OCCURRENCE AND MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA) IN BOVINE MASTITIS IN SETTLED FULANI HERDS IN KADUNA STATE, NIGERIA

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DECLARATION

I declare that the work in the thesis entitled **"Occurrence and Molecular Characterization of Methicillin resistant *Staphylococcus aureus* (MRSA) in Bovine Mastitis in Settled Fulani Herds in Kaduna State, Nigeria"** has been performed by me in the Department of Veterinary Public Health and Preventive Medicine under the supervision of Prof. J. K. P. Kwaga, Prof. M. Bello and Prof. M. A. Raji. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

Gali Abaka UMARU _____ _____

Signature Date

CERTIFICATION

This thesis entitled **"Occurrence and Molecular Characterization of Methicillin resistant *Staphylococcus aureus* (MRSA) in Bovine Mastitis in Settled Fulani Herds in Kaduna State, Nigeria"** by Gali Abaka UMARU meets the regulations governing the award of the degree of Doctor of Philosophy in Veterinary Public Health and Preventive Medicine of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this thesis to Almighty Allah, then to my mother, Hajiya Halima Audu, my wife, Mrs. Armata Umaru Gali and my children, Mubarak Umaru Gali and Aishatu Umaru Gali

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ABSTRACT

Staphylococcus aureus is an important pathogen causing superficial and systemic infections in humans and animals as nosocomial or community-acquired. *S. aureus* is a common causative agent of bovine mastitis in dairy herds. The persistent emergence of multidrug resistant *S. aureus*, particularly methicillin-resistant *S. aureus*, has caused high economic burden and concerns for public health, due to limited options of treatment of MRSA infections. Therefore, this study was designed to determine the occurrence and molecular characterization of Methicillin-resistant *Staphylococcus aureus* (MRSA) from bovine mastitis in settled Fulani herds in Kaduna State. A cross-sectional study was conducted to determine the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) and the molecular epidemiology of *S. aureus* and MRSA from bovine mastitis in settled Fulani herds in Kaduna State. Three hundred and sixty milk samples were collected from randomly selected herds or farms in Kaduna South, Igabi, Kagarko, Sabon-Gari and Zaria and examined for *Staphylococcus* species by phenotypic and molecular methods. The results revealed that the prevalence of mastitis at cow level was 26.9% (97/360), out of which 3.1% (11/360) were clinical mastitis and 23.9% (86/360) were subclinical mastitis respectively ($P<0.05$). The results also showed that out of the 97 mastitis positive samples, 44.3% (43/97) were associated with *S. aureus* and 24.7% (24/97) with coagulase negative staphylococci respectively. Of the 360 cow milk samples examined, 55 (15.3%) were positive for *S. aureus*, out of which 28 (7.8%) were found to be MRSA. Twelve (42.9%) of MRSA were associated with mastitis (4 each from

Kaduna South and Kagarko, 2 from Zaria and one each from Giwa and Igabi respectively). The resistance patterns of the 28 MRSA isolates showed high resistance to penicillin 28 (100.0%), amoxicillin 23 (89.3%), ampicillin 23 (89.3%), tetracycline 24 (85.7%) and erythromycin 20 (71.1%). The multiple drug resistance indices of the MRSA strains revealed that all the 28 MRSA strains were resistant to 5 or more antibiotics tested, 6 (21.4%) strains were resistant to 6 antibiotics, 6 (21.4%) were resistant to 7 antibiotics, 8 (28.6%) were resistant to 8 antibiotics and 4 (14.3%) were resistant to 9 antibiotics. Twenty six of the 28 MRSA (92.9%) exhibited unique resistance patterns with only 4 exhibiting similar patterns of multiple resistance. The two patterns encountered were AML,AMP,C,CN,E,NA,P,S,TE exhibited by isolates K23 and K42, and AML,AMP,E,P,TE,VA,W exhibited by isolates K3 and S26. The multiple antibiotic resistance classification results showed that significant number ($P<0.05$) exhibited the extensive drug classification pattern, while the MIC values of the antibiotics showed that all (100%) had values greater than 256 µg/mL against oxacillin, 15 (53.6%) had values greater than 256 µg/mL against vancomycin respectively. Of the 28 MRSA isolates examined by multiplex PCR for the presence of *mec* A, *mec* C and *fem* B genes, 3 were positive for *fem* B. None was positive for *mec* A and *mec* C genes respectively. Typing using MLST revealed that all the 3 isolates were MLST types 1 (ST 1) and clonal complex (CC) 1. All the 3 isolates (23448_1#128, 23448_1#129. 23448_1#131) were isolated from cases of subclinical mastitis and exhibited multidrug and extensive drug resistance (XDR) patterns. The three strains were 100 % homologous to each other with no divergence within the

specie level. The occurrence of mastitis coupled with the isolation of ST 1 (CC1) *S. aureus*; CONS and MRSA are of great public health concern and requires strict control and preventive measures. The study recommends proper hygienic measures during milking, environmental sanitation and culling of infected cows to prevent the spread of pathogens to uninfected cows and humans.

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LIST OF ABBREVIATIONS AND SYMBOLS

µg	-microgram
ARC	-antibiotic resistance classification
ATCC	-American Type Culture Collection
Bp	-base pair
CA	-community-acquired
CC	-clonal complex
CIP	-ciprofloxacin
CMT	-California mastitis test
CO_2	-carbon dioxide
CONS	-coagulase positive staphylococci
df	-degree of freedom
DNA	-deoxy ribonucleic acid
dNTP	- deoxy nucleotides triphosphate
E	-erythromycin
ET	-exfoliative toxin
G3	-Giwa number 3
GI	- genomic islands
H_2O_2-	-hydrogen peroxide
HA	-hospital acquired
HCl	-hydrochloric acid
I59	-Igabi number 59
K3	-Lere number 3

M.I.C.E.	-minimum inhibitory concentration evaluator
MARI	-multiple antibiotic resistance index
MDR	-multi-drug resistance
Ml	-minimum inhibitory
MLST	-multilocus sequence typing
MRSA	-Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	- microbial surfaces complex that recognize adhesive matrix molecules
NART	- number of antibiotics resistance to
NCTC	- National collection of type cultures
O_2	-Oxygen
^oC	-degree centigrade
ORSAB	-oxacillin resistance agar basal medium
PBP2a	-penicillin binding protein 2a
PCR	-polymerase chain reaction
PDR	-pan drug resistance
PFGE	-pulse field gel electrophoresis
pH	-hydrogen ion concentration
PNSG	- poly-*n*-succynyl-β-1, 6 glucosamine
PTSAgs	- Pyrogenic toxin super antigens
PVL	-Panton-Valentine Leukocidin
RAPD	- Random amplified polymorphic DNA
Rpm	-revolution per minute
S1	-Kaduna South number 1
SB24	-Sabon Gari number 24

SCC*mec*	-Staphylococcal cassette chromosome
SEs	-staphylococcal enterotoxins
Spa	-staphylococcal protein A
SSSS	-staphylococcal scalded skin syndrome
ST	-sequence type
tRNA	-transfer ribonucleic acid
TSS	-toxic shock syndrome
XDR	-extensive drug resistance
Z10	-Zaria number 10
χ^2	-chi square

CHAPTER ONE

1.0 INRODUCTION

Food borne diseases are one of the major threats to health of the society and *Staphylococcus aureus* (*S. aureus*) ranks third as the major cause involved in food borne diseases (Hennekinne *et al.,* 2012; Kadariya *et al.,* 2014). Growth of *S. aureus* in foods leads to the production of staphylococcal enterotoxins (SEs), a cause of food poisoning (Intrakamhaeang *et al.,* 2012; Hennekinne *et al.,* 2012). Therefore, contaminated milk and milk products have frequently been implicated in staphylococcal food poisoning (Argudın *et al.,* 2010; Vanderhaeghen *et al.,* 2010b; Hennekinne *et al.,* 2012).

Staphylococci are group of resistant bacteria which are dispersed (Mirzaei *et al.,* 2011). They are among the first known human pathogens that can colonize the skin and mucus membranes of humans and animals (Reinosa *et al.,* 2008; Kluytmans, 2010; Chaibenjawong and Foster, 2011). Among the different species, *S. aureus* which is the most important pathogen due to its innate ability and its capacity to acquire resistance against antibiotics that has now turned it into an organism of major concerns to public health (Helal *et al.,* 2015). *Staphylococcus aureus (S. aureus)* form part of the normal flora of the skin, intestine, upper respiratory tract and vagina (Kluytmans, 2010; Li *et al.,* 201; Chaibenjawong and Foster, 2011). The bacteria can become pathogenic when conditions such as pH, temperature and nutrient availability are

altered and become favourable for growth (Li *et al.,* 2011). The pathogenicity of *S. aureus* is determined by the production of toxins, such as the 33-kd protein-alpha toxin, exfoliatin A, exfoliatin B and Panton-Valentine Leukocidin (PVL) toxins (Argudín *et al.,* 2010). These toxins can be harmful to the host and cause skin diseases (carbuncles, boils, folliculitis and impetigo) and other complications, such as endocarditis, meningitis, toxic shock syndrome (TSS) as well as food poisoning (Argudın *et al.,* 2010; Hennekinne *et al.,* 2012). *S. aureus* is primarily separated from the majority of the other staphylococci by its production of the enzyme coagulase and the subsequent ability to coagulate plasma. Remaining staphylococci are hence referred to as coagulase-negative staphylococci (CoNS) and many of these are found as natural colonizers of the human skin and mucous membranes, whereas other CoNS are exclusively identified in animals (Becker *et al.,* 2014). *S. epidermidis* is the most important representative of the CoNS group, its entire genome has been determined (Mendes *et al.,* 2012), and it is associated with infections in patients with implanted medical devices or in immunocompromised patients (Mendes *et al.,* 2012), while *S. saprophyticus* is a common cause of urinary tract infections in women (Becker *et al.,* 2014). *S. haemolyticus* is a frequently found CoNS in human blood cultures and it may cause clinically relevant infections such as prosthetic joint infections, septicemia, peritonitis, otitis, and urinary tract infections, and this species has also been thoroughly studied by whole genome sequencing (Becker *et al.,* 2014). In addition, CoNS are the foremost cause of infections occurring in neonatal intensive care units (NICUs) where they may endemically persist for many years (Becker *et al.,* 2014).

Coagulase-negative staphylococci (CoNS) have become an increasing concern in veterinary medicine because they are associated with a variety of animal diseases, such as suppurative disease, arthritis and urinary tract infection in animals. Animals are common reservoir of and can carry CoNS on their skin, noses, upper alimentary and urogenital tract and intestinal tract and thus may transmit the bacteria to human by animal handling. Coagulase-negative staphylococci carried by food animals can also contaminate the animal products and enter the food chain, thus posing potential threat to food consumers and handlers.

S. aureus is one of the most common and economically important pathogens associated with intramammary infections in dairy herds (Pexara *et al.,* 2013; Joshi *et al.,* 2014), and has been the factor responsible for 30-40% mastitis cases (Reinosa *et al.,* 2008; Suleiman *et al.,* 2012b; Haram *et al.,* 2013). Therefore, the micro-organism can contaminate milk from udder with clinical or subclinical mastitis or from the environment during manipulation and processing (Reinosa *et al.,* 2008; Mirzaei *et al.,* 2011; Suleiman *et al.,* 2012b; Umaru *et al.,* 2013). It can easily grow and multiply in milk and related products and may produce enterotoxins (Intrakamhaeang *et al.,* 2012).

Since 1959, treatment of *S. aureus* infections was with the semi-synthetic penicillin drugs such as methicillin (Boucher *et al.,* 2010; Otter and French, 2010). However, in

1960 methicillin-resistant *S. aureus* (MRSA) was discovered (Enright *et al.,* 2002; Basset *et al.,* 2011). This organism is frequently resistant to methicillin and essentially all other β-lactam antibiotics (Pexara *et al.,* 2014; Schaumburg *et al.,* 2016; Fooster *et al.,* 2017). The resistance is caused by an alternative penicillin-binding protein, called PBP2a with a low affinity for β-lactamase, which is encoded by the *mec* A gene located in the mobile genetic element called Staphylococcal cassette chromosome (SCC*mec*) (Vanderhaeghen *et al*., 2010a; Li *et al.,* 2011; Kaito *et al.,* 2011). Since then, MRSA increased every decade and were identified as a major cause of nosocomial infections. The possibility of transmission of health-care associated MRSA (HA-MRSA) to community was unavoidable. Since, 1987, MRSA was increasingly found in the community (community-associated-methicillin-resistant *S. aureus*) (CA-MRSA) presenting with severe skin and soft tissues infections and necrotizing pneumonia (Boucher *et al.,* 2010; Otter and French, 2010).

The use of antibiotics on dairy farms as well as in other food animal production systems is a major concern in the emergence of resistant bacterial pathogens (Umaru *et al.,* 2013). Antibiotics on dairy operations are used to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy. Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly those with zoonotic potential (Haran*et al.,* 2012). The infections with methicillin-resistant *Staphylococcus aureus* (MRSA) have turned into major problems in treatment (Pexara *et al.,* 2013; Foster, 2017). MRSA has emerged as a

major cause of hospital-associated (HA) and community-associated (CA) infections (Faccioli-Martins and Souza da Cunha, 2012). Similarly, livestock-associated (LA) MRSA genotypically classified under clonal complex 398 (CC398) has been detected in pigs and swine farmers in the Netherlands and other countries (Vanderhaegen *et al.*, 2010a; Monaco *et al.,* 2013), and is known to cause infections in humans and animals and may be an important reservoir for human MRSA infections (Monaco *et al.,* 2013; Tegegne*et al.,* 2017).

MRSA isolates are multi drug resistant (MDR), which can result in high costs of treatment, longer treatment times, and higher rates of hospitalization and co-morbidities (Boucher *et al.,* 2010; Otter and French, 2010; Faccioli-Martins and Souza da Cunha, 2012). MRSA was first reported from bovine sources in 1975, although it has occasionally been reported since then in different parts of the world including Nigeria (Tùkyilmaz *et al.,* 2010; Mirzaen *et al.,* 2011; Kreausukon *et al.,* 2012; Haran *et al.,* 2012; Erdem and Tùkyilmaz, 2013; Paterson *et al.,* 2013; Unnerstand *et al.,* 2013; Umaru *et al.,* 2013; Omoshaba *et al.,* 2018). The presence of *S. aureus* and MRSA in bovine milk and dairy environment poses potential risk to farm workers, veterinariansand farm animals that are exposed to contaminated cattle (Intrakamhaeang *et al.,* 2012; Erdem and Tùkyilmaz, 2013) with cow to human transmission having been established (Juhasz-kaszanyitzky *et al.,* 2007; Erdem and Tùkyilmaz, 2013).

Genotypical analysis of *S. aureus* and MRSA from milk and bovine origin has been demonstrated by numerous authors from different parts of the world and some parts of Nigeria (Reinoso *et al.,* 2008; Tùkyilmaz *et al.,* 2010; Fessler *et al.,* 2010; Kreausukon *et al.,* 2012; Haran *et al.,* 2012; Erdem and Tùkyilmaz, 2013; Suleiman *et al.,* 2012b; Unnerstand *et al.,* 2013; Helal *et al.,* 2015; Ali, 2016; Odetokuna *et al.,* 2018), using Pulsed Field Gel Electrophoresis (PFGE), *Staphylococcus aureus* Protein A (*spa*) Typing, Multilocus Sequence Typing (MLST) and Staphylococcal Cassette Chromosome (SCC *mec*) typing. However, at present there is derth of information on the genetic spread or molecular epidemiology of *S. aureus* and MRSA in bovine milk or dairy environments in Kaduna State. Pulse Field Gel Electrophoresis is an acceptable gold standard for determination of clonal relations between strains while MLST is used to detect the strains whether originating from hospital or community (Bosch *et al.,* 2010; Moussa *et al.,* 2011; Turlej *et al.,* 2011; Szabo *et al.,* 2014). Recently, DNA sequence based methods for typing *S. aureus* such as MLST and *Staphylococcus aureus* Protein A (*spa*) typing have been developed and are widely used to establish clonal relationships between strains and to compare the geographical locations of *S. aureus* and MRSA clones (Catry *et al.,* 2010; Lamers *et al.,* 2012).

1.1 Statement of research problems

Mastitis is one of the diseases which causes high liabilities in milk production owing to decrease in quantity and quality of milk and milk products, medicine costs, increase

in labour expenses and in the time wasted during milk discarding after treatment, up to the total elimination of antibiotic residues employed. Among aetiological agents in mastitis, *Staphylococcus aureus* has a high prevalence in the cattle under analysis (Junaid *et al.,* 2011; Islam *et al.,* 2010; Intrakamhaeang *et al.,* 2012; Shittu *et al.,* 2012).

The determination of the origin of the organisms involved in the aetiology of bovine mastitis is highly relevant from the epidemiological point of view (Souza *et al.,* 2012; Szabo *et al.,* 2014). In such a context, the precise characterization of pathogens becomes mandatory for the detection of transmission methods and infection sources (Mongkolrattanothai, 2013; Bitrus *et al.,* 2016). It also provides monitoring of the dissemination of bacterial strains among animal populations (Basset *et al.,* 2011; Souza *et al.,* 2012; Sefanu *et al.,* 2012). Molecular characterization should be enhanced for the correct identification of microorganism species involved in mastitis and for the identification of genes which cause virulence in the main pathogens. In fact, they will help in epidemiological studies and will provide the tracing of pathogens throughout the milk production chain. The characterization of the genetic diversity of *S. aureus* isolated from milk-producing cattle is highly important for a better understanding of the pathogen's dispersion pattern. Such information may be of great help in the elaboration of more efficient strategies that decrease infection cases. Through molecular profiles, genetic relationships among different clones may be

inferred, genetic flow may be detected and infection dispersion routes in cattle may be traced (Pexara *et al.*, 2013; Szabo *et al.,* 2014).

MRSA is cosmopolitan in nature. Initially, the bacterium was found prominently in hospitals and health centers (HA-MRSA), because of the antibiotic use and susceptible patients, and hence as a health care associated problem. Now, more and more infections are community associated (Faccioli-Martins and Souza da Cunha, 2012; Samar *et al.*, 2018). The strains of health care-associated MRSA differ from the community-associated strains, indicating that they come from different sources. This needs to be identified and verified. Probably the most important reason for investigation of MRSA in dairy cows, milk and milk products is not the cows themselves, but public health implications. Many people are colonized by *S. aureus* in the nose or the skin, without being affected by it. They are so-called carriers. A growing percentage of these carriers are MRSA (Hennekinne *et al.*, 2012; van Balen *et al.*, 2013; Kadariya *et al.,* 2014).

Just as MRSA have been documented to be a cosmopolitan pathogen in humans, it is trying to follow suit in Veterinary Medicine (Graveland *et al.,* 2011; van Balen *et al.*, 2013; Tegegne *et al.*,2017), as has been detected in pigs (Smith *et al.,* 2013), horses (Weese, 2010), dogs and cats (Weese, 2010), chickens (Otalu *et al.*, 2011; Bala *et al.*, 2016) and sheep (Eriksson *et al.,* 2013). In Nigeria, a study on MRSA in fresh and fermented milk conducted by Umaru *et al.* (2013) in Zaria and Kaduna obtained a

prevalence of 4.8%, with 22.2% of the MRSA isolates positive for *mec* A gene. Similarly, Omoshaba *et al.* (2018) examined 200 raw milk and soft cheese (*wara*) in Abeokuta, Nigeria and discovered 50 (15%) to be MRSA. In 2011 MRSA with a divergent *mec* A homologue, name *mec C* (formally *mec* A_{LGA251}) was detected in milk samples from dairy cows in the UK and human clinical samples in the UK, Denmark and Ireland (Garcia-Alvarez *et al.,* 2011; Paterson *et al.,* 2013). The *mec* C has only ~70% similarity with *mec* A and is not identified with conventional confirmatory methods (Garcia-Alvarez *et al.,* 2011).

MRSA can spread from human to human (Samar *et al.,* 2018), from animal to human (Juhasz-kaszanyitzky *et al.,* 2007; Huber *et al.,* 2009), and most likely from human to animal (Juhasz-kaszanyitzky *et al.,* 2007; Cantry *et al.,* 2010; Cuny *et al.,* 2010). Because MRSA infections are so hard to treat and eradicate, efforts are now geared towards finding the different sources and risk factors related to MRSA infections. The focus on animals, farmers and foods as possible sources of MRSA is increasing.

Food may be contaminated with MRSA. Handling or eating contaminated food is also a potential means of transmission. In hospital outbreaks, contaminated food can disseminate the organisms to patient as well as to healthcare workers (Wendlandt *et al.,* 2013; Pexara *et al.,* 2013). Considering the increasing evidence of MRSA in food-producing animals, the concerns regarding MRSA contamination of food of animal origin may be reasonable (Weese, 2010; Wendlandt *et al.,* 2013). However, and

despite the reported increase in both the incidence of human community acquired-MRSA infections, there are no reports of a direct link between them (Weese, 2010). Further investigations are needed to determine the true role of food of animal origin in transmission of MRSA from animals to humans.

1.2 Justification of the study

Staphylococcus aureus are recognized worldwide as important pathogens that have a great versatility in their ability to colonize different hosts and cause infections at different anatomic sites (Hennekinne *et al.,* 2012; Kadariya *et al.,* 2014). The molecular processes responsible for disease and host specificity are poorly understood, but are presumed to be caused in part by differences in gene content and by allelic variations between strains (Reinoso *et al.,* 2008). Genotypical differences between *S. aureus* isolated from humans and bovines had been observed by numerous authors (Mirzaei*et al.,* 2011; Suleiman *et al.,* 2012b; Erdem and Tükyilmaz, 2013; Shitu *et al.,* 2014). In addition,genome sequencing of human and bovine strains identified notable differences in both genomes and provides a framework for the identification of specific factors associated with host specificity in this major human and animal pathogen (Harrison *et al.,* 2013). However, at present little is knownabout *S. aureus* populations isolated from differenthosts in different locations in the globe, but scarce in Kaduna State, Nigeria.

Antibiotics especially β-lactams are indiscrimately used as growth promoters in food animal production system and as preventive measures against several diseases (Bala *et al.,* 2016). This promotes the emergence of drug resistance pathogens such as methicillin resistant *S.aureus (*MRSA), which is now a global public health concern (Bala *et al.,* 2016). With increase in population density within a particular geographical location, the incidence of both communities associated and hospital associated MRSA has been observed to increase with time, regardless of hospital size and control measures due to drug abuse and zoonotic transfer of resistance gene through horizontal gene transfer. Until now, MRSA has been detected in different species such as swine, cattle, horses, dogs, cats and chickens (Otalu *et al.,* 2011; van Balen *et al.*, 2013). Pigs were the first food animal species in which MRSA colonization was linked to transmission to humans (Smith *et al.,* 2013), before being demonstrated in cattle, dogs, chickens, cows and recently milk. There is now increasing concern about the public health impact of MRSA associated with food producing animals, because MRSA and, consequently, their resistance genes can spread from animals to humans by direct contact or through the food chain (Kluytmans, 2010).

Molecular typing techniques have been used with increasing frequency in studies of *S. aureus* and methicillin-resistant Staphylococci (MRS), and also for a better understanding of the evolutionary relations among *S. aureus* and MRSA clones (Erdem and Tükyilmaz, 2013). It determines the prevalence and monitoring of

outbreaks as well as identifies the SCC*mec* types, subtypes and genotypes of *S. aureus* and MRSA that might be circulating in the dairy environments. The determination of the different antibiotypes of *S. aureus* and MRSA can assist in monitoring of the antibiotic resistance profile trends in the dairy environments, thus assisting with the correct implementation of antibiotic regiments for suspected *S. aureus* or MRSA infections. The most important techniques used to investigate the molecular epidemiology of *S. aureus* and MRSA are PFGE, MLST, *spa* typing and SCC*mec* typing (Catry *et al.,* 2010; Bosch *et al.,* 2010; Moussa *et al.,* 2011; Turle *et al.,* 2011); Lamers *et al.,* 2012. Pulsed Field Gel Electrophoresis (PFGE) of fragments obtained by macro-restriction is the most sensitive method. It may actually detect genetic variations among phylogenetically and epidemiologically related bacterial isolates, including *S. aureus* (Mousa *et al.,* 2011). Pulsed Field Gel Electrophoresis technique is the most discriminatory typing tool for *S. aureus*. In fact, it is not merely used to detect smaller genetic variations among epidemic strains, but it has proved to be an excellent method for the establishment of clonal relationships in epidemiological and molecular studies. The organization of the SCC*mec* can be determined with a number of PCR based methods, such as *mec* A specific multiplex PCR (Oliveira and Lencastre, 2002). The SCC*mec* typing may determine whether the clone is hospital associated (HA) and community-associated (CA) (Stefabi *et al.,* 2012), s*pa* typing uses DNA sequencing of short nucleotide repeats in the polymorphic X region of the *S. aureus* protein A gene *spa* (x-r) for discrimination of *S. aureus* (Turlej *et al.,* 2011;

Salehzadeh *et al.,* 2016). The *S. aureus* MLST scheme uses the allelic profile of seven (7) house keeping genes to establish the strain types (Enright *et al.,* 2000).

1.3 Research questions

i. Are *S. aureus* and MRSA not associated with bovine mastitis in settled Fulani herds in Kaduna State?

ii. Are *S. aureus* and MRSA isolates from milk samples in settled Fulani herds in Kaduna State multi-drug resistant?

iii. Are MRSA isolates from cow milk in settled Fulani herds in Kaduna State harbor *mec* A, *mec* C and *fem* B gene?

iv. Are MRSA isolates from cow milk in settled Fulani herds in Kaduna State belong to any sequence type (ST) or clonal complex (CC)?

v. Are MRSA isolates from cow milk in settled Fulani herds in Kaduna State from homologous with established isolates in the GenBank?

1.4 Aim of the study

To determine the occurrence and to characterize Methicillin-resistant *Staphylococcus aureus* (MRSA) from bovine mastitis in settled Fulani herds in Kaduna State using molecular techniques.

1.5 Objectives of the study

i. To determine the prevalence of bovine mastitis in settled Fulani herds in Kaduna State

ii. To determine the prevalence of *S.* aureus and MRSA in bovine mastitis in settled Fulani herds in Kaduna State using phenotypic and molecular methods.

iii. To determine the antibiotic susceptibilities of the *S. aureus* and MRSA isolated from cow milk in settled Fulani herds in Kaduna State

iv. To detect the presence of *mec* A, *mec* C and *fem* B genes in the MRSA isolates from cow milk in settled Fulani herds in Kaduna State by mulplex PCR.

v. To determine the sequence types (ST) and clonal comlex (CC) of *S. aureus* and MRSA present in cow milk in settled Fulani herds in Kaduna State by MLST and molecular analysis (evolutionary relationship.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Genus Staphylococci

The Staphylococci are group of bacteria that belong to the family *Staphylococcaceae*, genus *Staphylococcus* and consist of more than 40 species and subspecies (Lamers *et al.,* 2012). *Staphylococcus aureus* (*S. aureus*) is the most important of the staphylococcal genus and is capable of causing a broad variety of infections ranging from minor skin infections to fatal conditions such as osteomyelitis, bacteremia and infective endocarditis (Que and Moreillon, 2010; Joshi and Devkota, 2014; Piechota *et al.,* 2014). However, the bacteria form part of the normal flora of the skin, intestine, upper respiratory tract and vagina of humans and animals (Reinosa *et al.,* 2008; Kluytmans, 2010; Chaibenjawong and Foster, 2011; Li *et al.,* 2011). *S. aureus* can become pathogenic when conditions such as pH, temperature and nutrient availability are altered and become favourable for overgrowth. In addition, *S. aureus* is capable of producing a wide range of enzymes and toxins such as the 33-kd protein-alpha toxin, exfoliatin A, exfoliatin B, and Panton-Valentine Leukocidin (PVL) toxins (Argudín *et al.,* 2010; Piechota *et al.,* 2014). These toxins are associated with specific clinical conditions, such as food poisoning or the toxic shock syndrome, endocarditis, meningitis, carbuncles, boils, folliculitis and impetigo (Argudın *et al.,* 2010; Hennekinne *et al.,* 2012; Joshi and Devkota, 2014).

S. aureus differ from the other staphylococci by its ability to produce the enzyme coagulase which has the ability to coagulate plasma. The remaining Staphylococci groups are hence called coagulase-negative Staphylococci (CoNS) which are mainly found as natural colonizers of the human and animal skin and mucous membranes (Becker *et al.,* 2014). The *S. epidermidis* is the most important member of the CoNS group (Mendes *et al.,* 2012), which is associated with infections in patients with indwelling medical devices or in immunocompromised patients (Mendes *et al.,* 2012; Becker *et al.,* 2014), while *S. saprophyticus* is a common cause of urinary tract infections in women (Becker *et al.,* 2014). *S. haemolyticus* is a CoNS of clinical importance that causes infections such as prosthetic joint infections, septicaemia, peritonitis, otitis, and urinary tract infections, (Dimitriou *et al.,* 2011; Sievert *et al.,* 2013; Willcox *et al.,* 2013). Moreover, CoNS are the most common causes of infections occurring in neonatal intensive care units (NICUs) where they endemically persist for many years (Mendes *et al.,* 2012; Becker *et al.,* 2014). *S. aureus* and *S. epidermidis* are both important causes of hospital-acquired infections, with CoNS accounting for 50% of catheter related infections globally (Mendes *et al.,* 2012; Sievert *et al.,* 2013; Becker *et al.,* 2014).

2.2 History, morphology and general characteristics of *Staphylococcus aureus (S. aurues)*

S. aureus was discovered in Aberdeen, Scotland in 1880 by the surgeon Sir Alexander Ogston in pus from surgical abscesses (Shinefield *et al.,* 2009; Nottasorn, 2012; Bercker *et al.,* 2014). It is a facultative anaerobic, Gram-positive coccus, which

appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with haemolysis, when grown on blood agar plates (Lamers *et al.,* 2012; Obajuluwa, 2014). *S. aureus* is catalase positive (which is useful to distinguishing Staphylococci from enterococci and streptococci), and coagulase positive which is useful in differentiating it with other staphylococci (Bercker *et al.,* 2014). However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase.

S. aureus is a commensal and opportunistic pathogen that can cause wide spectrum of infections, from superficial skin infections to severe, and potentially fatal, invasive disease (Hennekinne *et al.,* 2012; Kadariya *et al.,* 2014).This ubiquitous bacteriumis an important pathogen due to combination of "toxin-mediated virulence, invasiveness, and antibiotic resistance", and emerged as a major pathogen for both nosocomial and community acquired infections (Argudín *et al.,* 2010; Piechota *et al.,* 2014). *S. aureus* does not form spores but can cause contamination of food products during food preparation and processing (Kadariya *et al.,* 2014). *S. aureus* can grow in a wide range of temperatures (7^{o} to 48.5^{o}C; optimum 30 to 37^{o}C), pH (4.2 to 9.3; optimum 7 to 7.5), and sodium chloride concentration up to 15% NaCl (Lamers *et al.,* 2012). *S. aureus* is a dessication tolerant organism with the ability to survive in potentially dry and stressful environments, such as the human nose and on skin and inanimate surfaces such as clothing and surfaces (Chaibenjawong *et al.,* 2011). These characteristics favor growth of the organism in many food products (Kadariya *et al.,* 2014)

2.2.1 Classification and taxonomyof *S. aureus*

The genus *Staphylococcus* consists of 47 species and 23 subspecies that are validly described (Que and Moreillon, 2010; Jenney *et al.,* 2014). Of these, 38 fulfill the categorisation of a coagulase-negative species, and one further species, *S. schleiferi*, includes both a coagulase-negative subspecies (*S. schleiferi* subsp. *schleiferi*) and a coagulase-positive subspecies (*S. schleiferi* subsp. *coagulans*). Most recently described CoNS species isolated from human clinical specimens comprise *S. jettensis*, *S. massiliensis*, *S. petrasii* (including *S. petrasii* subsp. *petrasii* and *S. petrasii* subsp. *croceilyticus*), and *S. pettenkoferi*. A further CoNS species, *S. pseudolugdunensis*, has been proposed (Que and Moreillon, 2010; Bercker *et al.*, 2014).

Meanwhile, two species previously considered to be CoNS were removed from this genus. *S. pulvereri*, was found to be identical to the previously described species *S. vitulinus* (Bercker *et al.,* 2014). *S. caseolyticus* was transferred to the newly established genus *Macrococcus*, comprising Gram-positive, catalase-positive cocci characterised by a higher DNA G+C content, the absence of cell wall teichoic acids, and larger cells than those of the *Staphylococcus* species (Jenney *et al.,* 2014).

2.2.2 Nomenclature of *S. aureus*

As with all generic names in binomial nomenclature, *Staphylococcus* is capitalized when used alone or with a specific species. Also, the abbreviations *Staph* and *S.* when

used with a species (*S. aureus*) are correctly italicized and capitalized. However, *Staphylococcus* is not capitalised or italicised when used in adjectival forms, as in a staphylococcal infection, or as the plural (staphylococci) (Lamers *et al.*, 2012).

The *S. saprophyticus* and *S. sciuri* groups are generally novobiocin-resistant, as is *S. hominis* subsp. *novobiosepticus*.

Members of the *S. sciuri* group are oxidase-positive due to their possession of the enzyme cytochrome c oxidase. This group is the only clade within the *Staphylococci* to possess this gene. The *S. sciuri* group appears to be the closest relations to the genus *Macrococcus*.

Staphylococcus pulvereri has been shown to be a junior synonym of *Staphylococcus vitulinus* (Lamers *et al.*, 2012).

Within these clades, the *S. haemolyticus* and *S. simulans* groups appear to be related, as do the *S. aureus* and *S. epidermidis* groups (Ghebremedhin *et al.*, 2008).

S. lugdunensis appears to be related to the *S. haemolyticus* group.

S. croceolyticus may be related to *S. haemolyticus*, but this needs to be confirmed.

The taxonomic position of *S. croceolyticus*, *S. leei*, *S. lyticans* and *S. pseudolugdunensis* has yet to be clarified.

2.2.3 Genome structure of *S. aureus*

The *Staphylococcus aureus* genome, which is the most common species among the *Staphylococcus* genome projects, is the most completed genome sequence compared to any other microbial species (Wan *et al.*, 2017), and was based on the strain NCTC 8325. The *S. aureus* genome consists of a singular circular chromosome of about 2.7 to 2.9 Mbp containing about 2600 genes composed of core and auxiliary (accessory) genes. (Craig, 2014). This strain has about 33% G+C content and an average gene length of 824 nucleotides with 85% coding sequence, similar to other *S. aureus* strains. Half the coding sequence is located predominantly on one replichore and the second half is located predominantly on the other replichore (Craig, 2014). The chromosome of the best recognised strain, NCTC 8325, is 2.8 mbp in length and after digestion with SmaI, exhibits 16 fragments. The succession of 15 of them has been elucidated. About 190 loci have been mapped within the chromosome of this strain (Craig, 2014).

Virulence factors are encoded by phages, plasmids, pathogenicity islands and *Staphylococcus* cassette chromosome. Increased resistance for antibiotics is encoded by a transposon (Tn 1546) that was inserted into a conjugated plasmid that also encoded resistance to other compounds including disinfectants (Novick *et al.*, 2010). Methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to the antibiotic methicillin, expresses a modified penicillin-binding protein encoded by *mec*

A gene (Malachowa *et al.*, 2010; Benson *et al.*, 2014). This was brought about by many evolutions thought horizontal gene transfer of *mec* A to a wide variety of methicillin susceptible *S. aureus* strains. The genes for antibiotic resistance in *Staphylococcus aureus* are located on plasmids or other similar structures (Harrison *et al.*, 2013).

2.2.4 Host range of *S. aureus*

Members of the genus *Staphylococcus* are known to colonize the skin and upper respiratory tracts of mammals and birds. Some species specificity has been observed in host range, such that the *Staphylococcus* species observed on some animals appear more rarely on more distantly related host species (Li *et al.*, 2011). Some of the observed host specificity includes:

S. arlattae – chickens, goats

S. aureus - humans

S. auricularis – deer, dogs, humans

S. capitis – humans

S. caprae – goats, humans

S. cohnii – chickens, humans

S. delphini – dolphins

S. devriesei – cattle

S. epidermiditis – humans

S. equorum – horses

S. felis – cats

S. fleurettii – goats

S. gallinarum – chickens, goats, pheasants

S. haemolyticus – humans, *Cercocebus*, *Erythrocebus*, *Lemur*, *Macca*, *Microcebus*, *Pan*

S. hyicus – pigs

S. leei – humans

S. lentus – goats, rabbits, sheep

S. lugdunensis – humans, goats

S. lutrae – otters

S. microti – voles (*Microtus arvalis*)

S. nepalensis – goats

S. pasteuri – humans, goats

S. pettenkoferi – humans

S. pseudintermedius – dogs

S. rostri – pigs

S. schleiferi – humans

S. sciuri – humans, dogs, goats

S. simiae – South American squirrel monkeys (*Saimiri sciureus*)

S. simulans – humans

S. warneri – humans, Cercopithecoidea, Pongidae

S. xylosus – humans.

2.2.5 Biology of of *S. aureus*

Staphylococci are spherical Gram-positive cocci 0.5-1.0 µm in diameter and occur singly, in pairs, in short chains, and most commonly, in irregular grape-like clusters (Patel *et al*., 2012; Bercker *et al.,* 2014). The staphylococci are strongly catalase positive and generally tolerate relatively high concentrations of sodium chloride (7.5-10%). This ability is often employed in preparing media selective for staphylococci (Cheesbrough, 2010; Patel *et al*., 2012; Bercker *et al.,* 2014). *Staphylococcus aureus* is distinguished from other staphylococci by the production of coagulase an enzyme that clots plasma. *S. aureus* produces a carotenoid pigment that imparts a golden colour to its colonies. This pigment enhances the pathogenicity of the organism by inactivating the microbicidal effect of superoxides and other reactive oxygen species within neutrophils. *S. aureus* haemolyzes red blood cells and is also able to ferment mannitol (Plata *et al*., 2009; Rath *et al.,* 2015).

