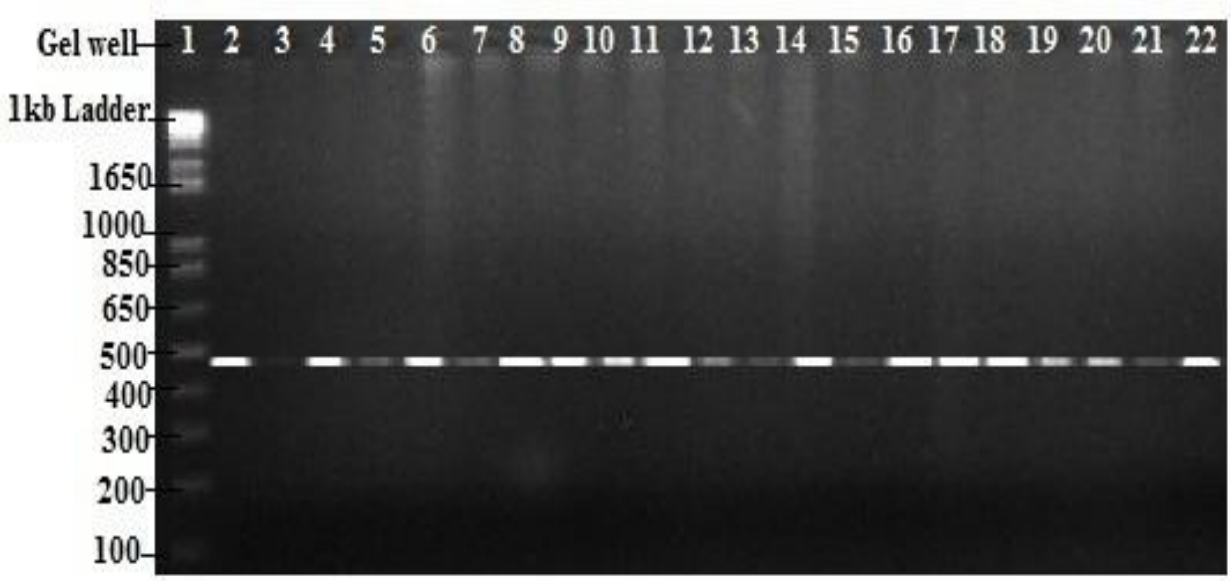


Plate 4.6: Amplification of PVL (lukS-luk F) gene in *Staph. aureus*

Lane 1: Ladder

Lane 2: MRSA PVL positive.

Lane 3: *Staph. aureus* from cloacae PVL negative.

Lane 4, 6*: Staph aureus* from cloacae PVL- positive.

Lane 7: *Staph.aureus* from cloacae PVL negative.

Lane8-11 MRSA PVL positive.

Lane 12 and 13: *Staph. aureus* from cloacae PVL negative.

Lane14: *Staph aureus* from nostril PVL- positive.

Lane 15: *Staph. aureus* from cloacanostril PVL negative.

Lane16-20: *Staph aureus* from nostril PVL- positive.

Lane22: *Staph aureus* from nosril PVL- positive.

None from farm workers.

4.14.7 Detection Of The Seven Housekeeping Genes

Three of the seven multilocus housekeeping genes were amplified **(***pta* (474bp), *gmk* (429bp) and *yqil*(516bp), at 43.3 %, 20 % and 16.7 % respectively. Four genes were not amplified in all the isolates using the primers (Plate 4.7).

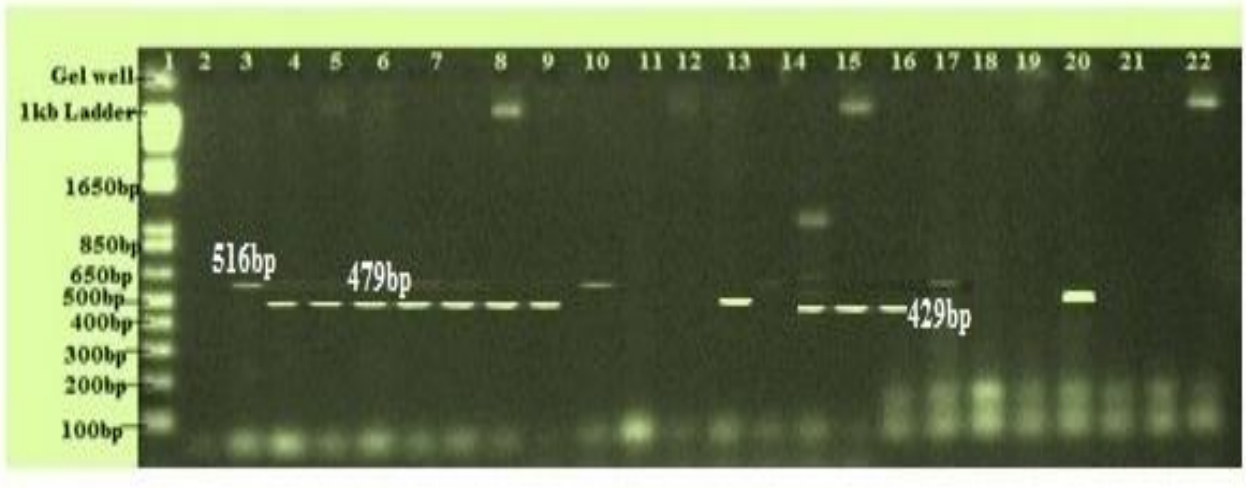


Plate 4.7: Molecular Characterization of the Seven House keeping genes

pta (474bp), *gmk* (429bp) and *yqil* (516bp).

Lane 1: 1kb ladder

Lane 2: *Staph aureus* from cloacae

Lane 3: *Staph aureus* from cloacae *yqil* positive

Lane 4- 9: *Staph aureus* from cloacae *pta* positive

Lane 10: *Staph aureus* from cloacae *yqil* positive

Lane 13: *Staph aureus* from cloacae *pta* positive

Lane 14-16: *Staph aureus* from nostril *gmk* positive

Lane 17: *Staph aureus* from nostril *yqil* positive

Lane 20: *Staph aureus* from nostril *yqil* positive

Lane 21-22: *Staph aureus* from nostril.

4.14.9 DNA Sequencing

Sixteen of the isolates were selected for DNA sequencing of the PCR product. The phylogenetic tree comparing the nucleotide sequence of the selected isolates with the other *Staph aureus* strain from the gene bank data base was shown in plate 4.8

As observed from the phylogenetic tree in this study, there are three main clusters based on their similarities and members within a cluster are more related than between clusters. It is also observed that isolates are mostly clustered together which further confirms their similarities except for two isolates (17 and 6) which may be either from where the sample originated or mutation of the gene. Plate 4.9 showed that they share the same evolutionary origin. There is a relationship between isolate number 28 which was from farm worker and the members of the cluster which were from the birds and the difference in the time of divergence is not wide. This showed that the organisms originated from the same source, indicating the possible transmission either from humans to birds or from birds to humans.

