ISOLAT-ION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM YOGHURT AND TRADITIONALLY FERMENTED MILK (NONO) IN ZARIA AND KADUNA METROPOLIS, NIGERIA

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JULY, 2015

DECLARATION

I, declare that the work in this dissertation entitled **"Isolation of Methicillin Resistant *Staphylococcus aureus* (MRSA) from Yoghurt and Traditionally Fermented Milk (Nono) in Zaria and Kaduna Metropolis, Nigeria"** has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine under the supervision of Prof. J.K.P Kwaga and Prof. J. Kabir. 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 in this or any other Institution.

Rehimat Zia USMAN _____ _____

Signature Date

CERTIFICATION

This dissertation entitled **"ISOLATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) FROM YOGHURT AND TRADITIONALLY FERMENTED MILK (NONO) IN ZARIA AND KADUNA METROPOLIS, NIGERIA''** by Rehimat ZIA USMAN meets the requirements for the award of the Master of Science in Veterinary Public Health and Preventive Medicine and is approved by Ahmadu Bello University, Zaria for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to Almighty Allah for his guidance and protection, my parents: Mr ZIA Usman and Mrs Rukayat Usman, Husband: Dr Banji Munirdeen Mustapha and lovely kids, Mariam, Mulikat and Mustapha, my only sister, Aminat ZIA Usman and my lovely brothers, Lateef ZIA Usman, Suleman ZIA Usman, Abdul Jabar ZIA Usman and Zulkifl ZIA Usman.

ABSTRACT

Contamination of food products such as milk by Staphylococci, have been known to occur post pasteurization. Poor sanitary practices have also contributed to gross contamination. Indiscriminate use of antibiotics in efforts to treat diseases has led to the emergence of resistant strains known as MRSA. This study was to determine the presence of *Staphylococcus* including the methicillin resistant strains in yoghurt and nono. Bacteriological and molecular techniques were carried out to determine the occurrence of *Staphylococcus aureus*. Resistance was determined by detection of the *mec*A gene by PCR, betalactamase and altered penicillin -binding protein (Pbp2a).Out of 560 samples of yoghurt (280) and nono(280) collected from Kaduna and Zaria, the overall prevalence of Staphylococcal species were 75(13.39%) . Out of this 52(69.3%) were presumptively identified as methicillin resistant using the Oxacillin resistant staphylococcal basal medium (ORSAB) while 73(97.3%) were methicillin resistant by the Kirby-Baeur disk diffusion method. The 75 staphylococcal isolates were further subjected to Microbact 12S identification system and 17(3.04%) were confirmed as *Staphylococcus aureus*.Out of this 12(70.59%) were presumptively identified as MRSA using ORSAB while 17(100%) were identified as MRSA using the Kirby-Baeur disk diffusion. The overall prevalence of MRSA was (3.04%). The occurrence of *Staphylococcus* was higher in Zaria 41(14.64%) than in Kaduna (12.14%) while prevalence of MRSA was (3.21%) and (2.86%) in Kaduna and Zaria respectively. There was no statistical significant difference between the occurrence of these organisms and the locations (P□ 0.05)The presence of the organisms was higher in nono 43(15.36%), and 12(4.29%) than in yoghurt 32(11.43%) and 5(1.79%) for Staphyloccocci and MRSA respectively but was not statistically significant (P□ 0.05)All the isolates were

resistant to more than one antibiotic but none resistant to all. The isolates of staphylococci and MRSA showed over 90% resistance to beta lactams such as penicillin, methicillin and oxacillin. The least resistance was to amikacin 34 (45.3%) and cefixime 7(41.2%) by staphylococcal species and MRSA respectively indicating that amikacin and cefixime are the drugs of choice. Resistance by the isolates was by a *mec* A independent mechanism. Eleven (64.7%) out of the 17 MRSA were positive for the penicillin – binding protein out of which 4(36.4%) were from yoghurt and 7(63.64%) from nono while 13(76.5%) were positive for betalactamase production out of which 4(30.77%) were from yoghurt and 9 (69.23%) from nono. The findings of this study, suggest that yoghurt and nono maybe vehicles for transmission of MRSA. Food safety should be advocated by relevant agencies and hygiene should be encouraged and enforced among the producers and sellers.

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LIST OF ABBREVIATIONS AND SYMBOLS

AK	Amikacin
bp	Base pair
C	Chloramphenicol
CIP	Ciprofloxacin
CN	Gentamicin
DNA	Deoxyribonucleic acid
E	Erythromycin
Kcl	Pottasium chloride
Mg	Magnesuim
$Mgcl_2$	Magnesuim chloride
Min	Minutes
ml	millilitres
mM	Millimolar
Mol/L	Mole per liter
MRSA	Methicillin-resistant *Staphylococcus aureus*
No	Number
^oC	Degree celcius
OX	Oxacillin
P	Penicillin

PCR	Polymerase chain reaction
Sec	Seconds
SXT	Sulphamethoxazole/Trimethoprim
Taq	*Thermus aquaticus*
TE	Tetracycline
Tris – Hcl	Tri- hydrochloric acid
VA	Vancomycin
χ	Chi-square
%	Percentage
µg	Microgram
µm	Micrometer
µL	Microliter

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Milk accounts for 16% of the total volume of all food products produced from livestock in sub-Saharan Africa (F.A.O., 1986). Fresh milk and its fermented products (nono, yoghurt, kindirmo) constitute good media for microbial multiplication (Jawatz *et al*., 1991) and hence for transmission of milk borne diseases such as staphylococcosis, salmonellosis, brucellosis and tuberculosis among others.

Studies approximate that 30-50% of the human population harbour *Staphylococcus aureus* on their bodies (Sowash and Uhlemann, 2014). It can survive for hours on dry environmental surfaces (Whitt and Salyers, 2002).

S. aureus produces a variety of extracellular protein toxins and virulence factors which include pyrogenic exotoxins such as staphylococcal enterotoxins (SE), extracellular toxins, toxic shock syndrome toxin1 (TSST-1), exfoliative toxins that are implicated in the staphylococcal scalded skin syndrome (SSSS) in infants, alpha toxins, beta toxins, delta toxins and bicomponent toxins like Panton valentine leukocidin which is associated with severe necrotizing pneumonia in children. The SE are proteins which when ingested induce gastroenteritic syndrome in humans and can cause toxic shock syndrome (Marrack and Kappler, 1990). They are aetiological agents of soft tissue infections and also isolated from anterior nares of healthy individuals. *S. aureus* cause several diseases such as suppurative disease, mastitis, arthritis and urinary tract infection in animals such as dogs, cats and horses and can cause bumble foot in chickens. In

humans, this organism cause foodborne intoxication, pneumonia, post operative wound infections and nosocomial bacteremia (Horan *et al*., 1998).

Staphylococcus aureus is known to be notorious in their acquisition of resistance to new drugs and continues to defy attempts at medical control (Talaro and Talaro, 2002). Many strains of *S. aureus* carry a wide variety of multi- drug resistance genes on plasmids (Jawatz *et al*., 1991; Graham *et al*., 2005; Ikeagwu *et al*., 2008).

MRSA strains are considered to be resistant to all cephalosporins, cephems, beta lactam drugs such as methicillin and oxacillin and other β lactams (such as ampicillin-sulbactam), amoxicillin-clavulanic acid, ticarcillin- clavulanic acid, piperacillin-tarzobactam and carbapenems (Clinical and Laboratory Standards Institute, 2006).