2.2.6 Epidemiology and transmission of *S. aureus* and MRSA

S. aureus is among the most common hospital acquired pathogens (Shitu *et al.,* 2011) colonizing the skin of both animals and humans (Chaibenjawong and Foster, 2011;

Shitu *et al.,* 2011). It can be spread through contaminated surfaces, through the air and through people. *S. aureus* can survive on domesticated animals such as dogs, cats, horses and chickens (Jason *et al.,* 2014; Jenney *et al.,* 2014). It can survive for some hours on dry environmental surfaces, but the importance of the environment in spread of *S. aureus* is currently debated. It can be the host for phages, such as the Panton-Valentine Leukocidin, that increase its virulence.

S. aureus infections can be spread through contact with pus from an infected wound, skin-to-skin contact with an infected person by producing hyaluronidase that destroy tissues, and contact with objects such as towels, sheets, clothing, or athletic equipment used by an infected person. Certain groups of individuals are more susceptible to *S. aureus* colonization than others including health-care workers, nursing home inhabitants, prison inmates, military recruits and children (Faccioli-Martins *et al.,* 2012.).

Transmission of MRSA occurs from person to person, animals to humans and *vice-versa* similar to the case of *S. aureus.* It can also occur by direct contact via the hands with colonized or infected humans or animals (Cantry *et al.,* 2010; Cuny *et al.,* 2010). In human hospitals environments, colonized and infected patients are the main sources of MRSA infection of uninfected patients. The routes of MRSA transmission are

similar to *S. aureus*. Carrier animals serve as reservoirs of MRSA and can transmit the organisms directly to other animals or humans (Cantry *et al.*, 2010; Cuny *et al.*, 2010).

It was thought that transmission of MRSA was solely from human to animal, with MRSA colonisation and infection typically occurring with contact between the hands of the human and anterior nares (nostrils) of animals (Monaco *et al.*, 2013). There is now increasing evidence that MRSA can be transmitted in both directions, from animal to human (Zoonotic) and from human to animal (reverse Zoonotic) (Huber *et al.*, 2009; Juhasz-kaszanyitzky *et al.*, 2007; Cantry *et al.*, 2010; Monaco *et al.*, 2013). Once exposed to MRSA, animals can become colonised, and may serve as reservoir to transmit the infection to other animals and also to their human handlers (Juhasz-kaszanyitzky *et al.*, 2007; Ferreira *et al.*, 2011). It has been shown that even apparently healthy animals may be reservoir, and therefore may pose a risk to their handlers (Juhasz-kaszanyitzky *et al.*, 2007; Ferreira *et al.*, 2011). This has been documented in the general community, and is becoming increasingly documented in health-care settings and in animal environments (e.g., veterinary clinics and hospitals, farms, and slaughtered houses) (Juhasz-kaszanyitzky *et al.*, 2007; Ferreira *et al.*, 2011). Furthermore, MRSA isolates can also be spread between humans and animals like dogs, cats and horses in veterinary hospitals and between companion animals and their owners in households (Cantry *et al.*, 2010; Smith *et al.*, 2013). MRSA can also be spread via contaminated food possibly during handling or eating contaminated food (Wendlandt *et al.*, 2013; Syne *et al.*, 2013). Reported food-borne MRSA outbreaks

have occurred through contamination by infected food handlers due to saphylococcal enterotoxin associated with food poisoning. Food sources of *S. aureus* have recently expanded to include retail meat products from food-producing animals, including swine, poultry, and cattle (Kluytmans, 2010; Jackson *et al.*, 2013). MRSA has also been isolated from retail meat and from meat-producing animals. Transmission of MRSA from food animals to humans was first realized for swine, since pig farmers and family members were found to be colonized with MRSA sequence type 398 (ST398) (Vanderhaegen *et al.*, 2010a; Monaco *et al.*, 2013).

2.2.7 Pathogenesis and virulence of *S. aureus* infections

S. aureus is the most pathogenic specie of the genus *Staphylococcus*, being implicated in both community-acquired and nosocomial infections. Itis known for its capacity to cause a broad range of important infections in humans and animals. Such capacity is related to the expression of an array of factors that participate in pathogenesis of infection, allowing this bacterium to adhere to surfaces/tissues, avoid or invade the immune system, and cause harmful toxic effects to the host (Chanda *et al.*, 2012; Costa *et al.*, 2013).

S. aureus produces many cellular components, enzymes, extracellular toxins and haemolysins more than any other bacterium (Piechota *et al.*, 2014; Macori *et al.*, 2017). The cell wall of *S. aureus* is composed of a thick peptidoglycan layer, which

contributes to the virulence of the bacterium (Azara *et al.,* 2014). They form spherical colonies in clusters in two planes and have no flagella. The peptidoglycan stimulates the production of cytokines by macrophages resulting in complement system activation and platelet aggregation (Azara *et al.,* 2014). Secretions are numerous, but include surface associated adhesins, endotoxins, exoenzymes, and capsular polysaccharides (Piechota *et al.,* 2014). The capsule is responsible for enhanced virulence of a mucoid strain (Piechota *et al.,* 2014).

S. aureus causes infection in two ways; i). The bacterium can enter the body through damaged endovascular points of the host where platelet-fibrin-thrombi complex have formed and attached via microbial surfaces complex that recognize adhesive matrix molecules (MSCRAMM) mediated mechanisms and ii). The bacterial cells can attach to endothelial cells via adhesion-receptors interactions or by bridging ligands, including serum components such as fibrinogen (Plata *et al*., 2009; Lin and Peterson, 2010). Upon entry into the host tissue, immune cells phagocytize *S. aureus* cells, which promote the production of proteolytic enzymes and toxins (Table 2.2) that facilitate the spread to adjoining tissues and the release of the staphylococci into the bloodstream resulting in bacteraemia. The infected endothelial cells due to immune response to infection produce tissue necrosis factor which results in necrosis and abscess formation (Lin, and Peterson, 2010). Some of the MSCRAMMs and other surface adhesions include coagulase, collagen-binding protein, clumping factors,

fibronectin-binding protein (FnBP), poly-*n*-succynyl-β-1, 6 glucosamine (PNSG) and protein A (Table 2.2) (Lin and Peterson, 2010; Que and Moreillon, 2010). Other important *S. aureus* proteins include enzymes such as beta-lactamase, which encodes resistance to beta-lactam antibiotics, thus facilitating invasion of viable cocci into the host (Costa *et al.,* 2013). The production of enterotoxins was partly regulated by a quorum sensing mechanism involving the *agr* gene (Azara *et al.,* 2014; Piechota *et al.,* 2014). The mechanism involves inter signaling of *S. aureus* cells through chemical production of extracellular products which control survival of the cell. The suppression of toxins is an important part in the treatment and management of *S. aureus* infections (Azara *et al.,* 2014).

2.2.8 Virulence factors associated with *Staphylococcus aureus*

2.2.8.1 Cell surface factors

S. aureus expresses several cell surface factors like microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsular polysaccharides, and staphyloxanthin (carotenoid pigment) which play a role in its virulence (Lin and Peterson, 2010; Patel *et al.,* 2010; Que and Moreillon, 2010).

2.2.8.2 *Toxins*

S. aureus is capable of secreting several toxins which are associated with specific diseases. The toxins protect the cell-wall and play active roles in disarming host immunity. Indeed, they disrupt host cells and tissues and interfere with the host immune system to release nutrients and facilitate bacteria dissemination (Que and Moreillon, 2010; Lin and Peterson, 2010). These secreted factors can be divided into four categories: superantigens, cytolytic (poreforming)toxins, various exoenzymes and miscellaneous proteins (Que and Moreillon, 2010; Lin and Peterson, 2010).

2.2.8.3 *Pyrogenic toxin super antigens (PTSAgs)*

Superantigens are a group of powerful secreted immune-stimulatory proteins capable of inducing a variety of human diseases, including toxic shock syndrome (TSS) (Costa *et al.,* 2013). This group includes the toxin TSST-1, which causes TSS associated with tampon use and the staphylococcal enterotoxins, which cause a form of food poisoning, are included in this group (Lin and Peterson, 2010; Argudın *et al.*, 2010; Hennekinne *et al.,* 2012).

2.2.8.4 *Toxic shock syndrome toxin*

The toxic shock syndrome (TSS) is a rare condition associated with infections in children related with wounds or burn damage, as well as in women using tampons during menstruation and these infections are characterized by a rapid onset with high

fever and multi organ failure (Que and Moreillon, 2010; Lin and Peterson, 2010). These serious conditions, as well as staphylococcal scarlet fever, is caused by *S. aureus* that are producing the toxic shock syndrome toxin- 1 (TSST-1), which belong to the group of staphylococcal super antigens that are capable of inducing a massive activation of T-cells (Lin and Peterson, 2010; Costa *et al.*, 2013). The gene encoding TSST-1 (*tst*) is located on the pathogenicity island designated SaPI 1 that is suggested to be a less transmissible mobile element, which would explain presence of *tst* in only a few restricted clones (Cista *et al.*, 2013).

2.2.8.5 *Staphylococcal enterotoxins*

The staphylococcal enterotoxins also belong to the group of staphylococcal super antigens and consist of several toxins of which some have been suggested to be involved in an increased virulence that have been reported in strains of nosocomial *S. aureus* (Jenney *et al.*, 2011; Piechota *et al.*, 2014). The SEA has been associated with more severe infections, such as staphylococcal food poisoning and septic shock, and induces a strong pro inflammatory response in comparison with other enterotoxins (Jenney *et al.*, 2011; Piechota *et al.*, 2014).

2.2.8.6 *Exfoliative toxins*

Exfoliative (EF) toxins are implicated in the disease staphylococcal scalded-skin syndrome (SSSS), which occurs most commonly in infants and young children. It may

also occur as epidemics in hospital nurseries (Bukowski *et al.,* 2010). The protease activity of the exfoliative toxins causes peeling of the skin observed with SSSS.

There are four serological isoforms of exfoliative toxins (ETA, ETB, ETC and ETD) of *S. aureus,* which may cause exfoliation of the skin epidermis followed by secondary infections. The exfoliative toxins A and B are the two most important isoforms in humans and those are associated with staphylococcal bullous impetigo and staphylococcal scalded skin syndrome (SSSS) that predominantly affects neonates and children (Bukowski *et al.,* 2010). The ETD has been associated with wound infections, while the ETC has not yet been associated with human disease (Bukowski *et al.,* 2010).

2.2.8.7 *Leukocidin toxins*

Staphylococcal toxins that act on cell membranes include alpha-toxin, beta-toxin, delta-toxin, and several bicomponent toxins (Vandenesch *et al.,* 2012). The bicomponent toxin Panton-Valentine Leukocidin (PVL) is associated with severe necrotizing pneumonia in children (Thomas *et al.,* 2011). The presence of PVL is associated with increased virulence of certain strains (isolates) of *Staphylococcus aureus*. It is present in the majority of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates studied and is the cause of

necroticlesions involving the skin or mucosa, including necrotic hemorrhagic pneumonia (Thomas *et al.*, 2011). PVL creates pores in the membranes of infected cells. PVL is produced from the genetic material of a bacteriophage that infects *Staphylococcus aureus*, making it more virulent. PVL causes leukocyte destruction and necrotizing pneumonia, an aggressive condition that can kill up to 75% of patients (Thomas *et al.*, 2011; Vandenesch *et al.*, 2012).

2. 2.8.8 *Hemolysins*

Hemolysins or haemolysins are lipids and proteins that cause lysis of red blood cells by destroying their cell membrane. Although the lytic activity of some microbe-derived hemolysins on red blood cells may be of great importance for nutrient acquisition, many hemolysins produced by pathogens do not cause significant destruction of red blood cells during infection (Que and Moreillon, 2010; Vandenesch *et al.*, 2012). However, hemolysins are often capable of lysing red blood cells *in vitro*.

The staphylococcal α-toxin (α-hemolysin) is encoded by the *hla* gene and shown to be associated with an increased virulence of *S. aureus*. The α-toxin creates pores in cell membranes and is capable of lysing erythrocytes and it may also stimulate apoptosis in lymphocytes (Kebaier *et al.*, 2012). In addition, the α-toxin is dermonecrotic and neurotoxic. Interestingly, β-hemolysin (*hlb*) displays the opposite effect by acting on the same sites of the erythrocyte membrane and thereby preventing α-toxin to attach

and create a pore (Que and Moreillon, 2010; Yamashita *et al.*, 2011). Yet, the hemolytic activity of β-hemolysin is enhanced if the erythrocytes are first refrigerated prior to the toxin treatment. The action and importance of the δ-toxin in staphylococcal virulence has not been fully investigated, but it is suggested to also display pore-forming activity (Que and Moreillon, 2010; Yamashita *et al.*, 2011).

2.2.8.9 Staphylococcal enzymes

Nearly all strains of *S. aureus* secrete several extracellular enzymes whose function is thought to be the disruption of host tissues and/or inactivation of host antimicrobial mechanisms to acquire nutrients for bacterial growth and facilitate bacterial dissemination (Que and Moreillon, 2010; Lin and Peterson, 2010). *S. aureus* produces enzymes such as coagulase (bound and free coagulases) which clots plasma and coats the bacterial cell, probably to prevent phagocytosis. Hyaluronidase breaks down hyaluronic acid and helps in spreading it. *S. aureus* also produces deoxyribonuclease, which breaks down the DNA, lipase to digest lipids, staphylokinase to dissolve fibrin and aid in spread, and beta-lactamase for drug resistance (Que and Moreillon, 2010; Lin and Peterson, 2010).

The function of the staphylococcal enzymes is primarily to produce nutrients that are necessary for the cell growth. However, some enzymes have also been proposed to play an important role in pathogenicity. For example, the proteases may be involved in

the inactivation of host defence peptides and perhaps also blocking of antibodies. Lipases may have an effect on the host immune response by inactivating the fatty acids that are intended to disrupt the bacteria (Que and Moreillon, 2010; Lin and Peterson, 2010).

2.2.8.10 Role of pigment in virulence

Some strains of *S. aureus* are capable of producing staphyloxantin - a carotenoidpigment that acts as a virulence factor (Otto, 2014). It has an antioxidant action that helps the microbe to evade killing with reactive oxygen used by the host immune system. It is thought that staphyloxantin is responsible for *S. aureus*' characteristic golden colour (Otto, 2014). Drugs designed to inhibit the bacterium's production of the staphyloxantin may weaken it and renew its susceptibility to antibiotics. Due to similarities in the pathways for biosynthesis of staphyloxantin and human cholesterol, a drug developed for lowering cholesterol was shown to block *S. aureus* pigmentation and disease progression in a mouse infection model (Otto, 2014).

2.3.8.11 *Role of protein A in virulence*

Protein A is a 42 kDa surface protein originally found in the cell wall of the *Staphylococcus aureus*. It is encoded by the *spa* gene and its regulation is controlled by DNA topology, cellular osmolarity, and a two-component system called ArlS-ArlR

(Morell *et al.*, 2010; Kobayashi and DeLeo, 2013). As a pathogen, *Staphylococcus aureus* utilizes protein A, along with a host of other proteins and surface factors, to aid its survival and virulence. To this end, protein A plays a multifaceted role:

i. By binding the Fc portion of antibodies, protein A renders them inaccessible to the opsonins, thus impairing phagocytosis of the bacteria via immune cell attack.
ii. Protein A facilitates the adherence of *S. aureus* to human von Willebrand factor (vWF)-coated surfaces, thus increasing the bacteria's infectiousness at the site of skin penetration.
iii. Protein A can inflame lung tissue by binding to tumor necrosis factor 1(TNFR-1) receptors. This interaction has been shown to play a key role in the pathogenesis of staphylococcal pneumonia.
iv. Protein A has been shown to cripple humoral (antibody-mediated) immunity which in turn means that individuals can be repeatedly infected with *S. aureus* since they cannot mount a strong antibody response.
v. Protein A has been shown to promote the formation of biofilms both when the protein is covalently linked to the bacterial cell wall as well as in solution (Morell *et al*., 2010; Kobayashi and DeLeo, 2013).

Table 2.1 Toxins and toxic components produced by *S. aureus*

Toxin	Toxin activity
Capsule	Antiphagocytic
DNase	Hydrolyses DNA
Enterotoxin (s)	Food poisoning toxins that cause vomiting and diarrhea
Epidermolytic toxins A and B	Epidermal splitting and exfoliation
Fibrinolysis	Digests fibrin
Haemolysin α, β and õ	Cytolytic; lyses erythrocytes of various animal species
Hyaluronidase	Breaks down hyaluronic acid
Leucocidin	Kills leucocytes
Protein A	Lypolytic (produce opacity in egg-yolk medium)
Toxic shock syndrome toxin-1	Shock, rash and desquamation

2.3 Pathology and diseases caused by *S. aureus*

Staphylococcus can cause a wide variety of diseases in humans and animals through either toxin production or penetration (Plata, 2009; Rasmussen *et al.,* 2011; Tong *et al.,* 2015). Staphylococcal toxins are a common cause of food poisoning, for they can be produced by bacteria growing in improperly-stored food items. *S. aureus* can infect several tissues when barriers have been breached (e.g., skin or mucosal lining). This leads to furuncles (boils) and carbuncles (a collection of furuncles). In infants, *S. aureus* infection can cause a severe disease, Staphylococcal scalded skin syndrome (SSSS) (Tong *et al.,* 2015).

2.3.1 Staphylococcal bacteremia

S. aureus is one of the common causes of blood stream infections and most of the *S. aureus* bacteraemia was attributed to cauterization and high nasal colonization (80%) in hospital settings (Rasmussen *et al.,* 2011). It is estimated that more than 50% of *S. aureus* bacteraemia are acquired in the hospital after surgery or from constant use of contaminated intravascular catheters. Other risk factors of HA-MRSA bacteremia include; immunosuppressive diseases, such as cancer, diabetes, human immune-deficiency virus (HIV) and the extensive use of corticosteroid and foreign bodies, which include prosthetic heart valves as well as central and peripheral venous catheters (Rasmussen *et al.,* 2011).

2.3.2 Atopic dermatitis

Skin infections manifest in various ways, including small benign boils, folliculitis, impetigo, cellulitis, and more severe, invasive soft-tissue infections (Hon *et al.,* 2012; Tong *et al.,* 2015). *S. aureus* is extremely prevalent in persons with atopic dermatitis, more commonly known as eczema. It is mostly found in fertile, active places, including the armpits, hair, and scalp. Large pimples that appear in those areas may exacerbate the infection if lacerated. This can lead to staphylococcal scalded skin syndrome, a severe form of which can be seen in newborns. The presence of *S. aureus* in persons with atopic dermatitis is not an indication to treat with oral antibiotics, as evidence has not shown this to give benefit to the patient (Hon *et al.,* 2012; Tong *et al.,* 2015). However, topical antibiotics combined with corticosteroids have been found to improve the condition. Colonization of *S. aureus* drives inflammation of atopic dermatitis; *S. aureus* is believed to exploit defects in the skin barrier of persons with atopic dermatitis, triggering cytokine expression and therefore exacerbating symptoms (Hon *et al.,* 2012; Tong *et al.,* 2015).

2.3.3 Toxic shock syndrome (TSS)

Toxic shock syndrome (TSS) is a toxin-mediated acute life-threatening illness, usually precipitated by infection with either *Staphylococcus aureus* and characterized by high fever, rash, hypotension, multiorgan failure and desquamation, typically of the palms

and soles, 1-2 weeks after the onset of acute illness (Tong *et al.*, 2015). The clinical syndrome can also include severe myalgia, vomiting, diarrhea, headache, and non-focal neurologic abnormalities. Toxic shock syndrome (TSS) is caused from intoxication by one of several related *Staphylococcus aureus* exotoxins. The most commonly implicated toxins include TSS toxin type-1 (TSST-1) and Staphylococcal Enterotoxin B (Tong *et al.*, 2015).

Almost all cases of menstrual TSS and half of all the non-menstrual cases are caused by TSST-1. Staphylococcal enterotoxin B is the second leading cause of TSS. Other exotoxins such as enterotoxins A, C, D, E, and H contribute to a small number of cases. Seventy to 80% of individuals develop antibody to TSST-1 by adolescence, and 90-95% have such antibody by adulthood. Apart from host immunity status, host-pathogen interaction, local factors (pH, glucose level, magnesium level), and age all have a direct impact on the clinical expression of this toxin-mediated illness (Tong *et al.*, 2015).

2.3.4 Staphylococcal endocarditis

Endocarditis is a life-threatening disease of the inner surface of the endothelium and the valves of the heart primarily restricted to younger patients (Hoer *et al.*, 2018). Symptoms may include fever, small areas of bleeding into the skin, heart murmur,

feeling tired, and low red blood cell count. Complications may include valvular insufficiency, heart failure, stroke, and kidney failure. Risk factors include valvular heart disease including rheumatic disease, congenital heart disease, artificial valves, hemodialysis, intravenous drug use, and electronic pacemakers. The number of people affected is about 5 per 100,000 per year (Hoer *et al.*, 2018). Rates, however, vary between regions of the world. Males are affected more often than females. The risk of death among those infected is about 25% (Hoer *et al.*, 2018).

2.3.5 Staphylococcal scalded skin syndrome

Staphylococcal scalded skin syndrome (SSSS) is a superficial blistering skin disorder caused by the exfoliative toxins of some strains of *Staphylococcus aureus* (Ton *et al.*, 2015). It is a syndrome of acute exfoliation of the skin typically following an erythematous cellulitis. Severity of staphylococcal scalded skin syndrome varies from a few blisters localized to the site of infection to a severe exfoliation affecting almost the entire body (Meshram *et al.*, 2018). Epidermolytic toxins are produced by the infecting *Staphylococcus* species; these toxins act at a remote site leading to a red rash and separation of the epidermis beneath the granular cell layer. Bullae form, and diffuse sheet like desquamation occurs. Two types of staphylococcal scalded skin syndrome are thought to exist: a localized form, in which there is only patchy involvement of the epidermis, and a generalized form, in which significant areas are involved, remote from the initial site of infection (Ton *et al.*, 2015).

Two exfoliative toxins (ETA and ETB) have been isolated and characterized, but the exact mechanism by which they caused exfoliation had until recently been uncertain. The toxins likely act as proteases that target the protein desmoglein-1 (DG-1), an important keratinocyte cell-to-cell attachment protein found only in the superficial epidermis (Ton *et al.*, 2015).The relative quantity of DG-1 in the skin differs with age and may partially explain the increased frequency of staphylococcal scalded skin syndrome in children younger than 5 years (Meshram *et al.*, 2018). It is theorized that immature renal function in this age group may contribute to impaired clearance of circulating exotoxins, contributing to more extensive disease (Ton *et al.*, 2015).

2.3.6 Food poisoning

Two-thirds of the 250 food borne diseases described in literature are caused by bacteria, and *S. aureus* is the leading cause of gastroenteritis resulting from the consumption of contaminated food (USFDA, 2013; CDC, 2016; Nakatsuji *et al.*, 2016). *S. aureus* food poisoning is due to the release of toxins in the food during its growth, causing symptoms ranging from abdominal pain to nausea, vomiting and sometimes diarrhoea but never alone (Jenney *et al.*, 2011; Piechota *et al.*, 2014; Nakatsuji *et al.*, 2016). The onset of *S. aureus* food poisoning is rapid, from 30 minutes to 8 hours after ingestion with spontaneous remission after 24 hours.

S. aureus enterotoxins (SEs) involved in food poisoning are highly stable and resistant to neutralization by proteolytic enzymes, such as pepsin or trypsin (Nakatsuji *et al.*, 2016). To date, there are 14 different SE types, which have similar structures. SEs are small proteins that are produced in food, soluble in water and are rich in lysine, aspartic acid and glutamic acid (Nakatsuji *et al.*, 2016). These SEs are more heat resistant in food than in laboratory medium. Various high sugars, protein and salt content foods are involved with *S. aureus* food poisoning including milk and milk products (cheese, ice cream, butter, etc), sausages, canned meat, salads (potato salads) and sandwich fillings (Umaru *et al.*, 2014; CDC, 2016). The foods that are involved in *S. aureus* food poisoning differ from one country to another. The main sources of contamination of these foods are food-handlers by manual contact, coughing or sneezing since up to 57% of the human population are *S. aureus* carriers (Umaru *et al.*, 2014; Nakatsuji *et al.*, 2016; CDC, 2016).

2.3.7 *Staphylococcus aureus* infections in animals

S. aureus has deleterious effects on animal health and has the potential of transmission from animals to humans and vice-versa (Juhasz-kaszanyitzky *et al.*, 2007; Cantry *et al.*, 2010; Cuny *et al.*, 2010; Peton and Le Loir, 2014). It thus has a huge impact on animal health and welfare and causes major economic losses in livestock production (Lozano *et al.*, 2016). Increasing attention is therefore being paid to both livestock and companion animals in terms of this pathogen. *S. aureus* can survive on dogs, cats, and

horses, and can cause bumblefoot in chickens. Some believe health-care workers' dogs should be considered a significant source of antibiotic-resistant *S. aureus*, especially in times of outbreak (Juhasz-kaszanyitzky *et al.,* 2007; Cantry *et al.,* 2010; Cuny *et al.,* 2010; Lozano *et al.,* 2016). *S. aureus* is one of the causal agents of mastitis in dairy cows. Its large polysaccharide capsule protects the organism from recognition by the cow's immune defenses.

2.3.8 Mastitis in cows

Mastitis is the most significant cause of economic loss in the dairy industry and *S. aureus* is known to be an important causative organism worldwide (Mirzaei *et al.,* 2011; Suleiman *et al.,* 2012a). The bacteria persist in mammary glands, teat canals, and teat lesions of infected cows and are contagious. The infection is spread at milking time when *S. aureus*-contaminated milk from an infected gland comes in contact with an uninfected gland, and the bacteria penetrate the teat canal. Recently studies have shown that 3 percent of all animals are infected with *S. aureus* (Mirzaei *et al.,* 2011).

S. aureus bacteria produce toxins that destroy cell membranes and can directly damage milk-producing tissue. White blood cells (leukocytes) are attracted to the area of inflammation, where they attempt to fight the infection. Initially, the bacteria damage the tissues lining the teats and gland cisterns within the quarter, which eventually leads

to formation of scar tissue. The bacteria then move up into the duct system and establish deep-seated pockets of infection in the milk secreting cells (alveoli). This is followed by the formation of abscesses that wall-off the bacteria to prevent spread but allow the bacteria to avoid detection by the immune system. The abscesses prevent antibiotics from reaching the bacteria and are the primary reason why the response to treatment is poor (Lozano *et al.,* 2016). During infection, destruction of alveolar and ductal cells reduces milk yield. These damaged cells may combine with leukocytes and clog the milk ducts that drain the alveolar areas, contributing to further scar tissue formation, occlusion of ducts, and decreased milk production. The ducts may reopen at a later time, but this usually results in a release of *S. aureus* organisms to other areas of the mammary gland. The spread of *S. aureus* within the gland results in the formation of additional abscesses that can become quite large and detectable as lumps within the udder (Lozano *et al.*, 2016).

Though most cases of *S. aureus* mastitis are subclinical, chronic cows usually have high SCC, abnormal mammary tissue, and recurrent cases of clinical mastitis (Mirzaei *et al.,* 2011). Clinically infected quarters often show moderate swelling and visible clots (chunks) in the milk, especially in forest rippings. Acute *S. aureus* infections generally develop late in the lactation (Peton and Le Loir, 2014). However, the clinical symptoms (udder swelling or hardness, changes in appearance of milk) do not show up until calving or early in the next lactation. It becomes difficult to successfully treat

an infection because drugs are not able to penetrate to all infection sites and because the bacteria can avoid contact with antibiotics while residing inside leukocytes (Lozano *et al.,* 2016).

2.3.8.1 Current status of bovine mastitis in Nigeria

Mastitis is one of the most costly devastating diseases in the dairy industry, and can be classified as clinical or subclinical based on clinical features of the disease (Shittu *et al.,* 2012). Subclinical mastitis occurrence is more prominent compared to clinical form and its prevalence is more than 20 to 50% of cows in given herds (Suleiman *et al.,* 2012). Both forms cause substantial financial losses due to reduced milk yield, degraded milk quality, drug costs and veterinary expenses, early culling of cows and increased labour costs for the farmers. The chief causal organisms of mastitis are cell-walled bacteria, even though mycoplasma, algae and yeast have also been identified to cause mastitis (Junaidu *et al.,* 2012). Numerous influencing factors like physiological, pathological, environmental or genetic factors may contribute to the occurrence of mastitis in dairy cattle.

Reports on bovine mastitis have been documented across Nigeria by several authors. Ameh *et al*. (1999) in Maiduguri, Borno State, Nigeria reported a prevalence of 2.1% with 34.6% S. *aureus* recovery rate from bovine mastitic milk, while prevalence rates of 31% and 3.2% were reported by Umoh *et al*. (1990). Similarly, Junaidu *et al.*

(2012) and Shittu *et al.* (2012) reported very high prevalence of 52% and 85.33% respectively in Sokoto and Savannah region of Nigeria. In Plateau State also, a prevalence of 30.9% was reported by Suleiman *et al.* (2013). Of recent Moru *et al.* (2018) determined the milk yield losses and cost of clinical mastitis in Friesian × Bunaji crossbred dairy cows in Zaria, Nigeria. They discovered a case of clinical mastitis at a base risk incidence of 35.2% to be ₦5, 005.85 ($15.87). This cost was found to have increased by 7.5% in a herd with 10% higher milk yield, while the revenue generated was higher by 10%. The cost was also found to be 1.64% higher in a herd with fifty per cent of it cows in first parity than a herd with fifty per cent of its cows in third parity. It was concluded in their study that improving milk production potential of cows resulted in more cases of clinical mastitis, although the increase in revenue overshadows the added costs of clinical mastitis, successful control of mastitis will also significantly reduce production costs and improve the farm revenue (Moru *et al.,* 2018).

2.4 Mechanism of antibiotic resistance in *Staphylococcus aureus*

Staphylococcus aureus resistance to penicillin is mediated by penicillinase (a form of β-lactamase), which is an enzyme that cleaves the β-lactam ring of the penicillin molecule, rendering the antibiotic ineffective (Schaumburg *et al.,* 2016; Fisher and Mobashery, 2017). Penicillinase-resistant β-lactam antibiotics, such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin, are able to resist degradation by staphylococcal penicillinase (Pexara *et al.,* 2013; Foster, 2017).

Resistance to methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome mec (SCC*mec*) and conferred by the *mec* A gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (Schaumburg *et al.,* 2016; Fisher and Mobashery, 2017; Foster, 2017).

Aminoglycoside antibiotics, such as kanamycin, gentamicin, streptomycin, etc., which were effective against staphylococcal infections were resisted due to inhibition of the aminoglycosides' action, which occurs via protonated amine and/or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosomal subunit (Yılmaz and Aslantaş, 2017). Three main mechanisms of aminoglycoside resistance mechanisms are currently and widely accepted: aminoglycoside modifying enzymes, ribosomal mutations, and active efflux of the drug out of the bacteria (Yılmaz and Aslantaş, 2017).

Glycopeptide resistance is mediated by acquisition of the *van* A gene, which originates from the enterococci and codes for an enzyme that produces an alternative peptidoglycan to which vancomycin will not bind (Reygaert, 2013). Vancomycin-resistant *S. aureus* (VRSA) is a strain of *S. aureus* that has become resistant to the glycopeptides (Kaleem *et al.,* 2012). Two forms of vancomycin resistance have been

documented. The first form involves the vancomycin intermediate *S. aureus* (VISA) strains with a minimum inhibitory concentration of 8-16 µgml (Kaleem *et al.,* 2012; Reygaert, 2013). The reduced susceptibility to vancomycin by *S. aureus* is hypothesized to be as a result of changes in peptidoglycan synthesis. There is a visible irregularly shaped and thickened cell wall in these VISA strains due to increased amounts of peptidoglycan (Kaleem *et al.,* 2012; Reygaert, 2013). Evidently, there is a decrease in cross-linking of the peptidoglycan strands resulting in the exposure of more D-alanyl-alanine residues (Reygaert, 2013).

The second form of vancomycin resistance involves vancomycin-resistant (*S. aureus*) with a minimum inhibitory concentration (MIC) of ≥ 128 µg/ml. The mechanism is hypothesized to be conjugation with vancomycin resistant *Enterococcus faecalis* (VRE). The process of conjugation results in the transfer of the *van* A operon of the *E faecalis* bacterium to the MRSA strain. The mechanism of resistance in VRSA is caused by alteration of the terminal peptide of D-Ala-D-Lac instead of D-Ala-D-Ala. The D-Ala-D-Lac synthesis occurs with minimal or low concentration of vancomycin.

Fluoroquinolone antibiotics (e.g. ciprofloxacin, ofcloxacin, norfolxacin) kill bacteria by inhibiting DNA synthesis (Pexara *et al.,* 2013; Foster, 2017)). Fluoroquinolones were initially developed for the treatment of Gram-negative bacteria, such as *Pseudomonas* species. Over the years, new fluoroquinolones with increased activity against Gram-positive cocci were developed including grepafloxacin, levofloxacin,

moxifloxacin, sparfloxacin and rovafloxacin (Foster, 2017). However, the use of these drugs has been highly regulated because of increased development of resistance by bacteria (Foster, 2017). *S. aureus* resistance to fluoroquinolones is suggested to be as a result of the exposure of bacteria to the drugs in mucosal and cutaneous surfaces in the nasal cavity. The DNA gyrase and topoisomerase, which are responsible for DNA replication, are the two enzymes targeted by fluoroquinolones (Pexara *et al.*, 2013). The DNA gyrase alters the supercoiling of the DNA whilst the topoisomerase IV separates DNA strands, which are interlocked to allow separation of the daughter chromosomes into daughter cells. The activity of the fluoroquinolones differs with the different types of dugs on the inhibitory activity against the two enzymes (Reygaert, 2013).

2.4.1 Methicillin-resistance in *Staphylococcus aureus*

Resistance to methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome *mec* (SCC*mec*). Resistance is conferred by the *mec* A gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (penicillins, cephalosporins and carbapenems) (Fisherand Mobashery, 2017; Foster, 2017). This allows for resistance to all β-lactam antibiotics and obviates their clinical use during MRSA infections (Schaumburg *et al.*, 2016). As such the glycopeptide, vancomycin, is often deployed against MRSA. The PBP2´ confers resistance to all clinically in use β-lactam antibiotics, i.e. the

penicillins, cephalosporins, carbapenems and monobactams. The β-lactam antibiotics damage bacteria by inactivating penicillin-binding proteins, that are essential in the assembly of the bacterial cell wall (Pexara *et al.,* 2013). Treated bacteria become osmotically fragile and easily lysed as a result of the weakened cell wall. The presence of the *mec* A gene has until recently been the 'gold standard' for the detection of MRSA worldwide. However, a novel *mec* A homologue that also confers methicillin resistance was identified in *S. aureus* isolates from dairy cattle and humans (Pexara *et al.,* 2013). This gene has been designated *mec C* (García-Álvarez *et al.,* 2011; Unnerstad *et al.,* 2013; Paterson *et al.,* 2014). Some strains of *S. aureus* possess an alternative resistance mechanism, attributable to the hyper-production of the *S. aureus* β-lactamase enzyme, which inactivates the antibiotic agents by hydrolysing the β-lactam ring of penicillin and cephalosporin compounds (Yılmaz and Aslantaş, 2017). However, some SCC*mec* types carry various additional genetic elements (e.g Tn*554*), which encodes resistance to macrolides, clindamycin and streptogramin B; *pT181*, which encodes resistance to tetracyclines. These genetic elements are especially common in HA-MRSA. Until recently, the only antibiotic available for treating MRSA infections was vancomycin, however, vancomycin-resistant MRSA strains, including CA-MRSA strains, have increasingly been reported (Pexara *et al*., 2013).

The expression of the *mec* A gene is regulated by the adjacent genes *mecR1* and *mecI*. The regulation of the *mec* A transcription is not completely understood but it involves the binding of the β-lactam antibiotics to an extracellular sensor domain and

subsequent cleavage of a sensor transducer (Schaumburg*et al.,* 2016) in a similar course of action to that described for β-lactamase. This leads to the activation of at least one intracellular domain that will cleave the repressor and enable transcription of the *mec* A gene. The induction of the PBP2´ is a slow process in presence of methicillin and oxacillin and the MRSA may appear susceptible in the laboratory. For this reason, cefoxitin is used for laboratory susceptibility testing of *S. aureus* since this substrate causes stronger induction (Schaumburg *et al.,* 2016). Additional genes might influence the expression of *mec* A, such as the *bla* regulatory genes (*blaR1* and *blaI*) and the factors essential for methicillin-resistance (*fem*) genes that encode peptidoglycan-modifying enzymes. However, the repressors (*mecI* and *blaI*) can repress either *mec* A or *blaZ*, whereas the inducers (*mecR1* and *blaR1*) are specific for their respective repressor (Schaumburg *et al.,* 2016).

2.4.2 *Staphylococcal cassette chromosomemec (SSCmec)*

Staphylococcal cassette chromosome *mec* (SCC*mec*) is a genomic island of unknown origin containing the antibiotic resistance gene *mec* A (Li *et al.,* 2011). SCC*mec* contains additional genes beyond *mec* A, including the cytolysin gene *psm-mec*, which may suppress virulence in HA-acquired MRSA strains (Kaito *et al.,* 2011). In addition, this locus encodes strain-dependent gene regulatory RNA called *psm-mec* RNA. SCC*mec* also contains *ccrA* and *ccrB*; both genes encode recombinases that mediate the site-specific integration and excision of the

Staphylococcal cassette chromosome *mec* (SCC*mec*) element from the *S. aureus* chromosome (Fisher and Mobashery, 2017; Foster, 2017). Currently, six unique SCC*mec* types ranging in size from 21–67 kb has been identified; they are designated types I-VI and are distinguished by variation in *mec* and *ccr* gene complexes (Turlej *et al.,* 2011). Owing to the size of the SCC*mec* element and the constraints of horizontal gene transfer, a minimum of five clones are thought to be responsible for the spread of MRSA infections, with clonal complex (CC) 8 most prevalent. SCC*mec* is thought to have originated in the closely related *S. sciuri* species and transferred horizontally to *S. aureus* (Turlej *et al.,* 2011).