(A) Phylogenetic Tree

This result showed the inter relationship between the isolates

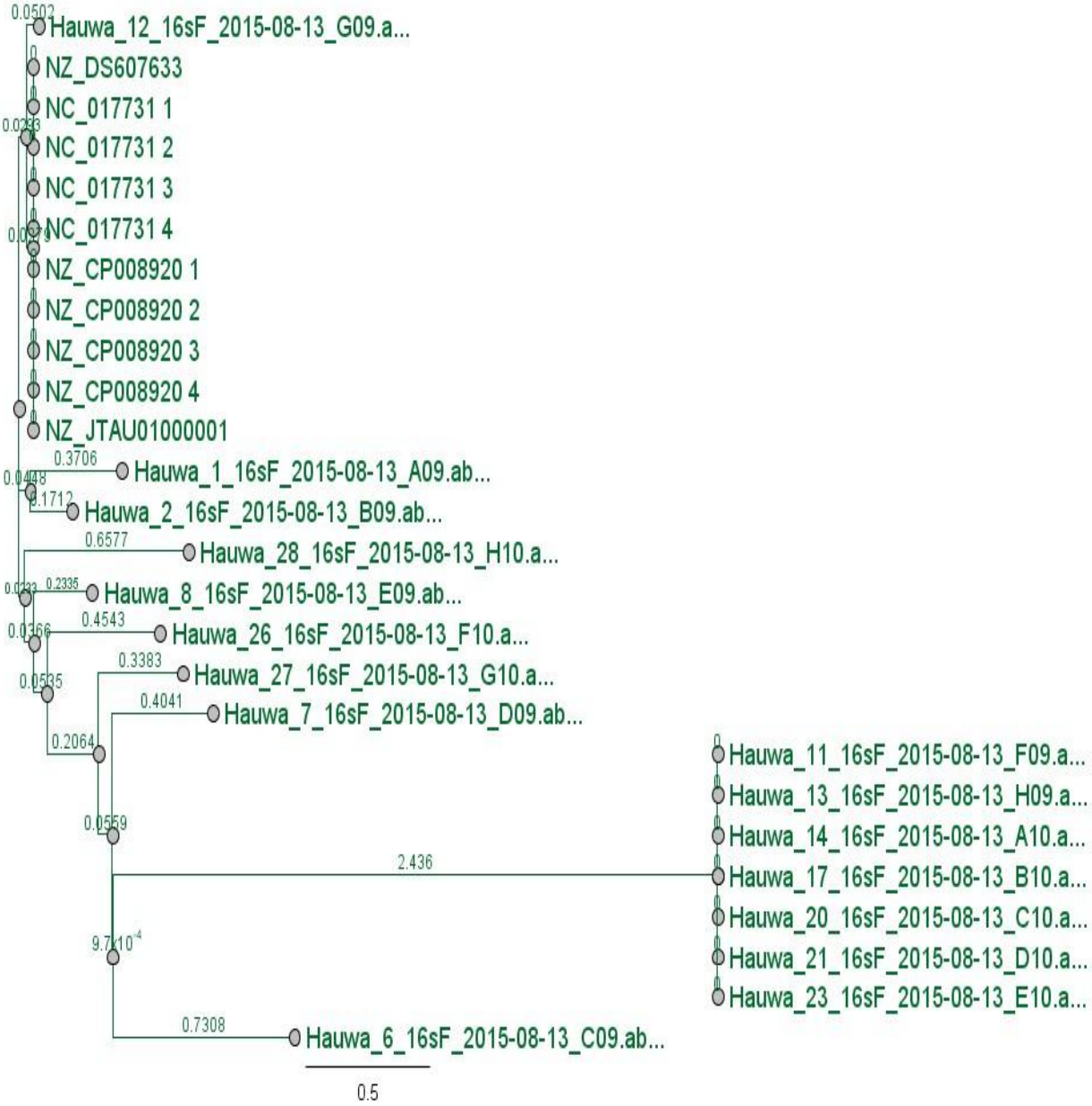


Plate 4.8: Phylogenetic tree of 16 *Staph. aureus* isolates.

(B) Variation Analysis

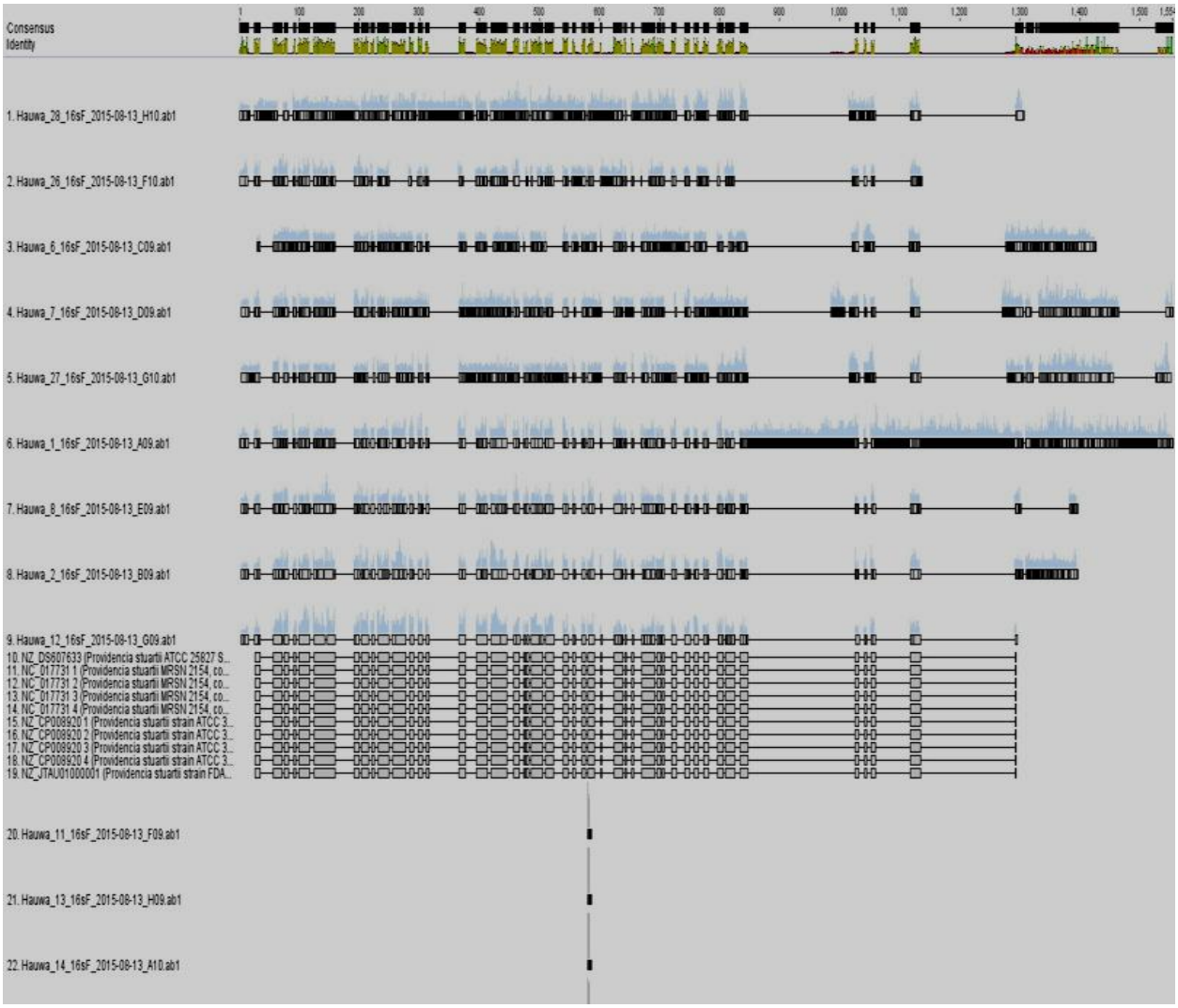


Plate 4.9:Variation analysis *Staph. aureus*

CHAPTER FIVE

5.0 DISCUSSION

Various studies have recognized *Staph. aureus* as an important pathogen isolated in poultry farms and other food producing animals. Occasional reports have been published of MRSA infection in domestic animals including dogs, cats, cattle, sheep, chicken, rabbit, and horses but in recent years, the number of cases appears to be increasing (Hartman *et al*., (1997); Tomlin *et al*., (1999); Lee, (2003a); Goni *et al*., (2004); Rich and Robert, 2004). Processed poultry products have been noted as favorable environments for the survival and transmission of various commensal, spoilage, and potentially pathogenic bacteria in the human food chain, with *Staphylococci* spp. accounting for one of the most predominant bacteria groups **(Huys *et al.,* 2005**). In view of this, isolation and molecular characterization of *Staph. aureus* from healthy chickens nostrils and cloacae, and farm workers nostril were evaluated in Kano State.

In this study 622 isolates were coagulase positive, out of which 290 were *Staph. aureus* using latex agglutination, and 98(33.8 %) isolates were finally confirmed to be *Staph aureus* from 12 poultry farms and farm workers using microgen staph identification kit. Statistical evaluation of our data showed that there was significant difference between the methods used for confirmation of *Staph. aureus* at confidence level $\alpha = 0.01$ since $3045.5 > 10.92$

The *Staph.aureus* recovered were from cloacae and nostril of the chickens and from nostril of poultry farm workers. Several studies have reported *Staph. aureus* in animal (Umaru *et al*., 2011) especially in their nares (Persons *et al.,* 2009) and in chicken droppings (Olayinka *et al*., 2010).The percentage of *Staph. aureus* isolated in this study is moderate

compared with the report of Suleiman *et al.,* (2013) who reported 83 % incidence of *Staph. aureus* from tracheal swabs of 100 apparently healthy chicken with 54 % being coagulase positive in Maiduguri. The isolation of *Staph. aureus* from poultry and human samples in this study further support the possibility of zoonotic infections in human (**Ellerbroek, 1997; Huys *et al.*, 2005).**

The distribution of *Staph. aureus* isolates in the three senatorial zones showed that the majority of the isolate were from Kano north (39.8 %), Kano central which had (35.7 %) and Kano south (24.5 %).

The entire twelve farms screened harbored *Staph. aureus* in varying number and percentages. The differences in the recovery of *Staph. aureus* amongst the farm could be attributed to the level and number of antibiotics used, contamination of the birds and hygiene level in each of the farms. Both cloacae and nostril yielded growth with more *Staph.aureus* from cloacae than in the nose. This was consistent with the report of Bassetti and Battegay, (2011) that *Staph.aureus* resides asymptomatically on the skin and in the nose of animals. *Staph. aureus* was found to be in varying proportion from the nares and the cloacae, with less number in the nostril.The percentage of *Staph.aureus* recovered from cloacae was 57.1 % while the ones that were isolated from nostril was 37. 8%. This might be associated with the feed which has been reported to carry contaminants including *Staph aureus* (Crump *et al*., 2002) and are shed in droppings part of which could be retained on the cloacae. Olayinka *et al*., (2010) reported dropping as a good specimen for recovery of *Staph. aureus* in poultry .In this study chicken droppings were not screened because the study birds were not caged as such it was difficult to established that a particular dropping was from a particular chicken. Statistical analysis showed that there was a significant difference between *Staph. aureus*

recovered from cloacae and nostril and nostril of farm workers at confidence level α=0.05 since 66.41>5.1 . It could therefore be inferred that cloacae was the best site for recovery of *Staph. aureus* in poultry than the nostril, an inference in agreement with Plentinckx *et al*., (2011) who reported statistical significant in cloacae than nostril.