MRSA was first reported in UK in 1961 after introduction of methicillin (Voss and Doebbeling, 1995). It is one of the most prevalent nosocomial pathogens and used to be a bacterium found mostly in hospital settings (Hospital associated methicillin resistant *Staphylococcus aureus* known as HA-MRSA) but in recent years, MRSA has infected healthy people who had contracted it within their communities (Community associated *Staphylococcus aureus* known as CA-MRSA) (www.cdc/eid.org, 2008). A few reports have been published on MRSA infections in dairy herds with mastitis, and in companion animals known as Livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) (Kwon*et al*., 2005).

MRSA infection and colonization have also been reported in dairy cows, horses, dogs, cats, birds and cattle (Lee, 2003; Duquette and Nuttal, 2004; Weese, 2005).

Transmission of MRSA between animals and humans have been reported (Simoons-Smit *et al*., 2000; Manian, 2003; O'Mahony *et al*., 2005) as have the recovery of human

MRSA strains from contact with animals (Manian, 2003; Weese, 2005). *S. aureus* has been detected in milk and other food products locally obtained in Zaria (Umoh, 1989; Umoh *et al*., 1990a; 1990b; 1990c). MRSA colonization and infection in humans have been reported in several parts of Nigeria including Abuja and Zaria (Ikeh, 2003; Taiwo *et al*., 2004; Onanuga *et al*., 2006a; 2006b; Fusi *et al*., 2007; Olonitola *et al*., 2007; Olowe *et al*., 2007).

1.2 Statement of the Research Problem

Poor sanitary practices were attributed to gross contamination of food products such as milk and its products with *S. aureus*. Milk has been reported to be contaminated with MRSA which is suspected to have been acquired from dairy animals or from post milking contamination (Umoh *et al.,* 1990).

Inadequate handling of milk up to the point of pasteurisation allows for the production of thermostable toxins which resist temperature as high as 100^{o}C for 30 minutes (Virgin *et al*., 2009). Poorly pasteurized milk could be a vehicle for zoonotic diseases and there is an inherent danger associated with this product, so the mere touching of raw milk that harbours the bacteria could result in an infection (www. cdc /eid.org, 2008).

Even fermentation process with the attendant drop in pH may not rid the product of *Staphylococcus.* (Adesiyun *et al*., 1986; Headrick *et al.,* 1998). Contamination of milk and its products during processing and distribution can occur and this is a potential threat for the acquisition of *Staphylococcus aureus* including methicillin resistant strains by those who handle foods, such as those who buy milk and its products, sellers of the milk and dairy workers.

A large hospital outbreak with MRSA due to contamination of food products (milk products) has been described (Kluytmans *et al*., 1995). In the U.S, among 8,987 cases of invasive MRSA reported in 2005, 1,234 (13.7%) were due to CA-MRSA and the rest involved health care associated (HA-MRSA), 85%, and unclassified (1.3%) infections (Klevens *et al*., 2007).

Attempts to control these diseases by chemoprophylaxis and chemotherapy through the use of antimicrobial agents particularly antibiotics have resulted in increased prevalence and resistance of *Staphylococcus* to some antibiotics (Ghali, 2009).

In Nigeria, antimicrobial agents are readily available to people in local drug stores over the counter, a practice which has led to misuse of antibiotics with the associated high prevalence of antibiotic resistance among *Staphylococcus* (Ndahi *et al*., 2011). The widespread indiscriminate use of antibiotics in animal feeds, without prescription among animal and human population has led to the development of resistance by these organisms to most antibiotics and these resistant strains can be shed into the environment and consequently contaminating milk products leading to infections in animals and humans (Lee, 2003).

In northern Nigeria, "nono" is mostly processed by nomadic cattle rearers who are always in close contact with their animals and who usually do not adhere to strict hygienic practices. Contamination of the product can occur through handling and storage (Umoh *et al*., 1989).

Environmental strains of *Staphylococcus* can re-contaminate milk products even after processing because of addition of poor quality water, from the seller's clothes and hands, exposure to air and use of the same utensils at point of sale (Umoh *et al*., 1989).

Yoghurt deteriorates faster when stored at a higher temperature than at a lower temperature (Hamann and Marth, 1984). Commercially processed yoghurt is assumed to be refined by the use of modern methods but quality control may be compromised due to unsatisfactory storage conditions occasioned by epileptic power supply and the mode of handling during distribution to the final consumers by retailers.

1.3 Justification

This study was necessitated by the fact that there is little recent information on contamination of yoghurt and "nono" in Nigeria and the potential hazard to human health as regards Staphylococcal infections. The tendency for *Staphylococcus aureus* to develop antibiotic resistance and reports of its presence in dairy animals necessitated the need for an extensive study on detection of MRSA in milk products.

Although, surveillance activities in Nigeria is poor, laboratory studies need to be extensive, and they have shown the presence of some food borne pathogens and high microbial load which have also been shown to have epidemiological links with illnesses in street vended foods like nono (Umoh and Odoba, 1999).This study was designed to detect the presence of the organism in yoghurt and nono by carrying out various tests in the laboratory.Yoghurt and nono are frequently taken as liquid refreshment because of their palatability and affordability suggesting that consumers of these products are at risk.

The occurrence of MRSA is expected to complicate the healthcare delivery system in Nigeria. Therefore the detection of staphylococci is epidemiologically essential. The results of this work will help in assessing the frequency of contamination of yoghurt and traditionally fermented milk "nono" with *S. aureus* and the methicillin resistant strains.

1.4 Aim

The aim of this study was to determine the presence of *Staphylococcus aureus*, including the methicillin resistant strains in traditionally fermented milk "nono" and yoghurt in Zaria and Kaduna metropolis.

1.5 Objectives

i. Determine occurrence of *Staphylococcus aureus* (MRSA) in yoghurt and "nono" by isolation and biochemical identification of *Staphylococcus aureus* using conventional biochemical tests and Microbact system 12S (OXOID).

ii. Determine the susceptibility of isolates to commonly used antimicrobial agents by the standardized single disk method and Oxacillin resistant *Staphylococcus aureus* Basal medium (ORSAB).

iii. Detection of *Staphylococcus aureus* harbouring staphyloccocal cassette chromosome *mecA* (SCC *mecA*) using PCR.

iv. Determine the presence of the penicillin- binding protein (PBP2a) and the enzyme, betalactamase among the MRSA isolates.

1.6 Research Questions

i. Are yoghurt and nono sold in Zaria and Kaduna areas contaminated with *Staphylococcus aureus?*

ii. Are there MRSA among the*S.aureus*isolated from yoghurt and nono sold in Zaria and Kaduna?

iii Do the MRSA harbor the *mec* A gene?

iv Do the MRSA produce the enzyme beta-lactamase and the protein Pbp2a

CHAPTER TWO

LITERATURE REVIEW

2.1 General Characteristics of *Staphylococcus*

Members of the genus *Staphylococcus* belong to the Kingdom, Bacteria; Phylum, Firmicutes; Class, Bacilli; Order, Bacillaces; Family, *Staphylococcaceae*; and Genus, *Staphylococcus* (Toder, 2004).Members are gram positive cocci usually in singles, pairs, or irregular grape-like clusters, 0.5-1.5µm in diameter, non motile,non spore-forming, facultative anaerobes, high salt tolerance (up to 10% NaCl), catalase positive (usually) , capsule variable except for *S. saccharolyticus* and *S.aureus* sub specie *anaerobius* whose growth is more rapid and abundant under aerobic conditions.They are also catalase negative (www.biotabs.tmcc.edu, 2013).