Different SCC*mec* genotypes confer different microbiological characteristics, such as different antimicrobial resistance rates. Different genotypes are also associated with different types of infections. Types I-III SCC*mec* are large elements that typically contain additional resistance genes and are characteristically isolated from HA-MRSA strains (Turlej *et al.,* 2011). Conversely, CA-MRSA is associated with types IV and V, which are smaller and lack resistance genes other than *mec* A. These distinctions were thoroughly explained by the fitness differences associated with carriage of a large or small SCC*mec* plasmid. Carriage of large plasmids, such as SCC*mec* I-III, is costly to the bacteria, resulting in compensatory decrease in virulence expression (Foster, 2017). MRSA is able to thrive in hospital settings with increased antibiotic resistance but decreased virulence. HA-MRSA targets immunocompromised, hospitalized hosts, thus a decrease in virulence is not maladaptive. In contrast, CA-

MRSA tends to carry lower fitness cost SCC*mec* elements to offset the increased virulence and toxicity expression required to infect healthy hosts (Foster, 2017).

2.4.3 Multidrug-resistance pattern in *Staphylococcus aureus*

The rapid spread of resistance to penicillin is an example of the unique adaptability of *S. aureus* to quickly respond to new environments and antibiotics (Pexara *et al.,* 2013; Foster, 2017). Several new antibiotics were introduced in order to treat the β-lactamase-producing staphylococci, however, until today, there is not one single group of antibiotics that have not been overcome by *S. aureus*, which begun with the inactivation of penicillin, followed by erythromycin, tetracycline and aminoglycosides, and more recently new agents such as linezolid and daptomycin (Magiorakos *et al.,* 2012; Yılmaz and Aslantaş, 2017). The glycopeptides, vancomycin has been considered as the antibiotic of "last resort" available for treatment of multidrug resistant staphylococcal infections, however there are now several reports of *S. aureus* showing intermediate or high level of resistance also to this drug (Kaleem *et al.,* 2012; Reygaert, 2013). There is a wide range of mechanisms altering in bacterial resistance to different groups of antibiotics, including enzymatic inactivation for example of penicillin caused by the action of β-lactamase (Magiorakos *et al.,* 2012; Fisher and Mobashery, 2016). Additional mechanisms include efflux pumps located in the cell membrane that transports the antibiotic outside the bacteria, alteration of the target site of the antibiotics, decreased cell permeability or capturing of the drug in a

thickened cell wall. The mechanisms of vancomycin resistance are not fully understood but are suggested to be multi-factorial and to vary in different strains (Kaleem *et al.,* 2012; Reygaert, 2013).

Acquisition of bacterial resistance could be mediated either by natural mutations in the chromosomal genes or by acquisition of additional genes by genetic exchange and the resistant bacteria are positively selected as the surrounding concentration of antibiotics increase (Kaleem *et al.,* 2012; Reygaert, 2013). We might face a worrisome future similar to the period prior to the penicillins because resistance to new agents arise and spread quickly and since it is both time consuming and expensive to develop new and effective drugs (Magiorakos *et al.,* 2012).

2.5 Discovery and evolution of methicillin-resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first discovered in 1961 in the United Kingdom in the same time period when methicillin was introduced into clinical use (Enright *et al.,* 2002; Basset *et al.,* 2011). The first MRSA originated when a SCC*mec* was integrated into the chromosome of a MSSA (Enright *et al.,* 2002; Peton and Le Loir, 2014; Samar *et al.,* 2018). One possible source is *Staphylococcus sciuri*, since one series of studies suggested that *mec* A is a native element in *S. sciuri;* a *mec* A gene, showing 88% overall similarity with *mec* A of MRSA, was present in each of more than 100 independent isolates from different ecological sources and

representing a wide variety of genotypes (Enright *et al.*, 2002; Baset *et al.*, 2011; Lozano *et al.*, 2016). However, the majority of these isolates showed no resistance to methicillin. The isolates which were resistant to methicillin had a further copy of *mec* A, identical to that of MRSA isolates (García-Álvarez *et al.*, 2011). The mode of transfer of the *mec* Agene from an unknown donor to *S. aureus* has been subject to a debate.

Initially, all MRSA strains were thought to be descendants of a single common ancestor, since they showed pigmentation different from MSSA strains, invariable survival capacity, and a typical resistance profile (Pexara *et al.*, 3013). Later, their clonality was studied in a more precise way by using a MRSA strain collection of 472 isolates from different geographic areas, and DNA probes to the *mec* Aregion and staphylococcal transposon Tn554. Twenty-nine Tn554 patterns and six different temporally ordered *mec* A patterns were found. Each Tn554 pattern associated with only one *mec* A pattern, suggesting that Tn554 polymorphism exist (Yılmaz and Aslantaş, 2017).

2.5.1 Methicillin-resistant *Staphylococcus aureus* (MRSA) organism

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacterium that is resistant to methicillin (a penicillin family) and many other β-lactam antimicrobials which include penicillins and cephalosporins (Fisher and Mobashery,

2016; Foster, 2017). The description "Methicillin-resistant" was first used in 1961, based on the discovery of a human *Staphylococcus aureus* infection in the United Kingdom that was resistant to methicillin (Peton and Le Loir, 2014; Samar *et al.*, 2018). Since that time, MRSA has emerged as a significant problem worldwide, and the term has evolved to include resistant to additional β-lactam antimicrobials. Currently, the term MRSA is often used to describe multi-drug resistant *S. aureus* (MDSA) or Oxacillin-resistant *S. aureus* (ORSA). There are many different strains of MRSA with differing degrees of immunity to the effects of various antibiotics. It does not mean that antibiotics are completely powerless against it, but simply require a much higher dose over a much longer period, or the use of an alternative antibiotic to which the bacteria has less resistance.

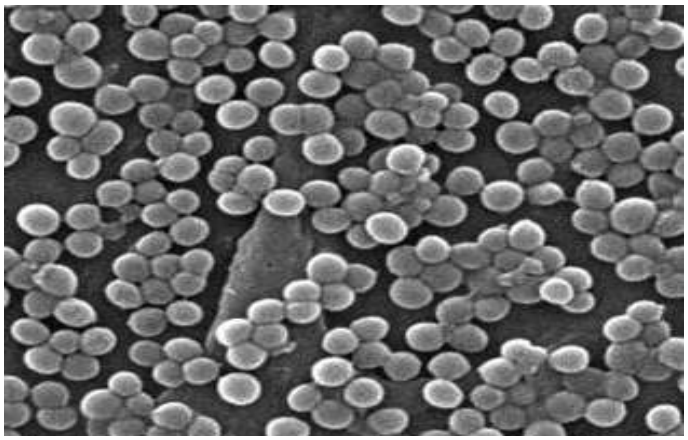

Plate 2.2 Electron micrograph of MRSA (CDC. 2010)

2.5.2 Strains of Methicillin-resistant *Staphylococcus aureus* (MRSA)

Acquisition of SCC*mec* in methicillin-sensitive *S. aureus* (MSSA) gives rise to a number of genetically different MRSA lineages (Monecke *et al.*, 2012; Stefani *et al.*, 2012). These genetic variations within different MRSA strains possibly explain the variability in virulence and associated MRSA infections (Stefani *et al.*, 2012). The first MRSA strain, ST250 MRSA-1 originated from SCC*mec* and ST250-MSSA integration (Stefani *et al.*, 2012). Historically, major MRSA clones: ST2470-MRSA-I, ST239-MRSA-III, ST5-MRSA-II, and ST5-MRSA-IV were responsible for causing hospital-acquired MRSA (HA-MRSA) infections. ST239-MRSA-III, known as the Brazilian clone, was highly transmissible compared to others and distributed in Argentina, Czech Republic, and Portugal (Calfee, 2011).

In the UK, the most common strains of MRSA are EMRSA15 and EMRSA16 (Vignaroli *et al.*, 2014). EMRSA16 is the best described epidemiologically: it originated in Kettering, England, and the full genomic sequence of this strain has been published (Vignaroli *et al.*, 2014). EMRSA16 has been found to be identical to the *ST*36:USA200, which circulates in the United States, and carry the SCCi>mec type II, enterotoxin A and toxic shock syndrome toxin I genes. Under new international typing system, this strain is now called MRSA252. It is not clear or entirely certain why this strain has become so successful, whereas previous strains have failed to persist. One explanation is the characteristic pattern of antibiotic susceptibility. Both EMRSA15 and EMRSA16 strains are resistant to erythromycin and ciprofloxacin. It is known that

S. aureus can survive intracellularly and these strains of *S. aureus* are therefore able to exploit an intracellular niche.

In the United States, most cases of CA-MRSA are caused by a CC8 strain designated ST8:USA300, which carries SCC<i>mec type IV, Panton-valentine leukocidin, PSM-alpha and enterotoxin Q and K and ST1:USA400(Monecke *et al.*, 2012). Other community-acquired strains of MRSA are ST8:USA500 and ST59:USA1000. In many nations of the world, MRSA strains with different predominant genetic background types have come to predominate among CA-MRSA strains. USA300 easily tops the list in the U.S. and is becoming more common in Canada. For example, in Australia ST93 strains are common, while in Continental Europe ST93 strains predominate. In Taiwan, ST59 strains, some of which are resistant to many non-bête-lactam antibiotics, have arisen as common causes of skin and soft tissue infections in the community.

A MRSA strain, CC398, is found in intensively reared production animals (primarily pigs, but also cattle and poultry), where it can be transmitted to humans as LA-MRSA (livestock-associated MRSA) (Graveland *et al.*, 2011; Stefani *et al.*, 2012).

2.5.3 Nomenclature and types of MRSA strains

Numerous strains of MRSA have been identified based on genetic analysis of the bacteria (Stefani *et al.,* 2012; Samar *et al.,* 2018). MRSA nomenclature varies worldwide, and a standard method for typing and naming MRSA strains has not yet been adopted; therefore, one genetic strain of MRSA may be referred to by several different names. The organism is often sub-categorized as Community-acquired (Community-associated) MRSA (CA-MRSA) or hospital-associated (Health care-associated) MRSA (HA-MRSA) although this distinction is complex (Monecke *et al.,* 2012; Graveland *et al.,* 2011; Stefani *et al.,* 2012; Samar *et al.,* 2018).

2.5.3.1 *Community-associated MRSA (CA-MRSA) in humans*

Community-associated MRSA (CA-MRA) strains were initially reported in the late 1980s among individuals living in remote communities in Western Australia with no previous history of hospitalization (Calfee, 2011; Samar *et al.,* 2018). The initial report was followed by similar reports from USA, New Zealand, and later in Europe. Initially, CA-MRSA were mostly associated with skin and soft tissue infections such as impetigo, cellulitis, folliculitis and boils and those at risk were the young patients. However, some CA-MRSA strains have been reported to cause severe infections including necrotizing fasciitis, post-influenza pneumonia, septic thrombophlebitis, septic arthritis, and bacteremia (Vignaroli *et al.,* 2014; Samar *et al.,* 2018).

CA-MRSA are usually susceptible to non-beta lactam antibiotics, carry smaller-sized SCC*mec* types IV, V and VI. In addition, CA-MRSA strains often express lower levels of resistance to oxacillin (MIC; 8–32 mg/L) and multiply faster than HA-MRSA strains with significantly shorter doubling times which may help CA-MRSA achieve successful colonization by enabling it to out-compete commensal bacterial flora (Vignaroli *et al.,* 2014; Samar *et al.,* 2018).

2.5.3.2 *Hospital-associated MRSA (HA-MRSA) or epidemic MRSA*

Healthcare-associated MRSA (HA-MRSA) were isolated from patients admitted to healthcare facilities such as nursing homes and long-term care facilities (Monecke *et al.,* 2012; Sievert *et al.,* 2013; Samar *et al.,* 2018). Healthcare-associated infections caused by HA-MRSA include bloodstream infections, urinary tract infections, respiratory tract infections, surgical-wounds and device-associated infections. Risk factors for acquiring HA-MRSA include previous admission to healthcare facilities, impaired immune system, use of multiple antibiotics, use of invasive medical devices and old age (Sievert *et al.,* 2013). Genetically, the HA-MRSA carried SCC *mec* types I, II and III, are usually multiresistant to antibiotics, and tend to multiply slowly in culture (Vignaroli *et al.,* 2014; Samar *et al.,* 2018).

2.5.3.3 Livestock-associated MRSA

Staphylococcus aureus is also an important cause of infections in livestock resulting in economic losses in the food industry (Graveland *et al*., 2011; Stefani *et al.,* 2012; Samar *et al.,* 2018). Livestock-associated MRSA (LA-MRSA) strains were initially identified because they were non-typeable by pulsed-field gel electrophoresis following digestion with *Sma*I restriction enzyme (Tegegne *et al.,* 2017; Samar *et al.,* 2018). Further molecular typing revealed that LA-MRSA defined to a new lineage of MRSA that belonged to clonal complex 398 (CC398). Although LA-MRSA ST398 was initially reported among livestock, it has also appeared in the community among human patients in contact with infected or colonized animals which is considered the major risk factor for LA-MRSA colonization (Tegegne *et al.*, 2017; Samar *et al.,* 2018). Other LA-MRSA lineages reported in humans include ST9, ST97 and ST433. LA-MRSA has also caused invasive infections including endocarditis, osteomyelitis, and ventilator-associated pneumonia in humans (Graveland *et al.,* 2011; Tegegne *et al.,* 2017).

2.6 Prevalence and molecular epidemiology of *S. aureus* and MRSA

The epidemiology of MRSA in the developed world has been extensively studied but remains under study in developing countries, and in Africa in particular (Mediavilla *et al.* 2012; Abdulgader *et al.,* 2017). MRSA can be categorized according to where the infection was acquired thus hospital-associated MRSA (HA-MRSA), community-

associated MRSA (CA-MRSA) and the recently discovered livestock-associated MRSA (LA-MRSA) (Sievert *et al.,* 2013; Graveland *et al.,* 2011; Tegegne *et al.,* 2017; Samar *et al.,* 2018). MRSA was initially reported as a nosocomial pathogen in human hospitals and was isolated from patients with compromised immune systems. In the 1990s, a major change in the epidemiology of MRSA occurred, with the appearance of cases affecting people with no epidemiological connection to hospitals. Strains that caused such infections were referred to as community-associated MRSA (EFSA, 2012). The CA-MRSA strains that cause non-hospital-associated infections are characteristically susceptible to many antibiotics, habour type IV SCC*mec*, which is smaller in size than other SCC*mec* elements, and contains no other antibiotic resistance genes except *mec* A (Samar *et al.,* 2018). In contrast to CA-MRSA, the majority of HA-MRSA strains carry one of two types of SCC*mec*, type II or III, and show characteristics of multidrug resistance (Sievert *et al.,* 2013). There is now increasing concern about the public health impact of MRSA associated livestock, because their resistant genes can spread to humans by direct contact or through the food chain (Kluytmans, 2010). Approximately 20% to 60% of humans are permanent or intermittent carriers of *S. aureus* and relevant sites include the anterior nares, axillae, perineum and vagina (Kluytmans, 2010). The prevalence of MRSA was lower than 50% in most of the African countries, although it appears to have increased since 2000 in many African countries, except for South Africa (Falagas *et al*., 2013).

2.6.1 Prevalence and molecular epidemiology of *S. aureus* and MRSA in humans

Several individual studies have been conducted across the globe to determine the prevalence and molecular epidemiology of MRSA in humans with varying prevalence and different types of clone detected (Abdulgader *et al.,* 2017; Faccioli-Martins and Souza da Cunha, 2012; Akilu *et al.,* 2013; Chu *et al.,* 2014; Gruteke *et al.,* 2015). For example, Aklilu *et al.* (2013) conducted a molecular epidemiology of Methicillin-resistant *Staphylococcus aureus* (MRSA) among veterinary students and personnel in Malaysia, where pandemic and widespread MRSA clones, ST5 and ST59 that were *spa* type t267 were detected. In Shangai, Chu *et al.* (2014) detected SCC*mec* III type ((81.3 %)), SCC*mec* II (15.9%) and IV (2.8 %) types strains in hospital. Similar study by Peters *et al.,* (2013) in Atlanta, Georgia observed that 13-15% of the HIV-infected patients were colonized with MRSA in the nares (41%), groin (21%) and both sites (38%).

In Hong kong, Gruteke *et al.,*(2015) investigated NL2007, NL2011, and NL2013 and HK2005 and HK2008 strains by using whole-genome sequencing. The results revealed the CC45/agr IV-MRSA V type with a total of 91.39% reading of NL2007 aligned with the published CC45/USA60 sequence. Alder *et al.* (2015) showed that 64% of the isolates were related to the global clones spa t001-SCCmec-I (n=99; 31%), t002-SCCmec-II (n=82; 26%), and t008-Sccmec-IV (n=21; 7%, five of which were identified as MRSA strain USA-300 while 17 strains unique to Israel were identified as SCCmec types IV and V that were common among hospital-acquired isolates.

2.6.2 Prevalence and molecular epidemiology of *S. aureus* and MRSA in animals

MRSA has been sporadically isolated initially from animals such as cows, small companion animals, pigs and horses,with the sources traced to humans (Holmes and Zadoks, 2011; Joshi and Devkota, 2014). MRSA was thought to be human pathogens and that animal husbandry was of little relevance to MRSA causing diseases in humans, but was rather a problem solely based on the antimicrobial use in human medicine (Samar *et al,* 2018). The situation later changed, with MRSA been reported in livestock, especially swine, dogs, horses and cattle with humans and animals to transmission also established (Holmes and Zadoks, 2011; Catry *et al.,* 2012; Joshi and Devkota, 2014). In general, MRSA strains recovered from companion animals (cats, dogs, or horses) are different from those recovered from food animals. In the first case strains are usually similar to human HA-MRSA, while in the second case they appear to belong to specific animal-adapted clones, unrelated to the most common HA-MRSA (Holmes and Zadoks, 2011; Catry *et al.*, 2012; Joshi and Devkota, 2014).

Pets have been shown to act as reservoirs of bacteria resistant to antimicrobials, and MRSA transmission between humans and animals has been described. For strains of MRSA with alow specificity to the host, the transference is likely to occur in both directions between humans and pets living at the same household (Juhansz-kaszanyitzky *et al.,* 2007). Cuny *et al.* (2010) observed the emergence of MRSA in animals such as horses, pet animals and productive livestock which was confirmed to

be of human origin. The Particular clonal lineages that are obviously specific for humans (e.g. ST15, ST25, and ST45) and other for ruminants (e.g. ST151) were isolated. MRSA of veterinary nosocomial infections (e.g. ST8 and ST254 in horses, ST22 in small animals) very likely have their origin in health care facilities. MRSA ST398 which became first known from widespread colonization in industrially raised pigs seems to have limited host specificity and is able to colonize and to cause infections in various hosts. Mechanisms of host adaptation and their genomic background are poorly understood. van Balen *et al.* (2013) performed a yearlong active MRSA surveillance program at The Ohio State University (OSU) Veterinary Medical Center to describe the spatial and molecular epidemiology of this bacterium in the small animal hospital. Overall, 13.5% of surfaces were contaminated with MRSA at 1 or more sampling times throughout the year. The majority of the environmental and canine isolates were fouind to be SCC*mec* type II and USA100, which were found to be closely relatedMRSA strains circulating in the hospital.

Vandendriessche *et al.* (2013) conducted a survey on MRSA in animals and humans on veal, dairy, beef and broiler farms and compared the risk for human MRSA carriage with that of strictly horticulture farmers. The results showed that MRSA carriage were higher ($P<0.01$) on veal farms (calves, 64%; farmers, 72%) compared to dairy (cows 1%), beef (cows, 5%; farmers, 11%), broiler (pooled broths, 5%; farmers, 3%) and horticulture (farmers, 3%) farms. The intensity of animal contact was identified as a risk factor for human MRSA carriage. The vast majority of MRSA

(n=344), including those from pigs, were CC398 (98%), SCCmec V(5C2), V95C2 & 5)c, IV(2B) and IV(2B & 5) predominate. MRSA CC599 carrying *mec* C were detected in beef and dairy cattle. MRSA from veal were significantly more resistant than MRSA from pigs (P<0.01) while a few including *mec* C-carrying MRSA, harboured pyogenic super antigen toxins (Vandendriessche *et al.*, 2013).

The first report on the isolation of MRSA in horses was reported in the 1990 from 13 mares with metritis in Japan (Catry *et al.*, 2010). The source was thought to be a stallion on a stud farm which 10 of the mares had visited. To date, MRSA has been isolated from horses in Europe, Asia and North America (Loeffler and Lloyd, 2010; Guérin *et al.,* 2017). Horses are colonized or infected by MRSA types quite different from the lineages predominant in humans in the corresponding areas (Loeffler and Lloyd, 2010). Horse MRSA commonly belong to ST8 and related STs within clonal complex (CC) 8. van Balen *et al.* (2014) examined the role of environmental contamination as a reservoir and a possible source of Methicillin-resistant *Staphylococcus aureus* (MRSA) for patients and personnel at equine veterinary hospitals. Overall, 8.6% of the surfaces and 5.8% of the horses sampled were positive for MRSA. Ninety percent of the isolates carried SCC*mec* type IV, and 62.0% were classified as USA500. Guérin *et al.* (2017) characterized 1393 *S. aureus* from horses and discovered 85 (6.1%) to be MRSA, which were recovered from skin and soft-tissue ($n = 39$), genital tract ($n = 20$), respiratory tract ($n = 8$), bone and joint ($n = 8$) and others ($n = 10$). None of the MRSA isolate harboured *pvl* and *tst* toxin genes.

MRSA infections in pets are mainly represented by skin and soft tissue infections and are sometimes associated with veterinary surgeries.

Pigs have been shown to be important reservoirs of community-associated (CA-MRSA) MRSA that colonizes and infects humans that are in close contact with them (Graveland *et al.,* 2011; Tegegne *et al.,* 2017. Guo *et al.,* 2018). ST398 is the most commonly reported MRSA sequence type among large livestock in Europe. These strains frequently carry genes encoding fornon-beta-lactam antimicrobial resistance, including a plasmid-borne gene with resistance to trimetoprim, *dfrK*, identified in an isolate from a pig from Germany. Furthermore, these isolates are referred to often as non-typeable by PFGE because their genome is not digestedby SmaI enzymes, several common *spa* types have been associated with them, carrying SCC*mec* types IV and V and they typically lack PVL genes (David and Daum, 2010). Horgan *et al.* (2010) evaluated the prevalence of MRSA in the swine population in Ireland. A total of 440 pigs were evaluated from 41 geographically distributed farms and 100 individuals involved in the pig industry. No MRSA isolate was recovered from pigs but two humans tested were identified as MRSA carriers. These individuals were working in the wider pig industry. These isolates belonged to ST22 and ST1307, indicating that during the period of study the porcine colonization by MRSA, in particular the animal-related strain ST398, was not common in Ireland. Similarly, Bardoň *et al.* (2012) surveyed herds of breeding pigs throughout the Czech Republic for MRSA, five cases,

were detected, with a prevalence of 1.8%. All the isolated strains belonged to a single sequence type ST398.

Despite that livestock-associated-MRSA appears be the predominant MRSA strain in pigs, some studiesmention the detection of non-LA-MRSA strains in these animals, possibly by transmission of human strains to the animals. In Singapore, ST22-MRSA-IV was isolated from pigs and this strain was previously found increasingly important in the hospital population there. Notably the ST22-MRSA-IV is also a major hospital clone, as is the UK-EMRSA-15 found inthe UK, with both strains indicating a contamination of human origin. In Canada, 14% of MRSA isolated from pigs appeared to belong to the human epidemic clone CMRSA-2 (Canadian epidemic MRSA-2, known as USA100), while 74.4% of isolates were LA-MRSA.The remaining strains belonged to rare clones, not related to LA-MRSA or CMRSA-2. Most reports on LA-MRSA in pigs originate from the Netherlands. In Europe, LA-MRSA has also been found in pigs in Germany, Denmark and Belgium, and beyond Europe, in Canada, Singapore and the USA (Vanderhaeghen *et al.*, 2010). In Jiangmen, China Guo *et al.* (2018) examined pigs for MRSA and discovered 9.5% of the pigs positive for *S. aureus*, out of which 3.3% were MRSA. The predominant genotype was CC9 (ST9) for *S. aureus* and MRSA isolates. The correspondence analysis showed a significant relationship between clonal complexes and resistance pattern or virulence genes. For example, CC9 was associated with extensive drug-

resistance and co-carrying *chp*, *sak*, and *hlb*, and CC1 was associated with multidrug resistance and co-carrying *sak* and *hlb*.

2.6.3 Prevalence and molecular epidemiology of *Staphylococcus aureus* and MRSA in milk

MRSA was initially reported as a nosocomial pathogen in human hospitals (or hospital associated MRSA, HA-MRSA). Since the 1990s, community-acquired or community-associated MRSA (CA-MRSA) infections have also been reported to affect people having no epidemiological connection with hospitals (Samar *et al.*, 2018). More recently, MRSA has been isolated from most food-producing animals and foods of animal origin, raising public health concerns. MRSA strains have been isolated from cows' or small ruminants' milk and various dairy products in many countries (Pexara *et al.*, 2013; Pexara *et al.*, 2018).

Milk is a medium for the growth of many bacteria, but pasteurization is known to eliminates *S. aureus* and MRSA (Umaru *et al.*, 2013; Pexara *et al.*, 2013; Ridwana *et al.*, 2017). Handling or consumption of contaminated raw milk and dairy products can lead to the transmission and spread of antimicrobial resistance genes of *S. aureus* and MRSA to humans (Haran *et al.*, 2012; Pexara *et al.*, 2013). Several studies were conducted on MRSA in milk with differences in prevalence among different countries or even regions within the same country (Pexara *et al.*, 2013). These differences were largely due to differences in animal production systemsamong the various countries

coupled with differences in national antimicrobial policies and regulations of various countries (Grave *et al.,* 2010). Other factors that affects the MRSA prevalence in milk are presence ofmultiple animal species in the same area that facilitate transfer of genetic material between *S. aureus* strains (Spohr *et al.,* 2011).

Data from literature review indicates that few reports of MRSA in dairy cattle are available (Ferreira *et al.,* 2014). Previous studies indicating that MRSA is rare in milk of dairy cattle in the United States (Ferreira *et al.,* 2014). Recent and sporadic isolation of MRSA and related staphylococci from cattle in countries other than the United States, and the similarity between some of the human and animal isolates found, provide rationale for monitoring MRSA occurrence in cattle (Ferreira *et al.,* 2014; Pexara *et al.,* 2018). High prevalence of MRSA has now been recorded in milk in most countries of the world including Africa and Nigeria in particular (Suleiman *et al.,* 2012b; Umaru *et al.,* 2013). In most European countries, the MRSA prevalence in milk and dairy products has been generally found to be low. The investigation of MRSA prevalence in milk may serve as a tool for assessing both the sanitary conditions employed in dairy herds and the health risks that humans may encounter when infected with antibiotic-resistant strains (Pexara *et al.,* 2018).

A study by Mirzaei *et al.* (2011) determined the prevalence rate of MRSA in raw milk, pasteurized milk, ice cream and traditional butter in Sarab to be 22 (24%) in raw milk samples, 4 (8%) in pasteurized milk, 9 (18%) in traditional butter samples and 12

(24%) in traditional cheese samples. Only 2 (50%) of pasteurized milk isolates and 2 (22%) of traditional cheese butter contained *mec* A gene. No *mec* A gene was detected in raw milk and traditional butter isolates. Haran *et al.* (2012) in a similar study detected 2 MRSA isolates from 150 Bulk Tank Milk, were identified as *spa* types t529, t034 and t121 Two of the MRSA isolates were MLST ST 5-PFGE USA100-unknown *spa* type, which has been reported among hospital-associated MRSA, while the second isolate carried the MLST ST 8-PFGE USA300-*spa* type t121 genotype, commonly identified among community-associated MRSA isolates. In Germany, Kreausukon *et al.* (2012) observed allthe MRSA isolates to be spa-types assigned to the clonal complex CC398 and were not positive forany typical virulence factors for *S. aureus* such as the Panton-Valentine leukocidin.

Papadopoulos *et al.* (2018) in Greece recorded 3% prevalence of *mec* A positive MRSA with none of the MRSA isolates positive for *mec* C or the Pandon-Valentine leucocidin (PVL) genes. Four *spa* types (t127, t3586, t1773, t4038) were identified among the MRSA isolates, with t127 being the most prevalent (7 out of 11). Two of them, t3586 and t1773, were isolated for the first time in Greece. Furthermore, Pulse-Field Gel Electrophoresis (PFGE) analysis indicated clonal circulation through the dairy production chain. In India Ridwana *et al.* **(**2017**)** isolated 34 *S. aureus* milk isolates, 15 (44.1%) of which were MRSA positive. Prevalence of *S. aureus* in curd was 12/30 (40%), in cheese was 6/25 (24%) and in ice-cream was 8/15 (53.33%).

In Uganda Asiimwe *et al*. (2017) found MRSA prevalence of 56.1% in bulk tank milk, with 91.3% being SCC*mec* type V; while up to 73.2% resistant to tetracycline. Five of the isolates were found to carry the *PVL* virulence gene, while PFGE typing revealed ten clusters (ranging from two seven isolates each) that comprised 83% of the sample, and only eight isolates with unique pulsotypes. The largest PFGE profile (E) consisted of seven isolates while t7753, t1398, and t2112 were the most common *spa*-types. In Tanzania Mohammed *et al.* (2018) examined 117 raw milk samples for MRSA, 3 *mec* A positive, but however, only one *mec* A positive strain among the three was typeable and belonged to spa type t2603.

In Nigeria, Umaru *et al.* (2013) observed an MRSA prevalence of 4.8% in raw cow milk in Zaria and Kaduna However, no MRSA was detected in any of the yogurt samples examined. The *mec* A was detected by polymerase chain reaction in 4 of the 18 MRSA isolates (3 in raw milk and 1 in 'kindirmo' samples).

2.6.4 Prevalence and Molecular Epidemiology of *Staphylococcus aureus* and MRSA in other Foods

MRSA in foods MRSA has been detected in a variety of foods from countries in North America, Europe and Asia (de Jonge *et al.,* 2010; Beneke *et al.,* 2011). Foods may be contaminated by human strains of MRSA present in meat processors and other food handlers.Meat may also be contaminated by MRSA carried in animals as

demonstrated by a study following pigs from lairage through slaughter to commercial pork products (de Jonge *et al.*, 2010; Beneke *et al.*, 2011). In one survey Pu *et al.* (2009) in Baton Rouge, Louisiana isolated MRSA from six meat samples (5 pork and 1 beef), which were identified as USA300 and USA100.

In Germany, Feßler *et al.* (2011) surveyed fresh chicken and turkey meat as well as chicken and turkey meat products for the presence of methicillin-resistant *Staphylococcus aureus* (MRSA). Thirt-two (37.2%) of 86 samples were MRSA positive with 28 of the MRSA belonging to clonal complex 398 (CC398), which is widespread among food-producing animals. These CC398 isolates carried SCCmec elements of type IV or V and exhibited spa type t011, t034, t899, t2346 or t6574 and either the known dru types dt2b, dt6j, dt10a, dt10q, dt11a, dt11v, and dt11ab or the novel dru types dt6m, dt10as, and dt10at. In addition, two MRSA sequence type 9 (ST9) isolates with a type IV SCC*mec* cassette, spa type t1430, and dru type dt10a as well as single MRSA ST5 and ST1791 isolates with a type III SCCmec cassette, spa type t002, and dru type dt9v were identified. All but two isolates were classified as multi resistant. A wide variety of resistance phenotypes and genotypes were detected. All isolates were negative for the major virulence factors, such as Panton-Valentine leukocidin, toxic shock syndrome toxin 1, or exfoliative toxins. In contrast to the MRSA CC398 isolates, the four ST9, ST5, or ST1791 isolates harbored the egc gene cluster for enterotoxin G, I, M, N, O, and U genes.

Jackson *et al.* (2013) in a similar study in Georgia isolated 3 MRSA from pork and 4 from beef which were SCCmec type IV and *spa* types (t002, t008, t012, t024, t179, t337, t548, t681, and t1062). One retail beef MRSA isolate belonged to ST8, while the remaining three were ST5 by MLST. In retail pork MRSA, ST5, ST9, and ST30 were observed.

Some studies detected primarily HA-MRSA strains in foods, indicating that humans were the probable source and others detected primarily LA-MRSA (Beneke *et al.,* 2011). Two reported outbreak sassociated with MRSA-contaminated food has been recorded, in Tennese and Dutch hospital (Pu *et al.,* 2009). The Tennese outbreak was caused by CA-MRSA recovered from 3 ill persons and produced enterotoxin C. The Dutch outbreak of MRSA occurred in a Dutch hospital and involved 27 patients and 14 health-care workers with 5 deaths. Epidemiological investigations indicated that a colonized food handler apparently contaminated food (a peeled banana tested positive for MRSA) served to hospital patients, and some nurses may have inadvertently spread the bacteria to different wards (Beneke *et al.,* 2011). MRSA does not appear to be transferred readily from meat to meat handlers. It was not detected on hands or in noses of 89 persons working in cold meat processing facilities or institutional kitchens in the Netherlands even though 14% of samples of meat (veal, pork, chicken) that they worked with did contain MRSA. Most of the MRSA isolates were identified as ST398, livestock-associated MRSA (de Jonge *et al.,* 2010).

2.6.5 MRSA as a mastitic agent

Staphylococcus aureus is one of the most important bacterial pathogens causing bovine mastitis, which causes significant economic losses in the milk industry (Pexara *et al.,* 2013; Joshi *et al.,* 2014). The first report of MRSA in bovine mastitis milk comes from Belgium where in 1972 Devriese and Hommez isolated strains that, using biotyping methods, appeared to be of human origin (Petonand Le Loir, 2014; Samar *et al.,* 2018). Cows with mastitis therefore, likely to harbor MRSA, and may be related to horizontal transfer via wet hands of colonized or infected dairy farm workers, and selection by the use of antibiotics to treat mastitis (Juhász-Kaszanyitzky *et al.*, 2007). The first known case of MRSA transmission between cows and a person was reported by Juhász-Kaszanyitzky *et al.* (2007). MRSA strains isolated from cows with subclinical mastitis were phenotypically and genotypically indistinguishable from the strain from the person who worked with these animals. These strains were determined as ST1, *spa* type t127, SCC*mec*IVa. After this report, and until recently, MRSA was only sporadically isolated from cow milk (Juhász-Kaszanyitzky *et al.*, 2007).).

In Germany, Feβler *et al.* (2010) detected 25 MRSA ST398 isolates from cases of bovine clinical mastitis and two isolates from farm workers. Nine major ApaI PFGE patterns, three *spa types* (t011, t034 and t2576) and two types of SCC*mec* (IV and V) were identified. Türkyılmaz *et al.* (2010) in a similar study in Aydin detected 14 MRSA from bovine milk which belonged to lineage MRSA ST-239-III in which one

was related to hospital-associated clones, and two strains were ST8/IV, which correspond to USA300 that causes severe community-acquired infections.

Erdem and Tükyilmaz (2013) in Aydın region, Turkey also identified 12 MRSA from mastitic cow milk and nasal swabs of cows and related workers were negative for PVL genes, but produced four distinct pulsotypes (PT) designated as PT A-D. The PT A (7 strains) detected sequence type (ST) were ST 239, spa type t030 and SCCmec III except one.

Peterson *et al.* (2013) in their study; prevalence and properties of *mec* C methicillin-resistant *Staphylococcus aureus* (MRSA) in bovine bulk tank milk in Great Britain detected *mec C* MRSA from 10 of 465 dairy farms sampled in England and Wales (prevalence 2.15%, 95% CI 1.17%–3.91%), but not from 625 farms sampled in Scotland (95% CI of prevalence 0%–0.61%). Seven isolates belonged to sequence type (ST) 425, while the other three belonged to clonal complex 130. Resistance to non-β-lactam antibiotics was uncommon. All 10 isolates produced a negative result by slide agglutination for penicillin-binding protein 2a. *mec* A MRSA ST398 was detected on one farm in England. They concluded that *mec* C MRSA is widely distributed among dairy farms in Great Britain, but this distribution is not uniform across the whole country. These results provide an important baseline data set to monitor the epidemiology of this emerging form of MRSA. Similarly, Unnerstad *et al.*

(2013) showed that all isolates with elevated minimum inhibitory concentrations of beta-lactams were positive for *mec C* in Swedish dairy cows. Three of the isolates, originating from milk samples collected in 2010, were of *spa*-type t524 (*spa* repeat succession 04–17) and ST130 (CC130) and had identical PFGE-patterns. One isolate, originating from a milk sample collected in 2011, was of *spa*-type t9111 (14-44-12-23-18-17-17-17-23-24), ST425 (CC425) and showed a PFGE-pattern different from the other three isolates. The PVL-genes were not detected in any of the isolates. All four isolates were resistant to beta-lactams, but susceptible to all other antimicrobials tested. The isolates were confirmed to produce penicillinase which is noteworthy because, to our knowledge, penicillinase production has not been reported in isolates with *mecC* despite presence of the beta-lactamase gene *blaZ*.

In Korea, Song *et al.* (2016) examined 1,222 raw milk samples, from which 23 (13.9%) MRSA were isolated from the 649 mastitic milk. All the MRSA isolates had staphylococcal enterotoxin genes, and two clusters of these genes were identified: seg-sei-sek-sem-sen-seo (20 isolates, 87%) and sed-seg-sei-sej-sem-sen-seo (3 isolates, 13%). Each MRSA-positive farm had only one spa-SCCmec type. Nine MRSA isolates (39.1%) with the t324-IVa genotype, which is related to community-acquired MRSA infection, were isolated from three dairy farms. Additional genotypes of t148-IVa and t002-II were detected and related to human MRSA strains. Most MRSA isolates had distinct pulsed-field gel electrophoresis subtypes, indicating they were not the same clones. Only two isolates collected from the same farm during different years

had an identical electrophoresis type, indicating persistence of the clone at this farm. In Jabalpur, Madhya Pradesh, Shrivastava (2017) observed the prevalence of MRSA to be 16.47%, and which were positive for *mec* A gene and virulence factors associated with pathogenicity.

2.6.6 Current situation of MRSA in Nigeria

MRSA infection remains a global problem and has been studied extensively in most developed countries (Abubakar *et al.*, 2018). However, in developing and low-income countries, where the burden of infectious diseases is high and effective antibiotics are not readily available, little information is available on the trends of MRSA infection and spread (Umaru *et al.*, 2011; Adetayo *et al.*, 2014). In Nigeria, studies and surveillance on MRSA have been conducted in different part of the country in mostly hospital environment and very little no hospiatal samples (Adetayo *et al.*, 2014; Ifeanyichukwu *et al.*, 2015; Aminu *et al.*, 2017; Onanuga *et al.*, 2018; Abubakar *et al.*, 2018; Osinupebi *et al.*, 2018). Despite this epidemiological data on MRSA in Nigeria, literatures are still relatively scarce when compared to literatures from developed countries.