The number of *Staph.aureus* isolates was found to be higher among broiler than the layers. Statistical analysis showed that there was significant difference in prevalence of *Staph. aureus* among broilers and layers in this study.Since 9.55 >1.17 with α=0.05.

The overall carriage of *Staph. aureus* amongst poultry, in this study was (7.8 %) n=1260. The prevalence of *Staph.aureus* in poultry in this study is low and low prevalence has also been found by Kitai *et al*., (2006) although they sampled chickens carcasses from the slaughter houses and did not find any livestock associated strain. This low prevalence is also in line with previous study by Dullweber, (2010) in Germany with 16.0 %.

Staph. aureus has also been isolated from the nasal swab of poultry farm workers with low prevalence of 5.1 % (n=60). The percentage carriage was low compared to the finding of Mulders *et al*., (2010) who reported a nasal carriage of 13.8% among rearers and Smith *et al*., (2009) who reported 45% carriage amongst swine workers. Carriage of 25-30 % of *Staph aureus* has been reported amongst farmers in the Netherlands (Van low *et al*., 2007).

Nasal carriage of *Staph. aureus* has been demonstrated as significant risk factor in nosocomial and community acquired infections in several populations (Kumar *et al*., 2011; Kock *et al*., 2013). Exposure to livestock has been demonstrated as a serious risk factor (Smith *et al*., 2009; Van Cleef *et al*., 2010).

Reports have also highlighted the possibility of poultry products contamination by MRSA which when in contact with human nasal or skin lesion may lead to colonization and

infection. Ingestion of poultry products contaminated with MRSA can cause gastrointestinal colonization or food poisoning leading to staphylococcal enterotoxin-associated diarrhea and subsequent extra-intestinal infection or transmission (Febler *et al.*,2010).

The nasopharynx of humans remains major niche of *Staph aureus* where they exist as normal commensals (Bien *et al*., 2011). As reported elsewhere, nasal carriage remains a major threat to human health as it may emerge from colonized sites to cause infection or to be transmitted to other persons (Rodrigues-Noriega *et al*., 2010). A carrier of *Staph. aureus* or MRSA is at a potential risk, especially when admitted to hospital, of developing an infection involving the colonizing pathogens and of transmitting the MRSA to other patients or health workers with risk factor for infection.

The antibiotics used in the farms included in this study were ampicillin, Augmentin^{R}, oxacillin, vancomycin, cefoxitin, sulfamethaxazol/trimethoprim, oxytetracycline, erythromycin, chloramphenicol, neomycin, ciprofloxacin, and gentamicin. Antibiotics such as Oxytetracycline, oxacillin and ampicillin were observed to have low activity against the test isolates, with 71.4 % of the isolates being resistant. Similar patterns of antimicrobial susceptibility have been reported in Zaria, Nigeria by Otalu *et al.*, (2011) and Olayinka *et al*., (2010), Italy (Pesavento *et al.,* 2007), United States (Waters *et al.,* 2011) and Ireland (Leonard and Markey, 2008). This might be as a result of extensive usage of these antimicrobial agents in animal husbandry over time, which has contributed to the selection of drug-resistant strains (Mostafa *et al.,* 2008).

One of the isolates was observed to be resistant to all the 12 antibiotics tested while another one was susceptible to all the antibiotics tested. This suggest that such resistant isolates originated from a high risk source of contamination where antibiotics are often used

or that a large proportion of the bacterial isolates have been pre-exposed to several antibiotics (Christopher *et al.,* 2013). Also the use of antibiotics as prophylaxes, growth promoters or inaccurate dosages given to sick flocks by unqualified personnel may likely result in plasma concentrations that are inconsistent with the desired objectives which might possibly influence resistance (Suleiman *et al.*, 2013).

Bacteria often develop resistance to β-lactam antibiotics by synthesizing β-lactamase, an enzyme that attacks the β-lactam ring.To overcome this resistance, β-lactam antibiotics are often given with β-lactamase inhibitors such as amoxicillin (β lactam antibiotic) and clavulanic acid (β-lactamase inhibitor). The clavulanic acid is designed to overwhelm all β-lactamase enzymes, bind irreversibly to them, and effectively serve as an antagonist so that the amoxicillin is not affected by the β-lactamase enzymes (Fukatsu *et al*., 1990). This might have accounted for the high percentage (69.4 %) of sensitivity to Augmentin^{R} in this study. Susceptibility to cefoxitin (69.4 %), followed by ciprofloxacin (64.3 %) and then gentamicin (60.2 %) was high. The high resistance level in the isolates to β-lactam drugs is to be expected in this study, as 89.8 % of the *Staph aureus* were β-lactamase producers. It is observed that *Staph aureus* isolates are resistant to a large number of commonly prescribed antibiotics with the β-lactam agent taking the highest proportion. It is believed that more than 80 % of staphylococcal isolates now produce penicillinase regardless of their origin (Pantosti *et al*., 2007).

The detection of β-lactamase in 89.8 % of the *Staph aureus* isolates tested point to the possible role of this enzyme in mediating resistance to β-lactam antibiotics than PBP2a production as 53.3 % of the isolates were PBP2a positive. This is because production of the enzymes coded for by a gene is a major mechanism of resistance to β-lactam antibiotics .The

level of β-lactamase production was determined to allow the identification of β-lactamase hyper producers. It was found that 11(36.7 %) were positive. Tenover *et al.,* (1994) stated that, the resistance in *Staph. aureus* mainly involve two mechanisms: The expression of β-lactamase and mecA gene. The methicillin resistance in some PBP2a negative *Staph aureus* strain in this study may be attributed to the hyper production of β-lactamase, indicating high rate of phenotypic MRSA compared to 16/30 isolates that was PBP2a positive.

Depending on the methicillin susceptibility testing result 30 (30.6 %) of the *Staph. aureus* were classified as MRSA, and 11 (37.9 %) of the isolates expressed mecA gene by PCR in addition to β-lactamase enzymes production. This result agreed with Fukatsu *et al*., (1990) who reported that 81.3% of *Staph. aureus* isolates were β-lactamase producers in Japan and is in conformity with the result of Al-Ruaily and Khalil (2001) who reported 86 % of *Staph. aureus* isolates to be β-lactamase producers.

Heteroresistance has been described in a wide range of microorganisms, but recent attention has been directed towards its expression in *Staph. aureus* which were first described in 1997 (Hiramatsu *et al*., 2001) shortly after the initial description of vancomycin intermediate susceptible strains (VISA). Erythromycin heteroresistant MRSA have also been isolated from Turkey 7 % (Wong *et al*., 2004). This is low as compared with the finding in this study where there was 13.7 % prevalence. Exact mechanisms of hetero resistance and its roles in adaptation to antibiotics stress are yet to be fully understood at the molecular level and single cell level (Wong *et al*., 2004). In *Staph. aureus,* heteroresistance to methicillin is dependent on the structural gene mecA which codes for a low affinity penicillin binding protein (PBP2a) (Ryffel *et al*., 1994). In addition to this low level resistance, mecA positive *Staph. aureus* possess an intrinsic ability to generate a small portion (10^{-8} – 10^{-4}) highly

resistant subclones (Stranden *et al*., 2003). The gene responsible for this has not yet been identified, but it has been shown that the conversion must be due to chromosomal mutation(s) that are not related to mecA (Ryffel *et al.,* 1994; Stranden *et al*., 2003). Heteroresistance to vancomycin have been reported in as many as 9 % of 129 MRSA isolates in Japan (Hiramatsu *et al*., 2001), UK (Howe *et al*., 1999). Heteroresistance to vancomycin was observed in 15.7 %, of the isolates and 17.6 % to gentamicin. Recently, heteroresistane has also been reported for teicoplanin (Park *et al*., 2000).

The prevalence of MRSA in this study was 30.6 % which is high compared to other studies in Morocco where 19.3 % and 35.4 % were reported at University Hospitals, in Rabat (Elhamzavol *et al*., 2009) and at Ibn Rochd University Hospital Center in Casablanca (Zriouil *et al*., 2012) respectively. Interestingly, the site of isolation of MRSA and specimen type has been found to be associated with prevalence of MRSA. In a survey on the prevalence of MRSA in Nigeria; Taiwo *et al*., (2004) obtained 34.7 % for Ilorin, while Olonitola *et al*., (2005) obtained 33.3 % from Federal Medical Center. Onanuga *et al*., (2006) reported a higher rate of 71.1 % from urine of healthy woman while Ikeh and Yakeu (2006) reported an alarming 92.6 % MRSA out of the *Staph. aureus* isolated from bacteria flora on the hands of nursing service workers .