2.1.1 Genome characteristics

Members of the genus *Staphylococcus* have a DNA G+C content of 30 to 39 %.The cell wall of *Staphylococcus* species contain peptidoglycan and teichoic acid susceptible to the action of lysostaphin. The genus is composed of over 32 species and 15 sub species (Ndahi *et al*., 2011).

2.1.2 Natural habitats and reservoirs of *Staphylococcus* species.

Staphylococci are wide spread in nature although they are mainly found living on the skin, skin glands and mucous membrane of mammals and birds. They may be found in the mouth, blood, mammary glands, intestinal, genitourinary and upper respiratory tracts of these hosts. Staphylococci are disseminated in pus and dried exudates discharged from skin lesions, large infected wounds, burns and in sputum.

Staphylococci found in humans and other primates include *S. aureus, S. epidermidis, S. capitis, S. caprae, S. saccharolyticus, S. hominis, S. lugdunensis, S. auricularis, S. saprophyticus, S. cohnii, S. xylosus* and S.*simulans* (Kloos, 1997). *S.xylosus* and *S.simulans* are usually transient on humans and are primarily acquired from domestic animals and their products. S. *schleiferi, S. intermedius, S. simulans*, *S.felis* and *S.hyicus* are commonly found living on carnivores and are of animal origin. However *S. schleiferi* may produce serious infection in humans and *S.intermedius* may produce infections in humans as a result of dog bites. *S.sciuri* and *S.xylosus* are residents on a variety of rodents (Ndahi, 2011).

2.1.3 Pathogenic members of the genus *Staphylococcus*

The pathogenic members include *S. aureus, S. epidermidis, S. haemolyticus, S. warneri, S. lugdunensis, S. saprophyticus,* in humans while *S.intermedius* and *S.hyicus* in animals.

2.1.4 General characteristics and history of *Staphylococcus aureus*

S.aureus, literally the"golden cluster or the "seed gold" and also known as "golden staph" and "oro staphira") is the most common of staphylococcal species. *S.aureus* was discovered in Aberdeen, Scotland in 1880 by the surgeon Sir Alexander Ogston in pus from surgical abscesses (Ogston, 1884). It is a facultative anerobe, gram positive cocci, grapelike clusters when viewed under the microscope and has large, round, golden-yellow colonies which shows haemolysis on blood agar plates (Ryan and Ray, 2004). The golden appearance is the etymological root of the name; *aureus* meaning "golden" in Latin. *S.aureus* produces catalase which is able to convert hydrogen peroxide to water and oxygen which makes the catalase test useful to dinstinguish staphylococci from

enterococci and streptococci.*S.aureus* is primarily coagulase positive, an enzymethat causes clot formation (Ryan and Ray, 2004). However, while the majority of *S.aureus* are coagulase positive some maybe atypical in that they do not produce coagulase and are known as coagulase negative *S.aureus*.

S. aureus lives on people and survive on inanimate objects and surfaces (fomites), such as bedding, clothing and doorknob. The organisms frequently colonise the anterior nares and are found in approximately 30 % of healthy individuals. They are well equipped to colonise the skin because they grow at high salt and lipid concentration. They produce enzymes, referred to as lipases and glycerol ester hydrolases that degrades skin lipids. The ability of *S. aureus* to colonize the skin and mucosal surfaces is also associated with bacterial cell surface proteins that bind to a variety of extracellular matrix proteins. Fibronectin binding proteins (Fnb PA and Fnb PB) have been identified on the surface of *S. aureus* which allows the bacteria to invade epithelial and endothelial cells and to attach to exposed fibronectin in wounds (Bannerman, 2003 and Tenover, 2005).

S.aureus infections are often acute and pyogenic. It can cause a range of illnesses from minor skin infections such as pimples, impetigo, boils(furuncles), cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses, to life- threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, myocarditis, pericarditis, meningitis, toxic shock syndrome(TSS), bacteremia, septicaemia and abscesses of the muscle, urogenital tract, central nervous system. The presence of enterotoxigenic strains of *S. aureus* in various food products is regarded as a public health hazard because of the ability of these strains to produce intoxication or food poisoning. It is still one of the five most common causes of nosocomial infections often causing post surgical wound infection (Kloos and Bannerman, 1995).

2.1.5 Epidemiology and transmission of *S.aureus*

S. aureus may occur as a commensal on human skin; it also occurs in the nose frequently and throat less commonly (Whitt and Salyers, 2002).

S. aureus infection can be spread through direct contact with pus from an infected wound, skin to skin contact with an infected person by producing hyaluronidase that destroys tissues, and contact with objects such as towels, sheets, clothing or athletic equipment used by an infected person. Prosthetic joints are a particular risk for septic arthritis and endocarditis (infection of the heart valves) and pneumonia, which maybe rapidly spread. Wild and domesticated animals may disseminate the organism from infected lesions (Curran and Al-Salihi, 1980). Higher rates are observed in injection drug users, persons with insulin- dependent diabetes, and patients with dermatologic conditions. Colonization may be transient or persistent and can last for years (Chambers, 2001). Air borne dissemination may also occur. *S. aureus* is an important secondary pathogen associated with patients recovering from influenza and para-influenza infections. Small discharging lesions on the hands of doctors and nurses are dangerous to their patients. Cross-infection is an important method of spread of staphylococcal disease, particularly in hospitals and scrupulous hand washing is essential to prevent the spread. Food handlers may similarly introduce entero-toxin producing food poisoning strains into food. Infants may be colonized with *S. aureus* shortly after birth, acquiring the organism from people in their immediate surroundings. *S. aureus* grows harmlessly on the moist skin of the nostrils in many healthy persons. This condition is referred to as colonization. Colonization frequently precedes infection in susceptible patients. The anterior nares are the principal sites of colonization with three distinct patterns in the population: persistent carriers (20 %), intermittent carriers

(60%), and non carriers (20%). Whereas, 10 -20% of healthy adults are persistently colonized with *S. aureus* , populations with higher colonization rates include patients with atopic dermatitis, surgical patients, hemodialysis patients, HIV infected patients and those with intra vascular devices. *S. aureus* is the leading cause of post operative wound infection and the second most frequent cause of nosocomial pneumonia and bacteremia. Health care workers who come in contact with patients colonized or infected with *S. aureus* have higher rate of nasal carriage and can serve as vehicles for transmission of *S. aureus* to patients. The morbidity and mortality rates of nosocomial and community acquired staphylococcal infections range between 19% and 34%. Some carriers called shedders disseminate exceptionally large number of staphylococci [Humphreys 2002; Foster 2004].

2.1.6 Virulence factors of *S.aureus*

2.1.6.1 Pyrogenic toxin super antigens (PTSAgs)

PTSAgs have superantigen activities that induce toxic shock syndrome (TSS).This group includes the toxin TSST-1, which causes TSS associated with tampon use. The staphylococcal enterotoxins, which cause a form of food poisoning, are included in this group.

2.1.6.2 Exfoliative toxins (EF toxins)

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.The protease activity of the exfoliative toxins causes peeling of the skin observed with SSSS. Some strains of *S.aureus* also produce an enterotoxin that is the causative agent of *S.aureus*gastroenteritis (Carter *et al*., 2000).