In Ibadan, Adetayo *et al.* (2014) investigated the prevalence of methicillin-resistant *Staphylococcus aureus* and antibiotic sensitivity pattern in clinical isolates in Ibadan, 23 *Staphylococcus aureus* were isolated, 52.2% from urine specimens, 30.4% from

wounds swab, 13.0% from ear swab and 4.3% from nasal swab. Seven (30.4%) out of the *Staphylococcus aureus* were MRSA which were resistant to Amoxicillin (100%), Augumentin (85.7%), Cotrimozaxole (71.4%), Tetracycline (57.1%), Gentamicin (42.9%), Ofloxacin (28.9%) and Ciprofloxacin (28.5%). Similar study was conducted by Ifeanyichukwu *et al.* (2015) in Afikpo, Ebonyi State in which 33 (43.4 %) were recovered from 200 nasal swabs examined. The MRSA showed high resistance to cefotaxime (100 %), ampicillin (100 %), clindamycin (9.0 %) and vancomycin (12 %). antibiotics.

In Kano, Aminu *et al.* (2017) examined hospital instruments for MRSA, 33/74 (44.5%) *S. aureus* were identified, from which 16/33 (48.5%) were MRSA. The results further revealed that invasive hospital instruments had the highest number of *S. aureus* (54.5%) and MRSA (68.8%) isolates respectively than instruments used for superficial assessment of patient body with *S. aureus* (18.2%) and MRSA isolates (12.5%) respectively. Furthermore, 16 of the MRSA isolates were generally resistant to the beta-lactam antibiotics tested with 7/16 (44%) being multi-drug resistant. Also 2 (20%) and 4 (40%) of the MRSA isolates were positive for *mec* A and blaZ gene respectively. Also, Onanuga *et al*. (2016) examined pregnant women for the presence of urobacteria in Yenagoa, Bayelsa State. All the *Staphylococcus*species exhibited 90% and 85% resistance to cefoxitin (methicillin resistant) and vancomycin respectively and exhibited multiple antibiotic resistance with extended-spectrum beta-lactamase production. Osinupebi *et al.* (2018) examined 338 clinical specimens of pus,

aspirate, ear and wound swabs for MRSA from three major health facilities in Abeokuta, Nigeria, 32.2% of the samples werefound to be positive for MRSA which were resistant to cotrimoxazole (93.9%), tetracycline (89.4%), augmentin (72.7%) and erythromycin (71.2%).

The trend and prevalence of MRSA in clinical samples were also conducted in Zaria and environs. For example, Obajuluwa *et al.* (2011) analyzed 185 *S. aureus* isolates from wounds, skins and beds of orthopaedic patients and atmospheric air of orthopaedic wards of Ahmadu Bello University Teaching Hospital, Shika, Zaria, Nigeria. The distribution of the *S. aureus* isolates was 44 (23.8%), 70 (37.8%), 65 (35.1%) and 6 (3.2%) from the wound, skin and bed of the orthopaedic patients and atmospheric air of the orthopaedic wards respectively. Of these *S. aureus* isolates, MRSA was recovered 75% (wound), 51.4% (skin) and 73.8% (bed); no MRSA strain was detected in the atmospheric air. The MRSA isolates showed high resistance to ampicillin, 100% in all the three sites, vancomycin, 81.8% wound, 83.3% skin, 79.2% bed, 90.9% wound, 72.2% skin, 66.7% bed, ceftriaxone 69.7% wound, 72.2% skin, 70.8% bed, gentamicin 54.5% wound, 52.8% skin, 37.5% bed and ciprofloxacin 51.5% wound, 47.2% skin, 35.4% bed. Currently, Obajuluwa *et al.* (2018) Out of the 104 Staphylococci isolates, 40 (38.5%) were coagulase positive, out of which 25 (62.5%) were methicillin-resistant *Staphylococcus aureus* (MRSA). Of the 64 (61.5%) coagulase negative Staphylococci, 49 (76.6%) were methicillin-resistant coagulase negative Staphylococci (MRCoNS).The coagulase positive isolates were more susceptible to antibiotics than the coagulase negative Staphylococci. Resistance pattern showed 11 (44%), 3 (12%) and 1 (4%)

of the MRSA isolates were MDR, XDR, and PDR respectively while 14 (28.6%), 3 (6.1%) and 2 (4.1%) of MRCoNS were MDR, XDR, and PDR respectively.

Just as MRSA in humans was extensively investigated, MRSA in animals and foods has been also investigated in in developed countries of the world. In Nigeria, very few data are available on the prevalence of MRSA in animals and foods (Umaru *et al.,* 2011). Data available include that of Suleiman *et al*. (2012b) who determined the prevalence of MRSA from subclinical mastitic milk. The study found 26 isolates to be MRSA, among which 2 contained *mec* A and *bla* Z genes.

In Maiduguri, Balami *et al.* (2016) conducted a study on the presence of MRSA in cattle in fattening lots in Maiduguri by examining 150 nasal swab samples from apparently healthy cattle, 78 (32%), 62 (42.6%) and 32 (21.3%) were positive for *Staphylococcus, S. aureus* and MRSA respectively. The MRSA isolates were highly resistance to Oxacillin (100%), Penicillin (100%), Tetracycline (100%), Cefoxitin (100%), Cephazolin (100%) and Sulphamethoxazole+Trimethoprin (86%) and were multidrug resistant (MDR).

The prevalence of MRSA in poultry was also investigated by Kwoji *et al.* (2017) who detected MRSA in 8.82% (n=12/136) of chickens and 14.29% (n=2/14) in farm personnel 136 chickens and 14 farm personnel examined. In a similar vain Bala *et al*. (2016) examined 250 samples of chicken droplets for the presence of MRSA within Zaria metropolis. Eighty eight (88) *S. aureus* isolates were detected, out of which 41.2% were observed to be MRSA and

produced β-lactamase. All the MRSA produced β-lactamase while 44.3% were multidrug resistant with 60% having MAR index of ≥0.4. Also, Ani *et al.* (2011) conducted a survey on the prevalence of *Staphylococcus aureus* in food samples selected at Aba town in Abia State. Microbial contaminants were incriminated in African salad (Abacha), Meat pie, Raw meat, Agidi and Moi-moi with African salad recording highest staphylococcal count among others. The highest *Staphylococcus aureus* counts of (85%) were obtained in African salad (abacha) when compared with other food samples as recorded in umungasi with (60%) in meat pie and African salad packaged with nylon. Similar study was conducted by Umaru *et al.* (2012) in which 84 (42%) were positive for *S. aureus* were recovered from soups from restaurants/food canteens and some selected families in Jalingo Metropolis, Taraba State, Nigeria. The occurrence of *S. aureus* was highest in green leaf ('Aleyehu') soup (65.0 %), followed by sorrel ('Yakuwa') (57.5 %), melon ('Eguisi') (40.0 %), stew (36.7 %), okro (25.0 %) and bitter leaf (0.0%). Of recent Odetokun *et al.* (2018) studied in *S. aureus* in two municipal abattoirs in Nigeria based on the risk perception, spread and public health implications in Ibadan and Ilorin. The prevalence of *S. aureus* was 6.5%, with MSSA and MRSA detected at a frequency of 5.4% and 1.1%. Molecular analysis of the isolates revealed 19 different spa types, including a novel spa type (t16751).

2.7 Diagnosis of *S. aureus* and MRSA

MRSA can be identified using phenotypic (antimicrobial susceptibility testing) or genotypic methods. In general, the phenotypic methods are easier to perform, easier to interpret, cost-effective and widely available; however, they are less discriminatory

(Stark *et al.,* 2013; Panda *et al.,* 2016. The genotypic methods are more discriminatory, but are expensive and technically demanding (Mehndiratta and Bhalla, 2012; Ismail *et al.,* 2016).

2.7.1 Phenotypic diagnosis of *S. aureus* and MRSA

Identification of *Staphylococcus* infections to a genus level is less tasking, as the organism grows well in a range of conditions. Genus identification is readily achieved with basic phenotypic tests such as Gram staining and the catalase test (Stark, 2013; Panda *et al.,* 2016). Identification to the species level is more challenging and requires a range of more advanced phenotypic tests or costlier but more accurate molecular tests (Ismail *et al.,* 2015; Nilima and Ghorpade, 2017). A Gram stain is first performed to guide the way, which should show typical gram-positive bacteria, cocci, in clusters (Pexara *et al.,* 2013; Nilima and Ghorpade, 2017). Secondly, the organism can be cultured in mannitol salt agar, which is a selective medium with 7–9% NaCl that allows *S. aureus* to grow producing yellow-colored colonies as a result of mannitol fermentation and subsequent drop in the medium's pH (Pexara *et al.,* 2013). Furthermore, differentiation to species level include catalase (positive for all *Staphylococcus* species), coagulase (fibrin clot formation, positive for *S. aureus*), DNase (zone of clearance on nutrient agar), lipase (a yellow color and rancid odor smell), and phosphatase (a pink color) (Nilima and Ghorpade, 2017).

Diagnostic microbiology laboratories and reference laboratories are keys for identifying outbreaks of MRSA. Proper MRSA diagnosis may be a potential problem since samples are sent to different diagnostic laboratories that may not be uniform in the testing of bacterial species and their antibiotic sensitivities. MRSA can be typed by both phenotypic and molecular methods (Stefani *et al.,* 2012; Szabó, 2014).

Phenotypic methods include; colonial characteristics, Gram-staining characterization, biochemical reactions (catalase, coagulase, DNase and sugar fermentation), susceptibility to various phages and toxin production (Lin and Peterson, 2010), and antibiotic susceptibility pattern and oxacillin resistance (EFSA, 2012; CLSI, 2014).

2.7.2 Antibiotic susceptibility testing (antibiogram)

The antibiotic susceptibility testing has been an epidemiological tool for typing of MRSA in which the antibiotic susceptibility profile is determined using selected sets of antibiotics (EFSA, 2012). The antibiotic susceptibility testing is important for the clinical laboratory and gives the first indication of MRSA (EFSA, 2012; CLSI, 2014). However, it should not be used as the only epidemiological instrument since the resistance determinants are often carried by mobile genetic elements, such as plasmids, and since these are subject to a selective pressure they are likely to undergo genetic rearrangements that might affect the susceptibility profile. The discriminatory power of the antibiogram is considered as poor because genetically unrelated strains

may carry the same resistance profile and, in contrast, two related strains may exhibit different phenotypes because of loss or gain of resistance determinants (Magiorakos *et al.*, 2012).

Determination of antibiotic susceptibility testing of the isolates is not only necessary for the optimal antibiotic treatment of infected patients, but for monitoring of the spread of MRSA strains or resistance genes throughout the hospital and the environment (Magiorakos *et al.*, 2012). Routine antibiotic resistance determination of MRSA strains includes; the determination of the Minimum Inhibitory Concentration (MIC) using traditional reference dilution methods such as agar dilution method and both dilution (CLSI, 2011). Nevertheless, there is conflicting recommendations regarding the most reliable method of identification of MRSA using the antibiotic susceptibility testing. This is because different strains of diverse heterogenicity are included in various studies and perform differently under specific test conditions. Several other antibiotic susceptibility testing methods include; automated methods such as the Viteck/Viteck2 (BioMérieux and Micrscan (Dade Behrin) (Magiorakos *et al.*, 2012; CLSI, 2014), conventional and screening methods (CLSI, 2011).

2.7.3 Confirmatory methods

2.7.3.1 The Latex agglutination assay for the diagnosis of S. aureus and MRSA

Rapid latex agglutination test (Oxoid, Basingstoke, England) is also a rapid laboratory test which detects the PBP2a protein (Delport *et al.* 2016). PBP2a is a variant penicillin binding protein that imparts the ability of *S. aureus* to be resistant to oxacillin. Later, the latex assay was developed to detect other *S. aureus* specific surface antigens (Delport *et al.* 2016). The assay is based on the detection of the PBP2a in approximately 20 minutes. PBP2a is mediated by the *mec* A gene (Delport *et al.* 2016). Monoclonal antibodies against PBP2a sensitize late particles of the isolated MRSA colonies. The PBP2a assay is a rapid and sensitive method of detecting MRSA isolates compared to other phenotypic methods such as the standard agar diffusion test (Delport *et al.* 2016). However, several reviewers showed that any test involving the clumping factor may give false positive results and also has poor sensitivity when applied directly on blood cultures for MRSA detection (Delport *et al.* 2016).

2.7.3.2 Chromogenic agar for diagnosis of S. aureus and MRSA

CHROMagar *S. aureus* ID (bioMérieux, La Balme Les Grottes, France) was recently developed for the identifying *S. aureus*, which employs chromogenic enzyme substrates in a selective agar medium, allowing the detection of *S. aureus* with a high degree of sensitivity and specificity (Moses *et al.*, 2017). On *S. aureus* ID, *S. aureus* forms distinctive green colonies due to production of α-glucosidase. Other

staphylococci generally form white colonies but occasionally produce pink colonies due to the hydrolysis of a second chromogenic substrate for β-glucosidase.

The new developed chromogenic agars have been utilized for the detection of MRSA. Chromogenic agars with various selective agents had variable sensitivity and specificity in screening for MRSA when compared with some of the more traditional media, but performance was good when cefoxitin was used as a selective agent (Moses *et al.*, 2017). There are several recently marketed chromogenic media containing cefoxitin (e.g. MRSA Select, Bio-Rad; CHROMagar MRSA, Bio Connections; MRSA ID, BioMerieux) and the manufacturers claim good performance with a high proportion of positives detected after incubation for 24 hours.

2.7.4 Molecular typing and identification of *S. aureus* and MRSA

Molecular typing techniques facilitates the epidemiological surveillance of MRSA and effective evaluation of prevention and control of infection as well as antibiotic prescribing measures and tracking their spread (Basset *et al.*, 2011; Bitrus *et al.*, 2016). Several typing methods have been developed and used in order to describe the origin and spread of MRSA (Basset *et al.*, 2011; Mongkolrattanothai, 2013; Szabó, 2014). Initially, phenotypical methods, such as phage typing and antibiogram, were used for the characterization of MRSA but have subsequently been replaced by the use of mainly molecular techniques, in order to gain enhanced specificity and to save time

(Stefani *et al.*, 2012). The molecular epidemiology of MRSA has contributed to the insights into the evolutionary history of MRSA, and also provided a rapid and unambiguous identification, characterization and tracking of the spread of MRSA at clinical microbiological laboratories (Stefani *et al.*, 2012; Mongkolrattanothai, 2013).

2.7.4.1 Polymerase chain reaction (PCR) methods for the identification of MRSA

The polymerase chain reaction (PCR) is an enzymatic reaction for production of large amounts of specific DNA fragments (Kumurya *et al.*, 2015; Ali, 2016). The PCR is used for diagnosis of many infectious microorganisms and provides fast results in the laboratory by detecting the presence of a specific DNA sequence. It requires two synthetic oligonucleotide primers, i.e. short DNA sequences, which will indicate the DNA region of interest and initiate the DNA synthesis, which is carried out by an enzyme, a heat-stable DNA polymerase (Ali, 2016). The PCR is carried out during repeated cycles of denaturation, annealing and polymerase extension at specific temperatures and times. The DNA is first denatured in order to separate the double stranded DNA and to make the region of interest accessible for the polymerase. A decreased temperature will allow each primer to anneal their complementary sites and initiate extension from each 3´-hydroxyl end directed toward the other. The polymerase generally has a temperature optimum of 72^{o}C, which will allow the DNA extension. These steps are repeated for 25-40 cycles and are carried out by an automated cycler, which can change the temperature of the tubes containing the reaction mixture in a short time (Liu *et al.*, 2016). There will be an exponential

increase in the amount of DNA produced since the newly made strands will act as templates during the following cycles. However, the PCR is a sensitive system and the efficiency is controlled by many parameters, such as primer concentration and stability, polymerase type, buffer type, deoxynucleoside triphosphate (dNTP) concentration, cycling parameters and the complexity as well as concentration of the starting template (Li *et al.,* 2016).

The conventional PCR requires subsequent separation of the PCR products by electrophoresis on an agarose gel in order to confirm that the expected DNA has been amplified. The DNA fragments are negatively charged and will migrate through the agarose gel towards the positive electrode when a voltage is applied. The mobility is determined by the size of the DNA fragments and its specificity is determined by comparison with a molecular size standard applied on the gel. The gel is subsequently stained with ethidium bromide and the DNA bands are visualized under ultraviolet illumination (Kumurya *et al.*, 2015; Ali, 2016).

2.7.4.1.1 Real time PCR for the detection of S. aureus and MRSA

Real time PCR amplification allows both amplification of the target DNA and subsequent detection of the product in a closed system (Kumurya *et al.,* 2015; Li *et al.,* 2016). In conventional PCR, you only evaluate the end product of the PCR-reaction,

while in real-time PCR a fluorescent dye binds to the amplified DNA at each cycle (Stark, 2013). The fluorescent signal is continuously registered and is directly proportional to the amplified amount of DNA, which allows interpretation of the reaction in "real time" (Stark, 2013). Real-time PCR has several advantages over traditional PCR. With real-time PCR it is possible to detect smaller variations in target copies compared to conventional PCR (Stark, 2013).

A melting curve analysis is performed subsequently to the PCR and ensures that the amplified nucleic acid has appropriate and specific characteristics of the expected product. Thereby, no post-amplification processing or gel electrophoresis is required (Kim *et al.,* 2013; Kumurya *et al.,* 2015). The Light Cycler PCR instrument is different from the conventional thermal cyclers since the heating and cooling is controlled by air instead of a heating block, which allows rapid temperature exchange rates (Kim *et al.,* 2013). In addition, the PCR reactions are performed in glass capillaries with a high ratio of surface area to volume for efficient heat transfer. The Light Cycler provides either SYBR Green I or hybridization probes as detection formats for real time PCR.The fluorescent SYBR Green dye binds double-stranded DNA and the fluorescence will 40 significantly increase upon binding. Since the dye binds the newly synthesized double-stranded DNA during amplification, a fluorescent signal that is proportional to the DNA concentration will be received. Characterization of the PCR products is performed by melting curve analysis subsequent to the

amplification run in order to increase the sensitivity of SYBR Green I detection. The temperature is raised slowly to the melting point of the DNA and the fluorescence is continuously monitored. Since SYBR Green I only bind double-stranded DNA, the fluorescent signal decreases as the melting temperature (Tm) of the DNA-helix is reached. The melting point of a DNA fragment depends for example on its individual length and its content of bases G and C (Kim *et al.*, 2013). Thus, the determination of the individual melting temperature for a DNA fragment can be used to characterize the amplification products. Melting peaks are derived from the initial melting curves (fluorescence [*F*1] versus temperature [*T*]) by plotting the negative derivative of fluorescence over temperature versus temperature (- *dF*1/*dT* versus *T*). The individual Tm for the amplification products of each sample are compared with the determined Tm of the control template that is included in all runs, to separate the specific product from primer-dimers and unspecific amplifications (Kumurya *et al.*, 2015; Li *et al.*, 2016).

2.7.4.1.2 Multiplex PCR (M-PCR) assay for characterization of MRSA

Multiplex PCR (M-PCR) utilizes multiple oligonucleotides primers all included in the same PCR reactions mixture to simultaneously amplify several target genes (Ghaznavi-Rad *et al.*, 2010; Ali, 2016). The number of the PCR primers is greatly influence by the PCR conditions including the annealing temperature, dNTP concentration, Mg^{2+} and DNA template concentration (Ali, 2016). Usually, M-PCR

primers are longer in base pairs than single PCR primers because of potential cross-reaction in a PCR amplification process (Ghaznavi-Rad *et al.,* 2010). Although M-PCR is an ideal DNA amplification method, several disadvantages including contamination, cross reaction due to non-specific binding due to the number of primers and extended pre-optimization time prior to actual reaction greatly impacts on the use of this PCR method in laboratories (Ghaznavi-Rad *et al.,* 2010).

M-PCR has been used to target several MRSA genes including the *S. aureus* specific 16S rRNA gene, the *S. aureus* methicillin resistance gene, *mec* A and the PVL conferring *luxS-PV* and *luxF-PV* (Ghaznavi-Rad *et al.,* 2010). Other *S. aureus* M-PCR protocols include the amplification of *S. aureus* specific toxins such as *etaA* and *etaB* responsible for exfoliative disease, TSST-1 for TSS disease and pyogenic toxin genes (Ghaznavi-Rad *et al.,* 2010).The M-PCR typing method is based on the characterization of MRSA's specific *ccr* gene complex, which encodes for site-specific recombinases responsible for the mobility of SCCmec. The *ccr* gene complex together with *mec* complexes which are classified into class A, Class B, Class C and class D can type MRSA isolates into different SCC*mec* types thus enabling researcher to distinguish between HA-MRSA and CA-MRSA (Ghaznavi-Rad *et al.,* 2010). Recently, another M-PCR assay was developed for the subtyping of SCCmec type IV into eight subtypes (Turlej *et al.,* 2011). The "SCC*mec* IV" M-PCR is important to trace clones of CA-MRSA characterized by SCC*mec* type IV to understand the

mechanism of SCC*mec* assembly and acquisition in this clone. The M-PCR assays can be useful in infection control strategies and be implemented for epidemiological studies to determine clonal relatedness during outbreaks in clinical settings (Ghaznavi-Rad *et al.,* 2010).

2.7.4.1.3 SCCmec typing of MRSA

Staphylococcal cassette chromosome (SCC*mec*) typing classifies SCC*mec* elements into types and subtypes on the basis of their structural differences. Most of the used methods rely on PCR mapping of cassette elements, such as the *mec* complex, the *ccr* complex and the J region (Mehndiratta and Bhalla, 2012; Ghanbari *et al.*, 2017). Until recently, eightmain types of SCC*mec* (type I to type VIII) along with many subtypes had been distinguished among MRSA isolates. Some of these types were more common than others. In the last few years, new types of SCC*mec* (IV to XI) were identified and additional subtypes and different variants of already existing ones were discovered (Turlej *et al.,* 2011). SCC*mec* type XI carries the recently described *mec C* gene (EFSA, 2012).

SCC*mec* typing provides information about the genetic origin of an isolate's methicillin-resistance and its ecological niche. Methicillin-resistance is conferred by an apparently unique mobile genetic element that carries the *mec* A gene, called the

staphylococcal cassette chromosome (SCC*mec*) (Vignaroli *et al.,* 2014; Ghanbari *et al.,* 2017). As well as carrying the *mec* A gene, SCC*mec* carries regulatory genes called cassette chromosome recombinase genes (*ccr*) that enable transfer of the element between bacteria (Vignaroli *et al.,* 2014). although their exact role in SCC*mec* transmission is yet to be determined. SCC*mec* typing uses DNA sequencing to analyse polymorphisms in the *mec* and *ccr* genes (Li *et al.,* 2011). In 2009, the International Working Group on the Classification of the Staphylococcal Cassette Chromosome Elements (IWG-SCC) described eight recognised SCC*mec* types in MRSA (Vignaroli *et al.,* 2014). As typing and PCR methods develop, more SCC*mec* types ar being discovered and there are currently at least 10 recognised SCC*mec* types (Li *et al.,* 2011; Mongkolrattanothai, 2013).

2.7.4.1.4 Multilocus sequence typing (MLST) of MRSA

Multilocus sequence typing (MLST) was first developed in 1998 and involves the sequencing of defined regions of seven genes (*arcC, aroE, glpF, gmk, pta*, *tpi* and *yqi)*. (Enright *et al.,* 2000; Liu *et al.,* 2013). Internal fragments of seven housekeeping genes of approximately 450 base pairs are amplified by PCR and subsequently sequenced on both strands using a single pair of primers and an automated DNA sequencer. For each gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST), which is an unambiguous description

of a clone (Enright *et al.,* 2000). Databases containing the allelic profiles and associated epidemiological data are available at the MLST website (*http://saureus.mlst.net/*) (Enright *et al.,* 2000; Szabó, 2014). MLST is a highly discriminatory and widely accepted technique for typing of *S. aureus* and MRSA epidemiology on the basis of sequence of 450 bp internal fragment of the seven house-keeping genes and it is basically a sequence based interpretation of the multilocus enzyme electrophoresis (MLEE) (Chua *et al.,* 2014; Enright *et al.,* 2000; Bitrus *et al.,* 2016). The technique is designed to evaluate and compare genetic polymorphism in a group of bacterial pathogens worldwide. Isolates are defined by distinct alleles assigned for the sequence of each house-keeping gene loci. Since there is more than one allele in each of the sequence of the seven house-keeping genes, the probability of having a sequence with a similar allele by chance is highly unlikely (Enright *et al.,* 2000). MLST provides important information about the variation in the nucleotide sequence of the bacterial core genome, the evolutionary origin of a clone, the phylogenetic relatedness between among strains and recombination rate (Basset *et al.,* 2011). Studies have shown that the majority of a bacterial population belongs to a limited number of related genotypes, referred to as clonal complexes (CC). Grouping is based on the similarity between sequence types in six of seven loci and singleton STs are defined as not to share six out of seven loci with any other STs in the dataset. The Based Upon Related Sequence Types (BURST) algorithm could be used to identify these groups of related genotypes in the population and attempts to identify the ancestral genotype of each group (Liu *et al.,* 2012; Szabó, 2014).

2.7.4.1.5 Staphylococcal protein (spa) A typing of S. aureus and MRSA

The staphylococcal protein A (*spa*) typing involves DNA sequencing of short nucleotide repeats in the polymorphic X region of the *S. aureus* protein A gene (*spa*). Different *spa* repeats are assigned an α-numerical code (r01, r02, etc.) and the repeat succession determines the *spa* type (e.g., t001, t002, etc.) by submission of the results to the RIDOM StaphType Database (*www.spaserver.ridom.de*) (EFSA, 2012; Bitrus *et al.,* 2016). *Spa* typing use of highly discriminatory genetic marker to characterize bacterial pathogen allowing the identification of closely related isolates with common ancestral origin (Chua *et al.,* 2014; Grundmann *et al.,* 2010). The *spa*-typing codes for protein A is a specie specific gene known for binding capacity for immunoglobulin G (IgG) (Basset *et al.,* 2011; Grundmann *et al.,* 2010). These loci have a high-level polymorphism due to a variation in the internal region of short tandem repeats, which vary in numbers and substitution within individual repeat unit (Basset *et al.,* 2011), a succession of number corresponding to each individual repeat unit of the X region represents the spa profile (Koreen *et al.,* 2014). This technique provides portable data that are unambiguous and biologically meaningful with great utility for epidemiological studies such as in transmission and outbreak investigation of *S. aureus* infection different within geographical location (Grundmann *et al.,* 2010). However, Spa typing might reflect homoplasy, the analysis complicated and its discriminatory power lower than PFGE (Basset *et al.,* 2011; Patrik *et al.,* 2016).

2.7.4.1.6 Random amplified polymorphic DNA fingerprinting of S. aureus and MRSA

Random amplified polymorphic DNA (RAPD), also known as arbitrarily primed-PCR (AP-PCR) technique uses a short primer of 10 base pairs (bp) (Ismail *et al.,* 2015). The short 10 bp primer with random sequences of nucleotides randomly amplifies DNA targets producing fragments, which serves as genetic markers (Mobasherizadeh *et al.,* 2016). The nucleic acid between the primer sites is amplified and the resulting fragments are separated by electrophoresis. They form a fingerprint based on the number and size of the amplified regions. RAPD will produce a genetic fingerprint with all MRSA and the results have been reproduciblewithin the same laboratory (Al-Zahrani, 2013). RAPD method was shown to have the ability to type non-phage type able MRSA strains in an outbreak setting proven a higher discriminatory power than phage-typing and PFGE (Al-Zahrani, 2013). The RAPD technique has also been shown to be suitable for routine genotyping of hospital-acquired staphylococci (Mobasherizadeh *et al.,* 2016).

The advantage of this method over others is the relative speed and simplicity of the technique. Discriminatory power, however, can be variable and depends heavily on the number and sequence of the primers employed (Mobasherizadeh *et al.,* 2016). Poor differentiation is achieved when a single primer is used but the application of three or more primers considerably increases the time needed to perform the test and still does not make RAPD as discriminatory as PFGE. On the other hand, RAPD has

clustered epidemiologically linked organisms and excluded unrelated isolates when used in an outbreak situation (Mobasherizadeh *et al.,* 2016).

2.7.4.1.7 *Coagulase typing of S. aureus and S. aureus and MRSA*

Production of coagulase is one of the defining characteristics of *S. aureus* and is thought to be an important virulence factor (Omar *et al*., 2014). The coagulase protein of *S. aureus* is an important virulence factor, which causes plasma to clot and has a polymorphic repeat region that can be used for differentiating *S. aureus* isolates (Salehzadeh *et al.,* 2016). The coagulase gene amplification discriminatory power relies on the heterogeneity of the region containing the 81 bp tandem repeats at the 3′ coding region of the coagulase gene which differs both in the number of tandem repeats and the location of *AluI* and *HaeIII* restriction sites among different isolates (Shakeri *et al.,* 2010; Omar *et al.,* 2014). Protein A has been coded by *spa* gene, where the repeated part is located at 3′end and identified as X region; the repetitive part of region X consists of up to 12 units each with a length of 24 nucleotides (Salehzadeh *et al.*, 2016). This 24-nucleotide region is highly polymorphic with respect to the number and sequence of repeats. Diversity of X region causes protein A variation (Shakeri *et al.,* 2010). In addition to its use as a marker, the number of repeats in the region X of *spa* has been related to the dissemination potential of MRSA, with higher numbers of repeats associated with higher epidemic capability (Salehzadeh *et al.*, 2016).

2.7.4.1.8 *Hyper-variable region typing of S. aureus ad MRSA strains*

One of the proposed typing methods involves hypervariable region (HVR) proliferation, or HVR typing, for MRSA strains through PCR in the investigation for polymorphisms in this region (Mirkarimi *et al.,* 2016). The DNA sequence between IS431*mec* and the *mec* A gene, a region responsible for resistance against methicillin, is known as the *HVR*. The *mec* HVR is composed of direct repeated units, each with a size of almost 40 base pairs (Mirkarimi *et al.,* 2016). However, since the number of these repeated units may be different between isolates, the *HVR* region can be used to type and classify MRSA strains. Compared to other molecular methods, *HVR* typing of MRSA offers greater speed, facility, reproducible and simple to perform, however, it has a lower reproducible than PFGE (Stefani *et al*., 2012).

2.7.4.1.9 Nucleotide sequencing of S. aureus and MRSA strains

Nuvcleotide sequencing was developed by Fred Sanger in order to determine the exact nucleotide sequence of the DNA (Pallen and Loman, 2011; Laabei *et al.,* 2014; Anderson *et al.,* 2014). The Sanger dideoxy-sequencing resembles the ordinary PCR method and employs a DNA polymerase, dNTPs and primersand is performed in a cyclic program. However, the DNA synthesis is performed using only one oligonucleotide primer in each tube, i.e. the DNA synthesis is initiated at a specific position on one strand separately. A fluorescence labeled ddNTPs (dideoxy-dNTP) that lack the 3´OH group is also added in the PCR mixturewhich will be incorporated randomly during the DNA synthesis, thereby terminating the continuing synthesis of

that specific strand (Parkhill and Wren, 2011; Pallen and Loman, 2011). The sequencing is performed in repeated cycles that will generate a mixture of DNA sequences differing in size and where each terminal ddNTP is labeled with four different fluorescent colors (R6G, ROX, R110, TAMRA). The products of the cyclic sequencing are subsequently separated by an automated capillary electrophoresis-based genetic analyzer. The samples are injected into capillaries that are filled with polymer and the electrophoresis is run by applying a high voltage that forces the negatively charged DNA to migrate through the polymer. The size-separated and fluorescently labeled DNA fragments will move through a laser that will emit the specific dye, which is detected by an optical device. It is possible to detect all four bases since these are labeled with different colours that emit light at different wavelengths. By using capillary electrophoresis, it is possible to resolve DNA molecules that differ in molecular weight by only one nucleotide and approximately 500 bp can be read from each reaction (Kim *et al*, 2014; Laabei *et al.,* 2014). Nucleotide sequencing has superior discriminatory power than all other molecular typing techniques (Parkhill and Wren, 2011; Pallen and Loman, 2011). It can be used in evolutionary studies, outbreak investigations and phylogeographic studies and therefore, better understanding of pathogens (Anderson *et al.,* 2014).

2.7.4.1.10 DNA Microarray of Staphylococcus aureus and MRSA

Microarray for bacterial typing can be carried out on a chip with a collection of DNA probes attached to a solid surface in a particular pattern (Sanchini *et al.*, 2011). These probes detect complementary nucleotide sequences in different bacterial isolates. The array is constructed to detect genes that serve as markers for specific bacterial strains, or to detect allelic variants of a gene that is present in all strains of a particular species (McCarthy and Lindsay, 2012). Microarrays for studies of gene expression are like arrays for genotyping, and are also based on DNA/RNA-hybridization (Sanchini *et al.*, 2011; Szabó, 2014). cDNA (250-5000 bp) or oligonucleotides (25-75 bp) corresponding to genes of interest, are attached to a solid surface, usually glass or a silica surface (Sanchini *et al.*, 2011). The RNA from the sample of interest (cell cultures, tissue or blood) is reverse-transcribed (RT) into cDNA, and then possibly transcribed to cRNA with incorporation of a fluorescent dye. The labeled cDNA/cRNA is then hybridized to the array. After stringent washing the array is scanned in a confocal microscopy scanner (McCarthy *et al.*, 2012; McCarthy and Lindsay, 2012). This kind of analysis allows for simultaneous study of thousands of genes in a single assay, e.g. all open reading frames in the human genome may be detected. However, the complexity of such wide-ranging analysis makes microarray more of a screening method. The data produced are then analyzed using dedicated software (Sabat *et al.*, 2013).

2.7.4. 2 Non polymerase chain reaction (PCR) for the identification of S. aureus and MRSA

Before the development of PCR, several efficient typing methods were used for *S. aureus* strain typing. These methods including bacteriophage typing, capsular typing, PFGE and zymotyping have been applied for discriminating between *S. aureus* and MRSA strains (Moussa *et al.,* 2011; Mehndiratta and Bhalla, 2012). Among these methods, PFGE is the most extensively used method to date for the typing of MRSA strains as it is the "gold standard". Most novel MRSA typing studies couple PFGE as a reference method for MRSA strain typing as it is the most sensitive and specific MRSA strain typing method to date (Chua *et al.,* 2014).

2.7.4.2.1 Pulsed field gel electrophoresis for genotyping of S. aureus and MRSA Strains

Pulsed field gel electrophoresis (PFGE) typing method for the analysis of *S. aureus* and MRSA epidemiology and is regarded as the "gold standard" or method of choice because of it's high discriminatory power (Basset *et al.,* 2011; Chua *et al.,* 2014). PFGE involves digestion of bacterial genomic DNA with *Sma*I restriction enzymes within an agarose plug and electrophoresis to resolve them gives a banding pattern of 8 to 20 bands which can be used to compare with PFGE profiles of other *S. aureus* isolates (Bosch *et al.,* 2010, Moussa *et al.,* 2011). PFGE is reproducible and allows for separation of larger fragments of genomic DNA. In addition, it can also detect acquisition or loss of mobile genetic elements and introduction and removal of

restriction sites due to mutation or gene deletion (Basset *et al.,* 2011; Chua *et al.,* 2014) and also allows for monitoring of MRSA transmission and has the ability to detect MRSA clones with potentials of causing outbreaks at a local and international level (Enright *et al.,* 2000).

The high discriminatory ability of PFGE allowed for the detection of minute changes in the *Staphylococcus* genome which allows tracking of strains within outbreak settings (Stefani *et al.,* 2012; Mongkolrattanothai, 2013). The downside of PFGE is that it requires multiple technical steps that can be performed several different ways, making it difficult to directly compare results between laboratories and thus hindering efforts to track epidemic strains across different geographic areas. Furthermore, the lack of a standardised protocol for *S. pseudintermedius* makes it difficult to compare results between laboratories (Mehndiratta and Bhalla, 2012; Mongkolrattanothai, 2013).

2.7.4.2.2 Bacteriophage typing of S. aureus and MRSA strains

Bacteriophage typing (BT)is the main method used for typing of *S. aureus* and MRSAto discriminate among strains related to outbreaks (Mehndiratta *et al.,* 2010). This method is based on virus infecting bacteria called bacteriophages that are able to infect *S. aureus*or MRSAand some of them can only infect a single strain of that species (Mehndiratta *et al.,* 2010; Velayudham *et al.,* 2016). The bacterial isolate is

grown on an agar plate. Inoculation of different phages on the agar surface is then performed. After incubation the susceptible phage regions will show a clearing where the bacteria have been lysed and this is used to differentiate the staphylococcal isolates from each other (Kali *et al.,* 2013). Phage typing has been used for differentiating between MRSA outbreak strains and monitoring the emergence and dissemination of MRSA strains in hospitals and the community (Velayudham *et al.,* 2016). However, this method has poor reproducibility, not possible to type all isolates and due to the large number of phages stocks needed, only a few reference laboratories could use the method (Velayudham *et al.,* 2016).

2.7.4.2.3 Capsular typing of S. aureus and MRSA strains

Many virulence factors, including surface-associated adhesins, cytotoxins, superantigens, exoenzymes, and capsular polysaccharides, contribute to the pathogenesis of staphylococcal infections (Verdier *et al.,* 2007; Sutter *et al.,* 2011). The capsular polysaccharide or capsule is a cell wall bacterial component that protects bacterium from phagocytic uptake and enhances microbial virulence (Sutter *et al.,* 2011). Although 11 capsular polysaccharide types are described, only two types, type 5 and type 8, are clinically relevant in that they are predominant among clinical infection isolates of varied geographic origin (Sutter *et al.,* 2011). Capsular typing is based on the reactivity of monoclonal antibodies to specific *S. aureus* antigen capsular polysaccharide (Verdier *et al.,* 2007).