A common factors in all these studies is the method (phenotypic detection using cefoxitin) of detecting MRSA which is generally regarded as less sensitive in detecting classical methicillin resistance mediated by mecA gene that code for the production of additional penicillin binding protein PBP2a which confers resistance to all β-lactam antibiotic (Brown *et al*., 2005).Out of the thirty MRSA, 16(53.3 %) were positive for penicillin binding protein which is encoded in mecA that is responsible for methicillin

resistant. This account for 16.3 % of the total staphylococcal isolates in this study. Also only 5 isolates out of 60 sampled from human source were found to be MRSA positive yielding a prevalence estimate of 8.3 % of the total sample.

Cefoxitin 30 µg was used and it is the best for determining mecA mediated resistance in *Staph aureus* (CLSI 2007). Cefoxitin will only detect MRSA with mecA mediated resistance mechanism (Swenson *et al*., 2007). Sixteen (53.3 %) out of the 30 cefoxitin resistant isolates carried mecA gene and contained the mecA gene product (PBP2a) determined by MRSA screen latex agglutination test. The binding specificity of the penicillin binding protein (PBPs) is altered in resistant strain and this is the type of resistance that is posing problem clinically, because resistance due to β-lactamase production can be prevented by using β-lactamase inhibitors, but does not work for alteration in PBPs. Resistance has been known to be caused by a gene called mecA, which codes for resistance to methicillin in *Staph aureus* (Louis *et al*., 2000), and the gene is located on the staphylococcal chromosomes cassette mec.

CLSI, (2010) has recommend that oxacillin be replaced by cefoxitin, a more potent inducer of mecA expression, that is less affected by test condition and hyper production of penicillinase (Brown *et al*., 2005). The gold standard for the detection of MRSA is the polymerase chain reaction (PCR) that detects mecA gene or alternatively detecting the mecA gene product PBP2a by latex agglutination test (Berger Bachi and Rocher 2002).

The result of vancomycin agar screening showed that 27(90 %) of the MRSA isolates were resistant to vancomycin on BHI agar supplemented with 6µg vancomycin. Isolation of VRSA threatens the most important treatment option to clinicians for infection caused by methicillin resistant *Staph aureus*. This is an alarming result due to the fact that the

vancomycin did not use selective pressure and is nonexistent in the environment. Since vancomycin is rarely prescribed and is not available over the counter.

In this study the disc diffusion method using standard 30µg vancomycin disc indicated high sensitivity (74.5 %) against staphylococcal isolates. Generally glycopeptides are highly effective with few cases of resistance. Their cost and route of administration might have prevented their ready availability over the counter and subsequent abuse that could have led to the strain development of resistance in community. Vancomycin is the drug for the treatment of MRSA infections. Now vancomycin is often combined with other antibiotics, most often rifampicin or gentamicin for the treatment of serious MRSA infections (Stan, 2009).

Vancomycin has been the most reliable therapeutic agent against methillin resistant *Staph aureus* and resistance to this glycopeptides is seen as serious problem in antimicrobial chemotheraphy of MRSA infection (Appelbaum, 2007).

Various studies carried out in diverse population and different setting have reported vancomycin resistance in MRSA, however in 1996, the first MRSA to acquire resistance to vancomycin was isolated from Japanese patient, subsequent isolation of several vancomycin resistance *Staph aureus* (VRSA) strain from USA, France, South Korea, Africa and Brazil ranging from 0-8 % has confirmed the emergence of VRSA a global issue (Hiramatsu *et al*., 2001). From 2002-2010 ten additional VRSA isolates were reported, eight from US one from Iran one from India (Gould, 2010).

By the end of 2013 VRSA isolates have been reported for the first time in Europe (ECDE) and Latin America. Whitener *et al*., (2004) had opinion that the use of vancomycin agar screening tests for MRSA may be the best reliable method to screen for Vancomycin

Intermediate *Staph. aureus* (VISA) or Vancomycin Resistant *Staph. aureus* (VRSA), the peculiar slower growth rate that is the characteristics of VISA strain may have created a problem for validity of test result which may be influenced by the temperature fluctuation during incubation and may have reduced the growth rate. Tenover *et al*., (1997) reported that the disc diffusion sensitivity testing using standard 30µg vancomycin disc frequency misclassify susceptible isolates as fully susceptible.

The observed activity of gentamicin in this study (60.2 %) suggests that it can be combined with vancomycin the treatment of MRSA infection. A number of studies demonstrated in vitro synergy between gentamicin and vancomycin against many MRSA isolates (Watanakunakorn and Glotzbecker, 1974). In fact the current guidelines for the treatment of prosthetic valve endocarditis (PVE) due to MRSA recommended the use of the three drug combination of vancomycin, rifampin and gentamicin, with the aminoglycoside administered for only the first 2 weeks of therapy (Baddour *et al*., 2005).The aim of this combination is to prevent the emergence of strains with reduced susceptibility to vancomycin. Prolonged exposure, both in vitro and in vivo, to vancomycin may lead to the emergence of reduced susceptibility to this glycopeptide antibiotic (Bennett, 2008; Mariani *et al.,* 2006; Mwanji *et al*., 2007). The addition of a second antibiotics that is rapidly bactericidal and that has a high threshold for the development of resistance could narrow the mutant selection window (Firsov *et al*., 2006) and has the potential to prevent the emergence of reduced susceptibility to vancomycin; enhancing tissue and intracellular penetration (vancomycin penetration into a number of compartments including the lungs), Lamers *et al*.,(2011); Moise Broder *et al*.,(2004), subcutaneous tissue (Skhirtladze *et al*., 2006), cortical bone (Garazzino *et al*., 2008), and cerebrospinal fluid (Jorgenson *et al*., 2007), is

limited, as is its intracellular activity (Yamaoka, 2007). Coadministration of drugs with more favorable penetrative characteristics, may have the potential to overcome these deficiencies reducing staphylococcal toxin production, since production of at least some toxins is reported to be increased by β-lactam antibiotics and to be diminished by clindamycin and linezolid, whereas vancomycin has no significant effect (Yamaoka, 2007; Stevens *et al.*, 2007; Dumistrescu *et al*., 2008).

The development of resistance to flouroquinolones may be connected with the increase 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). Ciprofloxacin interfere with DNA synthesis since bacterial resistance to the 4-quinolones agents is not plasmid mediated (Courvalin, 1986). The antibacterial effect of ciprofloxacin observed (64.3 %) in this study showed that it can be used to treat MRSA infection, even some of the VRSA isolates were sensitive to ciprofloxacin. In a study by Mullingan *et al*., (1987) on ciprofloxacin for eradication of methicillin resistant *Staph. aureus* colonization, ciprofloxacin 750 mg orally twice a day was used to treat 22 episodes of methicillin resistant *Staph. aureus* colonization among 20 patients. For the 18 patients who received at least two weeks of therapy, results of the cultures from 47 of the 56 colonized sites became negative.

The relative ineffectiveness of oxytetracycline, ampicillin and chloramphenicol might be due to wide spread use and high level of adulteration associated with these antibiotics. *Staph. aureus* is a good example of Gram positive bacteria that resist tetracycline (Harizawala *et al*., 2000). Resistance to tetracycline is known to be determined by small but

closely related multi copy plasmids. Chromosomally encoded tetracycline resistance is very rare. It is accepted that tetracycline resistance is due to decrease drug accumulation, but recently, several experiments have shown that the efflux mechanism seems to be the major cause of tetracycline resistance. As reported by Davies and Davies (2010), the huge prescription and extensive use of antibiotics without control and caution may account for widespread of antibiotic resistant organisms. High percentage (81.6 %) of the isolates were resistant to three or more antibiotics, MARI $\geq$ 0.3 indicated that the isolates originated from an environment where antibiotics were frequently used (Christopher, 2013).

The highest (20%) prevalent multidrug resistance pattern was observed against combined resistance to ampicillin, oxytetracycline, oxacillin, chloramphenicol and erythromycin. *Staph. aureus* with multi drug resistance has been reported in poultry in China with prevalence that varied from farm to farm in relation to severity of use of antibiotics. All the MRSA in this study exhibited higher multidrug resistance than the *Staph. aureus*. It has been documented that MRSA adaptability and propensity for acquiring multidrug resistance genes among species and from the environment by horizontal gene transfer accounts for its multidrug resistance (Lupo *et al*., 2012). One isolate showed 100 % resistance to all the antibiotics tested.

In all the farms screened, most of the antibiotics used in the study were commonly used as food supplements, prophylaxis, and treatment of diseased birds and this could be the reason for the resistance of these isolates to a good number of the antibiotics.

There has been a tremendous increase in the number of MRSA cases in the past few years. Therefore, several efforts have been made for quicker and early detection of MRSA. Molecular methods have now become the gold standard for rapid detection of MRSA.