2.1.6.3 Other toxins

Staphylococcal toxins that act on cell membranes include alpha toxin,beta toxin,delta toxin, and several bicomponent toxins.The bicomponent toxin Panton-Valentine Leukocidin (PVL) is associated with severe necrotizing pneumonia in children.The genes encoding the components of PVLare encoded on a bacteriophage found in community-associated MRSA strains(Carter *et al*.,2000). Other toxins include coagulase, haemotoxin, enterotoxin, fibrinolysin, hyaluronidase and dermonecrotoxin

2.1.6.4 Role of pigment in Virulence

Some strains of *S.aureus* are capable of producing staphyloxantin – a carotenoid pigment.It has an antioxidant action that helps the microbe to evade killing with reactive oxygen used by the host immune system.Staphyloxanthin maybe responsible for *S. aureus* characteristic golden colour (Zhu *et al*., 2008).The pigmented strain of *S.aureus* is more likely to survive dousing with an oxidizing chemical such as hydrogen peroxide and to survive when exposed to human neutrophils than mutant strains that lack the yellow coloration(Liu *et al*., 2008).

2.1.7 Molecular Diagnosis of *S.aureus*

Diagnostic microbiology laboratories and reference laboratories are key for identifying outbreaks and new strains of *S.aureus*.Recent genetic advances have enabled reliable and rapid techniques for identification and characterization of clinical isolates of *S.aureus* in real-time.These tools support infection control strategies to limit bacterial spread and ensure the appropriate use of antibiotics. These techniques include real- time PCR and quantitative PCR and are increasingly being employed in clinical laboratories (www.mrsaresources.com, 2013; Mackay, 2007; Francois and Schrenzel, 2008).

2.1.8 Treatment and Antibiotic Resistance

The treatment of choice for *S.aureus* infection is penicillin; but penicillin – resistance is extremely common and in some countries the first line of therapy is commonly penicillinase resistant penicillins such as oxacillin or flucloxacillin.Combination therapy with gentamicin may be used to treat serious infections like endocardiatis (Bayer *et al*., 1998), but its use is controversial because of the high risk of damage to the kidneys (Cosgrove *et al*., 2009). The duration of treatment depends on the site of infection and severity.

Antibiotic resistance in *S.aureus* was almost unknown when penicillin was first introduced in 1943 by Alexander Fleming of Imperial College London. By 1950, 40% of hospital *S. aureus* isolates were penicillin resistant; and by 1960, this had risen to 80% (Chambers, 2001).

Researchers from Italy have identified a bacteriophage active against *Staphylococcus aureus*, including methicillin resistant strains in mice and possibly humans (Cappareli *et al*., 2007)

2.1.9 Mechanism of antibiotic resistance

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 a unique penicillin binding protein PBP2a with reduced affinity for β-lactam rings (the primary active-site of the β-lactam antibiotics) and is transmitted by a mobile genetic element termed staphylococcal cassette chromosome *mec* (SCC*mec*) (Ballhausen *et al*., 2014). In addition, some strains of *S. aureus* express β-lactamase and some strains which possess a modification of existing penicillin-binding proteins (

rather than acquisition of a PBP2a) appear to be resistant to oxacillin despite being *mec*A- negative(Gunawardena *et al*., 2012**).** Resistance to penicillin by *Staphylococcus* is mediated by penicillinase (a form of β-lactamase) production; an enzyme which breaks down the β –lactam ring of the penicillin molecule.Penicillinase resistant penicillins such as methicillin, nafcillin, oxacillin, dicloxacillin and flucloxacillin are able to resist degradation by staphylococcal penicillinase.

Aminoglycosides such as kanamycin, gentamicin and streptomycin were once effective against staphylococcal infections until the organism evolved mechanisms to destroy the aminoglycosides action, which occurs via protonated amine and/or hydroxyl interactions with the ribosomal RNA of the bacterial Ribosome (Carter *et al*., 2000).

Glycopeptide resistance is mediated by acquisition of the *van*A gene.The *van*A gene originates from the enterococci and codes for an enzyme that produces an alternative peptidoglycan to which vancomycin will not bind.

S.aureus is known to exchange antibiotic resistance and virulence determinants between different strains.The genetic information for these determinants is usually found on the mobile genetic elements.It has been noted that exchangeable elements carry genes for either virulence or antibiotic resistance or both.Mobile genetic element – encoded cytolysin connects virulence to methicillin resistance in MRSA (Shu *et al*., 2009).The β-lactamase-resistant penicillins (methicillin,oxacillin, cloxacillin and flucloxacilin)were used to treat penicillin resistant *S.aureus*.Methicillin was introduced in 1959 but the first case of MRSA in England was reported only two years later (Jevons, 1961).Despite this, MRSA generally remained an uncommon finding even in hospital setting until the 1990s when there was increase in MRSA prevalence in hospitals where it is now endemic (Johnson *et al*., 2001).

MRSA infections are commonly treated with non β-lactam drugs such as clindamycin and co-trimoxazole but resistance to these drugs has led to the use of new, broad-spectrum anti-gram positive antibiotics such as linezolin. First line of treatment for invasive infections due to MRSA is currently glycopeptides antibiotics (vancomycin and teicoplanin).The shortcomings of these drugs include the need for intravenous injection, toxicity, need to monitor drug levels regularly and inability to penetrate very well into infected tissues such as infection associated with brain, meninges and heart. Glycopeptides must not be used to treat methicillin – sensitive S.*aureus* as outcomes are inferior (Blot *et al*., 2002). The Centers for Disease Control and Prevention, U.S.A have published guidelines for the appropriate use of vancomycin because of the potential for MRSA to develop resistance to vancomycin (CDC, 1995).

Vancomycin-resistant *S.aureus* (VRSA) is a strain of *S.aureus* that has become resistant to the glycopeptides.The first case of vancomycin –intermediate *S.aureus* (VISA) was reported in Japan in 1996(Hiramatsu *et al*., 1997), but the first case of resistance to glycopeptides antibiotics was reported in 2002 (Chang *et al*., 2003a).

2.2 Methicillin – Resistant *Staphylococcus aureus* (MRSA) Organisms

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global public health problem, associated with considerable morbidity and mortality (Pallab,, *et al*., 2011). MRSA is a gram positive bacteria that is resistant to methicillin and other β-lactam antimicrobials which include penicillins and cephalosporins.Currently, the term MRSA is often used to describe multi-drug resistant *S.aureus* (MDSA) or oxacillin-resistant *S.aureus* (ORSA).It doesn't mean antibiotics are completely ineffective but it simply requires a much higher dose over a longer period, or the use of an alternative antibiotic to which the bacteria has less resistance.

2.2.1 Community-associated MRSA (CA-MRSA) in humans

Community-associated MRSA (CA-MRSA) infects healthy individuals without any previous health- care contact. CA- MRSA is usually panton-valentine leucocidin (*pvl*) positive, susceptible to non-β-lactam antimicrobial drugs, and frequently manifests as skin and soft-tissue infections. However, this distinction between CA- and HA-MRSA is gradually fading owing to the emergence of *pvl* negative and/or MDR CA-MRSA clones, and its invasion into hospitals. The CA- MRSA has a higher transmissibility and virulence compared to HA-MRSA, and is epidemic in many geographical locations Pallab *et al*., 2011). CA-MRSA was first reported in the late 1990s; these cases were defined by a lack of exposure to the health settings.Risk factors for CA-MRSA include crowding, frequent contact, compromised skin, contaminated surfaces, shared items and poor hygiene.Presently,strains most commonly associated with CA-MRSA in humans is *USA300*(Naimi *et al*., 2003).