2.8 Treatment of *S. aureus* and MRSA infection

2.8.1 Treatment of *S. aureus* and MRSA infection in humans

The treatment of choice for *S. aureus* infection is penicillin, but in most countries, penicillin-resistance is extremely common and first-line therapy is most commonly penicillinase-resistant penicillin (for example, oxacillin or flucloxacillin) (Foster, 2017; Samar *et al.*, 2018). Combination therapy with gentamicin may be used to treat serious infections like endocarditis (Cosgrove *et al.*, 2009; Gould *et al.*, 2012).

In patients with histories of delayed-type penicillin allergy, a cephalosporin such as cefazolin or cephalothin can be administered as an alternative choice of treatment (David and Daum, 2013; Samar *et al.*, 2018). A semi-synthetic penicillin such as methicillin is indicated for patients with beta-lactamase producing staphylococci (David and Daum, 2013; Foster, 2017). Patients who have an MRSA infection are treated with a glycopeptide known as vancomycin (Samar *et al.*, 2018). Patients who are intolerable to vancomycin are treated with fluoroquinolone (ciprofloxacin), lincosamide (clindamycin), tetracycline (minocycline or sulphamethoxazole-trimethoprim (Gould *et al.*, 2012). Novel quinolones, such as ciprofloxacin with increased anti-staphylococcal activity are available but their use become limited due to the rapid development of resistance during therapy (Sievert *et al.*, 2013; Samar *et al.*, 2018).

MRSA is of special concern with regards to treatment because it is usually multi-drug resistant. In addition to most beta-lactams, MRSA is also commonly resistant to erythromycin, clindamycin, aminoglycosides, fluoroquinolones, co-trimoxazole and rifampin (Samar *et al.,* 2018). However, some strains remain sensitive to minocycline and recently, strains acquired outside of health-care settings remain susceptible to agents, such as clindamycin and gentamicin (Cosgrove *et al.*, 2009; Gould *et al.,* 2012).

Vancomycin continues to be the drug of choice for treating most MRSA infections caused by multi-drug resistant strains (Sievert *et al.*, 2013; Gurusamy *et al.,* 2013). Clindamycin, co-trimoxazole, fluoroquinolones or minocycline may be useful when patients do not have life-threatening infections caused by strains susceptible to these agents. For serious infections caused by strains that are susceptible to rifampin, adding this agent to vancomycin or fluoroquinolone may contribute to improved outcomes. Rifampin should not be used alone to treat MRSA infections due to the rapid development of resistance. The infecting strain always should be tested for susceptibility prior to initiating any of these therapies (Sievert *et al.,* 2013; Gurusamy *et al.,* 2013). The two newest antimicrobials, quinupristin-dalfopristin (Synercid®) and linezolid (Zyvox™), also are effective for MRSA infections, although routine use is generally discouraged to prevent further resistance to these agents. Other

investigational agents are in research stages but not yet approved for use (Gurusamy *et al.*, 2013; Samar *et al.*, 2018).

2.8.2 Treatment of *S. aureus* and MRSA infection in animals

Occasionally, antimicrobials less commonly used in human medicine may be useful (Catry *et al.*, 2010; Faccioli-Martins and da Cunha, 2012). If the infection is local in infected animals, meticulous management of the wound can avoid the necessity of antimicrobial use (Catry *et al.*, 2010). The risk of resistance development, the susceptibility profile of the isolate, the severity of the infection and the presence of systemic disease (fever, leukocytosis), and the patient's underlying disease or any comorbidity must be considered when choosing the antimicrobial treatment. In deciding to treat or eventually euthanize, veterinarians must consider the national veterinary guidelines available (Faccioli-Martins and da Cunha, 2012; Gould *et al.*, 2012).

2.9 Prevention and control of *S. aureus* and MRSA infection

2.9.1 Control and Prevention of *S. aureus* and MRSA in humans.

2.9.1.1 Avoidance of S. aureus and MRSA patients or carriers

Not making direct contact with skin, clothing, and any items that come in contact with either *S. aureus* and MRSA patients or *S. aureus* and MRSA carriers is the best way to prevent *S. aureus* and MRSA infection. In many instances, this situation is simply not practical because such infected individuals or carriers are not immediately identified (Skov *et al*, 2012; Gould *et al.,* 2012). What people can do is to treat and cover (e.g., antiseptic cream and a Band-Aid) any skin breaks and use excellent hygienic practices, e.g., hand washing with soap after personal contact or carriers, using disposable items when treating MRSA patients. Also available at most stores are antiseptic solutions and wipes to both clean hands and surfaces that may contact MRSA (Skov *et al*, 2012; Gould *et al.,* 2012).

The National Institutes of Health recommends that hospital workers with wound drainage that cannot be covered and contained with a clean, dry bandage and those who cannot maintain good hygiene practices be reassigned (Gould *et al.,* 2012; Banach *et al.,* 2018). Workers with active infections are excluded from activities where skin-to-skin contact is likely to occur (CDC, 2010). To prevent the spread of *S. aureus* or MRSA in the workplace, employers make available adequate facilities that encourage good hygiene. In addition, surface and equipment sanitizing conforms to

the Environmental Protection Agency (EPA)-registered disinfectants (Examples, Bleach- Sodium Hypochlorite 5.25%, Phenols, Quaternary Ammonium Compounds, Accelerated Hydrogen Peroxide (hydrogen peroxide /anionic surfactants), Botanicals-Benefect – Thymol and Silver Dihydrogen Citrate -PureGreen 24) (CDC, 2010). Health Departments recommend that preventing the spread of MRSA in the home can be to: launder materials that have come into contact with infected person separately and with a dilute bleach solution; reduce the bacterial load in your nose and on your skin; clean those things in the house that people regularly touch like sinks, tubs, kitchen counters, cell phones, light switches, doorknobs, phones, toilets, and computer keyboards (Gould *et al.,* 2012; Banach *et al*., 2018).

2.9.1.2 Screening programs

Patients screening upon hospital admission, with nasal cultures, prevents the cohabitation of MRSA carriers with non-carriers, and exposure to infected surfaces (Tacconelli *et al.,* 2009; Skov *et al,* 2012). In some UK hospitals screening for MRSA is performed in every patient, and all surgical patients except for minor surgeries are previously checked for MRSA (Tacconelli *et al.,* 2009).

MRSA can be identified by swabbing the nostrils and isolating the bacteria found inside the nostrils. Combined with extra sanitary measures for those in contact with

infected people, swab screening people admitted to hospitals has been found to be effective in minimizing the spread of MRSA in hospitals in the United States, Denmark, Finland, and the Netherlands (Tacconelli *et al.,* 2009).

2.9.1.3 Surface sanitization

Alcohol has been proven to be effective surface sanitizer against MRSA (CDC, 2010). Quaternary ammonium can be used in conjunction with alcohol to extend the longevity of the sanitizing action. In health care environments, MRSA can survive on surfaces and fabrics, including privacy curtains or garments worn by care providers (Samar *et al.,* 2018). Complete surface sanitation is necessary to eliminate MRSA in areas where patients are recovering from invasive procedures. Testing patients for MRSA upon admission, isolating MRSA-positive patients, decolonization of MRSA-positive patients, and terminal cleaning of patient's rooms and all other clinical areas they occupy is the current best practice for nosocomial MRSA (Gould *et al.,* 2012; Samar *et al.,* 2018).

2.9.1.4 Hand washing

The CDC suggested the prevention and spread MRSA infection in community settings, including incarcerated populations, childcare center employees, and athletes (CDC, 2010). To prevent the spread of MRSA the recommendations are to wash hands

using soap and water or an alcohol-based sanitizer. Additional recommendations are to keep wounds clean and covered, avoid contact with other people's wounds, avoid sharing personal items such as razors or towels, shower after exercising at athletic facilities, and shower before using swimming pools or whirlpools (CDC, 2010; Gould *et al.,* 2012).

At the end of August, 2004, after a successful pilot scheme to tackle MRSA, the UK National Health Service announced its Clean Your Hands campaign. Wards are required to ensure that alcohol-based hand rubs are placed near all beds so that staff can hand wash more regularly. Although alcohol-base rubs are somewhat effective, a more effective strategy is to wash hands with an anti-microbial cleanser with persistent killing action such as chlorhexidine (CDC, 2010; Gould *et al.,* 2012).

2.9.1.5 Decolonisation

After drainage or other treatment of MRSA, patients can shower at home using chlorhexidine (Hibiclens) or hexachlorophene (Phisohex) antiseptic soap from head to toe, and apply mupirocin (Bactroban) 2% ointment inside each nostril twice daily for 7 days, using a cotton-tipped swab (Gould *et al.,* 2012; Gurusamy *et al.,* 2013). Household members are recommended to follow the same decolonization protocol. Doctors may also prescribe antibiotics such as clindamycin, doxycycline or trimethoprim/sulfamethoxazole (Skov *et al,* 2012). However, there is very little

evidence that using more antibiotics actually has the effect of preventing recurrent MRSA skin infections (Gould *et al.,* 2012; Gurusamy *et al.*, 2013).

Care should be taken when trying to drain boils, as disruption of surrounding tissue can lead to larger infections, or even infection of the blood stream (often with fatal consequences) (Gurusamy *et al.,* 2013). Mupirocin 2% ointment can be effective at reducing the size of lesions. A secondary covering of clothing is preferred. As shown in an animal study with diabetic mice, the topical application of a mixture of sugar (70%) and 3% povidone-iodine paste is an effective agent for the treatment of diabetic ulcers with MRSA infection (Shi *et al.*, 2017).

2.9.1.6 Control of antibiotic use

Glycopeptides, cephalosporins, and, in particular, quinolones are associated with an increased risk of colonisation of MRSA. Reducing use of antibiotic classes that promote MRSA colonisation, especially fluoroquinolones, is recommended in current guidelines (Tacconelli *et al.,* 2009; Gould *et al.*, 2012).

2.9.1.7 Community settings

It may be difficult for people to maintain the necessary cleanliness if they do not have access to facilities such as public toilets with hand washing facilities. All work places and businesses should be provided with toilets for their employees, along with

washing facilities including soap or other suitable means of cleaning (CDC, 2010; Gould *et al.*, 2012).

2.9.2 Control and prevention of *S. aureus* and MRSA infections in animals

2.9.2.1 MRSA decolonisation of animals and owners

Collaboration between veterinarians and physicians is essential to coordinate swabbing and decolonization, particularly if simultaneous animal and human decolonization is to be attempted (Nes and Wulf, 2012). Clinically infected animals are often treated with co-trimoxazole and may be decolonized with topical decolonization lotions similar to those used in humans (Catry *et al.*, 2012; Nes and Wulf, 2012; Pantosti, 2012). Failure to eradicate MRSA carriage from humans may be due to re-acquisition of the strains from close human or animal contacts (Pantosti, 2012). Hence, the possibility of an animal reservoir should be considered, and if proven, decolonization of all carriers should be considered (Broens *et al.*, 2011).

2.9.2.2 Decolonisation of companion animals

Animal carriers such as pet dogs with history of hospital visits should be washed with chlorhexidine (Bootsma *et al.*, 2011; Catry *et al.*, 2012). Topical decolonization of

felines is especially difficult, and ingestion of disinfectants may be harmful to them. Hence, if attempted, animals should be rinsed thoroughly afterwards.

Official detailed guidance for and the potential for decolonization is available from the British Small Animals Veterinary Association (BSAVA) guidelines for management of MRSA in animals. The guidelines advocate decolonization only if the human companion is immune-suppressed or otherwise vulnerable, but suggested that regimens include antibacterial shampoos and 2% fusidic acid or 2% mupirocin intranasal cream two to three times daily (Weese and Van Duijkeren, 2010; Catry *et al.,* 2012).

2.9.2.3 Decolonisation of large animals

Topical chlorhexidine or 1% acetic acid has been used to decolonize horses with systemic and incisional infections (Broens *et al.,* 2011). However, overall, the efficacy of topical antimicrobials for decolonization is unclear, as sometimes instigating screening and good infection control standards alone may result in disappearance of carriage. The efficacy of any decolonization is more effective when re-exposure to MRSA is prevented (Nes and Wulf, 2012).

2.9.2.4 Precaution to be taken by veterinarians

i. As in human medicine, hand hygiene is an integral part of the intervention of the spread of MRSA between animals and humans. Frequent hand washing and proper disinfection of hard surfaces and equipment between patients is essential (Catry *et al.*, 2012; Nes and Wulf, 2012).
ii. Hand sanitizers should be provided in all consulting rooms and kennels to remind staff of the need for frequent hand sanitization (Catry *et al.,* 2012).
iii. Uniforms, gloves, disposable aprons and masks should be worn when changing dressings on infected wounds or to prevent potential contact with body fluids or contaminated tissues.
iv. Eye protection is indicated if splashing or aerosols are expected.
v. All surroundings in the clinic should be kept to a high standard of cleanliness. Although the cleanliness of floors does not appear to be as important as hand-touch sites in the control of human MRSA infections, the situation may be different in veterinary medicine because many animals are examined or treated on the clinic floor (Catry *et al.,* 2012).

2.9.2.5 The therapeutic animals and handlers

i. Good hand hygiene by all who encounter the animals, both before and after touching the animal (Catry *et al.,* 2012).

ii. Licking should be prevented, as well as "shaking paws"-even if the animal's paw is clean before they enter the health-care facility, the floors may be contaminated (Nes and Wulf, 2012).

iii. Handlers are restricted to interaction only with the patients and their families.

iv. When placing an animal on a bed, a clean towel or absorbent pad should be placed between the pet and the bed linens.

v. No animals should be allowed to visit patients in isolation units (Nes and Wulf, 2012).

2.9.3 Vaccine for the prevention of *S. aureus* infection

There is currently no acceptable vaccine against *S. aureus* infection (Anderson *et al.,* 2010; Parker, 2018). However, a vaccine was shown to protect mice against staph pneumonia by inducing antibody protection against Hla (Wardenberg and Schneewind, 2008). The mice did not show any sign of *S. aureus* infection despite receiving a dose of bacteria that caused disease in unprotected mice. Antibodies against Hla were transfused into mice who were given an infective dose of *S. aureus*, but were also protected. The preformed antibodies were later shown to protected human lung cells in culture from the destruction by *S. aureus*. The vaccine was then presumed to be the one scientist have been waiting for (Daum and Spellberg, 2010).

Further clinical trials were conducted for several vaccines candidates such as Nabi's StaphVax and PentaStaph, Intercell's / Merck's V710, VRi's SA75 and others (Begier *et al.,* 2017; Parker, 2018). Some of the trial vaccine candidates have shown immune responses, while others were shown to aggravate an infection by *S. aureus*. To date, none of these candidates provides protection against a *S. aureus* infection (Torre *et al.*, 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study areas

This research was conducted in Kaduna State, North Central Nigeria. Kaduna State is located at the centre of Northern Nigeria with the coordinate's *10°31'N 7°26'E10.517°N 7.433°E*. It has a political significance as the former administrative headquarters of the North during the colonial era. The State shares boundaries with Niger State to the west, Zamfara, Katsina and Kano States to the North, Bauchi and Plateau States to the East and FCT Abuja and Nasarawa States to the South (Fletcher *et al.,* 1996). Kaduna State is located in the Savanna zone, which is characterised by short wet season and long dry season. Agriculture is the main stay of the economy with about 80% of the people engaging in farming. Cash and food crops cultivated and produced include: cotton, groundnut, tobacco, maize, beans, guinea corn, millet, ginger, rice and cassava. Another major occupation of the people is animal rearing, namely cattle, sheep, goats and pigs (Kaduna State, 2010). Kaduna state has an estimated population of 1,144,000 cattle, 832,000 sheep and 988,000 goats (KDSG, 2008).

The study was conducted in the six (6) selected Local Government Areas of the State that have significant population of settled Fulani herds, namely; Sabon-Gari, Giwa and Zaria in Northern senatorial zone, Kaduna South and Igabi in the central senatorial zone and Kagarko in the Southern senatorial zone (Fig. 3.1)

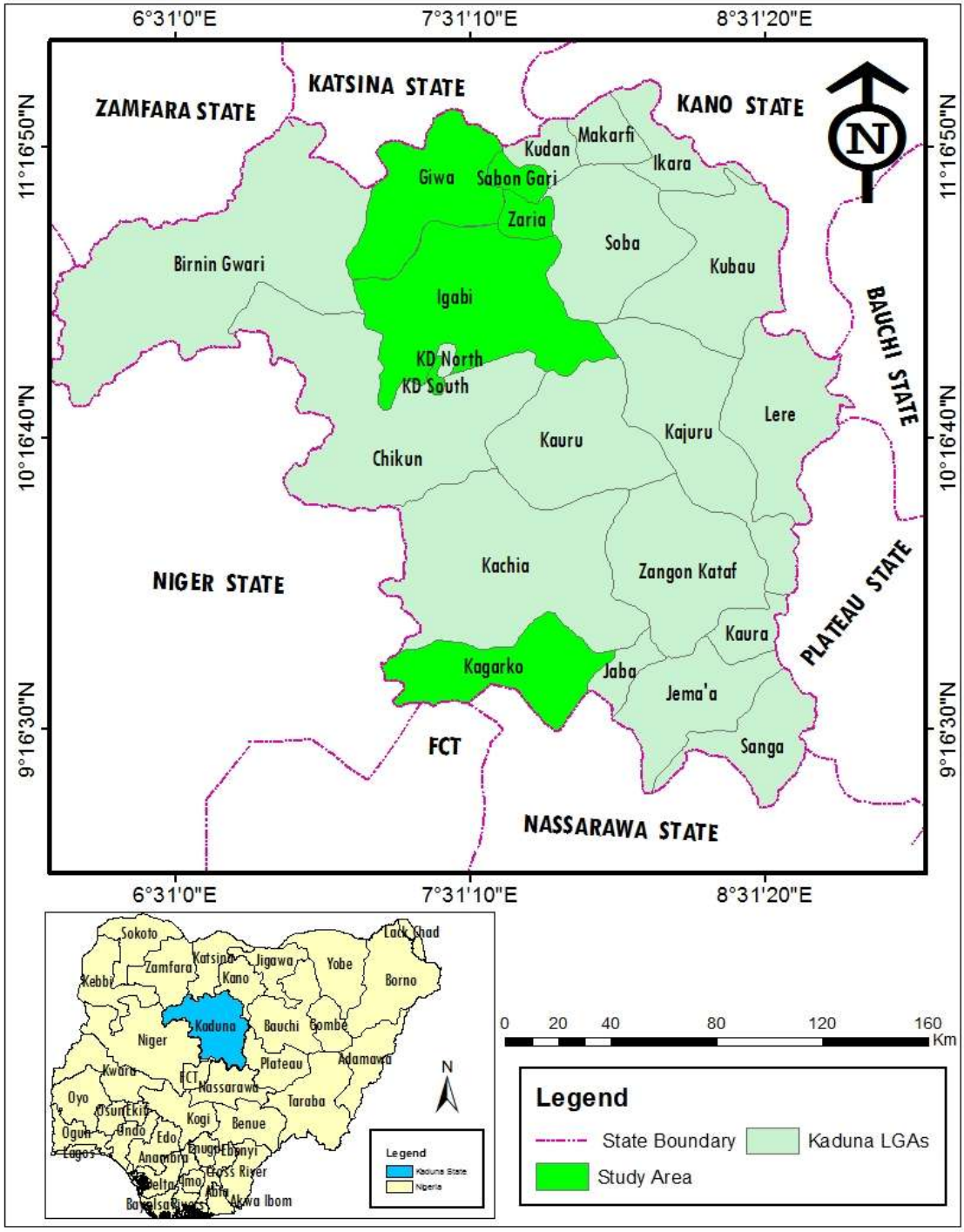

Figure 3.1: Map of Kaduna State showing the study areas

Source: GIS Laboratory Department of Geography and Environmental Management, A.B.U. Zaria

3.2 Study design

A descriptive cross-sectional was conducted involving milk produced by cows raised by settled Fulani herds population. Six (6) Local Government Areas of the State that have significant population of settled Fulani herds, namely; Sabon-Gari, Giwa and Zaria in Northern senatorial zone, Kaduna South and Igabi in the central senatorial zone and Kagarko in the Southern senatorial zone were selected and the study conducted.

3.3 Determination of sample size

The sample size was determined using the Fisher's formula (Kasiulevičius *et al.*, 2006)

$$n = \frac{Z^2 P (1-P)}{e^2}$$

Where:

n = Desired sample size for the study

Z = Desired confidence level (1.96 for 95% confidence level)

P = Prevalence of MRSA (35.6%, Suleiman *et al.*, 2012a)

1 – p = Proportion of cows without MRSA (1-0.048)

e^2 = Desired level of precision (5% or 0.05)

Therefore, n = 352, using the prevalence rate of 35.6%, from bovine mastitic milk in Plateau State, Nigeria (Suleiman *et al.,* 2012a).

Using a confidence level of 95%, an expected prevalence of 35.6% (Suleiman *et al.,* 2012a) of MRSA from bovine mastitic milk in Plateau State, Nigeria, and an accepted error of 0.5%, a minimum of 352 milk samples will have to be collected. However, the number of samples was increased to 360 for conveniency.

3.4 Sample collection

Milk samples were collected from settled Fulani herds in the six (6) Local Government Areas. The Local Government Areas were selected using purposive sampling using information on population of local Government with high settled Fulani herd population obtained from the peri-urban dairy programs (MILCOPAL in Kaduna and NAPRI in Shika). Only consenting herds were involved in the study in each of the selected Local Government Areas. Herds that had a exaclty of ten (10) milking cows were taotally sampled, that is all the ten cows, while simple randomsampling was used to select exactly ten (10) cows when the number of milking cows exceeded ten (10).

Each of the herd was visited during milking time between 6 and 8 A. M. Ten (10) milk samples were collected aseptically from each cow after washing and disinfection of the udder. The farmers were given demonstration and instructions on how to aseptically collect the milk samples. The teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry before milking manually. Samples were collected from all the quarters and dispensed in the wells of the Carlifornia Mastits test (CMT) paddle, and the second part of the milk pooled in to a composite sample with one composite sample representing a cow. The first streams (2 ml) of the milk samples were discarded; five (5) ml of the composite milk samples was collected into sterile plastic sample bottle. All the samples collected were placed on ice and transported to the Bacterial Zoonoses laboratory, Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria for the isolation of *S. aureus*.

3.5 Mastitis screening

Data related to husbandry system, age, parity, udder and milk abnormalities (injuries, blindness, swelling, milk clots and other abnormal udder secretion, etc.) were noted and recorded. Age of the study animals was determined from owners and categorized as young adults (3 to 6 years), adults (> 6 to < 10 years), and old (10 yrs and above) (Mekibib *et al.*, 2010). All the cows and milk samples were assessed for clinical and subclinical mastitis.

3.5.1. Determination of clinical mastitis

Clinical mastitis was detected by observing changes in the udder and milk characteristics (Erdem and Tùkyilmaz, 2013). Changes in the udder associated with mastitis included pain, swelling, warmth, and abnormal appearance (blood tinged milk, watery secretions, clots, pus) of milk.

Cows that did not have clinical mastitis were subjected to further examination for subclinical mastitis by examining the milk using California Mastitis Test (CMT) as described below by Dingwell *et al.* (2003).

3.5.2 California Mastitis Test (CMT)

The CMT reagent was purchased from Kruuse, Denmark, and stored in refrigerator prior to it's use. Fore-milk from each quarter was milked into cups of four-cup plastic paddle. The paddle was tilted to equalize milk quantities in the cups at about half teaspoon (2.5 ml) each. Then equal volume of the test reagent was added to each cup and the paddle rotated to mix thoroughly. Changes in colour and gel formation were observed within 10-15 seconds after mixing. Milk from normal quarter remained liquid and flowed freely, while gel formation that formed and breaked into clumps indicted moderate reaction. Milk samples that reacted strongly formed a gelatinous mass that clinged together as the paddle was rotated.

3.5.3 Interpretation of CMT score result

The CMT result was interpreted according to Dinwel *et al.* (2003). If there is no reaction; the CMT result was taken as negative, if the CMT score was -/+, the result was taken as Trace and if the CMT score was 1, the result was taken as weak positive. Also, when the CMT score was 2, the result was taken as distinct positive, and when the CMT score was 3, the result was taken as strong positive.

3.6 Isolation of *Staphylococcus aureus*

Two (2) ml of each milk sample was suspended in 5 ml Tryptone Soya Broth (TSB, Oxoid) containing 6.5% sodium chloride and was incubated for 24 hours at 37° C for selective enrichment of staphylococci (Moses *et al.*, 2017). Baird Parker medium supplemented with egg yolk and potassium tellurite was prepared according to Oxoid's instruction. Enriched milk samples were then streaked on to the surface of the agar plate to obtain discrete colonies according to the standard protocol for testing of foods for *Staphylococcus aureus* (Cheesbrough, 2006). The plates were then incubated aerobically for 24-48 hours at 37^{o} C, and observed for growth (Tamagnini *et al.*, 2006).

Typical coagulase-positive *S. aureus* colonies appeared as black and were surrounded by an opaque halo of precipitation which signifies the coagulase reaction (Panda *et al.*, 2016).

3.6.1. Storage of the isolates

Nutrient agar medium was prepared according to the manufacturer's instruction. Five milliliters were transferred to Bijou bottles, which were slanted and allowed to solidify after autoclaving. The isolates were then inoculated onto nutrient agar slants, incubated at 37^{o}C overnight and stored in refrigerator at 4^{o} C for further analysis and characterization.

3.7. Identifications of *Staphylococcus. aureus*

3.7.1. Conventional methods

3.7.1.1 Colonial morphology

Typical colour, shapes and sizes of the colonies of *S. aureus* as described by Parker (1963) were checked. Typical coagulase-positive *S. aureus* colonies appear as black, grey or white and are surrounded by an opaque halo of precipitation which signifies the coagulase reaction (Panda *et al.*, 2016).

3.7.1.2 Gram staining

Some suspected colonies were picked with a sterile wire loop and emulsified in the drop of the normal saline, and then smeared. The smear was allowed to air dry before fixing by passing over flame. The slide was then placed on a rack over a sink and flooded with crystal violet, and allowed for 2 minutes. The crystal violet was then washed off with running tap water. The slide was then flooded with lugol's iodine, which was allowed for 1 minute, before flushing with running water. Acetone was applied, and then washed off immediately, to decolorize the stain. The stained slide was allowed to air-dry. The dried stained slide was mounted and examined under microscope using oil immersion (×100) objective (Cheesbrough, 2010).

3.7.1.3 Catalase test

A colony of the suspected organism was picked and emulsified in one drop of the 3% hydrogen peroxide on a clean dry grease free slide. The organism that produced immediate bubbling and effervescence by liberation of oxygen was recorded as catalase positive (Cheesbrough, 2010).

3.7.1.4 Coagulase test

The tube agglutination test was performed by pipetting and placing 0.5ml of the EDTA diluted plasma (1 in 10 ratio) in two (2) test tubes. 0.5ml of 24 hours culture of

test organism was inoculated into in tube 1. Tube 2 contained the positive control, and therefore served as a control. The tubes were incubated in water-bath at 37^{o}C for 4 hours, and observed for clot formation at hourly interval. If there was no clot formation by the 4th hour, the tubes were then removed from the water and kept at room temperature overnight. Positive result was indicated by agglutination which did not re-emulsify (Cheesbrough, 2010).

3.7.1.5 Haemolysis test

Pure overnight culture was streaked on 5% sheep blood agar (Oxoid, Basingstoke, UK), incubated at 37^{o}C for 24 hours and observed for haemolysin production. Positive haemolysis was indicated by clear zone (Panda *et al.*, 2016).

3.7.1.6 DNase test

An overnight broth culture of the *S. aureus* was inoculated onto DNase agar (Oxoid Basingstoke, United Kingdom) plate and incubated for 24 hrs at 37 ^{0}C. The plates were flooded with 1 N HCl and the excess decanted. The HCl was left to act for about ten minutes after which the plates were observed for clear zones around each streak of *S. aureus.* The presence of a clear zone indicated a positive result, showing that DNase was produced which digested the DNA (Cheesbrough, 2010).

3.7.1.7 Sugar fermentation test

A volume of 1.25 ml of sterile bromothymol blue solution was added to 100ml of sterile peptone water in a conical flask. Then 5ml of 1% sugar solution (sucrose, glucose, lactose and mannitol) prepared, and was added and mixed well. Then the isolates were inoculated and incubated at 37^{o}C for 24 hours before observing for any change of colour. They were then left for 48 hours under incubation for the final reading (Cheesbrough, 2010).

3.7.2 Commercial Identification System

3.7.2.1 Microbact 12S Staphylococcal Identification System

All coagulase positive *S. aureus* were confirmed further using commercial identification system, Microbact 12S Staphylococcal Identification System (Oxoid, Basingstoke, UK) according to the manufacturer's instruction. Four discrete colonies were picked from 24 hour pure culture and emulsified in 3 ml of staphylococcal suspending medium. This was mixed thoroughly to form a homogenous suspension. The test strip was removed from the foil pouch, placed in the holding tray and labeled appropriately. The lid from the test strip was then removed. Four drops (100 µl) of the bacterial suspension was added to the wells of each test strip using a sterile Pasteur pipette. Two (2) drops of Mineral oil (code MB1093A) was overlaid in well.7, arginine (indicated by a black circle on the test strip) and the lid replaced. One (1)

drop of the inoculum was placed onto TSA agar for purity check and incubated at 35^{o}C for 24 hours. The test strip was then incubated at 35^{o}C for 24 hours. The fast blue reagent (code MB1588A) was reconstituted by adding the entire contents of the dilluent bottle to the reagent bottle and mixed thoroughly. The test strip was removed from the incubator and the lid removed. One drop of the fast blue reagent was added to well No 12 indicated by a green circle on the test strip (Beta-galactosidase) and observed for colour change. All the test results were recorded onto the Microbact™ Organism ID Report Forms. The colour chart on the inside flap of the pad was used in the interpretation of the colour changes in each well of the 12S strip. Each block of three reactions was then concerted into numeric value, and the three numbers added together to obtain a digit of the Microbact™ code which was compared to the profile register or entered into the computer package. The results of the test reactions via the five digit code number were entered into the Microbact™ Computer Aided Identification Software which produced a probable species ID of the *Staphylococcus* in question.

3.8 Antimicrobial susceptibility testing

All confirmed *S. aureus* were tested for resistance to a panel of 13 antibiotics using the disc diffusion method outlined by the Clinical Laboratory Standard Institute (CLSI, 2011; 2014). Also, all the MRSA isolates were tested further for resistance to other antibiotics. The following antibiotics were obtained commercially from Oxoid (Oxoid

Basingstoke, United Kingdom): amoxicillin (30 µg), ampicillin (30 µg), cefoxitin (30 µg), chloramphenicol (12 µg), ciprofloxacin (5 µg), erythromycin (5 µg), gentamicin (10 µg), nalidixic acid (30 µg), oxacillin (1 µg), penicillin (10 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), and vancomycin (30 µg).The sealed packages of the disks were stored at 4 ^{o}C before use.

3.8.1 Inoculum preparation

To standardize the inoculum density for a susceptible test, a Barium sulphate ($BaSO_4$) turbidity standard, equivalent to a 0.5 McFarland standard was used. Brain Heart Infusion Broth was prepared according to the manufacturer's instruction. Three well-isolated colonies of the same morphological type were selected from a blood agar plate culture which was incubated for 24 hours. The top of each colony was touched with a wire loop, and the colony was transferred into a tube containing 5ml of Brain Heart Infusion Broth (BHIB). The broth culture was then incubated at 35^{0} C for 6 hours until the turbidity approximately to 0.5 McFarland standard was observed. The turbidity of the actively growing culture was adjusted with sterile saline to obtain turbidity optically comparable to that of the 0.5 McFarland standards (CLSI, 2014).

3.8.2 Inoculation of test plates

Mueller-Hinton Agar was prepared according to the manufacturer's instruction. A sterile cotton swab was dipped into the adjusted suspension within 15 minutes after

adjusting the turbidity of the inoculum suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of the Mueller-Hinton Agar was inoculated by streaking the swab over the entire sterile agar surface. The above procedure was repeated by swabbing two more times, rotating the plate approximately 60^{o} each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

3.8.3 Application of discs to inoculated agar plates

The application was performed with the aid of antibiotic disk dispenser (Oxoid Basingstoke, United Kingdom). After applying the disks, the plates were allowed to dry, and placed in an incubator at 35^{o} C within 15 minutes after the discs were applied.

3.8.4 Reading of plates and interpretation of results

Zones of inhibitions were observed in some plates that were uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition (as judged by unaided eye) were measured, including the diameter of the disc. The petri plate was held a few inches above a black, non reflecting background and illuminated with reflected light. Zones were measured to the nearest whole millimeter, using a ruler, which was held on the back of the inverted petri plate. The sizes of the zones of inhibition were interpreted by comparing with the normal breakpoints as described by

Clinical Laboratory Standard Institute (CLSI, 2014). *S. aureus* (ATCC 33591) strains positive control was obtained from a known methicillin resistant *Staphylococcus aureus* (Courtesy of Prof. B. Ghebremedhin, Otto-von-Guericke-University Clinic, Medical Microbiology, Magdeburg, Germany).

3.8.5 Minimum inhibitory concentration (MIC) of the antibiotics

The minimum inhibitory concentrations (MIC) values of oxacillin and vancomycin for the MRSA were determined by the standard broth micro dilution method (CLSI 2014) using a commercially available M.I.C. Evaluator Strips (Oxoid Basingstoke, United Kingdom). According to the break points defined by CLSI (CLSI, 2014), isolates with MIC ≥4 μg/mL and ≥ 16 μg/mL were respectively considered oxacillin and vancomycin resistant.

3.8.5.1 Inoculum preparation

McFarland inoculum level was prepared by emulsifying discrete colonies from a pure culture on Mueller-Hinton broth, and the turbidity compared to o.5 McFarland standard. A sterile cotton swab was dipped into the suspension and inoculated onto Muller-Hinton agar plates by swabbing in three different directions. The plates were allowed to dry before the application of the M.I.C.E stips (CLSI, 2014).

3.8.5.2 Application of the M.I.C.E.

The M.I.C.E. strips were removed from the satchel using sterile forceps. The strip was placed in contact with the agar with the scale facing upwards and the antibiotic gradient downwards. The plates were then incubated at 35^{o}C for 24 hours CLSI, 2014).

3.8.5.3 Interpretation of results

If the growth intersects the strip on the white section, the MIC is read as the value in that section.

If the growth touches the strip on the black section, then for clinical interpretation, the MIC is read as a value in the next white section above where the growth finished.

If there is growth along the entire length of the strip (no zone of inhibition), the MIC should be read as greater than the highest value on the strip. If large zone of inhibition is obtained, and the growth of the organism does not intersect with the strip, the MIC should be read as less than the lowest value on the strip (CLSI, 2014).

3.9 Isolation and identification of MRSA isolates

3.9.1 Oxacillin resistance agar base (ORSAB) medium

All confirmed coagulase-positive *S. aureus* were screened for methicillin/Oxacillin resistance using cultures on Oxacillin Resistance Agar Base (ORSAB) medium (Oxoid, Basingstoke, United Kingdom) (Moses *et al.*, 2017). The samples were grown in broth enrichment comprising of Mueller-Hinton broth supplemented with sodium chloride (NaCL). Then the medium was prepared according to the manufacturer's instruction, and all plates were inoculated with the samples. The plates were then incubated at 37^{o} C for 24 hours (Paterson *et al.*, 2013).

3.9.2 Slide agglutination test for PBP2a

All the confirmed MRSA isolates were tested with the Penicillin Binding Protein (PBP2') Latex Agglutination Test (Oxoid Basingstoke, United Kingdom). The *mec* A-positive strain NCTC 12493 was used as a positive control.

3.9.2.1 Inoculum preparation

A broth suspension of the organism equivalent to 1 McFarland standard was prepared, and a lawn of the suspension was streaked on blood agar and incubated at 37^{o} C for 24 hours.

3.9.2.2 PBP2a extraction procedure

Four drops of the extraction reagent 1 was added to the micro centrifuge tube. Five micro litre (5µl) of the suspension was removed using 5µl loop and suspended in the micro centrifuge tube, vortexed and observed for clumps. The tube was placed into boiling water and heated for 3 minutes. The micro-centrifuge tube was removed and allowed to cool at room temperature. One drop of the extraction reagent 2 was added to the tube and mixed thoroughly. The tube was then centrifuged at 3000 rpm at 15cm rotation radius, and the supernatant separated (Nilima and Ghorpade, 2016).

3.9.2.3 Latex agglutination procedure

One circle of the test card was labeled for each suspension for testing with Test Latex and another for testing with Control latex. The latex reagent was mixed thoroughly by inversion several times, and one drop of Latex Test and Control Test was added to each of the labeled circle. Five (5) µl of the supernatant was placed on the Test circle and Control circle, and then mixed thoroughly with a mixing stick. The card was then rocked for 3 minutes and agglutination was observed under light. The results for the tests and control reactions were then recorded (Paterson *et al.*, 2013).

3.9.2.4 Strength of agglutination reaction

Negative (-) = a homogeneous suspension of particle with no viable clumping

Weak positive (+) = small but definite clumps against a clouded background

Strong positive (+) = large and small clumps against a slight clouded background or large clump against a very cloud background.

3.9.3 Molecular characterization of MRSA isolates

3.9.3.1 Storage and transportation of MRSA

S. aureus isolates were inoculated onto Mannitol Salt agar and Tryptone Soya agar slants in cryo vials and also spotted on Whatman filter Paper, placed in a container with ice and shipped to The Wellcome Trust, Sanger Institute, United Kingdom for the molecular characterization.

3.9.3.2 Detection of mec A, mec C and fem B genes by multiplex polymerase chain reaction

DNA extraction

Two pure colonies of bacterial cultures were picked up from overnight growth cultures on blood agar and melted with 40µl water (DNase free water). This was then heated for 5 minutes at 95º C in a thermocycler (McLauchlin *et al.*, 2000). The mixture (2.5 µl) was used as a DNA template for Multiplex PCR procedures.