Several studies have reported the use of mecA marker for detection of methicillin resistance and 16SrRNA and nuc for identification of *Staph. aureus* species. However, in Nigeria there are reported cases of detection of mecA gene in MRSA isolates from different parts of the country. In a research in Benin City, Nigeria, 4 isolates representing 11 % were confirmed to carry mecA gene according to molecular technique (Obasuyi, 2013), while Clement, (2009) confirmed only one MRSA isolate from healthcare institutions from Ekiti and Ondo states. In another study 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 (South western Nigeria). In an international study of the prevalence of MRSA among Veterinarians, Wulf *et al*., (2008) reported that 12.5 % of participants from 9 countries carried MRSA and transmission of MRSA from pig to staff appears to be an international problem, creating a new reservoir for community-acquired MRSA (CA-MRSA) in humans in Europe and possibly worldwide. Faires *et al*., (2009), in a study of the prevalence of concurrent MRSA colonization in people and pets in the same household, reported that 17.9 % humans, 8.3 % dogs, and 10 % cats were colonized with MRSA.

Molecular analysis of the isolates from bird's cloacae and nostril showed that all the isolates that were cefoxitin resistant are *Staph. aureus.* They were positive for the species specific marker 16SrRNA. An intriguing finding in this study was the non-amplification of nuc the thermostable nuclease in most (63.6 %) of the isolates when screened, as compared to 16rRNA which showed more specificity and sensitivity. However, the absence of nuc gene in 14 *Staph. aureus* isolates, is suggestive of likely mutations or deletions in the nuc gene. Thermostable nuclease gene nuc was reported to have 100% sepcificty and sensitivity for the identification of *Staph. aureus* isolates (Brackstad *et al*., 1992; Cai, *et al*., 2007). In India

only a few studies have reported the use of nuc along with mecA as molecular targets for identification of *Staph. aureus* and characterization of MRSA (Mathews *et al*., 2010; Chakraborty *et al*., 2010).

The results showed that there is a correlation between phenotypic cefoxitin resistance and carriage of *mecA* gene. The percentage carriage of mecA amongst poultry (cloacae and nostril) was 53.6 %. Only one isolate expressed mecA from the poultry farm workers. This carriage is regarded as very low (3.3 %), as compared to the result of Van Cleef *et al*., (2010) who reported 5.6 % overall prevalence of nasal MRSA carriage amongst the population of 3 large pig slaughter house, workers in the Netherlands. This was higher than in the general population, suggesting that working with live pigs was the single most important factor for being MRSA positive (Van Cleef *et al*., 2010). Smith *et al*., (2009) reported 45 % carriage of MRSA amongst swine workers in the Midwestern United State of America.

A total of 21 isolates were screened for PVL (lukS-lukF) associated with virulence in *Staph. aureus*. PVL is a toxin composed of two components lukS-lukF. It is one of the virulence factors that have a major role in pathogenicity of *Staph. aureus*. Thirteen isolates were PVL positive (66.7 %). Several studies have reported an association between PVL genes and invasive diseases and are mostly associated with community acquired infections. PVL has been reported in China not only in CA-MRSA but HA-MRSA, which suggests that PVL is not an exclusive marker of CA-MRSA (Deurenberg *et al*., (2007); David and Daum, (2010). All the PVL positive isolates in this study were from poultry. The finding of PVL genes amongst poultry isolates in this study portend great risks to those working in close contact with poultry birds due to its association with necrosis and skin infections. PVL positive isolates appeared to be more successful pathogens in skin and soft tissue infections

(SSTIs) and because of its high transmissibility, this strains could cause serious clinical conditions if it makes its way into the community and hospital setting just like MRSA USA 300 (Omuse *et al*, 2013).

Staph. aureus encodes many virulence factors including the surface Ig-binding protein A(Spa). Spa is an important virulentce factor which enables *Staph. aureus* to evade host immune responses; it acts as an immunological disguise. Ten percent of the MDR isolates express Spa typing (Plate 4.5) with varying length. It is known that spa gene shows a variation in lenghth in different strains. The length of the spa gene depends either on resistance to Methicillin or the source of *Staph. aureus* isolation.

MLST is a powerful tool for understanding the evolutionary dynamics of pathogens and to gain insight into their genetic diversity. Evolutionary analyses on the seven multilocus sequence typing (MLST) genes that encode for proteins of central metabolic functions which can only evolve through mutation and gene replacement, influencing genetic variation and causes phenotypic differences among population genetic (Enright *et al., (*2000); Lamers *et al.,* 2011) was also carried out on the MDR MRSA to analyze the genetic diversity present in the isolates. The result showed that 3 of the seven housekeeping genes (*pta, gmk* and *yqil*) were also present in the MDR isolates at 43.3 %, 20 % and 16.7 % respectively. Four genes were not amplified in all the isolates using the primers possibly due to alteration in the binding site of the primers.

CHAPTER SIX

6.1 SUMMARY

The potential for poultry meat acting as a vehicle for transferring MRSA to humans is a cause of concern for public health. In the current study 30.6% of the staphylococcal isolates were phenotypically identified as the methicillin resistant *Staph.aureus* in the poultry farms studied. Applications of antibiotics in poultry production bring about an increase in resistance to antibiotics not only in pathogenic bacterial strains but also in commensal bacteria. It is supposed that the transmission of antibiotic resistant bacteria to people who got in contact with these sources through direct ingestion or handling results in an increased in the human reservoir of these strains which can rapidly spread to community.

This study was carried out to isolate and molecularly characterize methicillin resistant *Staph. aureus* in poultry and poultry farm workers. It showed that 33.8 % of the isolates were *Staph. aureus.* Phenotypic identification using microgen StaphID characterized staphylococcal isolates to specie level as compared with the conventional biochemical characterization. MecA was detected in 59.0 % % of the MRSA from poultry. There was a correlation between the cefoxitin resistance and detection of meecA gene. All MRSA isolates

were multidrug resistant. Eighty three point seven (83.7%) of the total staphylococcal isolates had MARI above 0.3 indicating that they originated from an environment where antibiotics were frequently used. Augmentin^{R}, vancomycin, cefoxitin and ciprofloxacin were the most effective antibiotics. Highest resistance was observed against ampicillin and oxytetracycline, oxacillin and chloramphenicol.

Eighty nine percent of the isolates were β-lactamase producers and 63.3% were β-lactamase hyper producers. More *Staph. aureus* were isolated from broilers (67.3%) than layer (32.7%).Panton valentine leukocidin was also detected. Amplification of the 16SrRNA confirmed all the isolates as *Staph. aureus*, while sequencing of the spa PCR product showed that the isolates are closely related.

6.2 CONCLUSIONS

The presence of *Staph. aureus* amongst poultry and poultry farm workers poses serious health hazards to those in close contact with the poultry and the community at large,. The high prevalence of *Staph. aureus* means an extensive distribution of the organism amongst poultry farms. It is concluded that the use of conventional biochemical method characterizing *Staph. aureus* is no longer reliable.Results showed that PCR is a specific and effective method for classifying and identifying *Staph. aureus* which demonstrated increasing resistance against many antibiotics.MRSA was isolated and mecA was detected from poultry isolates, detection of mecA gene remain the gold standard for the detection of methicillin resistance in *Staph. aureus*. Isolates are generally resistant to β- lactam antibiotic and oxytetracycline. Coadministration of β-lactam antibiotic with β- lactamase inhibitor proved effective.

The high resistance observed against tetracycline and ampicilllin may be due to misuse and over use of these antibiotics in poultry and humans. All the MRSA in this study were multi drug resistant. There was a correlation between the phenotypic MRSA and detection of mecA gene. Panton valentine leukocidin was detected in poultry isolates. No LA- MRSA CC 398 was detected. DNA sequencing showed that the isolates were closely related.

6.3 CONTRIBUTION TO KNOWLEDGE

- To the best of our knowledge, this is the first time of detecting Methicillin resistant *Staph aureus* (MRSA) from poultry and poultry farm workers, in Kano State, Nigeria.
- mecA is detected in 13 of the phenotypic MRSA isolates.
- This is the first report of PVL positive *Staph. aureus* isolates from poultry in Kano State, Nigeria
- This is the first report of detection of *nuc, spa,* and 3 house keeping genes from *Staph. aureus* isolates in Kano State, Nigeria.
- Ninety percent of the phenotypic MRSA isolates were vancomycin resistant.

6.4 RECOMMENDATIONS

From the findings of the current study the following recommendation were made,

- There should be a careful consideration of the epidemiology of MRSA in animals, human interaction with animals especially food producing animals and companion animals.
- Use of antimicrobial drugs as supplement for higher meat and milk production in food producing animals must be reviewed and reduce to the minimum level.
- To provide a better understanding of the prevalence and epidemiology of this novel strain (LA-MRSA), rapid and accurate detected of methicillin resistance in *Staph*. *aureus* is important for the use of appropriate antimicrobial therapy and for the control of HA-MRSA,CA-MRSA and LA-MRSA.
- High hygienic standards in farms are recommended to avoid contamination and spread of MRSA. Hand washing remains the single most important infection control that prevents the spread of infectious diseases.
- There is need for more rational use of antibiotics in animals and more prudent use in humans.
- Alternatives to antibiotics should be explored such as the application of probiotics in poultry for production of safe edible products.
- Funding individual and institute researches by providing and equipping the laboratories with standard materials.