2.2.2 Hospital – associated MRSA (HA-MRSA) in humans

Hospital-associated MRSA (HA-MRSA) characteristically colonizes or infects hospitalized individuals with predisposing risk factors, usually harbours SCC*mec* type I, II or III, and also multi-drug resistant (MDR). The HA-MRSA is endemic in many hospitals worldwide (Pallab *et al*., 2011).Risk factors for HA-MRSA include hospitalization, surgery, dialysis, long-term care, indwelling devices and history of previous MRSA infections.Currently, the MRSA strains most commonly associated with HA-MRSA in humans is *USA 100*.

2.2.3 Resistance and virulence factors associated with MRSA

Resistance is mediated by *mec* A gene that encodes the production of an altered penicillin-binding protein (PBp2a), which does not allow for the binding of β-lactams to the bacterial cell wall (Weese, 2005). Since β-lactams exert antibacterial activity by binding and inhibiting enzymes necessary for bacterial cell wall synthesis,these antibacterials are not effective against MRSA.However, in a study in Zaria, Nigeria, Olonitola *et al*.(2007) observed that all the MRSA isolated from nasal swabs of healthy individuals were *mec*A negative but β-lactamase producing and concluded that resistance in MRSA is due to the presence of β-lactamase.Other studies have revealed the likelihood of novel strains of MRSA which were *mec*A negative but may have other homologues responsible for marked antibiotic resistance.

Novel MRSA isolates that are less likely to be resistant to antimicrobials other than β-lactams have been identified and associated with epidemic CA-MRSA infection.Also the genes encoding the pore-forming, bicomponent cytotoxin, Panto-valentine Leukocidin (PVL), are present in novel CA-MRSA.However, evidence from animal studies has been contradictory in assessing the importance of PVL in the virulence of these isolates (Labandeira-Rey *et a*l., 2007; Voyich *et al*., 2006).

In addition to pvl genes,strains that cause CA-MRSA infections typically carry Staphylococcal chromosomal cassette *mec*(SCC *mec*)types 1V and V,small genetic resistance elements that are presumably mobile.The predominant CA-MRSA strain is USA 300 (defined by pulsed-field gel electrophoresis),corresponding to sequence type (ST) 8 by multilocus sequence typing (MLST) in many centers in the United States and China (Pan *et al*., 2005; Moran *et al*.,2006;Furtado *et al*.,2006;Han *et al*.,2010).The reason for the dominance of USA300 is not clear.

2.3 Transmission of MRSA

2.3.1 Transmission of MRSA in humans

Transmission is thought to occur primarily from colonised or infected persons to others (Opal, 1990;Muder *et al*., 1991).MRSA is usually transmitted by direct skin-to skin contact or contact with shared items or surfaces that have come into contact with someone else's infections (e.g. towels,used bandages). Health care providers transfer of infection to patients is common.In one hospital in Texas, 12% of the staff were carriers of MRSA, and so also 15% of the staff in an Illinois emergency room (Ibarra *et al*., 2008; Bisaga *et al*., 2008).

Milstone *et a*l.(2010) in a similar study characterized the degree to which CA-MRSA strains were imported into and transmitted in paediatric intensive care units (PICU).They performed a retrospective study of the children admitted to the Johns Hopkins Hospital PICU .They found that 72(6%) of 1,674 PICU patients were colonized with MRSA, and colonized patients were more likely to be younger (median age of 3years Vs 5years;p=0.02 and African American (P<0.001)and to have been hospitalized within 12 months(P<0.001)than were none colonized patients.MRSA isolates from 66 (92%) colonized patients were fingerprinted; 40(61%) were genotypically CA-MRSA strains. Epidemic CA- associated strains are becoming endemic to PICU's, can be transmitted to hospitalized children and can cause invasive hospital-acquired infections.

2.3.2 Zoonotic nature of MRSA and transmission in animals

MRSA is now an urgent problem in veterinary medicine with infections in horses, cats, dogs, pet birds, cattle and pigs (Lee 2003; Weese, 2005; Baptiste *et al*., 2005).CA-

MRSA in humans is thought to be a major factor in the rise of MRSA infection in companion animals (Baptiste *et al*., 2005; Weese, 2005; Huijsden *et al*., 2006; Hanselman *et a*l., 2006). Infection with the MRSA strain USA100 (CA-MRSA-2, ST-MRSA-SCC *mec* II) has been shown to be more predominant in small animals, while infection with the MRSA strain USA 500(CA-MRSA-5, ST8-MRSA SCC *mec* IV) has been shown to be more predominant in large animals (Hanselman *et al*., 2006)

There is now increasing evidence that MRSA can be transmitted from animal to humans (zoonotic) and from humans to animals (reverse zoonotic).Healthy animals can become colonized and may serve as reservoir to transmit the infection to other animals and their human handlers (Weese, 2005; Huijsden*et al*., 2006; Hanselman *et al*., 2006; Baptiste *et al*., 2005; Weese *et al*., 2005b; DeNeeling *et al*., 2007).

With human to animal transmission of MRSA, there is a possibility that until the animal is free of the infection, re-transmission from animal and subsequent human transmission can occur (Manian, 2003; Baptiste *et al*., 2005). In one reported case of possible dog to human transmission, a diabetes mellitus patient and his wife experienced recurrent MRSA infections with difficulty in elimination (decolonization). A nares culture of the family dog revealed it was colonized with MRSA, and long term decolonization for the man and his wife occurred only after the dog was treated (Manian, 2003).

Lee (2003) determined the molecular epidemiological relatedness of 15 animal MRSA isolates from humans by random amplified polymorphic DNA (RAPD).The result showed that the RAPD patterns of 6 of the isolates from animals were identical to the patterns of certain isolates from humans.Also Lewis *et al*. (2008) and Kreziwanek *et al*. (2009) showed that MRSA clonal complex (cc) 398 and clonal type ST 398 associated with animals, particularly pigs were found in humans in Denmark and upper Austria.

In Malaysia, studies reviewed showed a widespread occurrence of MRSA in animal species which includes pigs, horses, dogs, cats, chickens and humans with several studies showing transmission in both directions.

2.3.3 Transmission of MRSA through milk

Behari *et al*. (2004) determined the potential source of MRSA colonization and infection among infants in a neonatal intensive care unit (NICU) using molecular analysis of breast milk samples. Routine active surveillance was performed on preterm triplets delivered at 26 weeks via caesarean operation in a NICU.Surveillance consisted of swabbing the throat,nose and umbilicus (tnu) weekly.Althoughtwo infants initially had negative tnu swabs,repeat cultures were positive for MRSA.The infants developed sepsis and conjunctivitis.Both infants were fed breast milk via nasogastric tube.Cultures of breast milk sampled for both infants were positive for MRSA.All MRSA isolates had identical results on antibiotic susceptibility testing.PFGE demonstrated identical banding patterns for the MRSA isolates from the blood culture of both infants.The mother did not develop evidence of mastitis or other local breast infection.They concluded that MRSA can be passed from mother to preterm infant through contaminated milk,even in the absence of maternal infection.

Similarly, Juhasz-Kaszanyitzky *et al*.(2007) isolated MRSA from cows with subclinical mastitis and a person who worked with the animals in Hungary.The bovine and human strains were indistinguishable by phenotyping and genotyping methods.They also concluded that there maybe transmission of MRSA between cows and humans.

2.4 Prevalence of MRSA

2.4.1 Prevalence of MRSA in humans

A population- based study of the incidence of MRSA infections in San Francisco during 2004-2005 demonstrated that nearly 1 in 300 residents suffered from such an infection in the course of a year and that greater than 85% of these infections occurred outside the healthcare settings (Liu *et al*., 2008).