3.9.3.2.1 PCR Procedures

Reaction Mixture

	x 1 reaction	
MyTaq Red Reaction Buffer 5U/µL(Bioline, UK) *	5µL	
2W2X [Primer *mecA*-F + *mecA*-R (10µM)]	1µL	3µL**
1A1B [Primer *mecC*-F + *mecC*-R (10µM)]	1µL	
1I1J [Primer *femB*-F + *femB*-R (10µM)]	1µL	
MyTaq DNA polymerase5U/µL (Bioline, UK)	0.5µL	
Dist. MilliQ H_2O	14.5µL	
DNA template (50ng/µl)	2.5µL	
Total volume	2**5µL**	

* The MyTaq Red Polymerase Buffer included in the PCR reaction mixture contains an inert red dye that enables users to load samples directly onto a gel after the PCR without the need to add loading buffer.

** Primers were synthesised at Sigma-Aldrich and stocks were stored at -20°C at a concentration of 100µM/µL. Solutions of 10µM/µL were prepared from the stocks with distilled water as diluent (also stored at -20°C). For multiplex reactions, a mixture containing equal volumes of of primers was used so that a 1x reaction contained 0.5µL of each Primer. This mixture of primers was stored at -20°C.

3.9.3.2.2 DNA Amplification

Multiplex Polymerase Chain Reaction to detect *mec* A, *mec* C and *fem* b genes were performed using DNA template, with the following amplification mixture: 200 μM of dNTPs, 1.5 mM magnesium chloride (Promega Corporations, USA), 0.25 μM of both forward and reverse primers of each gene, and 2.5 μL of template in a total volume of 25 μL. The amplification was carried out in MyCycler Thermal Cycler (Bio-Rad) with an initial denaturation at 94 ℃ for 30 s, 30 cycles of denaturation temperature of 94 ℃ for 45 s, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 2 min, and final extension at 72 ℃ for 2 min. *S. aureus* ATCC43300 and ATCC25932 were used as positive and negative controls respectively.

3.9.3.2.3 Agarose gel electrophoresis

Molecular biology grade Agarose (2% w/v) was dissolved by heating in a microwave in 1×TAE buffer (40mM Tris, 20mM Acetate and 2mM EDTA pH 8.1). Sybr® Safe DNA gel stain (Thermo Fisher, UK) was added to the melted and cooled agarose solution. Each 100ml of 2% agarose contained 2.5μL of Sybr® Safe which was poured into the gel tray (with 1mm 25 well comb from PeqLab) to set. A 10μL of PCR product was loaded on to each well. The molecular marker (5μL) used was HyperLadder^{TM} 100bp, consisting of 100-1000bp ladder (Bioline, United Kingdom). Electrophoresis was performed in a Sigma-Aldrich electrophoresis tank with 1 ×TAE

at 80V for 75 minutes. Electrophoresed gels were visualised under blue-light and their images captured using the GelDoc^{TM} XR System Imager (BioRad).

Table 3.2: Oligonucleotide primers of MRSA and *S. aureus mec* A, *mec* C and *fem B* genes

Name	Sequence (5'-3')	Size (bp)	Gene	Reference
mecA-F	TGG TAT GTG GAA GTT AGA TTG GGA T	155	*mec*A	(Nakagawa *et al.*, 2005)
mecA-R	CTA ATC TCA TAT GTG TTC CTG TAT TGG C			
mecCA-F	CAT TAA AAT CAG AGC GAG GC	188	*mec*C	(Paterson *et al.*, 2014)
mecCA-R	TGG CTG AAC CCA TTT TTG AT			
femB-F	CAT GGT TAC GAG CAT CAT GG	531	*fem*B	(Paterson *et al.*, 2014)
femB-R	AAC GCC AGA AGC AAG GTT TA			

3.9.3.3 Multilocus Sequence typing (MLST) of MRSA isolates

Primers (*arcC*-Up, *arcC*-Dn, *aroE*-Up, *aroE*-Dn, *glpF*-Up, *glpF*-Dn, *gmK*-Up, *gmK*-Dn, *pta*-Up, *pta*-Dn, *ipi*-Up, *ipi*-Dn, and *yqiL*-Up, *yqiL*-Dn) were purchased commercially and the DNA sequences of 7 housekeeping genes obtained (M. Burnham of SmithKline Beecham, U.K). The sequences of their gene products were c ompared with those in the EMBL/GenBank database by using BlastP (*http://www.ncbi .nlm.nih.gov/blast/blast.cgi*). Each primer pair amplified an internal fragment of the housekeeping gene (about 500 bp) and was allowed accurate sequencing of ~450-bp fragments of each gene on both strands (Enright *et al.*, 2000).

The following seven housekeeping genes were used in the final MLST scheme and the fragments was amplified by using the primers shown in Table 3.3.

Table 3.3: Sequences of primers used in the PCR

Gene	Primer	Size of product (bp)	Sequence (5′-3′)
Carbamate kinase (*arcC*)	*arcC*-Up	456	TTGATTCACCAGCGCGTATTGTC
	arcC-Dn		AGGTATCTGCTTCAATCAGCG
Shikimate dehydrogenase (*aroE*)	*aroE*-Up	456	ATCGGAAATCCTATTTCACATTC
	aroE-Dn		GGTGTTGTATTAATAACGATATC
Glycerol kinase (*glpF*)	*glpF*-Up	465	CTAGGAACTGCAATCTTAATCC
	glpF-Dn		TGGTAAAATCGCATGTCCAATTC
Guanylate kinase (*gmk*)	*gmk*-Up	429	ATCGTTTTATCGGGACCATC
	gmk-Dn		TCATTAACTACAACGTAATCGTA
Phosphate acetyltransferase (*pta*)	*pta*-Up	474	GTTAAAATCGTATTACCTGAAGG
	pta-Dn		GACCCTTTTGTTGAAAAGCTTAA
Triosephosphate isomerase (*tpi*)	*tpi*-Up	402	TCGTTCATTCTGAACGTCGTGAA
	tpi-Dn		TTTGCACCTTCTAACAATTGTAC
Acetyl coenzyme A acetyltransferase (*yqiL*)	*yqiL*-Up	516	CAGCATACAGGACACCTATTGGC
	yqiL-Dn		CGTTGAGGAATCGATACTGGAAC

DNA amplication of target genes was carried out with 50-µl reaction volumes containing 0.5 µl of chromosomal DNA (approximately 0.5 µg), 0.5 µg of each primer, 1 U of *Taq* DNA polymerase (Qiagen, Crawley, United Kingdom), 5 µl of 10× buffer (supplied with the *Taq* polymerase), and 0.2 mM deoxynucleoside triphosphates (Perkin-Elmer Applied Biosystems; Foster City, Calif.). The PCR was performed in a PTC-200 DNA engine (MJ Research, Boston, Mass.) with an initial 1 min denaturation at 95°C, followed by denaturation at 95°C for 5 min, 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1 min, followed by a final extension step of 72°C for 5 min. The amplified products were precipitated with 20% polyethylene glycol, 8000-2.5 M NaCl. The fragments were then sequenced from both strands using the same primers and sequencer. The product were then precipitated with sodium acetate in 70% ethanol, resuspended in 10 µl Hi-Di. formamide before loading in a sequencer (Enright *et al.*, 2000).

For each locus, the sequences obtained from all the isolates were compared and the different sequences were assigned allele numbers. For each isolate, the alleles at each of the seven loci defined the allelic profile which corresponded to its ST. The clustering of isolates was achieved by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) from the matrix of the percentage of pair wise differences between the allelic profiles of the isolates by using Statistica (StatSoft, Tulsa, Okla.). The non-randomness in the distribution of variable sites along the

sequence of each gene fragment was examined by the method of Sawyer (Sawyer, 1989). Polymorphic sites were displayed by using Sequence Output, a Macintosh program available from the MLST website (*http://mlst.zoo.ox.ac.uk)*.

The origins of the strains were examined by the algorithm eBURST, in order to group the received allelic profiles into clonal complexes. Grouping was based on the similarity between sequence types in six of seven loci and singleton STs are defined as not sharing six out of seven loci with any other STs in the data set. Comparison with all *S. aureus* isolates in the database at http://www.*mlst.net* revealed the presence of only one clonal complex (CC1).

3.9.3.4 Phylogenetic studies and analysis of the isolates

The phylogenetic relationship was established using the nucleotide sequences of the type strains defining the genus *Staphylococcus* (BX571857, BA000033, AP015012, CP017115 and CP01780). The BLAST search for previously reported sequences that are identical to the three (3) local isolates was done using NCBI GenBank (*http://www.ncbi.nlm.hih.gov/*). Multiple alignments were carried out using cluster algorithm. Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar *et al.,* 2001; *http://www.megasoftware.net*). The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985) and 1000

bootstrap replications were used. A bootstrap value of 70% was considered signficant evidence for phylogenetic grouping.

The nucleotide sequences of the *Staphylococcus aureus* 23448_1#128, 23448_1#12 and 23448_1#1311genes were used for the final construction of both Neighbour-Joining (NJ) phylogenetic trees. The methods produced a topology revealing species clustering of *Staphylococcus aureus* obtained in this study and those obtained from the Genbank.

3.9.4 Data analysis

All data were statistically analyzed using SPSS package (Version 16.0). Probability values of statistical significance among prevalences of mastitis, *S. aureus* and MRSA in milk from different locations were determined using Chi-square and Fisher's exact test at 5% level of confidence. Statistical significance was defined as a probability value of ≤ 0.05. A P value of $P < 0.05$ was considered as significant.The 95% confidence intervals for sensitivities and specificities were performed with the NCSS 2000 program (NCSS, Kaysville, Utah) under the assumption of a binomial distribution of the results.

Antimicrobial susceptibility results were analyzed for *S. aureus* and MRSA to observe trends in resistance for each tested antibiotic. Multiple antimicrobial resistance index (MARI) was determined for isolate using the formula: MARI= x/y, where, x is the number of antibiotics to which an isolate is resistant and y is the total number of antibiotics tested. Association between mastitis, *S. aureus*, MRSA,antimicrobial susceptibility, MDR, *mec* A, *mec* C, *fem* B and MLST were also assessed.

The results of MLST analysis of the three strains were compared to a database of *S. aureus* STs available on-line (*http://saureus.mlst.net*). The sequences were processed using CLC Main Workbench 7 (CLC Bio-Qiagen, Denmark) and analyzed using the *S. aureus* MLST database to assign the allele type and thus the ST. The profiles obtained including the new alleles and new profiles were submitted to the MLST database to contribute to the resource for *S. aureus* global epidemiology.

Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar *et al.*, 2001; *http://www.megasoftware.net*). The reliability of the trees was estimated by bootstrap confidence values and 500 bootstrap replications were used.

CHAPTER FOUR

4.0 RESULTS

4.1 Occurrence and prevalence of mastitis in cow milk in Kaduna State, Nigeria

The results obtained from the study revealed that the prevalence of mastitis at cow level was 26.9% (97/360), out of which 3.1% (11/360) and 23.9% (86/360) were clinical and subclinical mastitis respectively (Table 4.1). The prevalence of mastitis at quarter level was 21.7% (312/1440), out of which 1.9% (28/1440) were clinical mastitis and 19.7% (284/1440) subclinical mastitis respectively (Table 4.2). Statistical analysis showed that there was significant difference in the occurrence of mastitis among the age groups ($P<0.05$). The prevalence at quarter level was 25.2% (157/624) in adult cows, 20.2% (97/480) in young adults, and 17.3% (58/360 in old cows (Table 4.2). Similar pattern of occurrence was recorded for both clinical and subclinical mastitis as the adults had rates of 21.8% and 3.4 %, young adults had 19.8% and 0.4% and old cows had 15.8% and 1.5% respectively ($P<0.05$).

Samples from Igabi, Kagarko and Kaduna South had the highest rates of mastitis (35.0%, 31.7% and 30.0%) than those from Zaria (26.7%), Giwa (25.0%) and Sabon-Gari (13.3%) ($P<0.05$) respectively. Similarly, both clinical and subclinical mastitis were highest in Igabi (30.0% and 5.0%), followed by Kaduna South and Zaria with 26.7% each for clinical mastitis, while the least was Sabon-Gari with 13.3%. In

contrast, clinical mastitis was significantly higher (P<0.05) in Kagarko (6.7%) and lowest in Giwa (3.3%) and Kaduna South (3.3%) respectively. No case of clinical mastitis was recorded in Sabon-Gari and Zaria (Table 4.1).

The CMT results showed that 55 (15.3%) of the milk samples had a score of 1+, 26 (7.2%) had a score of 2+, 3 (0.8) had a score of 3+ while only 2 (0.6%) had a score of "Trace" (Table 4.3). Two hundred and seventy four (274) of the milk samples examined were negative for mastitis by CMT (Table 4.3).

The results also showed that 67 (69.1%) of the mastitis positive samples showed growth after culturing, out of which 44.3% (43/97) were *S. aureus* and 24.7% (24/97) were coagulase negative staphylococci (Table 4.4). The prevalence of both *S. aureus* and coagulase negative staphylococci (CONS) was significantly higher (P<0.05) in subclinical mastitis (35.1% and 21.6%) than in clinical mastitis (9.3% and 3.1%) respectively (Table 4.4).

Table 4.1: Prevalence of clinical in cows and sub clinical mastitis in fresh cow milk samples in settled Fulani herds in Kaduna State, Nigeria (N= 360)

Location	No. of samples examined	No. (%) with Clinical mastitis	No. (%) with Subclinical mastitis	Mastitis
Giwa	60	2 (3.3)	13 (21.7)	15 (25.0)
Igabi	60	3 (5.0)	18 (30.0)	21 (35.0)
Kaduna South	60	2 (3.3)	16 (26.7.0)	18 (30.0)
Kagarko	60	4 (6.7)	15 (25.0)	19 (31.7)
Sabon-Gari	60	0 (0.0)	8 (13.3)	8 13.3)
Zaria	60	0 (0.0)	16 (26.7	16 (26.7)
Total	**360**	**11 (3.1)**	**86 (23.9)**	**97 (26.9)**
	$\chi^2 = 41.722$	df = 15		P < 0.001

Table 4.2: Occurrence of mastitis according to the quarters and age of the cows examined in settled Fulani herds in Kaduna State, Nigeria

Age	No. of cows/ quarters examined	No. (%) clinical mastitis	No. (%) subclinical mastitis	Total
Young adults	120/480	2 (0.4)	95 (19.8)	97 (20.2)
Adults	156/624	21 (3.4)	136 (21.8)	157 (25.2)
Old	84/336	5 (1.5)	53 (15.8)	58 (17.3)
Total	**360/1440**	**28 (1.9)**	**284 (19.7)**	**312 (21.7)**

$\chi^2 = 49.988$ df = 18 P= 0.009

Table 4.3: California mastitis test scores of cow milk according to the gel formation scores in Kaduna State, Nigeria

Location	No. of samples examined	No. (%) CMT score				
		Negative	**Trace**	**1+**	**2+**	**3+**
Giwa	60	47	-	4	8	1
Igabi	60	42	-	10	7	1
Kaduna South	60	44	2	12	2	-
Kagarko	60	45	-	12	3	-
Sabon-Gari	60	52	-	6	2	
Zaria	60	44	-	11	4	1
Total	**360**	**274**	**2 (0.6)**	**55 (15.3)**	**26 (7.2)**	**3 (0.8)**

4.2 Prevalence of staphylococci in fresh milk in in settled Fulani herds Kaduna State, Nigeria

Of the 360 cow milk samples examined, 55 (15.3%) isolates were presumed to be *S. aureus* by coagulase test. The highest isolation of *S. aureus* was from Kagarko L.G.A. (26.7%) followed by Kaduna South (18.3%) while the least was from Giwa L.G.A. (5.0%) ($P<0.005$) (Table 4.5). Coagulase negative staphylococci recorded an overall prevalence of 3.3% with samples from Kagarko having the highest prevalence of 6.3% (Table 4.5). Kaduna South and Igabi recorded a prevalence of 3.3% each while samples from Giwa were negative for coagulase positive staphylococci (Table 4.5).

Biochemical test results showed that all the *S. aureus* were coagulase positive using rabbit plasma, while significant number ($P<0.05$) 22 (40.0%) produced alpha haemolysin, 25 (45.5%) produced beta haemolysin and 8 (14.5%) produced gamma haemolysin respectively (Table 4.6). The results also revealed that all (n= 55) of the *S. aureus* were DNase positive and fermented Mannitol where as significant number ($P<0.05$) 32 (58.2%) also showed pigmentation on Mannitol salt agar (MSA) (Table 4.6).

Table 4.4: Prevalence of staphylococci in the mastitic milk in Kaduna State, Nigeria

Mastitis	**No. (%) positive for mastitis**	**No. (%) with *S. aureus* (N=55)**	**No. with (%) CONS**	**Total**
Clinical	11 (2.8)	9 (20.0)	3 (18.2)	12 (17.9)
Subclinical	86 (21.5)	34 (58.2)	21 (12.8)	55 (82.1)
Total	**97 (24.3)**	**43 (44.3)**	**24 (24.7)**	**67 (69.1)**

Key: CONS= Coagulase negative staphylococci

$\chi^2 = 18.916$ df = 12 P =0.091

Table 4.5: Prevalence of staphylococci in cow milk in settled Fulani herds, Kaduna State, Nigeria (N= 360)

Location	No. of milk examined	No. (%) positive for *S. aureus*	No. (%) positive for CONS
Giwa	60	3 (5.0)	0 (0.0)
Kaduna South	60	11 (18.3)	2 (3.3)
Igabi	60	7 (11.7)	2 (3.3)
Kagarko	60	16 (26.7)	5 (6.3)
Sabon-Gari	60	10 (16.7)	1 (1.7)
Zaria	60	8 (13.3)	2 (3.3)
Total	**360**	**55 (15.3)**	**12 (3.3)**

Key: CONS= Coagulase negative staphylococci

$\chi^2 = 22.855$ df = 10 P =0.011

Table 4.6: Haemolytic and biochemical characteristics of *S. aureus* isolates from cow milk in settled Fulani herds, Kaduna State, Nigeria (N=55).

Test	**No. positive**	**% positive**	**P value (<0.05)**
Alpha haemolysis	22	40.0	0.05
Beta haemolysis	25	45.5	0.05
Coagulase	55	100.0	0.011
DNase	55	100.0	0.032
Gama haemolysis	8	14.5	0.05
Mannitol fermentation	55	100.0	0.032
Pigmentation	32	58.2	0.068

4.3 Antibiotic susceptibility of *Staphylococcus aureus* isolated from cow milk in Kaduna State, Nigeria

The resistance patterns of the 55 *S. aureus* isolates showed that all the isolates were resistant to three or more antibiotics (Table 4.7 and 4.9). Two isolates (3.6%) were resistant or of intermediate resistance each to 3 antibiotics while 7 (12.7%) were each resistant or of intermediate susceptibility to 10 or more antibiotics respectively. Multidrug resistance was detected in all (100.0%) of the *S. aureus* isolates (Table 4.8 and 4.9). A number of the *S. aureus* isolates were resistant to commonly used antibiotics like penicillin 55 (100.0%), ampicillin 50 (90.9%), tetracycline 45 (81.8%) and erythromycin 41 (75.5%). High resistance rates were also recorded against vancomycin 34 (61.8%), nalidixic acid 22 (40.0%), streptomycin 18 (32.7%), oxacillin and cefoxitin 16 (29.1%) (Table 4.7).

The multiple drug resistance indices of the *S. aureus* strains are shown in Table 4.8 and 4.9 respectively. It is defined as resistance of an isolate to 3 or more antimicrobials. The results revealed that all the 55, representing 100.0% of the *S. aureus* strains were resistant to 3 or more antibiotics tested, 2 strains were resistant to 3 antibiotics while 7 isolates were resistant to 10 or more antibiotics respectively (Tables 4.8 and 4.9).

The multiple resistant pattern of the *S. aureus* showed that only 4 (7.3%) showed common pattern against AML, AMP, E, P, TE, VA, 3 (5.5%) against AML, AMP, E, P, TE, VA, W and 2 (3.6%) against AML, AMP, P, TE, VA, W respectively (Table 4.8). The remaining 46 (83.6%) *S. aureus* exhibited different types of resistance pattern to each other.

Table 4.7: Antibiotic susceptibility of *S. aureus* isolated from cow milk in settled Fulani herds, Kaduna State, Nigeria (N=55)

Antibiotic	No. (%) susceptible	No. (%) intermediate	No. (%) resistant
Amoxicillin	8 (14.5)	0 (0.0)	47 (85.5)
Ampicillin	5 (9.1)	0 (0.0)	50 (90.9)
Cefoxitin	27 (49.1)	0 (0.0)	28 (56.9)
Chloramphenicol	36 (65.5)	7 (12.7)	12 (21.8)
Ciprofloxacin	51 (92.7)	2 (3.6)	2 (3.6)
Erythromycin	13 (23.6)	1 (1.8)	41 (75.5)
Gentamicin	39 (70.9)	3 (5.5)	13 (23.7)
Nalidicic acid	33 (60.0)	0 (0.0)	22 (40.0)
Oxacillin	27 (49.1)	0 (0.0)	28 (56.9)
Penicillin	0 (0.0)	0 (0.0)	55 (100.0)
Streptomycin	37 (67.3)	0 (0.0)	18 (32.7)
Tetracycline	10 (18.2)	0 (0.0)	45 (81.8)
Trimethoprim	32 (58.2)	8 (14.5)	15 (27.3)
Vancomycin	21 (38.2)	0 (0.0)	34 (61.8)

Table 4.8: Multiple antibiotic resistance indices (MARI) of *S. aureus* isolated from cow milk in settled Fulani herds, Kaduna, State, Nigeria (N=55)

MAR Index	No. of isolates	% isolates
0.2	2	3.6
0.3	1	1.8
0.4	14	25.5
0.5	10	18.2
0.6	21	38.2
0.7	5	9.1
0.8	1	1.8
0.9	1	1.8
Total	**55**	**100.0**

Table 4.9: Multiple antibiotic resistance profiles of *S. aureus* isolated from cow milk in Kaduna State, Nigeria

Antibiotic (s)	No. of isolates resistant	% of isolates resistance
AML, AMP, C, CIP, CN, E, FOX, NA, OX, P, S, TE	1	1.8
AML, AMP, C, CN, E, FOX, NA, OX, P, S, TE	1	1.8
AML, AMP, C, CN, E, FOX, NA, OX, P, TE	1	1.8
AML, AMP, C, CN, E, FOX, NA, P, S, TE	1	1.8
AML, AMP, C, CN, E, FOX, P, TE, VA	1	1.8
AML, AMP, C, CN, E, P, S, W	1	1.8
AML, AMP, C, CN, FOX, NA, OX, P, S, TE	1	1.8
AML, AMP, C, E, FOX, NA, P, TE, VA, W	1	1.8
AML, AMP, C, E, NA, P, TE, VA, W	1	1.8
AML, AMP, C, E, OX, P, TE, VA	1	1.8
AML, AMP, C, E, P	1	1.8
AML, AMP, C, E, P, S, TE, W	1	1.8
AML, AMP, C, E, P, TE, VA	1	1.8
AML, AMP, C, NA, OX, P, TE, VA, W	1	1.8
AML, AMP, CIP, E, FOX, NA, P, TE	1	1.8
AML, AMP, CN, E, FOX, NA, P, S, TE	1	1.8
AML, AMP, CN, E, NA, OX, P, TE, VA, W	1	1.8
AML, AMP, CN, E, OX, P, VA	1	1.8
AML, AMP, CN, E, P, S, TE, VA	1	1.8
AML, AMP, CN, E, P, TE, VA, W	1	1.8
AML, AMP, CN, FOX, NA, OX, P, S, TE	1	1.8
AML, AMP, CN, FOX, NA, P, S	1	1.8
AML, AMP, E, FOX, NA, OX, P, TE	1	1.8
AML, AMP, E, FOX, NA, P, TE, VA, W	1	1.8
AML, AMP, E, FOX, P, S, TE, VA	1	1.8
AML, AMP, E, NA, P, TE, VA, W	1	1.8
AML, AMP, E, OX, P, S, TE, VA, W	1	1.8
AML, AMP, E, OX, P, TE, VA	1	1.8
AML, AMP, E, OX, P, TE, VA, W	1	1.8
AML, AMP, E, P, S, TE, VA	1	1.8
AML, AMP, E, P, TE, VA	4	7.3
AML, AMP, E, P, TE, VA, W	3	5.5
AML, AMP, E, P, VA, W	1	1.8
AML, AMP, FOX, NA, OX, P, TE	1	1.8
AML, AMP, FOX, P, VA, W	1	1.8
AML, AMP, NA, OX, P, W	1	1.8
AML, AMP, P	1	1.8
AML, AMP, P, S, TE	1	1.8
AML, AMP, P, TE, VA, W	2	3.6
AML, AMP, P, W	1	1.8
AML, C, E, P, TE, VA	1	1.8
AML, E, OX, P, TE, VA	1	1.8
AML, E, OX, P, TE, VA, W	1	1.8
AML, E, P	1	1.8
AMP, C, E, NA, P, TE, VA, W	1	1.8
C, CIP, CN, E, NA, P, S, TE, W	1	1.8
C, CN, E, NA, OX, P, S, TE	1	1.8
C, CN, OX, P, S, TE, VA	1	1.8
C, E, NA, P, S, TE	1	1.8
Total	**55**	**100.0**

4.4 Determination of Methicillin-resistant *Staphylococcus aureus* and their association with mastitis in Kaduna State, Nigeria

Out of the 360 cow milk samples examined, 28 were found to be MRSA positive using ORSAB screening, 1 µg oxacillin discs and 30 µg cefoxitin discs respectively. This gives an overall MRSA prevalence rate of 7.8 % (Table 4.10). Kaduna South and Kagarko recorded the highest prevalence rate of 13.3% each, while Sabon Gari and Zaria followed suite with 6.7% prevalence each (Table 4.10). Giwa recorded 5.0% prevalence while Igabi the recorded the least (1.7%) (Table 4.10).

Occurrence of MRSA in respect to mastitis showed that 12 (42.9%) of the 28 MRSA were associated with mastitis (Table 4.11). Four (4) of the MRSA from Kaduna South and Kagarko were associated with mastitis, 2 isolates were associated with mastitis in Zaria and 1 each from Giwa and Igabi respectively. None of the 4 MRSA isolates from Sabon Gari is associated with mastitis (Table 4.11).

Table 4.10: Prevalence of *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA) in cow milk in Kaduna State, Nigeria

Location	**No. of milk examined**	**No. (%) positive for *S. aureus***	**No. (%) positive for MRSA**
Giwa	60	3 (5.0)	3 (5.0)
Kaduna South	60	11 (18.3)	8 (13.3)
Igabi	60	7 (11.7)	1 (1.7)
Kagarko	60	16 (26.7)	8 (13.3)
Sabon-Gari	60	10 (16.7)	4 (6.7)
Zaria	60	8 (13.3)	4 (6.7)
Total	**360**	**55 (15.3)**	**28 (7.8)**

Table 4.11: Prevalence of MRSA in relation to mastitis in cow milk in settled Fulani herds in Kaduna State, Nigeria

Location	No. of milk examined	No. (%) with mastitis	No. (%) with MRSA	No. (%) of MRSA with mastitis
Giwa	60	15 (25.0)	3 (5.0)	1 (33.3)
Kaduna South	60	21 (35.0)	8 (13.3)	4 (50.0)
Igabi	60	18 (30.0)	1 (1.7)	1 (100.0)
Kagarko	60	19 (31.7)	8 (13.3)	4 (50.0)
Sabon-Gari	60	8 13.3)	4 (6.7)	0 (0.0)
Zaria	60	16 (26.7)	4 (6.7)	2 (50.0)
Total	**360**	**97 (26.9)**	**28 (7.8)**	**12 (42.9)**

4.5 Antibiotic susceptibility of Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from cow milk in Kaduna State, Nigeria

The resistance patterns of the 28 MRSA isolates showed that high frequency of resistance was recorded against penicillin 28 (100.0%), amoxicillin 23 (89.3%), ampicillin 23 (89.3%), tetracycline 24 (85.7%) and erythromycin 20 (71.1%) (Table 4.12). High resistance was also recorded against nalidixic acid 15 (53.6%) and streptomycin 12 (42.9%) (Table 4.12).

The multiple drug resistance indices of the MRSA strains are shown in Table 4.13, and defined as the number of antibiotics to which an isolates is resistance divided by total number of antibiotics tested. The results revealed that all the 28 MRSA strains were resistant to 5 or more antibiotics tested, 6 (21.4%) strains were resistant to 6 antibiotics, 6 (21.4%) were resistant to 7 antibiotics, 8 (28.6%) were resistant to 8 antibiotics and 4 (14.3%) were resistant to 9 antibiotics (Table 4.13).

The multiple resistance patterns of the MRSA encountered showed that 26 (92.9%) exhibited different resistance patterns to each other, while only four (4) of the isolates exhibited common patterns of multiple resistance. The 2 patterns encountered were AML,AMP,C,CN,E,NA,P,S,TE exhibited by isolates K23 and K42 and AML,AMP,E,P,TE,VA,W exhibited by isolates K3 and S26 (Table 4.14). The multiple antibiotics resistance (MDR) classification revealed that 19 (67.9%) exhibited the extensive drug classification pattern while the rests exhibited mltidrug resistance patterns (Table 4.13).

Table 4.12: Antibiotic susceptibility of MRSA isolated from cow milk in settled Fulani herds, Kaduna State, Nigeria (N=28)

Antibiotic	No. (%) isolates susceptible	No. (%) isolatesintermediate resistant	No. (%) isolates resistant
Amoxicillin	3 (10.7)	0 (0.0)	25 (89.3)
Ampicillin	3 (10.7)	0 (0.0)	25 (89.3)
Chloramphenicol	17 (60.7)	3 (10.7)	8 (28.6)
Ciprofloxacin	26 (92.9)	2 (7.1)	0 (0.0)
Erythromycin	7 (25.0)	1 (3.6)	20 (71.4)
Gentamicin	16 (57.1)	1 (3.6)	11 (39.3)
Nalidicic acid	13 (46.4)	0 (0.0)	15 (53.6)
Penicillin	0 (0.0)	0 (0.0)	28 (100.0)
Streptomycin	16 (57.1)	0 (0.0)	12 (42.9)
Tetracycline	4 (14.3)	0 (0.0)	24 (85.7)
Trimethoprim	18 (64.3)	3 (10.7)	7 (25.0)
Vancomycin	13 (46.4)	0 (0.0)	15 (53.6)

Table 4.13: Antibiotic resistance profiles (antibiogram), antibiotic classification and multiple antibiotic resistance index of MRSA isolated from cow milk in Kaduna State, Nigeria (N=28)

MRSA isolate	Resistance pattern	NART	ARC	MARI
K3	AML,AMP,E,P,TE,VA,W	7	XDR	0.6
K14	AML,AMP,C,CIP,CN,R,P,S,TE	9	XDR	0.8
K20	AML,AMP,E,P,S,TE,VA	7	XDR	0.6
K23	AML,AMP,C,CN,E,NA,P,S,TE	9	XDR	0.8
K32	C,CN,E,NA,P,S,TE	7	MDR	0.6
K34	AML,C,E,P,TE,VA	6	MDR	0.5
K42	AML,AMP,C,CN,E,NA,P,S,TE	9	XDR	0.8
K57	AML,AMP,C,E,P,TE,VA	7	XDR	0.6
S1	AML,AMP,C,CN,E,P,TE,VA	8	XDR	0.7
S2	AML,AMP,C,CN,E,NA,P,TE	8	XDR	0.7
S5	C,CN,P,S,R,VA	6	MDR	0.5
S6	AML,AMP,C,CN,NA,P,S,TE	8	XDR	0.7
S20	AML,AMP,CIP,E,NA,P,TE	7	XDR	0.6
S26	AML,AMP,E,P,TE,VA,W	7	XDR	0.6
S42	AML,AMP,E,P,W	5	MDR	0.4
S60	AML,AMP,E,NA,P,TE	6	MDR	0.5
I59	AML,AMP,C,E,P,TE,VA,W	8	XDR	0.7
SB24	AML,AMP,CN,E,NA,P,S,TE	8	XDR	0.7
SB34	AML,AMP,CN,NA,P,S,TE	7	XDR	0.6
SB51	AML,AMP,E,NA,P,TE,VA,W	8	XDR	0.7
SB60	AML,E,P,TE,VA,W	6	MDR	0.5
G3	AML,AMP,NA,P,S	5	MDR	0.4
G23	AML,AMP,NA,P,TE	5	MDR	0.4
G25	AML,AMP,P,VA,W	5	MDR	0.4
Z10	AML,AMP,CN,E,P,VA	6	MDR	0.5
Z39	AML,AMP,E,P,S,TE,VA,W	8	XDR	0.7
Z47	AML,AMP,C,NA,P,TE,VA,W	8	XDR	0.7
Z49	AML,AMP,CN,E,NA,P,TE,VA,W	9	XDR	0.8

Key: NART = number of antibiotics resistant to, ARC = antibiotics resistance classification, MARI = multiple antibiotics resistance index.

4. 6 Determination of minimum inhibitory concentration (MIC) of two antibiotics

The Minimum Inhibitory Concentration (MIC) results of oxacillin and vancomycin showed no zone of inhibition along the entire length of M.I.C.E. strips for all the MRSA against oxacillin, the MIC was read as greater than the highest value on the strip (256 μg/mL), i.e. the MIC values of the antibiotics showed that all (100%) had values greater than 256 μg/mL against oxacillin, hence all isolates were resistant to oxacillin by MIC test (Table 4.14). Fifteen (53.6%) of the MRSA isolates showed no zone of inhibition along the entire length against vancomycin; i.e they have values greater than 256 μg/mL against vancomycin (Table 4.12). Thirteen (46.4%) had MIC values <4 μg/mL against vancomycin and were considered susceptible (Table 4.14).

4.7 Determination of penicillin binding protein 2a (PBP2A) among Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from cow milk in Kaduna State, Nigeria

All the 28 MRSA isolates were tested with the PBP2a Latex Agglutination Test for Penicillin Binding Protein 2a (PBP2a) (Table 1.14). Nineteen (67.9%) gave strongly positive (3+) reactions in the Latex Agglutination Test, 5 (1.9%) were weakly positive (1+) in the Latex Agglutination Test, while 4 (14.3%) were negative. These isolates were considered LAT negative in the data analysis.

Table 4.14: Penicillin Binding Protein 2a (PBP2a) and minimum inhibitory concentration (MIC) of oxacillin and vancomycin of the MRSA isolated from cow milk in settled Fulani herds, Kaduna State, Nigeria (N=28)

	PBP2a		**MIC (µg/mL) OX**		**MIC (µg/mL) VA**	
No. (%) Positive	No. (%) weak Positive	No. (%) Negative	No. (%) =256	No. (%)= <256	N0. (%)= 256	No. (%)= <4
19 (67.9)	**5 (17.9)**	**4 (14.3)**	**28 (100)**	**0 (0)**	**15 (53.6)**	**13 (46.4)**

4.8 Molecular characterization of *Staphylococcus aureus* and MRSA isolates

4.8.1 Detection of *mec* A, *mec* C and *fem* B genes by multiplex polymerase chain reaction

Twent eighty (28) MRSA isolates were examined for the presence of *mec* A, *mec* C and *fem* B genes, of which only 3 isolates were positive for *fem* B gene. None of the isolates was positive for *mec* A and *mec* C genes (Plate 4.11).

4.9. Bioinformatics

4.9.1 Multilocus Sequence typing (MLST) of the MRSA isolates

Fourteen housekeeping gene fragments were sequenced from three *S. aureus* isolates. These seven housekeeping gene fragments, which were about 450 bp (Table 4.15), were then sequenced from each of the isolates. For each isolate, the sequences obtained at each of the seven loci were compared with those of every other isolate, and the alleles were numbered consecutively. Sequences were assigned as distinct alleles even if they differed at a single nucleotide site; no weighting was applied to reflect the number of nucleotide differences between alleles (Table 4.15). The *S. aureus* genes were relatively uniform; there were no polymorphic (variable) nucleotide sites at the seven loci. Visual inspection of the sequences suggested that there was no distribution of polymorphic sites along each gene fragment.

Typing, using MLST revealed that all the 3 isolates were MLST type 1 (ST 1) and which had similar allelic profiles at all seven loci, hence they showed high degree of monomorphism (Table 4.15). All the three (3) isolates (23448_1#128, 23448_1#129 and 23448_1#131) were isolated from cases of subclinical mastitis (Table 4.16).

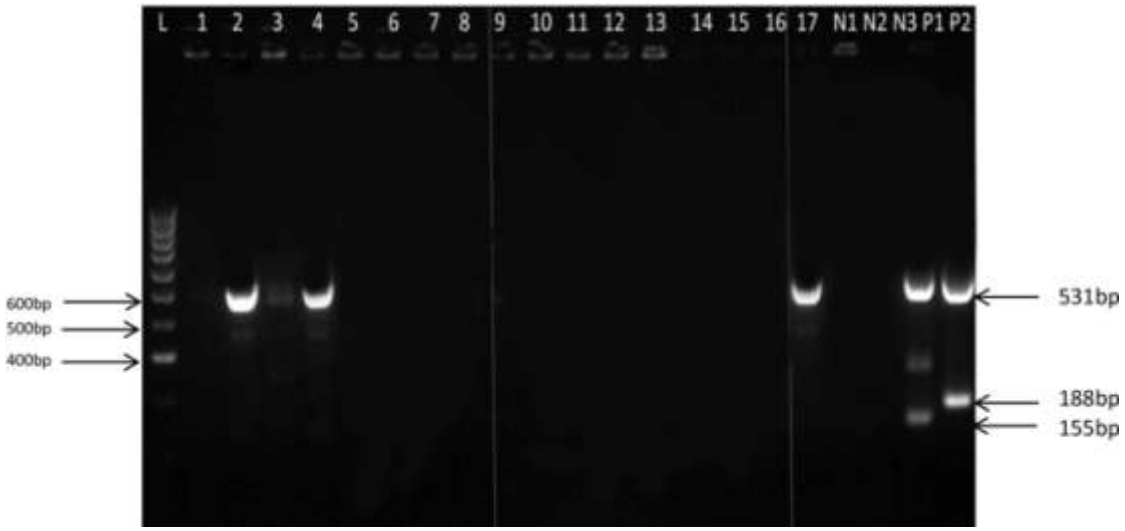

Plate 4.1: *mec* A (188bp), *mec* C (155bp) and fem B 531 (bp) multiplex PCR. L= Molecular Ladder, N1= *mec* A negative control, N2= *mec* C negative control, N3= *fem* B negative control, P1= *fem* B positive control, P2= *mec* A positive control and number 1 to 17 are samples both positive and negative. Lane 2, 4 and 17 are *fem* B positive

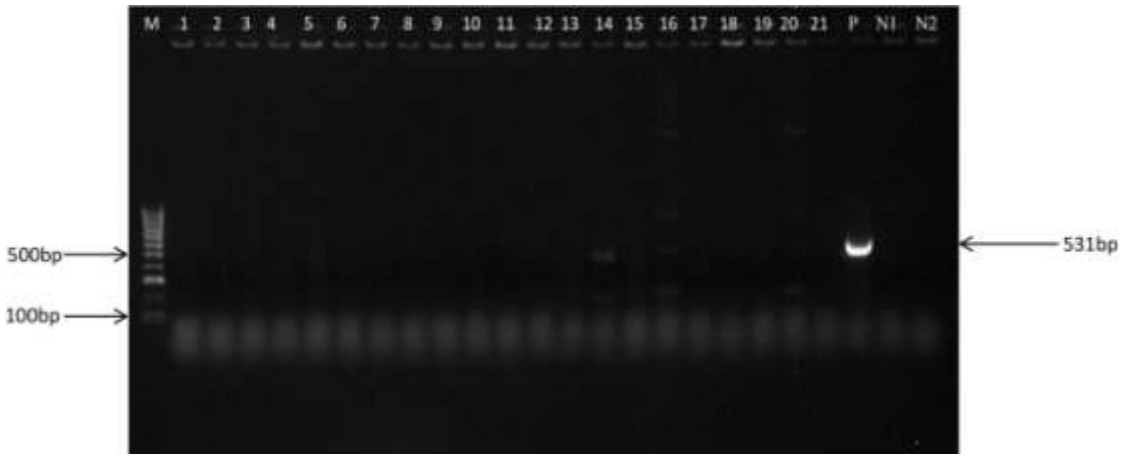

Plate 4.2: M= Molecular ladder, Lane 1-21= Samples P= *fem* B Positive control N1 and N2= *fem* B negative controls. All isolates are negative for *mec* A, *mec* C and *fem* B genes.