No staphylococcal isolate should be characterized as MRSA unless the phenotypic resistance determined by disc agar diffusion is confirmed by PCR amplification of the detection of the mecA gene and/or detection of the mecA product PBP2a by latex agglutination test.

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APPENDICES

Appendix 1

A) PHOSPHATE BUFFER PH7.0

Stock solution "A"

Sodium dihydrogen phosphate dehydrate	4.3g
Distilled water to	250ml

Stock solution "B"

Disodium hydrogen phosphate dodecahydrate	8.95g
Distilled water to	250ml

To obtain 100ml of the phosphate buffer ph 7.0 the two solutions were mixed in the following proportions

Stock solution 'A'	39.0ml
Stock solution 'B'	61.0ml

(W.H.O. 2003)

B) PHOSPHATE BUFFERED SOLUTION (PBS)

Stock solution 'A'	28ml
Stock solution 'B'	72ml
Sodium chloride	1.7g
Distilled water	100ml

Appendix 2

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 $1.5x10^8$ cfu/ml (Cheesbrough, 2002).

Appendix 3

INTERPRETATIVE CHART FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antibiotics	Disc Potency	Diameter of zone of growth inhibition (mm)		
		Susceptibility	Intermediate	Resistance
Vancomycin	30µg	≥15	_	_
Cefoxitin	30µg	≥22	_	≤21
Amoxicillin clavulanic acid	30µg	≥20	_	≤19
Ampicillin	10µg	≥29	_	≤28
Gentamicin	10µg	≥15	13-14	≤12
Oxacillin	1µg	≥13	11-12	≤10
Erythromycim	15µg	≥23	14-22	≤13
Neomycin		≥17	_	≤16
Ciprofloxacin	5µg	≥21	15-12	≤15
Sxt	25µg	≥16	11-15	≤10
OXT	30µg	≥15	12-14	≤11

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Appendix 4

A: PCR COCKTAIL MIX

The DNA was subjected to the following cocktail mix and condition for the PCR

10× PCR buffer	1.0
25mM Mgcl2	1.0
5pMol forward primer	0.5
5pMol reverse primer	0.5
DMSO	1.0
2.5Mm DNTPs	0.8
Taq 5u/ul	0.1
10ng/µl DNA	2.0
H2O	3.1
	10µL

B: PCR CONDITION

Initial den.	Den.	Ann. Tempt	Extension	No. of circles	Final Extension	Hold tempt
94˚c	94˚c	56˚c	72˚c	36	72˚c	10˚c
5min	30sec	30sec	45sec		7min	∞

The amplicon from the reaction above was loaded on 1.5% agarose gel and the gel picture is attached as PCR. The ladder used is 1kbplus ladder from Invitrogen. The expected base pair of the amplicon is around 1500bp.

Appendix 5

PCR PRODUCT PURIFICATION

*Add 2vol (20ul) of absolute ethanol to the PCR product

*Incubate at room temperature for 15minutes

*Spin down at 10000rpm for 15minutes

*Decant supernatant

*Spin down at10000rpm for 15minutes

*Add 2ol (40ul) of 70% ethanol

*Decant supernatant

*Air dry

*Add about 10ul of ultrapure water

*Check for amplicon on1.5% agarose

Appendix 6

Biochemical characteristics of the 98 *Staph. aureus* isolates

S/N	Isolate	A	B	C	D	E	F	G
1	135C	+	+	+	+	+	+	+
2	97C	+	+	+	+	+	+	+
3	54C	+	+	+	+	+	+	+
4	12C	+	+	+	+	+	+	+
5	63N	+	+	+	+	+	+	+
6	52N	+	+	+	+	+	+	+
7	7N	+	+	+	+	+	+	+
8	79N	+	+	+	+	+	+	+
9	83C	+	+	+	+	+	+	+
10	88N	+	+	+	+	+	+	+
13	93C	+	+	+	+	+	+	+
14	127C	+	+	+	+	+	+	+
15	136C	+	+	+	+	+	+	+
16	151C	+	+	+	+	+	+	+
17	67N	+	+	+	+	+	+	+
18	81N	+	+	+	+	+	+	+
19	112N	+	+	+	+	+	+	+
20	73N	+	+	+	+	+	+	+
21	141C	+	+	+	+	+	+	+
23	94N	+	+	+	+	+	+	+
24	63C	+	+	+	+	+	+	+

25	51C	+	+	+	+	+	+	+
26	88C	+	+	+	+	+	+	+
27	52C	+	+	+	+	+	+	+
28	11N	+	+	+	+	+	+	+
29	86C	+	+	+	+	+	+	+
30	65N	+	+	+	+	+	+	+
31	33C	+	+	+	+	+	+	+
32	91N	+	+	+	+	+	+	+
33	138N	+	+	+	+	+	+	+
34	68C	+	+	+	+	+	+	+
35	9C	+	+	+	+	+	+	+
36	21N	+	+	+	+	+	+	+
37	139C	+	+	+	+	+	+	+
38	126C	+	+	+	+	+	+	+
39	14C	+	+	+	+	+	+	+
40	67N	+	+	+	+	+	+	+
41	38N	+	+	+	+	+	+	+
42	62N	+	+	+	+	+	+	+
43	36C	+	+	+	+	+	+	+
44	122N	+	+	+	+	+	+	+
45	75C	+	+	+	+	+	+	+
46	121C	+	+	+	+	+	+	+
47	62C	+	+	+	+	+	+	+

48	12N	+	+	+	+	+	+	+
49	92N	+	+	+	+	+	+	+
50	61N	+	+	+	+	+	+	+
51	115N	+	+	+	+	+	+	+
52	111C	+	+	+	+	+	+	+
53	56C	+	+	+	+	+	+	+
54	21C	+	+	+	+	+	+	+
55	25C	+	+	+	+	+	+	+
56	95N	+	+	+	+	+	+	+
57	91N	+	+	+	+	+	+	+
58	39C	+	+	+	+	+	+	+
59	90C	+	+	+	+	+	+	+
60	76C	+	+	+	+	+	+	+
61	80N	+	+	+	+	+	+	+
62	58N	+	+	+	+	+	+	+
63	31N	+	+	+	+	+	+	+
64	145C	+	+	+	+	+	+	+
65	38C	+	+	+	+	+	+	+
66	27C	+	+	+	+	+	+	+
67	69C	+	+	+	+	+	+	+
68	77C	+	+	+	+	+	+	+
69	19C	+	+	+	+	+	+	+
70	3C	+	+	+	+	+	+	+

71	64N	+	+	+	+	+	+	+
72	28N	+	+	+	+	+	+	+
73	26N	+	+	+	+	+	+	+
74	45N	+	+	+	+	+	+	+
75	30N	+	+	+	+	+	+	+
76	40N	+	+	+	+	+	+	+
77	182C	+	+	+	+	+	+	+
78	184C	+	+	+	+	+	+	+
79	170N	+	+	+	+	+	+	+
80	153C	+	+	+	+	+	+	+
81	66N	+	+	+	+	+	+	+
82	93N	+	+	+	+	+	+	+
83	95C	+	+	+	+	+	+	+
84	102C	+	+	+	+	+	+	+
85	108C	+	+	+	+	+	+	+
86	41C	+	+	+	+	+	+	+
87	35C	+	+	+	+	+	+	+
88	39N	+	+	+	+	+	+	+
89	128C	+	+	+	+	+	+	+
90	189C	+	+	+	+	+	+	+
91	119N	+	+	+	+	+	+	+
92	18C	+	+	+	+	+	+	+
93	16C	+	+	+	+	+	+	+