In a study of MRSA infection in a state prison in Missisipi,Culpeper *et al*.(2000) showed that 86(4.9%) of the 1,757 inmates sampled were MRSA carriers. More women,73 of 1,251(5.9%) were carriers than men.Huijsdens *et al*.,(2008) observed an outbreak of CA-MRSA USA 300 in a beautician and two of her customers.Eight otherpersons, who were either infected or colonized, were linked to the outbreak, including a family member,a household contact and partners of customers.Also Tattevin *et al*.(2009) performed an analysis of MRSA USA 300 clone in long- term care facility in San Francisco Laguna hospital in 2006.Thier report revealed that USA 300 clone increased from 11.3% of all MRSA isolates in 2002 to 64.0% in 2006 and was mostly recovered from skin lesions on the face,characterized by rapid evolution to furuncles and small abscesses.Cultures of the abscesses yielded an MRSA isolate that was confirmed to be CA-MRSA belonging to the USA400 clone.

The proportion of MRSA isolates from invasive infections that were either USA300 or USA400 increased significantly from 1999 – 2005 through 2006 ($P<0.0001$).During 2006, the incidence of invasive community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections was highest in the summer ($P = 0.0004$).Age <69 years was associated with an increase risk for invasive CA-MRSA

infection(odds ratio =5.1, 95% CI 2.06-12.64)and hospital exposure was associated with decreased risk (OR 0.07,95% CI 0.01-0.51).

Worldwide, an estimated 2 billion people carry some form of *S.aureus*; of these up to 53million(2.7% of carriers)are thought to carry MRSA.

2.4.2 Prevalence of MRSA in Nigeria

AMRSA prevalence rate of 43% was observed at Jos University Teaching Hospital (Ikeh, 2003) while Onanuga *et al*.(2006a) showed a prevalence rate of 76.7% and 68.5% from urine samples in Abuja and Zaria respectively among healthy women.The prevalence rate of 20% was also recorded in Zaria from non-hospital sources (Olonitola *et a*l., 2007).Also Taiwo *et al*.(2004), Fusi- Ngwa *et al*.(2007) and Olowe *et al*.(2007) in separate studies observed a prevalence rate of 34.7%,54.9% and 47.8% in Ilorin, Lagos and Oshogbo respectively. Ghebremedhin *et al*.(2009) provided a comprehensive overview of the molecular epidemiology and genetic diversity of *S.aureus* strains at the largest university clinic in Ibadan,Nigeria.From 1300 patients, clinical samples collected at the University Teaching Hospital in Ibadan, Nigeria, in one year surveillance in 2007,346 non duplicate *S.aureus* isolates were obtained. All isolates underwent antibiotic susceptibility testing, toxin gene analysis,multilocus sequence typing, *agr* group typing and *spa* typing. For methicillin resistant *S.aureus*(MRSA), *Staphylococcus* cassette chromosome *mec*(SCC *mec*) typing was also performed.Of the 346 isolates, 20.23% were methicillin resistant.Thirty three isolates(47.15%) fulfilled the definition criteria for CA-MRSA according to a review of the medical charts.The ST88 strain was resistant to trimethoprim-sulphamethoxazole as well as to penicillin and oxacillin.

Similarly, Okon *et al*.(2009) characterized phenotypically and genotypically ninety-six clinical isolates of *S.aureus*.Twelve multi-drug resistant *S.aureus*(MRSA) isolates carrying a new staphylococcal cassette chromosome *mec* element and a high proportion of Panton-Valentine Leukocidin(PVL)-positive methicillin-susceptible *S.aureus*(MSSA) isolates were observed.The occurrence of multidrug-resistant MRSA and PVL positive MSSA isolates entails the risk of emergence of a multidrug resistant PVL-positive MRSA clones.

Also,Fadeyi *et al*.(2010)worked on MRSA carriage amongst health workers of the critical care units in a Nigerian Hospital.Of the 198 health workers screened,104 had MRSA either in the nose,hand or both giving a carriage rate of 52.5%,nasal carriage(38.9%)was higher than hand (25.3%).Doctors(22.75%) and nurses(16.7%) were the predominant carriers.

In Kano, Nwankwo *et al*.(2010) studied MRSA and their antibiotic sensitivity patterns.Their results showed that out of the 185 *S. aureus* tested, 53(28.6%) were found to be methicillin resistant.

2.4.3 Prevalence of MRSA in animals

Few data on MRSA colonization rates in non-clinically affected animals are available, the routine screening of all animals is not practicable, and so there remains the possibility that a small percentage of colonized animals will remain undetected upon admission to a veterinary clinic or hospital (Leonard and Markey, 2008).

Studies have also shown a low prevalence of MRSA in dogs (0.6%) (Abbott *et al*., 2006). A study reported that 20 out of 140 horses with suspected wound infections were positive for MRSA strains of ST1, ST25 and AT398 (Cluny *et al* ., 2009).

Studies by Khann *et al*. (2008) and Smith *et al*.(2009) found MRSA colonization rates of 25% and 70% in pigs in Europe and US respectively.In the Netherlands,Wulf and Voss(2008) revealed that 39% of the slaughtered pigs were positive for an unusual strain of MRSA ST398.From poultry flocks ,Pearsons *et al*.(2009) showed that the number of positive samples varied between 1/5(20%) and 5/5(100%)

Studies have also shown the occurrence of MRSA strains in a goat breeding farm. Eight MRSA isolates were detected.Five of them originated from goat milk and three were from one human carrier of the farm personnel.

Similarly, Huber *et al*. (2010) worked on the prevalence of MRSA in humans in contact with farm animals, in livestock and in food animal such as pigs, calves and cattle in Switzerland.

2.4.4 Prevalence of MRSA in milk

Novak *et al*. (2000) analysed 500 samples of human milk from five Brazilian cities, and discovered that 57 strains (11.4%) of MRSA were isolated.Also *mec*A gene responsible for resistance was detected in all 57 strains using specific molecular probe.The study concluded that breast milk can be a reservoir of multiresistant *S.aureus* epidemic clones.

Kwon *et al*. (2005) showed that MRSA were isolated from the milk of cows. SCC*mec* of some of the MRSA strains was designated as a new subtypeIVgl. The strains shared panton-Valentine Leukocidin (*pvl*) and staphylococcal enterotoxin, SED, SEI and SEJ genes. The strains had community-acquired MRSA (CA-MRSA) characteristic.The study concluded that MRSA isolated from bovine milk harboured a unique SCC*mec* subtype.

Reddy *et al*. (2007) in a case-control study documented a relative increase in methicillin resistance among *Staphylococcus aureus* associated postpartum mastitis during 1998-2005.Majority (95%) contained the SCC*mec* typeIV gene which suggests that the increase is due to CA-MRSA.

Also, Tuirkyilmaz *et al*.(2009) identified MRSA strains from milk samples in Aydin region in Turkey.Among 93 *S. aureus* isolated from bovine milk with mastitis,16(17.2%)were resistant to methicillin.The MRSA strains were multi-drug resistant, with susceptibility rates to antimicrobial tested as 0%, 0%, 0%, 0%, 6.25%, 16.25% and 57.25% for erythromycin, clindamycin, chloramphenicol, gentamicin, tetracycline, ciprofloxacin and vancomycin respectively.