TABLE 4.15: Sequence variation at the seven loci

Gene	Sequence length (bp)	No. of alleles	No. of polymorphic sites
Arc	456	17	No
aroE	456	17	No
glpF	465	11	No
Gmk	429	11	No
Pta	474	15	No
Tpi	402	14	No
yqiL	516	16	No

Table 4.16: **Properties of the 3 sequence types**

Isolate	ST	Allelic profile (allele no) [a]							CC	Origin
		*aro*C	*aro*E	*glp*F	*gm*k	*Pta*	*tpi*	*yqi*L		
23448_1#128	1	1	1	1	1	1	1	1	1	Community
23448_1#129	1	1	1	1	1	1	1	1	1	Community
23448_1#131	1	1	1	1	1	1	1	1	1	Community

[a]Allelic profiles are read from left to right and correspond to genes *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *ygiL*.

[b]CC, clonal complex

Table 4.17: Resistance profile and Multi-locus sequence iype (MLST) of the Isolates

Isolate	Type of mastitis	Resistance profile	MLST
23448_1#128	Subclinical	AML,AMP,CN,E,FOX,NA, OX,P,S,TE	1
23448_1#129	Subclinical	AML,AMP,E,FOX,NA,OX,P,TE,VA,W	1
23448_1#131	Subclinical	AML,AMP,CN,E,FOX,NA,OX,P,TE,VA,W	1

Table 4.18: Phenotypic properties of the 3 *S. aureus* isolates from cow milk in settled Fulani herds, Kaduna State, Nigeria

Isolate	Cat.	Coag.	DNase	Haemol	Mannitol ferm	Pigm
23448_1#128	+ve	+ve	+ve	Alpha	+ve	+ve
23448_1#129	+ve	+ve	+ve	Alpha	+ve	+ve
23448_1#131	+ve	+ve	+ve	Alpha	+ve	+ve

Key:
Cat- Catalse
Coag- Coagulase
Haemo- Haemolysis
Ferm- Fermentation
Pigm- Pigmentation

4.10 Phylogenetic studies of the isolates

The phylogenetic relationship was established using the nucleotide sequences of the type strains defining the genus *Staphylococcus* (BX571857, BA000033, AP015012, CP017115 and CP01780). A blastn search of all nucleotide sequence of 23448_1#128, 23448_1#129 and 23448_1#1311 using default parameters revealed 99 % identity with the available *S. aureus* (BX571857, BA000033, AP015012, CP017115 and CP01780) genomes in the database (Fig. 4.11). In addition, the nucleotide sequences of three (3) *S. aureus* isolates (23448_1#128, 23448_1#129, 23448_1#131) were extracted, and a neighbor joining phylogenetic tree was constructed with Mega v.6.06 (Fig. 4.11). The three illustrates the close relationship of *S. aureus* 23448_1#128, 23448_1#129 and 23448_1#1311 with *S. aureus* isolates from BX571857, BA000033, AP015012, CP017115 ad CP01780 (Fig. 4.11). The position relative to other species within the genus *Staphylococcus*is also illustrated. *Enterococcus faecium* strain SM KX430929) was used as outgroup for the genus *Staphylococcus*.It can also be shown from the tree that all the 3 *Staphylococcus aureus* obtained were 100 % homologous to each other with no divergence within the specie level. They clustered together more tightly as compared to those obtained from Genbank. However, those obtained from Genbank showed some levels of divergence ranging from 11 to 19 %. The sequence of *Staphylococcus aureus* from the present study were in different branch from those in the GenBank signifying a significant level of within specie divergence between our isolates with those of the Genbank.

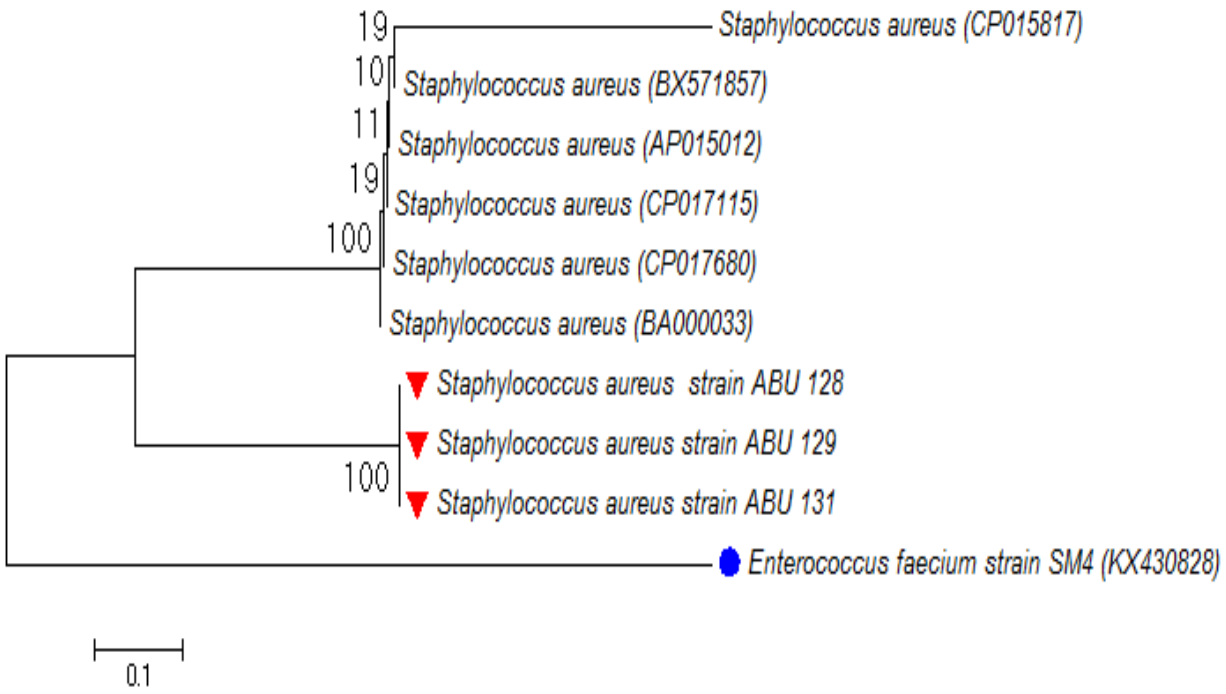

Fig. 4.1: Neighbour-joining phylogenetic tree (1000 bootstrap replicates) of *Staphylococcus aureus* based on nucleotide residues of the ABU 128, ABU 128 and ABU 131 genes computed using the p-distance model (Felsenstein, 1985; Saitou and Nei, 1987). The *Staphylococcus aureus* haplo types isolated from milk in this study are indicated (ABU 128, ABU 128 and ABU 131 series) and those *Staphylococcus aureus* obtained from the NCBI GenBank are with the accession numbers in parenthesis. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*,2013).

CHAPTER FIVE

5.0 DISCUSSION

The present study was carried out on settled Fulani cattle herds in Kaduna State to determine the occurrence of Methicillin resistant *S. aureus* (MRSA) in fresh cow milk and its association with mastitis and to characterize the isolates using molecular techniques. The status of mastitis in cow/quarters was first assessed by California Mastitis test (CMT) and microbiological analysis of both suspected and apparently healthy milk samples for *Staphylococcus* spp. The prevalence of clinical mastitis at cow level was 26.9% (n=97) (Table 4.1). The prevalence of subclinical mastitis as detected by CMT was 23.9% while that of the clinical mastitis was 3.1% (Table 4.3). The prevalence in the present study is lower than the 52% reported by Junaidu *et al.* (2012) in Sokoto, Nigeria, 85.33% by Shittu *et al.* (2012) in the Savannah region of Nigeria, 30.9% by Suleiman *et al.* (2013) in Plateau Sate, Nigeria and 71.0% by Mekibib *et al.* (2010) in Ethiopia. However, the result is higher than 2.1% reported by Ameh *et al.* (1999) in Maiduguri, Borno State, Nigeria and those reported elsewhere (19.9%, 2.12% and 21.6% respectively in Bangladesh, Sirajganj and Iran) (Islam *et al.,* 2010; Hashemi *et al.,* 2011). The discrepancies in the results above could be due to differences in the management systems, stage of lactation, parity and breed of the dairy animals as has been reported also by Hashemi *et al.* (2011).

The over all quarter prevalence of mastitis in the present study was 21.7% (312/1440) with 6.5% (28/1440) as clinical mastitis and 19.7% (284/1440) as subclinical mastitis respectively. This is comparable with the reports of Ameh *et al.* (1999), but differ with the 44.9% and 43.25% overall quarter prevalences reported by Mekibib *et al.* (2010) and Shittu *et al.* (2012) in Holeta town, Central Ethiopia and Savannah region of Nigeria respectively.

The traditional method and protocol of milking cows was the method practiced in all the herds sampled, thus milking hygiene was generally not satisfactory in some of the farms sampled. Mastitis and infection preventive measures like the use of udder antisepsis, hand disinfectants, post-milking teat dipping, dry cow therapy and intensive surveillance were also not practiced in these herds. This may be the possible reasons for the high prevalence of mastitis in these areas. Another reason could be the traditional practice of keeping calves away from the dam for long periods and only allowed to suckle for a short period coupled with low milk supply that resulted in the calves suckling vigorously leading to teat injuries which subsequently predisposed to infection (Junaidu *et al.,* 2013).

In this study, the prevalence of subclinical mastitis (21.5%) was higher than clinical mastitis (2.8%) as observed by many researchers (Ameh *et al.,* 1999; Mekibib *et al.,* 2010; Islam *et al.,* 2010; Hashemi *et al.,* 2011; Suleiman *et al.,* 2013). The possible

explanation is that clinical mastitis is characterized by gross visible pathologies which are familiar to the farmers and so are treated as soon as noticed. It was also shown that subclinical mastitis is normally found to be higher than clinical mastitis due to host's defense mechanism that reduces the severity of the disease (Mekibib *et al.,* 2010). The CMT results showed that 55 (64.0%) of the milk samples had a score of 1+, 26 (30.2%) had a score of 2+, 3 (3.5) had a score of 3+, while only 2 (2.3%) had a score of "Trace" (Table 2). This agrees with the acceptable fact that the CMT score of 1+ which corresponds to somatic cell count SCC of 500, 000 is the threshold index of intra mammary infection (IMI).

Statistical analysis showed that there is significant difference in the occurrence of mastitis among the age groups ($P<0.05$). The prevalence at quarter level was 25.2% (157/624) in adult cows, 20.2% (97/480) in young adults and 17.3% (58/3360 in old cows (Table 4). Similar pattern of occurrence was recorded for both clinical and subclinical mastitis as the adults had 21.8% and 3.4 %, young adults had 19.8% and 0.4% and old cows had 15.8% and 1.5% respectively. This trend is in line with the reports by Ameh *et al.* (1999) who recorded high occurrence of mastitis in adult cows (n=50) than in young cows (n=25) and old (n=8). Mekibib *et al*. (2010) also showed that the likelihood of mastitis in adult cows was two-foldmore than in young adults. They reported the prevalence to be 56.0% in adults, 33.5% in young adults and 7.3% in old cows. It was observed that there was increase in cases of mastitis (clinical and subclinical) with advancing age. This trend was observed in the present study as the

prevalence of mastitis increased from young adults to adult cows and decreased when the cows were old.

Even though, other pathogens were not identified, laboratory results showed that *Staphylococcus aureus* and coagulase negative staphylococci (CONS) were associated with mastitis in this study. Sixty seven samples (69.1%) of the CMT positive samples were found to be culture positive, out of which 56.7% (55/97) were *S. aureus* and 12.3% (12/97) were coagulase negative staphylococci (Table 3). Ameh *et al.* (1999) showed that coagulase negative staphylococci and *S. aureus* occurred predominantly in both clinical and sub-clinical bovine mastitis. In this study, 56.7% and 12.3% of *S. aureus* and CONS were respectively isolated from the 97 mastitic milk samples, showing that 69.0% of the mastitis was associated with staphylococci. This may mean that staphylococci were not the only bacterial agents responsible for both clinical and subclinical mastitis in this study, hence other pathogens and agents were likely responsible for the remaining 31.0%. This result is of public health concern due to the involvement of *S. aureus* in food poisoning outbreaks. The study is also in agreement with Mekibib *et al.* (2010) who revealed that *S. aureus* and CONS were the predominant bacteria isolated in mastitic milk in Ethiopia with a 47.1% and 30.1% prevalence rates respectively. A report by Suleiman *et al.* (2013) showed that the prevalence of *S. aureus* in bovine mastitic milk was 98.1% in Plateau State, Nigeria and suggested that most of the *S. aureus* were of human origin since *S. aureus* of animal origin coagulate bovine plasma, produced beta-hemolysis and were less often

enterotoxigenic than the human isolates. Several studies have also confirmed that staphylococci are the predominant bacteria isolated from bovine mastitis, and thus remains the leading cause of clinical and subclinical mastitis (Ameh *et al.,* 1999; Hashemi *et al.,* 2011; Junaidu *et al.,* 2011; Ridwana *et al.,* 2017; Shrivastava *et al.,* 2018).

Milk is a good medium for the growth and multiplication of several bacteria among which staphylococci ranks the first (Alien *et al.,* 2012). Contamination of milk by staphylococci is normally through infection of the mammary gland via teat canal, milkers, human handlers, milking equipment and from the environment (Alien *et al.,* 2012). The contamination and presence of staphylococci are of public health significance due largely to their role in food poisoning.

The presence and antibiotic resistant of *S. aureus* in cow milk, milk products and other foods have been studied and documented in Nigeria and elsewhere (Fagundes *et al.,* 2010; Daka *et al.,* 2012; Alien *et al.,* 2012; Umaru *et al.,* 2012; Maduka *et al.,* 2013; Maduka *et al.,* 2014; Ridwana *et al.,* 2017; Shrivastava *et al.,* 2018). In this study, out of the 360 cow milk samples examined, 55 were positive for *S. aureus* giving a prevalence of 15.3% (Table 4.5). This is in line with previous studies in which 12.6% and 13.2% prevalence of *S. aureus* were respectively detected in milk samples in Zaria and Kaduna, Nigeria and in Iran (Umaru *et al.,* 2012; Alien *et al.,* 2012). However, the

situation was different in Sao Paulo, Brazil, Hawassa Area, South Ethiopia, Kashmir, India, and North-Western Greece, where Fagundes *et al.* (2010), Daka *et al.* (2012), Ridwana *et al.* (2017) and Papadopoulos *et al.* (2018) reported a prevalence of 6.7%, 48.8%, 46.15% and 57.8% of *S. aureus* in milk samples respectively. The differences could be largely due to differences in type of husbandry system, the breeds of the cattle, the sanitary conditions and the milking procedures. In the present study, the traditional hand milking was the method used in all the herds sampled and no hygienic measures such as teat dipping and disinfection of utensils, containers and personnel hands was practiced prior to milking procedures. In several cases, untreated ground water was used to wash the utensils, containers and hands of the milkers. This may probably have resulted in cross contamination which contributed to the high occurrence of *S. aureus* in the milk samples. Therefore, improving the general herd hygiene, milking environment, containers and utensils can reduce the contamination of milk by *S. aureus* and possibly prevent its spread to other animals and human population.

In this study, it was observed that all the *S. aureus* isolates coagulated rabbit plasma and produced DNase, while significant number produced beta haemolysin (45.5%) and alpha haemolysin (40.0%) and relatively small percentage produced gamma haemolysin (14.5%) (Table 4.6). Such results show the potential of virulence and pathogenicity of these isolates and therefore of serious public health concern. This trend was also reported by Omoshaba *et al.* (2018) who showed that haemolysis of 52

Staphylococcus aureus isolates on 5% sheep blood agar revealed that 28 (53.9%) showed complete haemolysis, 15 (28.9%) partial haemolysis and 9 (17.3%) no haemolysis. In contrast, the DNAse result showed that 20 (38.5%) of the *Staphylococcus aureus* isolates were positive while 32 (61.5%) were negative. Fourty (40) (76.9%) of the *S. aureus* isolates were positive for slide coagulase while all the 52 (100%) of the isolates were positive for tube coagulase.

It can be inferred from this study that most of the *S. aureus* isolates are of human biotypes, since they are known to produce alpha haemolysin which are more toxigenic than the animal biotypes which are known to produce beta haemolysin and less toxigenic. It can also be concluded from this study that the contamination of the cow milk may have been from both human and animal sources. This contamination of milk by alpha and beta haemolytic *S. aureus* have been reported by Umoh (1989) in Fura-da-nono in Zaria, Nigeria and Oranusi *et al.* (2006) in food contact surfaces and foods prepared by families in Zaria, Nigeria.

The antibiotic resistance profiles of the 55 *S. aureus* isolates showed that all the isolates were resistant to two or more antibiotics (Tables 4.7, 4.8 and 4.9). The highest resistance was recorded against penicillin (100.0%), ampicillin (90.9%), amoxicillin (85.5%), tetracycline (81.8%) and erythromycin (75.5%) (Table 4.7). This report is in line with our previous studies and those of others in which resistance to β-lactam

antibiotics such penicillin, ampicillin, tetracycline and oxacillin were prevalent. (Umaru *et al.,* 2012; Alien *et al.,* 2012; Daka *et al.,* 2012). In our previous study, (Umaru *et al*., 2012) involving fresh and fermented milk in Kaduna and Zaria, Nigeria, the highest rate of resistance was recorded against penicillin (100%), oxacillin (46.8%), and amoxicillin (44.7%) while the lowest was demonstrated against amikacin (2.1%), chloramphenicol (4.3%) and sulphamethoxazole/trimethoprim (6.4 %). Alien *et al.* (2012) showed in their study on bovine, sheep and goat raw milk in Iran that resistance to ampicillin was the most common (54.3%), followed by oxacillin (28.3%), tetracycline (26.1%), penicillin G (23.3%) and erythromycin (23.9%). Also, Daka *et al.* (2012) in their study observed resistance in penicillin G (67.9%), ampicillin (70.9%), oxacillin (60.3%), amoxicillin (30.9%) and erythromycin (32.1%). The findings are also similar to those reported by Omoshaba *et al.* (2018) who reported the rate of antimicrobial resistance among 52 isolates of *Staphylococcus aureus* in fresh milk and *wara* as follows: ceftazidime (100%), ampicillin (98.1%), augmentin (96.2%), cefoxitin (96.2%), colistin (96.2%), oxacillin (96.2%), sulphamethoxazole (82.7%), tetracycline (76.9%), gentamicin (61.5%), doxycycline (50%) and streptomycin (38.5%). They reported that 15 (88.2%) out of 17 *S. aureus* isolates from fresh milk and all the 35 (100%) isolates from *wara* showed resistance to oxacillin and cefoxitin, suggesting methicillin-resistance attribute.

The high rate of resistance to penicillin, ampicillin, amoxicillin and tetracycline in the present study may be due to abuse, misuse and mismanagement of these drugs because

they are readily available and in common use in both human and veterinary medicine in the study areas. What is of great concern is the high number of the isolates resistant to erythromycin (75.5%). Erythromycin is not frequently used in veterinary medicine in this area, and so high resistance could be due to transfer of genes carried on plasmid between different strains of bacteria (Olayinka *et al*, 2009; Alien *et al.*, 2012).

The multiple drug resistance of the *S. aureus* strains, defined as resistance of an isolate to 3 or more antibiotics revealed that all the 55 *S. aureus* strains were resistant to 3 or more antibiotics tested, 2 strains were resistant to 3 antibiotics while 7 isolates were resistant to 10 or more antibiotics respectively (Tables 4.8 and 4.9). This result differs from our previous study in which 37 (78.7%) of the isolates exhibited multi-drug resistant pattern with tetracycline- penicillin (53.2%), tetracycline- penicillin-erythromycin (31.9%) and tetracycline- penicillin-erythromycin-oxacillin-vancomycin (25.5%) (Umaru *et al.*, 2013). The reasons for the disparity could be due to increased number of antibiotics tested in the present study and also the fact that only fresh milk was examined compared to the previous one in which both fresh and fermented milk were examined. This observation is of serious concern and may mean that the sources of milk contamination in the study areas may be reservoirs of multidrug resistant *S. aureus.* The abuse and indiscriminate use of antibiotics in animals and also their use in animal husbandry should be discouraged because resistant pathogens such as *S. aureus* can be transmitted to humans through the consumption of foods contaminated by such resistant pathogens (Alien *et al.*, 2012).

The present study revealed that cow milk in the study areas are contaminated with resistant strains of *S. aureus* which were considered as animal and human biotypes and can serve as means of subsequent spread to humans through the consumption and handling of raw milk. This risk can be eliminated through improving the general herd hygiene, proper management practices, proper milking hygiene and pasteurization of milk.

Out of the 360 cow milk samples examined, twenty-eight (28) MRSA were isolated using ORSAB screening, 1 µg oxacillin discs and 30 µg cefoxitin discs respectively, giving an overall MRSA prevalence rate of 7.8 % (Table 4.10). Kaduna South and Kagarko recorded the highest prevalence rate of 13.3% each, while Sabon Gari and Zaria followed with 6.7% prevalence each (Table 4.10). Giwa recorded 5.0% prevalence while Igabi recorded the least (1.7%) (Table 4.10).

The prevalence of MRSA (7.8%) was somewhat higher when compared to 1.4% in raw milk in Switzerland (Huber *et al.,* 2010), 4% in Bulk Tank Milk from Minnesota Dairy Farms (Haran *et al.,* 2012), 2.15% in bovine bulk tank milk in Great Britain (Paterson *et al.*, 2013), 4.8% in our previous study in Zaria and kaduna respectively (Umaru *et al.,* 2013), and 4.4% in bovine milk in Tanzania (Mohammed *et al.,* 2018). The prevalence is however lower than 11.25% in dairy farms of Pokhara, Nepal (Joshi *et al.,* 2014), 35.6% in Plateau State, Nigeria (Suleiman *et al.*, 2012a), and 22.30% in

bovine milk, in Kashmir, India (Ridwana *et al.,* 2018). Similarly, the present result (7.8%) is lower than the 25% obtained by Omoshaba *et al.* (2018) in raw milk and soft cheese (*wara*) sold in Abeokuta, Nigeria and 56.1% in raw milk and bulk tank milk in South-west Uganda (Assinwe *et al.,* 2017). The difference in prevalence was statistically significant, although the reasons for this are not obvious, especially as MRSA has been isolated from milk and other animals in Nigeria including humans (Umaru *et al.,* 2013; Bala *et al.,* 2016). Possible explanation for the significant occurrence of MRSA in milk in this part of Nigeria may be due to unrestricted and uncontrolled use of antibiotics in animals and farming, an unsatisfactory health status of cattle herds compared to many countries in Europe and U.S.A., and also the fact that greater percentage of cattle herds are extensively managed, which exposed them to contaminated environment. Also, the two study areas have the largest hospitals in Kaduna State, and MRSA of hospital origin being nosocomial pathogens may be transferred from the hospitals to the environment from where animals can then be infected.

Occurrence of MRSA in respect to mastitis showed that 12 (42.9%) of the 28 MRSA were associated with mastitis (Table 4.11), 4 each from Kaduna South and Kagarko, 2 from Zaria and 1 each from Giwa and Igabi respectively. None of the 4 MRSA isolates from Sabon Gari was found to be associated with mastitis (Table 4.11). This may represent the first report of MRSA strains being reported from cases of bovine mastitis in the study areas to the best of our knowledge. The finding agrees or

coincides with several other studies. For example, Türkyilmaz *et al.* (2010) reported that 16 of the 93 *S. aureus* isolated from bovine mastitic milk were resistant to methicillin. Also, in a study by Itrakamhaeng *et al.* (2012), it was discovered that 4 of the 375 *S. aureus* from bovine mastitis cases were methicillin resistant. Similar report was observed in Nigeria by Suleiman *et al.* (2012a) who revealed that of the 103 *S. aureus* isolated from mastitic milk, 26 (35. 6%) were resistant to oxacillin. Also, MRSA associated with cases of mastitis in the human has been documented. Similarly, Nam *et al.* (2011) in Korea discovered 6.6% of the isolated MRSA to be associated with mastitis. MRSA has also been reported to be associated with mastitis in humans. For example, Reddy *et al.* (2007) reported 21 MRSA in 48 cases of *S. aureus* postpartum mastitis during 1998-2005 in Chicago, Illinois, USA. The present study also concurs with the report of Helal *et al.* (2015) who confirmed 7 (77.8%) MRSA isolates out of total 9 *S. aureus* isolates from cow mastitic milk, while, only 2 (22.2%) *S. aureus* isolates showed non-MRSA isolates. *S. aureus* is one of the most important bacterial pathogens in bovine mastitis, a disease that causes significant economical losses in the milk industry; thus, *S. aureus* in general and MRSA in particular have been the focus of several studies in dairy cattle.

The occurrence and spread of MRSA in milk and dairy animals is of serious public health concern because of possible transmission between cows and humans (Itrakamhaeng *et. al.,* 2012). According to the report of Juhász-Kaszanyitzky *et al.* (2007), Erdem and Türkyilmaz (2013) and Helal *et al.* (2015) MRSA strains of bovine

and human origins from their studies were epidemiologically related and indistinguishable, which indicated that transmission from either cow to human or human to cow occured. In addition, MRSA may contaminate foods and represents a source of MRSA infection and intoxication. The significant number of MRSA isolated from cow milk in these areas was probably caused by excessive therapeutic use of antibiotics in both human and veterinary practice. It is widely recognised that the widespread use of antibiotics has generated selective pressures on *S. aureus* that have driven the emergence of methicillin resistant by acquisition of *mec* A gene. This gene encodes a Penicillin Binding Protein (PBP2a) with low affinity for β-lactams (Fisher and Mobashery, 2017; Foster, 2017).

The resistance patterns of the MRSA isolates showed high rate of resistance against penicillin 28 (100.0%), amoxicillin 23 (89.3%), ampicillin 23 (89.3%), tetracycline 24 (85.7%) and erythromycin 20 (71.1%) (Table 4.12). High resistance rates were also recorded against nalidixic acid 15 (53.6%), streptomycin 12 (42.9%) and gentamicin 11 (39.3%) (Table 4.12). This report is similar to our previous study (Umaru *et al.*, 2013) in the same area in which high resistance rate was recorded against Penicillin (100%), Tetracycline (55.5%), Oxacillin (55.6%), Vancomycin (44.4%), Amoxicillin (38.9%) and Erythromycin (27.8%). Türkyilmaz *et al.* (2010) also showed the MRSA strains to be multi-drug resistant with susceptibility rates to antimicrobials to be 0%, 0%, 0%, 0%, 6.25%, 16.25% and 56.25% for erythromycin, clindamycin, chloramphenicol, gentamicin, tetracycline, ciprofloxacin and vancomycin,

respectively. All tetracycline and gentamicin resistant strains carried *tet* (M) and *aac* (6)-aph (2) genes, respectively, and among macrolide-resistant isolates, nine had *erm* (A), and seven had both *erm* (A) and *erm* (B) genes. Bala *et al.* (2016), in their study on molecular characterization of methicillin resistant *S. aureus* from poultry farms in Kano State, Nigeria observed that the methicillin resistant isolates exhibited 100% resistant to ampicillin and amoxicillin, 93.3% to oxytetracycline, 90% to chloramphenicol, 80% to erythromycin, 76.7% to oxacillin, 63.3% to trimethoprim/sulphamethoxazole, 30% to ciprofloxacin and 26.7% to gentamicin. Similarly, Safarpoor *et al*. (2017) in their study discovered that all the MRSA isolated from soup, salad and rice samples in hospital to be resistant to penicillin (100%), ceftaroline (100%), tetracycline (100%), erythromycin (89.18%) and trimethoprim-sulfamethoxazole (83.78%).

The multiple drug resistance profiles of the MRSA strains are shown in Table 4.13, and defined as resistant of an isolate to 3 or more classes of antibiotics. All the MRSA strains were found to be resistant to 5 or more antibiotics tested. The multiple antibiotic resistance was classified as Extensive Drug Resistance (XDR), which is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and Pan Drug Resistance (PDR) which is the non-susceptibility of an isolate to all antimicrobial agents listed (Magiorakos *et al.*, 2012). The results showed that 19 (67.9%) exhibited the extensive drug classification pattern while the rests (32.1%)

exhibited multidrug resistance natures (Table 4.13). This finding also concur with the report of Bala *et al.* (2016) which showed that 83.3% of their MRSA isolates were MDR and had MARI of > 0.3 respectively while 16.7% had MARI of ≤ 0.3 showing that the isolates have been pre-exposed to antibiotics tested in this study with high multidrug resistance potential. This might be as a result of uncontrolled usage of antibiotics in poultry feeds for growth and diseases. Magiorakos *et al.* (2012) showed that emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for infections caused by these bacteria. Gram-positive and Gram-negative bacteria are both affected by the emergence and rise of antimicrobial resistance. As this problem continues to grow, harmonized definitions with which to describe and classify bacteria that are resistant to multiple antimicrobial agents are needed, so that epidemiological surveillance data can be reliably collected and compared across healthcare settings and countries. In the strictest sense, multidrug-resistant organisms (MDROs) are labeled as such because of their *in vitro* resistance tomore than one antimicrobial agent. Infections with MDROs can lead to inadequate or delayed antimicrobial therapy, and are associated with poorer patient outcomes.

The MIC values for oxacillin and vancomycin for the MRSA isolates showed that all (100%) had values greater than 256 µg/mL against oxacillin, whle 15 (53.6%) had values greater than 256 µg/mL against vancomycin respectively. Thirteen 13 (46.4%)

had MIC values <4 μg/mL against vancomycin (Table 4.14). This indicates that all the 28 MRSA isolates were oxacillin-resistant, while 46.4% were vancomycin- resistant, respectively. Also the Penicillin Binding Protein 2a (PBP2a) test for the 28 MRSA isolates using the PBP2a Latex Agglutination Test (LAT) kit, showed that 19 (67.9%) gave strongly positive (3+ to 4+) reactions in the Latex Agglutination Test, 5 (1.9%) were weakly positive (1+) in the Latex Agglutination Test, while 4 (14.3%) were negative (Table 4.14). These tests for isolates were considered LAT negative in the data analysis (Table 4.14).This trend was also reported by Akilu *et al.* (2013) in which MIC values for oxacillin of MRSA isolates were found to be greater than 256 μg/mL, with only 5 isolates showing MIC values of <4 μg/mL. Similar result was also observed by Bressler *et al.* (2005) which showed that 249 (64%) of the isolates were resistant to oxacillin based on the MIC results and all gave strong positive (3+ to 4+) reactions in the LAT. Jackson *et al.* (2013) determined the MICs (μg/ml) for *S. aureus* and discovered that multidrug resistance (resistance to ≥3 antimicrobials of different classes) was prevalent among the isolates with ampicillin (AMP), erythromycin (ERY), gatifloxacin (GAT), oxacillin (OXA), penicillin (PEN) the most common resistance patterns. In the same vain, Omoshaba *et al.* (2018) obtained 50 oxacillin- and cefoxitin-resistant isolates which tested positive to PBP2a using PBP2´ latex agglutination test.

MRSA and *S. aureus* develop resistance to the beta lactam antibiotics by acquiring the *mec* A gene, which needs intense investigations through genotyping and other

advanced methods which give a compressive information on them. In this study, twenty-eight (28) local isolates of MRSA strains were genotyped by using multiplex PCR for the presence of *mec* A, *mec* C and *fem* B genes which are characteristic for MRSA, Only three (3) isolates were *fem* B positive, while none of the isolates were positive for *mec* A and *mec* C genes (Plate 4.11). This is comparable to study by Olaiyinka *et al.* (2009) who isolated 139 (54.7%) MRSA from human sources in Zaria, but none of the isolates contained the *mec* A gene by PCR. In contrast Suleiman *et al.* (2012a) found only 2 (7.6%) of the 26 MRSA from subclical mastitis to contain *mec* A gene. Similarly, Shittu *et al.* (2016) found only 3/200 (1.5%) of the oxacillin strains to be MRSA by PCR detection of the *mec* A gene, while Umaru *et al.* (2013)in similar study in Nigeria detected *mec* A gene in 22.2% of the isolates from fresh and fermented milk samples. In the same vain, Papadopoulos *et al.* (2018) found 11 (3%) of the isolated MRSA to carry the *mec* A gene by PCR. A factor common to all these studies is the use of disc diffusion method of detecting MRSA. This is generally regarded as less specific in detecting classical methicillin resistance mediated by *mec* A gene that codes for the production of penicillin binding protein, PBP2a, which confers resistance to all β-lactam antibiotics (Ali, 2016). Methicillin resistant isolates with alterations to the existing PBP2a have been reported (Olayinka *et al.,* 2009), and are termed moderately resistant *S. aureus* (MODSA). Under some test conditions it has also been reported that isolates which produce large amounts of penicilinase (β-lacatmase hyper producers) show low level resistance to oxacillin and have been referred to as Borderline oxacillin-resistant *S. aureus* (BORSA).

Detection of *mec* A by polymerase chain reaction (PCR) is regarded as the gold standard for methicillin resistance, or alternatively detecting the gene product PBP2a by latex agglutination tests, but, the expression of the *mec* A gene is highly variable and dependent on different factors. The staphylococcal cassette chromosomes (SCC) are mobile genetic elements that are mainly associated with the carriage of the *mec* A gene, encoding methicillin resistance in staphylococci (Kaito *et al.,* 2011). However, several SCC*mec* elements have been found to carry other sets of genes besides *mec* A. The *SCCmec* element containing the *mec* A gene can be unstable after incorporation into the strain due to excision. This can result in loss of *mec* A gene. Excision of a SCC*mec* from the chromosome of a MRSA results in a susceptible phenotype. It has been shown that the excision of the SCC*mec* is not always done precisely and that parts of the SCC*mec* may remain in the chromosome of the post-MRSA (Turlej *et al.,* 2011; Kaito *et al.,* 2011). Also, storage and transportation of MRSA isolates for long period has resulted in the loss of *mec* A gene. This was shown by Kumurya *et al.* (2003) that 25% of the 100 MRSA lost the *mec* A gene on storage for two (2) years. Another possible explanation is that the detection of *mec* A gene is governed by gene expression. If for any reason, factors necessary for gene expression are affected, the *mec* A gene can not be expressed and as such can not be detected by PCR.

Although PCR amplification of the *mec* A gene fragment has been applied to the identification of MRSA, the method proved to be unreliable because the *mec* A gene is also found in some coagulase negative staphylococci (Kobayashjf *et al.,* 1994;

Kumurya *et al.*, 2013; Ali, 2016). As a means for excluding *mec* A positive CNS in the identification of MRSA, the simultaneous detection of *mec* A and *fem* B genes has been proposed previously (Yamashita and Kagawa, 1992). In this study, only 3 of the isolates were *fem* B positive (Plate 4.11). This work is not different from others in which *fem* B was not foud among all isolates. For example, Ali (2016) found 13 of the 35 samples to be positive for *fem* B gene, while Kobayashjf *et al.* (1994) in their report showed that, *fem* genes were possessed by almost all MRSA strains, whereas 18 strains (17.8 %) of *fem* negative methicillin-sensitive *S. aureus* and one (1I.1%) *fem* B positive *S. epidermidis* were also detected.

However, it has also been recognized that detection of a certain markers which are specific for *S. aureus* is needed to distinguish MRSA from methicillin-resistant CNS, in addition to demonstrating the *mec* A gene by PCR. Besides the *mec* regulator genes, *fem* A and *fem* B genes on the chromosome have been shown to encode proteins which considerably affect the level of methicillin resistance of *S. aureus* (Kobayashjf *et al.*, 1994). Although, *fem* genes were suggested to be specific for *S. aureus* (Kobayashjf *et al.*, 1994), distribution of these genes in staphylococci has not been fully established.

A novel methicillin resistance mechanism gene, a divergent *mec* A homologue named *mec* C (formerly mecALGA251), was described in *Staphylococcus aureus* (García-

Álvarez *et al.,* 2011; Holmes, 2013). This gene has been recovered from *S. aureus* in humans, ruminants, pets, and other animals such as rats, seals, and guinea pigs (García-Álvarez *et al.,* 2011; Walther *et al.,* 2102; Holmes, 2013). However, none of the isolates from this study was positive for *mec* C (Plate 4.1). The *mec*ALGA251 was 70% identical to *S aureus mec* A homologues and was initially detected in 15 *S aureus* isolates from dairy cattle in England. These isolates were from three different multilocus sequence type lineages (CC130, CC705, and ST425); *spa* type t843 (associated with CC130) was identified in 60% of bovine isolates. When human *mec* A-negative MRSA isolates were tested, the *mecA*LGA251 homologue was identified in 12 of 16 isolates from Scotland, 15 of 26 from England, and 24 of 32 from Denmark. As in cows, t843 was the most common *spa* type detected in human beings (García-Álvarez *et al.,* 2011; Holmes, 2013).