94	55C	+	+	+	+	+	+	+
95	190N	+	+	+	+	+	+	+
96	144N	+	+	+	+	+	+	+
97	166N	+	+	+	+	+	+	+
98	199C	+	+	+	+	+	+	+

KEY

A --- Gram staining

B --- Growth on selective media

C --- Catalase test

D--- Deoxyribonuclease test

E --- Coagulase test

F --- Agglutination test

G --- Microgen staph ID

Appendix 7

Antibiotic Sensitivity Result for 98 *Staph. aureus*

S/N	Isolate	CN	CIP	AMP	AMC	OT	FOX	SXT	VAN	E	OX	N	C	
1	135C	S	R	S	S	R	S	S	S	R	R	S	S	
2	97C	S	R	S	S	R	S	S	S	R	R	S	S	
3	54C	S	R	S	S	R	S	S	S	R	R	S	S	
4	12C	S	R	S	S	R	S	S	S	R	R	S	S	
5	63N	S	R	S	S	R	S	S	S	R	R	S	S	
6	52N	S	R	S	S	R	S	S	S	R	R	S	S	
7	7N	S	R	R	R	S	R	S	R	R	R	R	R	
8	79N	S	R	R	R	S	R	S	R	R	R	R	R	
9	83C	S	S	R	R	R	R	S	R	R	S	S	R	
10	88N	S	R	R	S	R	R	S	S	R	S	S	S	
11	3C	S	R	R	S	R	R	R	S	R	R	S	R	
12	90C	S	R	R	R	R	R	S	R	R	R	R	R	
13	93C	S	S	R	R	R	R	S	R	R	R	R	R	
14	127C	R	S	S	S	R	S	R	R	R	R	R	R	
15	136C	S	R	R	S	R	S	R	R	S	S	R	S	
16	151C	R	R	R	S	R	R	R	R	S	S	S	R	
17	67N	S	R	R	S	R	R	R	R	S	R	R	S	
18	81N	R	S	R	S	R	S	R	S	R	R	S	S	
19	112N	R	S	S	S	R	S	S	S	S	S	R	R	
20	73N	R	R	R	R	S	S	R	R	R	R	R	R	
21	141C	S	S	R	S	S	S	R	R	R	R	R	S	
22	73C	R	R	R	R	R	R	R	R	R	R	R	R	
23	94N	S	S	S	R	R	S	R	R	R	R	R	R	
24	63C	S	R	R	S	R	S	S	R	R	R	R	R	
25	51C	S	S	R	S	R	S	S	R	R	R	R	R	
26	88C	R	R	R	R	R	S	S	R	R	R	R	R	
27	52C	R	R	R	S	R	S	R	R	R	R	R	R	
28	11N	S	R	R	R	R	R	R	R	R	R	R	R	
29	86C	S	R	R	R	R	R	R	R	R	S	R	R	
30	65N	S	S	R	R	R	S	R	R	R	R	R	R	
31	33C	S	S	R	S	R	S	S	R	R	R	R	R	
32	91N	R	R	S	S	R	S	S	R	R	R	R	R	
33	138N	S	R	R	S	S	S	R	R	R	R	R	R	
34	68C	R	R	R	S	R	R	S	S	S	R	R	R	
35	9C	S	S	R	S	R	S	R	S	R	R	R	R	
36	21N	S	R	R	S	R	S	R	S	S	R	R	R	
37	139C	S	R	S	R	R	S	R	S	R	R	R	R	38
	126C	S	S	R	S	R	S	R	S	S	R	R	R	
39	14C	R	R	R	R	R	R	R	S	R	R	R	R	

40	67N	R	S	R	S	R	S	S	S	R	R	R	R
41	38N	S	R	S	R	R	S	S	S	S	R	R	R
42	62N	R	S	R	R	R	S	R	S	R	S	R	R
43	122N	R	S	R	R	R	S	R	S	R	R	S	R
44	36C	S	R	R	S	R	S	S	S	R	R	S	R
45	75C	R	R	R	S	R	S	R	S	R	R	S	S
46	121C	S	R	S	S	R	S	R	S	R	R	R	R
47	62C	S	R	S	S	S	S	R	S	R	R	R	R
48	12N	S	S	R	R	S	R	S	S	R	R	R	R
49	92N	S	S	R	S	R	S	S	S	S	S	S	R
50	61N	S	S	R	R	R	S	S	S	R	R	R	R
51	115N	R	S	S	S	R	S	R	S	R	R	S	R
52	111C	S	R	R	S	R	S	R	S	R	R	S	R
53	56C	R	S	R	R	R	S	R	S	R	R	S	R
54	21C	S	R	R	R	R	S	R	S	R	R	R	R
55	25C	S	R	R	R	R	S	S	S	R	R	R	R
56	95N	S	R	R	R	R	S	R	S	R	R	R	R
57	91N	R	S	R	S	S	S	S	S	R	R	S	R
58	39C	R	R	R	R	R	R	R	S	R	R	S	R
59	90C	S	S	R	R	R	R	R	S	R	R	S	R
60	76C	R	R	R	R	R	R	R	S	R	R	S	S
61	80N	R	S	S	S	S	S	R	S	R	R	S	R
62	58N	S	S	R	R	R	S	R	S	R	R	S	R
63	31N	S	R	R	R	S	R	R	S	R	S	S	R
64	145C	S	R	S	S	R	R	S	S	R	R	S	R
65	38C	S	R	R	R	S	S	S	S	S	R	S	R
66	27C	S	S	S	S	S	S	R	R	R	R	R	R
67	69C	R	S	R	R	R	R	S	S	S	R	S	S
68	77C	R	R	S	S	R	S	S	S	S	R	R	S
69	19C	R	S	S	R	S	R	S	S	R	R	S	R
70	3C	S	R	R	S	R	S	R	S	S	S	R	S
71	64N	S	R	R	S	R	R	S	S	S	S	R	S
72	28N	R	R	R	S	R	S	S	S	S	R	S	S
73	26N	R	R	S	S	S	S	R	S	S	R	S	S
74	45N	S	S	S	S	S	S	R	S	S	S	S	S
75	30N	S	S	S	R	S	R	S	R	S	S	S	S
76	40N	R	R	S	S	S	S	S	S	S	R	S	S
77	182C	R	R	R	S	S	R	S	S	S	S	S	S
78	184C	S	R	S	S	R	R	R	S	S	R	S	S
79	170N	S	S	R	S	R	S	S	S	S	S	S	S
80	153C	R	R	R	S	S	S	R	S	S	R	S	S
81	66N	S	S	R	S	S	S	S	S	S	S	S	R
82	93N	R	S	S	S	S	S	S	S	S	R	S	R
83	95C	S	R	R	S	S	R	S	S	S	S	S	S
84	102C	S	R	S	S	S	S	R	S	S	S	S	S
85	108C	R	R	R	S	R	S	R	S	S	R	S	S

86	41C	S	R	S	S	S	R	S	S	S	S	S	R
87	35C	R	R	R	S	R	R	R	S	R	S	S	S
88	39N	S	R	S	S	S	R	S	S	S	S	S	S
89	128C	R	R	R	S	S	S	R	S	S	R	S	R
90	189C	R	R	R	S	R	S	S	S	S	S	S	S
91	119N	S	R	S	S	R	S	S	S	S	R	S	S
92	18C	S	R	R	S	S	S	S	S	S	R	S	S
93	16C	R	R	R	S	R	S	S	S	S	S	S	S
94	55C	S	R	R	S	R	S	R	S	S	S	S	S
95	190N	R	R	S	S	R	R	S	S	S	S	S	S
96	144N	R	R	S	S	S	S	S	S	S	S	S	R
97	166N	R	R	R	S	S	S	S	S	S	S	S	S
98	199C	R	R	S	S	S	R	S	S	S	S	S	S

KEY

CN --- gentamicin 10µg

OX --- oxacillin 1µg

OT---oxytetracycline 15µg

E --- erythromycin 15µg

C---chloramphenicol 30µg

N- neomycin 30µg

FOX--- cefoxitin 30µg

SXT---trimethoprim/sulfamethaxazole 25µg

CIP--- ciprofloxacin5µg

AMP---ampicillin 10µg

AMC--- amoxicillin-cloxacillin 30µg

VAN---vancomycin 30µg

S--- sensitive

R---resistant

Appendix 8

Detection of *Staph. aureus* 16S rRNA, *nuc*, *mecA* gene, *S. aureus spa* typing and PVL (*lukF-lukS*) by Multiplex PCR

16S rRNA, *nuc* and *mecA* gene multiplex reagents		***S. aureus spa* typing**		**PVL (*lukF-lukS*) PCR of *S. aureus***	
Reagent	Quantity	Reagent	Quantity	Reagent	Quantity
Nuclease free water	17.8ul	Nuclease free water	39.7ul	Nuclease free water	39.7ul
HotStart 10x buffer	2.5ul	10X Buffer	5ul	10X Taq Buffer	5ul
10mM dNTPs	0.5ul	10uM dNTPs	1ul	10mM dNTPs	1ul
10uM Nuc F□	0.5ul	10uM spa F□	1ul	10uM F primer	1ul
10uM NucR□	0.5ul	10uM spa R□	1ul	10uM R primer	1ul
				Taq DNA	
10uM mecA F□	0.5ul	Taq polymerase	0.3ul	polymerase	0.3ul
10uM mecA R□	0.5ul	DNA template	2ul	Sample DNA	2ul
10uM 16S F□	0.5ul				
10Um 16S□	0.5ul				
HotStartTaq	0.2ul				
DNA template	1ul				

APPENDIX 9: Analysis of Variance (ANOVA) Summary Table

TABLE A: Anova between Coagulase, Agglutination And Microgen Tests.

Source	Sum of squares	d.f	Mean square	F
Between	6960084.52	2	3480042.26	3045.5
Within(error)	6854.8	6	1142.5	
Total	6966939.32	8		

P = 0.01

TABLE B: Anova on *Staph. aureus* recovered from broiler and layers.

Source	Sum of squares	d.f	Mean square	F
Between	60.34	2	30.17	1.17
Within (error)	77.67	3	25.89	
Total	138.01	5		

P = 0.05

TABLE C: Anova on *Staph. aureus* from cloacae,nostril andnostril of farm workers.