2.5 Clinical Signs of MRSA Infections

2.5.1 Clinical Signs of MRSA in humans

The initial presentation of MRSA is small red bumps that resembles pimples,spider bites or boils that may be accompanied by fever and occasionally rashes.Within a few days the bump becomes larger,more painful and eventually opens into deep,pus-filled lesion.About 75% of CA-MRSA infections are localized to skin and soft tissue and usually can be treated effectively.However,CA-MRSA strains display enhanced virulence,spreading more rapidly and causing illness such as pneumonia, mastitis, meningitis,osteomyelitis and endocardiatis much more severe than traditional HA-MRSA infections,and they can affect vital organs and lead to wide spread infections(sepsis),toxic shock syndrome and necrotizing(“flesh-eating”) pneumonia.This is thought to be due to toxins carried by CA-MRSA strains,such as PVL and PSM (Gunawardena,*et al*., 2012).

CA-MRSA often results in abscess formation that requires incision and drainage.Before the spread of MRSA into the community, abscesses were not considered contagious because it was assumed that infection required violation of skin integrity and the introduction of *Staphylococcu*s from normal skin colonization.However, new emerging CA-MRSA is transmissible.

2.5.2 Clinical signs of MRSA in animals

Not all animals that encounter MRSA develop clinical signs.While research is still ongoing,it appears only a small percentage becomes ill,while most eliminate the organism or become colonized without developing clinical signs.The most commonly reported clinical signs are postoperative and wound infections,with less reported incidence of intravenous catheter site infections,urinary tract infections,pneumonia,skin and ear infections(Weese,2005;Vitale *et al*., 2006).CA-MRSA infection may present as red, swollen,painful site with drainage (Vitale *et al*., 2006; Oehler *et al*., 2009).

2.6 MRSA Infections in HIV/AIDS Patients

Whether HIV increases the risk of CA-MRSA needs more study. In a study presented at the XVI International AIDS Conference in August, 2006, investigators stated that HIV patients had an 18-fold increase in their risk for CA-MRSA (Ball, 2005).However, several studies have found that HIV does not increase the risk significantly.The reports of MRSA having higher HIV rates than the general population does not imply that HIV patients are at a higher risk for MRSA (Ball, 2005).

2.7 Diagnosis of MRSA

Diagnostic microbiology and reference laboratories are keys for identifying outbreaks of MRSA.Improper 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.Phenotypic methods include:colonial characteristics,biochemical reactions,antibiotic susceptibility patterns,and the susceptibility to various phages and toxin production.

Diagnosis should involve the identification of coagulase-positive staphylococci to the species level,and all *S.aureus* should then be tested for oxacillin resistance,since methicillin is less stable invitro (Lee, 2003; Ike, 2003; Taiwo *et al*., 2003; Hanselman *et al*., 2007; Fusi Ngwa *etal*., 2007;Onanuga *et al*., 2006b;Olonitola *et al*., 2007).

Another common laboratory test is a rapid latex agglutination test which detects the PBP2a protein. PBP2a is a variant penicillin binding protein that imparts the ability of *S. aureus* to be resistant to oxacillin (Seiken, 2009). If there is a recurrent or persistent case of skin infection in the animal, a small biopsy of either the infected skin or a sample of the exudates from the site may be submitted for lab diagnosis. If *S. aureus* is isolated, further tests are needed to determine if it is MRSA and start appropriate antibiotic selection (Lee, 2003).

2.7.1 Molecular diagnosis of MRSA

The most common molecular detection methods are, pulsed field gel electrophoresis (PFGE), multilococcus sequence typing (MLST), Real time-PCR and Quantitative-PCR (Mackey, 2007; David *et a*l., 2008; Francois and Schrenzel, 2008; Persoons *et al*., 2009; Denis,2009;Tattevin *et al*.,2009), simple and multiplex PCR , DNA sequencing, hybridization based techniques which includes macro and micro assays (www.

Specialtylabs.com). Commercial amplification assays such as IDI-MRSA are also available (Derek, *et al*., 2005).

2.8 Treatment of MRSA Infections

2.8.1 Treatment of MRSA in humans

CA-MRSA has a greater spectrum of antimicrobial susceptibility,including sulfa drugs,tetracycline and clindamycin.HA-MRSA is resistant even to these antibiotics and is often susceptible only to vancomycin.Vancomycin and teicoplanin are glycopeptide antibiotics used to treat MRSA infections (Gunawardena,*et al*., 2012).Teicoplanin is a structural congener of vancomycin that has a similar activity spectrum but a longer half-life(Rybak *et al*., 1991).The oral absorption of vancomycin and teicoplanin is low and are therefore, usually administered intravenously.Moreover,many clinicians believe that the efficacy of vancomycin against MRSA is inferior to that of anti-staphylococcal beta-lactam antibiotics. Some strains of MRSA show antibiotic resistance to even vancomycin and teicoplanin and are known as vancomycin intermediate *S.aureus* (VISA) or vancomycin resistant *S.aureus* (VRSA) (Sieradzki and Thomas, 1997; Schito, 2006).

Linezolid, quinupristin/dalfopristin, daptomycin and tetracycline are used to treat more severe infections that do not respond to glycopeptides such as vancomycin(Mongkolrattanothai *etal.*, 2003).MRSA can be treated with oral agents including, rifampicin-fusidic acid, rifampicin + fluoroquinolone,pristinamycin, co-trimoxazole (trimethoprim –sulfamethoxazole), doxycycline or minocycline and clindamycin (Birmingham *et al*., 2003). Mupirocin can potentially eliminate MRSA from mucus membrane colonization

A new antibiotic called platensimycin has been demonstrated to be useful against MRSA (Bayston *et al*., 2007; Wang, 2007).In a study at the University of East London and University of New York, allicin a compound in garlic and small quantities of silver carbonate was found to successfully treat MRSA.

2.8.2 Treatment of MRSA infections in animals

When animals are found to be colonized with MRSA based on nasal swabs,there is currently no recognised method of decolonizing these animals,but based on clinical cases observed some experts have indicated that companion animals are transient carriers of MRSA,and decolonization is either unnecessary or the treatment could be simply isolation from the individuals who maybe transferring the MRSA back to the animals(Weese,2008).Weese *etal*.(2005b) suggested that decolonization of horses with MRSA can be achieved without use of antimicrobial therapy,only by segregation and repeated screening.

Skin infections are the most common manifestation of MRSA in animals and combination of systemic therapy with topical antimicrobial treatment may have some effect(Lloyd,2006).It has been suggested that while systemic therapy is needed, topical treatment with antimicrobials may be impractical based on cost and difficulty in adequate application of topical ointment into the nasal cavity. It has been speculated that both treatment may lead to further microbial resistance (van Duijkeren *et al*., 2004; Weese, 2008)

Lee (2003) observed that MRSA isolates from chicken and cattle were susceptible to amikacin, trimethoprim-sulfamethoxazole, norfloxacin, ofloxacin and tetracycline. Persoons *et al*. (2009) also reported that MRSA strains isolated from poultry in Belgium

were susceptible to chloramphenicol, ciprofloxacin, linezolid, mupirocin, quinopristin-dalfopristin, rifampin and sulphonamides.

Researchers investigated six Nigerian medicinal plants (*Terminalia avicenniodes, phylantus discoideus, Bridella, ferruginea, Argeratum conyzoides, Ocimum grassimum and Acalypha wilkesiana)* commonlyused by traditional medical practitioners in Western Nigeria for the treatment of several ailments of microbial and non-microbial origin and for invitro anti-MRSA activity.The results of the study indicated that four (*Terminalia avicenniodes, Phylantus discoideus, Ocimum grassimum and Acalypha wilkesiana)* out of the six medicinal plants commonly used by the traditional medical practitioners to cure skin and respiratory tract infections such as pneumonia, carbuncle, papule, impetigo and tonsillitis were active against hospital strains of MRSA (Kabir *et al*., 2005).