All the isolates examined from this study were *mec* C negative, which is similar to the report of Papadopoulos *et al.* (2018) in which all the *mec* A positive and negative MRSA were negative for *mec* C. In contast, Paterson *et al.* (2013) and Unnerstad *et al.* (2013) detected *mec* C MRSA in bulk tank milk samples and cows in Sweden. MRSA isolates identified by Garcia-Alvarez *et al.* (2011) from clinical bovine mastitis were shown to belong to CC130 and contained *mec C*.

Typing using MLST in this study revealed that all the 3 isolates were MLST types 1 (ST 1) and clonal complex (CC 1), which had similar allelic profiles at all seven loci (1 1 1 1 1 1 1) (Table 4.15), and were all isolated from cases of subclinical mastitis (Table 4.16). Even though the 3 isolates exhibited multidrug and extensive drug resistance (XDR) resistance patterns, they showed different resistance profiles (Table 4.16). *S. aureus* is one of the main challenges in dairy farm industry as it is considered one of the main agents causing mastitis especially the subclinical type (Ali, 2016). The emergence of *S. aureus* strain which is resistant to a wide array of antibiotics especially the beta lactam antibiotics is of public health concern. This study agrees with several others in which ST1 strain was detected. For example, Juhász-Kaszanyitzky *et al.* (2007) in Hungary typed one human and four bovine MRSA isolates by *spa* typing and MLST, and found all to be *spa* type t127 and MLST sequence type (ST) 1.

MRSA strains or bovine MRSA strains already present in small numbers and selected for by the frequent use of long-acting antimicrobial preparations, especially β-lactams. *S. aureus* usually shows limited host specificity, and transfer between different host species may occur. The transmission of milk-associated *S. aureus* strains between cows and humans was suggested by Juhansz-kaszanyitzky *et al.* (2007), whose study showed MRSA in milk samples with comparable antibiotypes as those in humans, but the transfer to humans was not proven. Rhee and Woo (2010) also detected ST1 in foods in strain Korea and reported that the *sea* gene which is an ST1 strain was the

most epidemic clone found in staphylococcal food poisoning incidents in Korea, and may suggest the evidence of considerable transmission of antimicrobial-resistant pathogens and the origin of staphylococcal food poisoning in Korea. ST1 MRSA was reported as the most common clone identified in the homeless people and injection drug users who shared the same homeless shelter, which suggests a possible focus of community transmission. Tegegne *et al.* (2017) detected CC/ST1 (t127) in cattle in the Czech Republic, which are commonly identified in Europe and are known as human strains. Detecting human strains from food of animal origin may be a sign of cross-contamination during processing (Weese *et al.*, 2010). However, colonization of livestock with human strains and jumping of animal strains to human host are increasing. In addition to this, host specific strains are widening their host range. Thus, colonization of pigs and other livestock animals with MRSA no matter which strain it belongs to, will be potential for the evolvement of new strains. Therefore, it is of paramount importance to assess the existing situation, and close monitoring of animal husbandry and food of animal origin processing line to safeguard the public and minimize the risk of development of wide host range antibiotic resistant strains. Of recent, Dittmann *et al.* (2017) typed 66 *S. aureus* isolates by MLST to determine if these isolates were persistent or continuously reintroduced in Brazilian Dairy Industries. Seven known sequence types (STs), ST1, ST5, ST30, ST97, ST126, ST188 and ST398, and four new ST were identified, ST3531, ST3540, ST3562 and ST3534. Clonal complex (CC) 1 (including the four new ST), known as an epidemic clone, was

the dominant CC. However, there were no indications of persistence of particular ST (Dittmann *et al.,* 2017).

Sequence type 1 MRSA has also been isolated from hospital settings. Earls *et al.* (2017) in their study noted that the recent emergence in hospitals of multidrug-resistant community-associated sequence type 1 and *spa* type t127 methicillin-resistant *Staphylococcus aureus* investigated by whole-genome sequencing.

According to the MLST database and previous studies on milk and dairy products, ST1 was not previously isolated in Nigeria, but was reported in other countries (Juhász-Kaszanyitzky *et al*., 2007; Rhee and Woo, 2010; Silva *et al.,* 2013, 2014; Dittmann *et al.,* 2017). Although several other studies have found large diversity regarding *S. aureus* contamination, Silva *et al.* (2013) also found that CC1 (ST1) was the main contaminant of milk in cows infected with mastitis due to MSSA and also identified a new ST belonging to CC1 in most countries of the word.. So far, the present data represents the preliminary study on ST 1 associated with bovine mastitis in Nigeria. The only available studies are the ones reported by Ghebremedhin *et al.* (2009) in Southwest Nigeria and Shittu *et al.* (2014) in Ile-Ife, Ibadan and Lagos (South-West Nigeria), and Maiduguri (North-East Nigeria. Both studies used human clinical samples from hospitals, but detected ST1 clones which were identified as community-acquired strains.

The phylogenetic relationships was established using the nucleotide sequences of the type strains defining the genus *Staphylococcus* (BX571857, BA000033, AP015012, CP017115 and CP01780). A blastn search of all nucleotide sequence of 23448_1#128, 23448_1#129 and 23448_1#1311 using default parameters revealed 99 % identity with the available *S. aureus* (BX571857, BA000033, AP015012, CP017115 and CP01780) genomes in the database (Fig. 4.12). It can be inferred that the three (3) isolates and those obtained from database might have originated from common ancestry. From the tree, it can be seen that all the isolates clustered more tightly together as compared to those obtained in Genbank. They were 100 % homologous to each other with no divergence within the specie level indicating that they originated from the same ancestry or phylogeny. The present study is similar to that of Ali (2016) who performed the phylogenetic analysis of *S. aureus* and showed high degree of similarities between the local isolates of *S. aureus* those retrieved from the Gene Bank. Similarly, Zubair *et al.* (2015) determined the sequence type of the *S. aureu s*isolate ILRI_Eymole1/1 from a Kenyan dromedary cameland a BLASTn search of all five copies of 16S rRNA sequence using default parameters revealed 99–100 % identity (with 98–100 % coverage) with all available *S. aureus* genomes in the database.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The results obtained from the present study revealed the prevalence of mastitis at cow level to be 26.9% (97/360), out of which 3.1% (11/360) and 23.9% (86/360) were clinical and subclinical mastitis respectively. The prevalence of *S. aureus* and MRSA were 15.3% and 7.8 % respectively with 12 (42.9%) of the MRSA associated with mastitis, and therefore regarded as causative agents of mastitis.

The resistance patterns of the MRSA isolates showed high frequency of resistance to penicillin 28 (100.0%), amoxicillin 23 (89.3%), ampicillin 23 (89.3%), tetracycline 24 (85.7%) and erythromycin 20 (71.1%). The multiple drug resistance index of the MRSA strains revealed that all the MRSA strains were resistant to 5 or more antibiotics tested (5 to 9 antibiotics); while the multiple resistance patterns showed that 26 isolates (92.9%) exhibited different resistance patterns from each other with only four (4) exhibiting common patterns of multiple resistance. The 2 patterns encountered were AML,AMP,C,CN,E,NA,P,S,TE exhibited by isolates K23 and K42 and AML,AMP,E,P,TE,VA,W exhibited by isolates K3 and S26 (Table 4.14).

The multiple antibiotic resistance (MDR) was classified as Extensive Drug Resistance (XDR) and Pan Drug Resistance (PDR) and the results showed that 19 (67.9%) of the isolates exhibited the extensive drug classification pattern while the rests exhibited multidrug resistance.

Typing using MLST revealed that all the 3 isolates were MLST types 1 (ST 1) and CC 1, which had similar allelic profiles at all seven loci, and showed high degree of monomorphism. All the three (3) isolates (23448_1#128, 23448_1#129 and 23448_1#131) were isolated from cases of subclinical mastitis and exhibited multidrug and extensive drug resistance (XDR) patterns.

The phylogenetic relationship established using the nucleotide sequences revealed 99% similarity of the three (3) strains with *Staphylococcus* BX571857, BA000033, AP015012, CP017115 and CP01780 established in the GenBank. This can be infered that the isolates might have originated from the same ancestry. The three (3) ST1 (CC1)clone strains clustered tightly together and were therefore 100% homologous to each other with no divergence within the specie level indicating that they are from the same clone.

This study therefore, established *S. aureus* and MRSA as one of the causative agents of bovine mastitis in settled Fulani herds in Kaduna State. It also revealed the possible

emergence of MDR MRSA and ST1 (CC1) clone responsible for subclinical mastitisin cows in settled Fulani herds in Kaduna State.The high prevalence of both clinical and subclinical mastitis coupled with the isolation of *S. aureus*, CONS, MRSA ST1 (CC1) clones are of great public health concern and requires strict control and preventive measures.

6.1.1 Contribtion to knowledge

i. The study confirmed the occurrence of mastitis in cows in Kaduna State, and detected *S. aureus* and MRSA as one of the causative organisms as reported by several researchers.

ii. The study showed that greater percentage of the isolated *S. aureus* and MRSA were multi-drug resistant or exhibited the extensive drug resistance patterns. This is of public health importance because of limited or lack of antibiotics for the treatment of infections due to these strains.

iii. The study also reported the occurrence of *S. aureus* sequence type 1 strains responsible for subclinical mastitis in Kaduna State, which was not previously reported in Nigeria according to MLST database.

6.2 Recommendations

This study recommends:

i. Adoption of standard hygienic measures and proper management practices during milking procedures by farmers and herders to reduce or eliminate infection of dairy cows with resistant *S aureus* or MRSA.

ii. Screening, isolation and culling of chronically infected cows to avoid spread of resistant *S aureus* or MRSA to uninfected animals or humans who are in contact with these animals.

iii. Pasteurisation of cow milk prior to human consumption or processing into other milk products, or totally desist from the consumption of fresh raw milk to avoid transmission of pathogenic bacteria to humans through milk or milk products .

iv. That the Fulani herdsmen and milkers should be educated on proper personal and environmental hygiene to reduce cross contamination of milk by *S. aureus* or MRSA.

v. Direct contact between human and milk, animal and milk should be avoided or properly maintained to avoid cross-infection with *S. aureus* or MRSA.

vi. That the use of antibiotics in human and veterinary medicine should be controlled and regulated by relavant regulatory agencies of government so as to reduce chances of abuse and misuse.

vii. Awareness or enlightment of healthcare professionals that animals and foods are a possible sources of MRSA to humans.

viii. Collaboration between infectious disease specialists, microbiologists, and epidemiologists on how to prevent both human and animal infections by *S. aureus* and MRSA.

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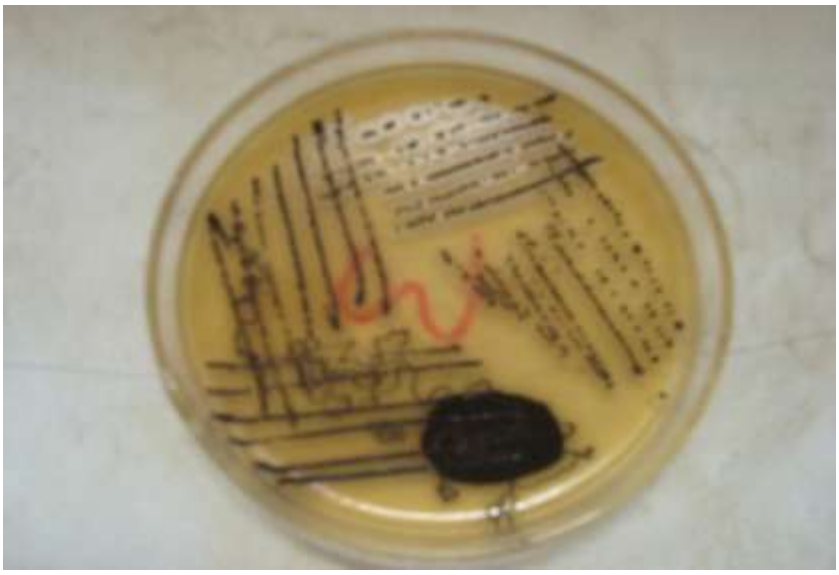
APPENDIX



I. Isolate showing grey black colonies on Baird Packer Medium

II. Confirmation of *S. aureus* on Microbact 12S Staphylococcal Identification System (Oxoid, Basingstoke, U

III. Isolates showing susceptibility to antibiotics

IV. MIC test on isolate

V. PBP2a test using Latex Agglutination kit

VI. Phenotypic isolation of *S. aureus* and MRSA

S/N	Sample No	Mastitis	Cat	Coa	DNase	Haem	Glu	Rab	Man	Malt	Pigm	PBP2a	ORSAB
1	K3	+ve SM	+ve	+ve	+ve	Alpha	+ve	-ve	-ve	+ve	-ve	Weak +ve	+ve
2	K8	-ve	+ve	+ve	+ve	Beta	+ve	-ve	-ve	+ve	+ve	-ve	-ve
3	K11	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	-ve	-ve	-ve
4	K14	+ve SM	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	Weak +ve	+ve
5	K17	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	+ve	-ve
6	K19	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	-ve
7	K20	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	-ve	+ve	-ve
8	K21	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ve	+ve	-ve
9	K23	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	+ve	-ve
10	K32	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	-ve	+ve
11	K34	+ve CM	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	+ve
12	K37	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	-ve	-ve
13	K42	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	Positive	+ve

14	K46	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	-ve	+ve	-ve
15	k52	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ve	-ve	-ve
16	k57	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	+ve	+ve
17	S1	+ve SM	+ve	+ve	+ve	Beta	+ve	-ve	-ve	+ve	+ve	+ve	-ve
18	S2	+ve SM	+ve	+ve	+ve	Beta	+ve	-ve	-ve	+ve	+ve	Positive	+ve
19	S5	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	Weak +ve	+ve
20	S6	-ve	+ve	+ve	+ve	Alpha	+ve	-ve	-ve	+ve	+ve	-ve	+ve
21	S14	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ve	+ve	-ve
22	S17	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ve	+ve	-ve
23	S20	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	Weak +ve	-ve
24	S26	-ve	+ve	+ve	+ve	Alpha	+ve	-ve	-ve	+ve	+ve	+ve	+ve
25	S27	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve		-ve
26	S42	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ve	Weak +ve	+ve
27	S60	+ve SM	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	-ve	+ve	+ve
28	I2	+ve CM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	-ve	-ve
29	I5	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	-ve

30	I18	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ve	-ve	-ve
31	I23	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	-ve
32	I34	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	-ve	-ve
33	I41	-ve	+ve	+ve	+ve	Alpha	+ve	-ve	-ve	+ve	-ve	-ve	-ve
34	I59	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ve	+ve	-ve
35	SB22	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	-ve	-ve	-ve
36	SB24	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ive	+ve	-ve
37	SB33	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ive	-ve	-ve
38	SB34	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	-ive	+ve	+ve
39	SB48	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	-ve
40	SB49	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ive	-ve	-ve
41	SB51	-ve	+ve	+ve	+ve	Beta	+ve	-ve	-ve	+ve	-ive	+ve	-ve
42	SB54	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	-ive	-ve	-ve
43	SB56	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	-ve
44	SB60	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	+ve	+ve
45	G3	+ve SM	+ve	+ve	+ve	Alpha	+ve	-ve	-ve	+ve	+ve	-ve	-ve

46	G23	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	+ve	+ve
47	G25	+ve SM	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	-ve
48	Z6	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ive	Weak +ve	-ve
39	Z8	-ve	+ve	+ve	+ve	Beta	+ve	-ve	-ve	+ve	-ive	-ve	-ve
50	Z10	-ve	+ve	+ve	+ve	Alpha	+ve	+ve	-ve	+ve	+ve	-ve	+ve
51	Z21	-ve	+ve	+ve	+ve	Alpha	+ve	-ve	-ve	+ve	-ve	-ve	-ve
52	Z22	-ve	+ve	+ve	+ve	Beta	+ve	-ve	-ve	+ve	+ve	-ve	-ve
53	Z39	-ve	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	-ve	+ve
54	Z47	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	+ve	+ve	+ve
55	Z49	+ve SM	+ve	+ve	+ve	Beta	+ve	+ve	-ve	+ve	-ive	-ve	+ve

Key:

Cat- Catalase

Coa- Coagulase

Haem- Haemolysis

Glu- Glucose

Rab- Rabinose

Man- Mannitol

Mal- Maltose

Pigm- Pigmentation

PBP2a- Penicillin Binding Protein 2a

ORSAB- Oxacillin- Oxacillin Resistance Screening Basal medium

VII. Antibiotic susceptibility of the *S. aureus* isolate

S/N	Sample No	AML	AMP	C	CIP	CN	E	FOX	NA	OX	P	S	TE	VA	W	MIC (OX)	MIC (VA)
1	K3	R	R	S	S	S	R	S	S	R	R	S	R	R	S	256	256
2	K8	S	S	R	R	R	R	S	R	S	R	R	R	S	R		
3	K11	R	R	S	S	S	R	S	R	S	R	S	R	R	I		256
4	K14	R	R	R	I	R	R	R	R	R	R	R	R	S	S	256	
5	K17	R	R	S	S	S	R	S	S	S	R	S	R	R	S		256
6	K19	R	R	I	S	S	R	S	S	S	R	S	R	R	S		256
7	K20	R	R	S	S	S	R	R	S	S	R	R	R	R	S		256
8	K21	R	R	S	S	S	R	S	S	S	R	S	R	R	R		256
9	K23	R	R	R	S	R	R	R	R	S	R	R	R	S	S		
10	K32	S	S	R	S	R	R	S	R	R	R	R	R	S	S	256	
11	K34	S	S	I	S	S	R	S	S	R	R	S	R	R	S	256	256
12	K37	R	R	S	S	S	R	S	S	S	R	S	R	R	S		256
13	K42	R	R	R	S	R	R	R	R	R	R	R	R	S	S	256	
14	K46	R	R	S	S	S	R	S	S	S	R	S	R	R	R		256
15	k52	R	R	I	S	S	R	S	R	S	R	S	R	R	R		256
16	k57	R	R	I	S	S	R	S	S	R	R	S	R	R	S	256	256
17	S1	R	R	I	S	I	R	R	S	S	R	S	R	R	S		256
18	S2	R	R	R	S	R	R	R	R	R	**R**	S	R	S	S	256	
19	S5	S	S	R	S	R	S	S	S	R	R	R	R	R	S	256	256
20	S6	R	R	R	S	R	S	R	R	R	R	R	R	S	S	256	
21	S14	R	R	I	S	S	R	S	S	S	R	S	R	S	S		
22	S17	R	R	I	S	S	R	S	S	S	R	R	R	R	I		256
23	S20	R	R	S	I	S	I	R	R	S	R	S	R	S	S		
24	S26	R	R	S	S	S	R	S	S	R	R	S	R	R	I	256	256
25	S27	R	R	S	S	S	R	S	S	S	R	S	R	R	S		256

26	S42	R	R	S	S	S	S	S	R	R	R	S	S	S	I	256	
27	S60	R	R	S	S	S	R	R	R	R	R	S	R	S	S	256	
28	I2	R	R	S	S	S	R	S	S	S	R	S	R	R	S		256
29	I5	R	R	S	S	S	R	S	S	S	R	S	S	R	R		256
30	I18	R	R	S	S	S	R	S	S	S	R	S	R	R	I		256
31	I23	S	S	R	S	S	R	S	R	S	R	R	R	S	S		
32	I34	R	R	S	S	I	R	S	S	S	R	R	R	R	S		256
33	I41	R	R	I	S	S	R	S	R	S	R	S	R	R	R		256
34	I59	*R*	*R*	*R*	*S*	*S*	*R*	*R*	*R*	*S*	*R*	*S*	*R*	*R*	*R*		256
35	SB22	R	S	S	S	S	R	S	S	S	R	S	S	S	S		
36	SB24	R	R	S	S	R	R	R	R	S	R	R	R	S	S		
37	SB33	R	R	S	S	S	S	S	S	S	R	S	S	S	R		
38	SB34	R	R	S	S	R	S	R	R	R	R	R	R	S	S	256	
39	SB48	R	R	S	S	I	R	S	S	S	R	S	R	R	R		256
40	SB49	S	R	S	S	S	R	S	S	S	R	S	R	R	S		256
41	SB51	R	R	S	S	S	R	R	R	S	R	S	R	R	I		256
42	SB54	R	R	R	R	S	R	S	S	S	R	R	S	S	R		
43	SB56	R	R	S	S	S	S	S	S	S	R	R	R	S	S		
44	SB60	S	R	S	S	S	R	S	S	R	R	S	R	R	R	256	256
45	G3	R	R	S	S	R	S	R	R	S	R	R	S	S	S		
46	G23	R	R	S	S	S	S	R	R	R	R	S	R	S	S	256	
47	G25	R	R	S	S	S	S	R	S	S	R	S	S	R	R		256
48	Z6	R	R	S	S	S	S	S	S	S	R	S	R	R	I		256
39	Z8	R	R	S	S	S	S	S	S	S	R	S	R	R	I		256
50	Z10	R	R	S	S	R	R	S	S	R	R	S	S	R	S	256	256
51	Z21	R	R	S	S	S	S	S	S	S	R	S	S	S	S		
52	Z22	R	R	S	S	S	R	S	S	S	R	S	R	R	S		256
53	Z39	R	R	S	S	S	R	S	S	R	R	R	R	R	R	256	256
54	Z47	R	R	R	S	S	S	S	R	R	R	S	R	R	R	256	256
55	Z49	R	R	S	S	R	R	S	R	R	R	S	R	R	R	256	256

Key:

MIC- Minimum Inhibitory Concentration

AML- Amoxicillin

AMP- Ampicillin

C- Chloramphenicol

CIP- Ciprofloxacin

CN- Gentamicin

E- Erythromycin

FOX- Cefoxitin

Na- Nalidixic acid

OX- Oxacillin

P- Penicillin

S- Streptomycin

TE- Tetracycline

VA- Vancomycin

W- Trimethoprim

+ve SM- Positive subclinical mastitis

+ve CM- Positive clinical mastitis

-ve- Negative

VIII. Penicillin Binding Protein 2a (PBP2a) and minimum inhibitory concentration (MIC) of oxacillin and vancomycin of the MRSA isolated from cow milk in settled Fulani herds, Kaduna State, Nigeria (N=28)

MRSA isolate	**PBP2a**	**MIC (µg/mL)**	
		OX	**VA**
K3	Negative	256	256
K14	Weak positive	256	<4
K20	Positive	256	256
K23	Positive	256	256
K32	Positive	256	<4
K34	Positive	256	256
K42	Positive	256	<4
K57	Positive	256	256
S1	Positive	256	256
S2	Positive	256	<4
S5	Weak positive	256	256
S6	Positive	256	<4
S20	Weak positive	256	<4
S26	Positive	256	256
S42	Weak positive	256	<4
S60	Positive	256	<4
I59	Positive	256	256
SB24	Positive	256	<4
SB34	Positive	256	<4
SB51	Positive	256	256
SB60	Positive	256	<4
G3	Weak positive	256	<4
G23	Positive	256	<4
G25	Positive	256	256
Z10	Negative	256	256
Z39	Negative	256	256
Z47	Positive	256	256
Z49	Negative	256	256

IX. MLST allele results

"Isolate"	ST	New ST	Contamination	arc	aroE	glpF	gmk	pta	tpi	yqiL
"23448_1#128	contigs_velvet"	"1"	"" ""	"1"	"1"	"1"	"1"	"1"	"1"	"1"
"23448_1#129	contigs_velvet"	"1"	"1"	"1"	"1"	"1"	"1"	"1"	"1"	"1"
"23448_1#131	contigs_velvet"	"1"	"1"	"1"	"1"	"1"	"1"	"1"	"1"	"1"

X. Sequencing summary

Sample	Lane	Assembly type	Total length	No contigs	Av contigs length	Largest contigs	N50	Contigs in 50	N60
SB60	23448_1#128	Scaffold: Velvet + Improvement	2700299	10	270029.9	1382630	1382630	1	676479
G3	23448_1#129	Scaffold: Velvet + Improvement	2743368	13	211028.31	1382624	1382624	1	715711
G25	23448_1#131	Scaffold: Velvet + Improvement	2742321	12	228526.75	1382614	1382614	1	715707

XI. MLST genomic results

Isol	MLST genomic sequence
128	"TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAGGGTATGATAGGCTATT GGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAATCGTTACACGTGTGGAAGTAGATAAAGATGA TCCACGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTATACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTA AAGAAGATGCAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGT AAAAATATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG" "AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGAAATTATTTCGAAAAAAGAAT TAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACA CTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAAT GCATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAG AACGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATTAGCAGATGCTGAAAAGTATTTA" "GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACC CAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAG CAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAG AATTACTTTGCCAACTTTTTAAGTGAGATTATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAA ATCCTTTAATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT" "CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGATGGCGTAGATTACTT TTTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCA ATATGTTAAAGATACAATGGACGAAGGTCATGATGTATTTTTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCG CTATTTATTTTCTTAGCACCTCCAAGTTTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTAT TAACGAAGCGCGTAAAGAAGTTGAAATGATGAATTTA" "GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATAT TTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAG AACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTTGTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACAT

	TCAACAGGAGACACTGTGCGTCCAGCTTTACAAATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGA TGTACAATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCAAAATCAGCATTA" "CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAG CGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAGTTGTAATTGC TTATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGATGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACT TATCAAGCAAAGAAGTATCAGAAGCAACTCGTATTCAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATT GATGGGGCATTAGTAGGTGGCGCA" "GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAG TTATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTT ACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGG TATGGAGAATATGTCTCAGTCACCAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATG ATGGTTTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA TTTGCTGTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA"
1 2 9	"TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAGGGTATGATAGGCTATT GGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAATCGTTACACGTGTGGAAGTAGATAAAGATGA TCCACGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTATACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTA AAGAAGATGCAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGT AAAAATATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG" "AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGAAATTATTTCGAAAAAAGAAT TAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACA CTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAAT GCATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAG AACGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATTAGCAGATGCTGAAAAGTATTTA" "GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACC CAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAG CAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAG AATTACTTTGCCAACTTTTTAAGTGAGATTATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAA ATCCTTTAATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT" "CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGATGGCGTAGATTACTT TTTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCA

	ATATGTTAAAGATACAATGGACGAAGGTCATGATGTATTTTTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCG CTATTTATTTTCTTAGCACCTCCAAGTTTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTAT TAACGAAGCGCGTAAAGAAGTTGAAATGATGAATTTA" "GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATAT TTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAG AACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTTGTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACAT TCAACAGGAGACACTGTGCGTCCAGCTTTACAAATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGA TGTACAATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCAAAATCAGCATTA" "CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAG CGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAGTTGTAATTGC TTATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGATGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACT TATCAAGCAAAGAAGTATCAGAAGCAACTCGTATTCAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATT GATGGGGCATTAGTAGGTGGCGCA" "GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAG TTATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTT ACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGG TATGGAGAATATGTCTCAGTCACCAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATG ATGGTTTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA TTTGCTGTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA"
1 **3** **1**	"TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAGGGTATGATAGGCTATT GGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAATCGTTACACGTGTGGAAGTAGATAAAGATGA TCCACGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTATACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTA AAGAAGATGCAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGT AAAAATATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG" "AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGAAATTATTTCGAAAAAAGAAT TAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACA CTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAAT GCATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAG AACGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATTAGCAGATGCTGAAAAGTATTTA" "GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACC

	CAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAG CAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAG AATTACTTTGCCAACTTTTTAAGTGAGATTATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAA ATCCTTTAATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT" "CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGATGGCGTAGATTACTT TTTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCA ATATGTTAAAGATACAATGGACGAAGGTCATGATGTATTTTTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCG CTATTTATTTTCTTAGCACCTCCAAGTTTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTAT TAACGAAGCGCGTAAAGAAGTTGAAATGATGAATTTA" "GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATAT TTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAG AACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTTGTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACAT TCAACAGGAGACACTGTGCGTCCAGCTTTACAAATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGA TGTACAATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCAAAATCAGCATTA" "CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAG CGTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAGTTGTAATTGC TTATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGATGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACT TATCAAGCAAAGAAGTATCAGAAGCAACTCGTATTCAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATT GATGGGGCATTAGTAGGTGGCGCA" "GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAG TTATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTT ACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGG TATGGAGAATATGTCTCAGTCACCAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATG ATGGTTTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA TTTGCTGTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA"

128	"TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAGGGTATGATAGGCTATT GGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAATCGTTACACGTGTGGAAGTAGATAAAGATGA TCCACGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTATACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTA AAGAAGATGCAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGT AAAAATATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG"
	"AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGAAATTATTTCGAAAAAAGAAT TAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACA CTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAAT GCATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAG AACGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATTAGCAGATGCTGAAAAGTATTTA"
	"GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACC CAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAG CAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAG AATTACTTTGCCAACTTTTTAAGTGAGATTATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAA ATCCTTTAATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT"
	"CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGATGGCGTAGATTACTT TTTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCA ATATGTTAAAGATACAATGGACGAAGGTCATGATGTATTTTTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCG CTATTTATTTTCTTAGCACCTCCAAGTTTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTAT TAACGAAGCGCGTAAAGAAGTTGAAATGATGAATTTA"
	"GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATAT TTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAG AACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTTGTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACAT TCAACAGGAGACACTGTGCGTCCAGCTTTACAAATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGA TGTACAATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCAAAATCAGCATTA"
	"CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAGC GTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAGTTGTAATTGCT TATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGATGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACTT ATCAAGCAAAGAAGTATCAGAAGCAACTCGTATTCAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATTG ATGGGGCATTAGTAGGTGGCGCA"

	"GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAG TTATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTT ACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGG TATGGAGAATATGTCTCAGTCACCAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATG ATGGTTTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA TTTGCTGTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA"
129	"TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAGGGTATGATAGGCTATT GGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAATCGTTACACGTGTGGAAGTAGATAAAGATGA TCCACGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTATACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTA AAGAAGATGCAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGT AAAAATATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG" "AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGAAATTATTTCGAAAAAAGAAT TAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACA CTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAAT GCATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAG AACGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATTAGCAGATGCTGAAAAGTATTTA" "GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACC CAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAG CAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAG AATTACTTTGCCAACTTTTTAAGTGAGATTATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAA ATCCTTTAATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT" "CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGATGGCGTAGATTACTT TTTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCA ATATGTTAAAGATACAATGGACGAAGGTCATGATGTATTTTTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCG CTATTTATTTTCTTAGCACCTCCAAGTTTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTAT TAACGAAGCGCGTAAAGAAGTTGAAATGATGAATTTA" "GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATAT TTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAG AACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTTGTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACAT TCAACAGGAGACACTGTGCGTCCAGCTTTACAAATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGA

	TGTACAATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCAAAATCAGCATTA" "CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAGC GTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAGTTGTAATTGCT TATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGATGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACTT ATCAAGCAAAGAAGTATCAGAAGCAACTCGTATTCAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATTG ATGGGGCATTAGTAGGTGGCGCA" "GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAG TTATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTT ACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGG TATGGAGAATATGTCTCAGTCACCAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATG ATGGTTTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA TTTGCTGTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA"
1 3 1	"TTATTAATCCAACAAGCTAAATCGAACAGTGACACAACGCCGGCAATGCCATTGGATACTTGTGGTGCAATGTCACAGGGTATGATAGGCTATT GGTTGGAAACTGAAATCAATCGCATTTTAACTGAAATGAATAGTGATAGAACTGTAGGCACAATCGTTACACGTGTGGAAGTAGATAAAGATGA TCCACGATTCAATAACCCAACCAAACCAATTGGTCCTTTTTATACGAAAGAAGAAGTTGAAGAATTACAAAAAGAACAGCCAGACTCAGTCTTTA AAGAAGATGCAGGACGTGGTTATAGAAAAGTAGTTGCGTCACCACTACCTCAATCTATACTAGAACACCAGTTAATTCGAACTTTAGCAGACGGT AAAAATATTGTCATTGCATGCGGTGGTGGCGGTATTCCAGTTATAAAAAAAGAAAATACCTATGAAGGTGTTGAAGCG" "AATTTTAATTCTTTAGGATTAGATGATACTTATGAAGCTTTAAATATTCCAATTGAAGATTTTCATTTAATTAAAGAAATTATTTCGAAAAAAGAAT TAGATGGCTTTAATATCACAATTCCTCATAAAGAACGTATCATACCGTATTTAGATCATGTTGATGAACAAGCGATTAATGCAGGTGCAGTTAACA CTGTTTTGATAAAAGATGACAAGTGGATAGGGTATAATACAGATGGTATTGGTTATGTTAAAGGATTGCACAGCGTTTATCCAGATTTAGAAAAT GCATACATTTTAATTTTGGGCGCAGGTGGTGCAAGTAAAGGTATTGCTTATGAATTAGCAAAATTTGTAAAGCCCAAATTAACTGTTGCGAATAG AACGATGGCTCGTTTTGAATCTTGGAATTTAAATATAAACCAAATTTCATTAGCAGATGCTGAAAAGTATTTA" "GGTGCTGATTGGATTGTCATCACAGCTGGATGGGGATTAGCGGTTACAATGGGTGTGTTTGCTGTCGGTCAATTCTCAGGTGCACATTTAAACC CAGCGGTGTCTTTAGCTCTTGCATTAGACGGAAGTTTTGATTGGTCATTAGTTCCTGGTTATATTGTTGCTCAAATGTTAGGTGCAATTGTCGGAG CAACAATTGTATGGTTAATGTACTTGCCACATTGGAAAGCGACAGAAGAAGCTGGCGCGAAATTAGGTGTTTTCTCTACAGCACCGGCTATTAAG AATTACTTTGCCAACTTTTTAAGTGAGATTATCGGAACAATGGCATTAACTTTAGGTATTTTATTTATCGGTGTAAACAAAATTGCCGATGGTTTAA ATCCTTTAATTGTCGGAGCATTAATTGTTGCAATCGGATTAAGTTTAGGCGGTGCTACTGGTTATGCAATCAACCCAGCACGT" "CGAATATTTGAAGATCCAAGTACATCATATAAGTATTCTATTTCAATGACAACACGTCAAATGCGTGAAGGTGAAGTTGATGGCGTAGATTACTT TTTTAAAACTAGGGATGCGTTTGAAGCTTTAATCAAAGATGACCAATTTATAGAATATGCTGAATATGTAGGCAACTATTATGGTACACCAGTTCA ATATGTTAAAGATACAATGGACGAAGGTCATGATGTATTTTTAGAAATTGAAGTAGAAGGTGCAAAGCAAGTTAGAAAGAAATTTCCAGATGCG

	CTATTTATTTTCTTAGCACCTCCAAGTTTAGAACACTTGAGAGAGCGATTAGTAGGTAGAGGAACAGAATCTGATGAGAAAATACAAAGTCGTAT TAACGAAGCGCGTAAAGAAGTTGAAATGATGAATTTA" "GCAACACAATTACAAGCAACAGATTATGTTACACCAATCGTGTTAGGTGATGAGACTAAGGTTCAATCTTTAGCGCAAAAACTTGATCTTGATAT TTCTAATATTGAATTAATTAATCCTGCGACAAGTGAATTGAAAGCTGAATTAGTTCAATCATTTGTTGAACGACGTAAAGGTAAAGCGACTGAAG AACAAGCACAAGAATTATTAAACAATGTGAACTACTTCGGTACAATGCTTGTTTATGCTGGTAAAGCAGATGGTTTAGTTAGTGGTGCAGCACAT TCAACAGGAGACACTGTGCGTCCAGCTTTACAAATCATCAAAACGAAACCAGGTGTATCAAGAACATCAGGTATCTTCTTTATGATTAAAGGTGA TGTACAATACATCTTTGGTGATTGTGCAATCAATCCAGAACTTGATTCACAAGGACTTGCAGAAATTGCAGTAGAAAGTGCAAAATCAGCATTA" "CACGAAACAGATGAAGAAATTAACAAAAAAGCGCACGCTATTTTCAAACATGGAATGACTCCAATTATTTGTGTTGGTGAAACAGACGAAGAGC GTGAAAGTGGTAAAGCTAACGATGTTGTAGGTGAGCAAGTTAAGAAAGCTGTTGCAGGTTTATCTGAAGATCAACTTAAATCAGTTGTAATTGCT TATGAGCCAATCTGGGCAATCGGAACTGGTAAATCATCAACATCTGAAGATGCAAATGAAATGTGTGCATTTGTACGTCAAACTATTGCTGACTT ATCAAGCAAAGAAGTATCAGAAGCAACTCGTATTCAATATGGTGGTAGTGTTAAACCTAACAACATTAAAGAATACATGGCACAAACTGATATTG ATGGGGCATTAGTAGGTGGCGCA" "GCGTTTAAAGACGTGCCAGCCTATGATTTAGGTGCGACTTTAATAGAACATATTATTAAAGAGACGGGTTTGAATCCAAGTGAGATTGATGAAG TTATCATCGGTAACGTACTACAAGCAGGACAAGGACAAAATCCAGCACGAATTGCTGCTATGAAAGGTGGCTTGCCAGAAACAGTACCTGCATTT ACAGTGAATAAAGTATGTGGTTCTGGGTTAAAGTCGATTCAATTAGCATATCAATCTATTGTGACTGGTGAAAATGACATCGTGCTAGCTGGCGG TATGGAGAATATGTCTCAGTCACCAATGCTTGTCAACAACAGTCGCTTCGGTTTTAAAATGGGACATCAATCAATGGTTGATAGCATGGTATATG ATGGTTTAACAGATGTATTTAATCAATATCATATGGGTATTACTGCTGAAAATTTAGTGGAGCAATATGGTATTTCAAGAGAAGAACAAGATACA TTTGCTGTAAACTCACAACAAAAAGCAGTACGTGCACAGCAA"