Source	Sum of squares	d.f	Mean square	F
Between	442.3	2	221.15	66.41
Within (error)	20	6	3.33	
Total	462.3	8		

P = 0.05

APPENDIX 9:

Concentration of extracted DNA using Nano drop result

This is used to detect the quality and quantity of the extracted DNA. The value ranges between 1.7 - 1.9. Nineteen (65.5%) of the DNA had a value of 1.7-1.8. The value determined total quality of each DNA extracted and electrophoresed. The result is shown in table 4.15. **Table 4.15: Nano drop result**

Sample ID	Nucleic Acid	260/280	10µg/µl	Water	25µg/µl	Water
Sample 1	12.5	1.89	80	20	200	-100
Sample 2	20.2	1.84	50	50	124	-24
Sample 3	30.8	0.91	32	68	81	19
Sample 4	36.2	1.81	28	72	69	31
Sample 5	73.3	1.11	14	86	34	66
Sample 6	16.5	1.71	61	39	152	-52
Sample 7	16.1	1.77	62	38	155	-55
Sample 8	11.2	1.85	89	11	223	-123
Sample 9	7.3	3.8	137	-37	342	-242
Sample 10	31.8	1.24	31	69	79	21
Sample 11	17.7	1.84	56	44	141	-41
Sample 12	13.4	1.88	75	25	187	-87
Sample 13	24.8	1.82	40	60	101	-1
Sample 14	36.8	1.75	27	73	68	32
Sample 15	29.4	1.92	34	66	85	15
Sample 16	14.4	1.77	69	31	174	-74
Sample 17	7.7	1.99	130	-30	325	-225
Sample 18	10.1	1.67	99	1	248	-148
Sample 19	9.3	1.74	108	-8	269	-169
Sample 20	7.5	2.08	133	-33	333	-233
Sample 21	12.2	1.58	82	18	205	-105
Sample 22	5.6	2.23	179	-79	446	-346
Sample 23	5.6	2.61	179	-79	446	-346
Sample 24	21.2	1.77	47	53	118	-18
Sample 25	66.2	1.74	15	85	38	62
Sample 26	54.9	1.89	18	82	46	54
Sample 27	19.6	1.73	51	49	128	-28
Sample 28	17.5	1.59	57	43	143	-43
Sample 29	105.1	1.82	10	90	24	76

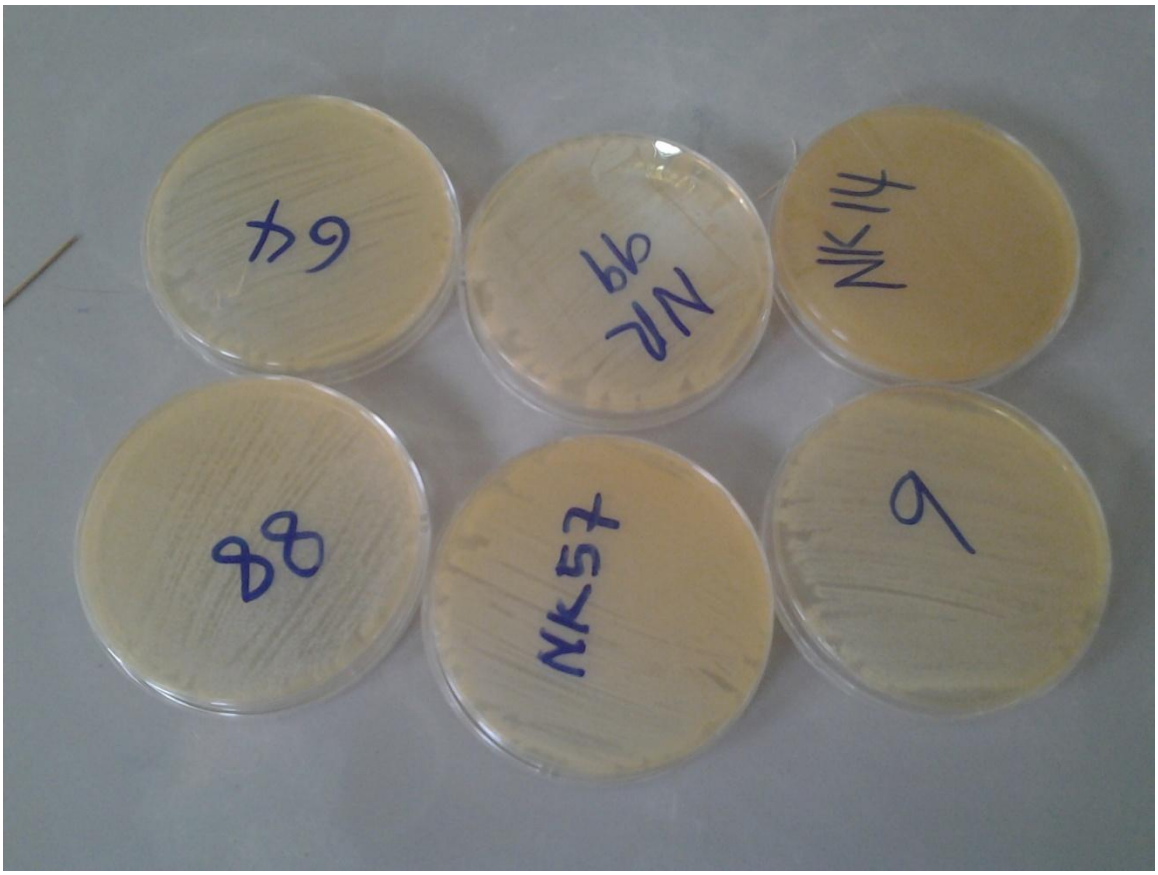


Plate 2: Vancomycin Agar Screening (Resistant Isolates)

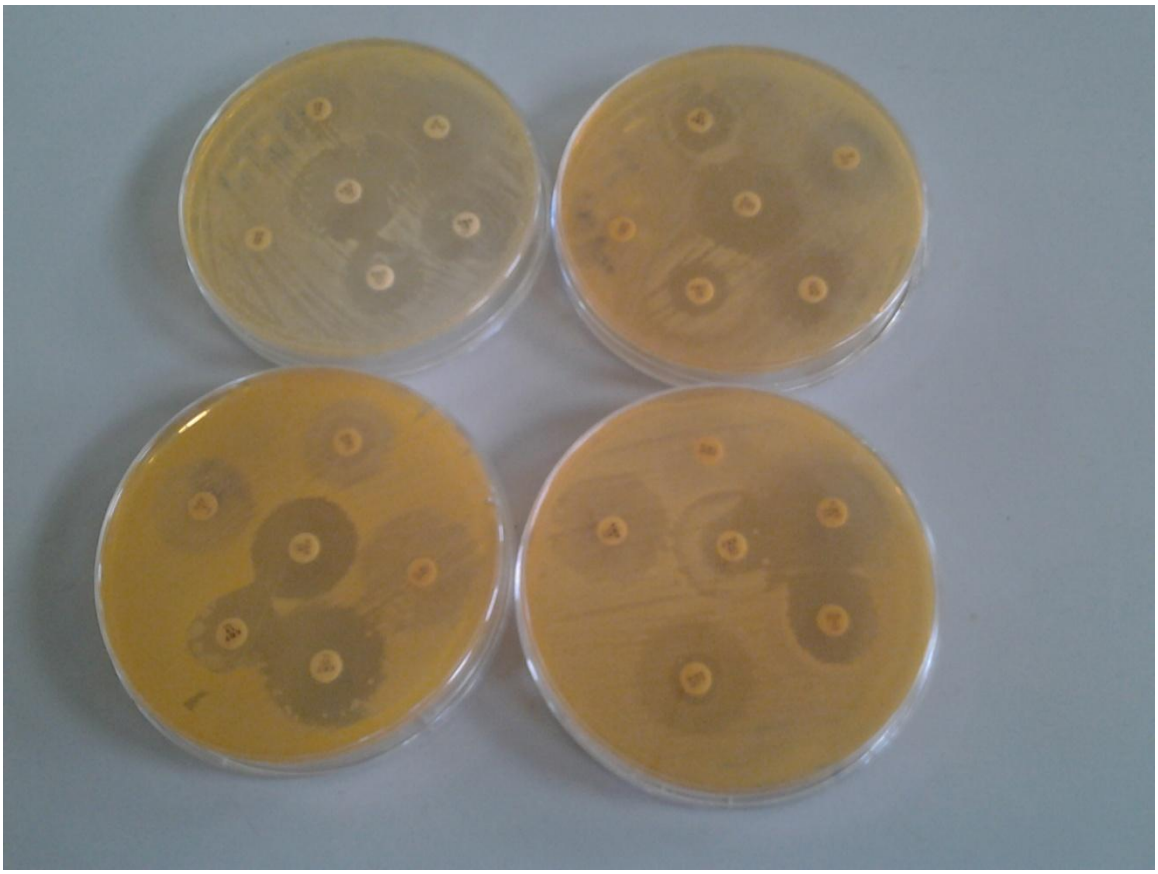


Plate 3: Zones of inhibition for Antibiotic Susceptibility Test



Plate 4: Results of Microgen StaphID



Plate 5: Staph.agglutination reaction. 121 and 9 are positive