2.8.3 Phage therapy against MRSA infections

Reports from Italy have identified a bacteriophage, *Msa* active againt *S.aureus* including Methicillin-sensitive strains in mice.Following simultaneous inoculation of both *Msa* and lethal and non- lethal dose of *S.aureus*, results showed that *Msa* rescued 97% of mice from death and fully cleared mice of non-lethal bacterial infections.These suggest a potential use of the phage for the control of both local and systemic human *S.aureus* infections (Cappareli *et al*., 2007).

2.9 Control and Prevention of MRSA Infections

2.9.1 Control and prevention of MRSA in humans

Control and Prevention of MRSA in humans is basically as follows:

1. Avoidance of MRSA patients and carriers by not making direct contact with skin, clothing and any items in contact with either MRSA patient or carrier and also by treating and covering skin breaks with antiseptic cream and a Band –Aid, maintaining hygienic practices such as hand washing with soap, antiseptic solutions disposing items, and wipes after contact with patients and carriers.

2. Patients screening upon hospital admission, with nasal cultures, prevents the cohabitation of MRSA carriers and non-carriers, and exposure to infected surfaces (Leonard and Markey, 2008).

3. Surface sanitization using alcohol is a proven effective surface sanitizer against MRSA.Quaternary ammonium can be used in conjunction with alcohol to extend the longetivity of the sanitizing action.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 best practice for nosocomial MRSA (Leonard and Markey, 2008).

4 Hand washing was announced by the UK National Health Service after a successful pilot scheme of the clean your hand campaign to tackle MRSA at the end of august 2004.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 based rubs are somewhat effective, a more effective strategy is to wash hands with an anti microbial cleanser with persistent killing action such as chlorhexidine (Buckingham, 2008).

5. Decolonization: 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 seven days.Household members are recommended to follow the same protocol.Doctors may also prescribe antibiotics such as clindamycin,doxycycline or trimethoprim/sulfamethoxazole. However, there is very little evidence that using more antibiotics actually has the effect of preventing recurrent MRSA skin infections (Buckingham, 2008).

6. Tea tree oil vaporizer is a vaporizer spraycontaining some essential oils (including tea tree oil) which is sprayed into the atmosphere and it reduced airborne bacterial counts by 90% and kept MRSA infections at bay (Leonard and Markey,2008).

2.9.2 Control and prevention of MRSA infections in animals

Precautions to be taken by Veterinarians, animal handlers and animal owners are as follows;

- Frequent hand washing and proper disinfection of hard surfaces and equipments between patients
- Provision of hand sanitizers in consulting rooms and kennels.
- Wear protective clothes like uniforms, gloves, disposable aprons and masks when changing dressings on infected wounds or to prevent potential contact with body fluids or contaminated tissues
- Protect eye if splashing or aerosols are expected
- Clinic, homes and farm surroundings should be kept to a high standard of cleanliness
- Licking and “shaking paws” should be avoided even if the animal’s paw is clean

- Handlers are restricted to interaction only with the patients and their families
- When placing an animal on a bed,clean towel or absorbent pad should be placed between the pet and the bed linens,
- No animal should be allowed to visit patients in isolation unit

2.9.3 Vaccines against MRSA infections

The surface of bacterial cell is decorated with variety of proteins that can interact with components of tissues and blood.One of such protein is the clumping factor(Clf) that binds to soluble fibrinogen in plasma and to fibrin in blood clots.It is conceivable that Clf on bacterial surface binds fibrinogen to coat the cell surface and prevent access by factors that normally enhance phagocytosis.Alternatively Clf could make cells clump together and prevent access.This is being investigated further with a bacterial variant that expresses a mutant form of Clf which cannot bind fibrinogen but which is otherwise expressed normally onthe bacterial surface (Foster, 2001).

The Clf is also a promising protein for a novel vaccine to prevent *S.aureus* infections in hospital patients.Mice were immunized with a part of Clf protein made by recombinant DNA technology.The immunized mice were much less susceptible to infection.This proves that resistance to infection was mediated solely by antibodies and may not involve the cellular arm of the immune system (Foster, 2001).

2.10 Yoghurt Preparation

The composition of milk is modified and standardized by reducing the fat content and increasing total solids.The fat is removed from milk by machines known as seperators, a process termed centrifugation.Stabilizers such as gelatin and pectin are added and the

milk is then pasteurized at 10^{o}C to 95^{o}C for 15secs to 20mins. Following pasteurization, the milk is homogenized using a homogenizer or viscolizer to get consistently dispersed particles which will yield smooth and creamier end product.The milk is then cooled to 42^{o}C to 46^{o}C for inoculation and fermentation.Starter cultures such as freeze dried bacteria are inoculated into milk at a concentration of 2%.It is held at this temperature for about 3 to 4 hours while incubation takes place and a p^{H} value of 4.4 and 0.9% acidity is reached as a result of the development of lactic acid.It is then cooled to 2^{o}C to 7^{o}C to stop fermentation.Additives such as fruits, flavours, emulsifiers and preservatives are added at this point and then packaged (www.dairyconultant.co.uk,2015).

2.11 Nono Preparation

Nono is a fermented food drink derivatives gotten from cow milk. As a general practice among Fulani Herdsmen, the milking is done between the third and sixth months of lactation. Until the third month, the calves are left to consume milk. Cows are only milked at night and since no milking is possible during the day calves roam with the dam. Traditionally, nono is prepared by inoculating freshly drawn cow milk with a little of the leftover as starter and then is allowed to ferment naturally for twenty four hours at room temperature. During fermentation, some of the lactose is converted to the lactic acid. At the end of the fermentation period, the milk butter is removed by churning for further use and the remaining sour milk, nono is a delicious and refreshing beverage. Most of the organisms involved in the fermentation process are usually of three main groups; bacteria, yeast, and mould. Of these, *Lactobacilus* (*L. acidophilus and L. bulgaris*), *Lactococcus* species (*L. cremoni, and L. lactis*), *Streptococcus thermophilus, Leuconostoc* species *and Saccharomyces* species seems to be the most prominent, each giving the product a characteristic flavor (Egwim *et al*., 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Kaduna and Zaria metropolis. Zaria and Kaduna are the two largest and most cosmopolitan cities in Kaduna state. Kaduna is the capital of Kaduna State in North Central Nigeria with the coordinates 10°31' N 7°26' E 10.517°N 7.433°E (Kaduna state , 2010). Zaria is comprised of Zaria and Sabongari local government areas. It lies between latitude 7° and 11° north and Longitude 7°44'east (Zaria, 2010.)Four major markets were randomly selectedby simple random method from among the major markets within Zaria metropolis (Samaru, Sabongari, Tudun wada and Zaria city) as well as from Kaduna metropolis (Barnarwa, Station, Central and Kawo). Markets in these cities are among the largest and enjoy commerce from people within the cities, surrounding cities and from other states. Major markets are those visited on all days of the week with a high population of buyers and sellers of nono and yoghurt (i.e where fulanis congregate).

3.2 Sample Size Determination

The sample size was calculated using the formula as described by Thrushfield (1997).

$$N = \frac{Z^2pq}{d^2}$$

where N= sample size for the study,Z= standard normal deviation(1.96),p = anticipated prevalence of MRSA = (4.8%) Ghali (2009)

q=1 – p, d = degree of freedom (5% or 0.05) = 70.2. Based on this a total of 560 nono and yoghurt samples were obtained.

3.3 Sample Size

Five hundred and sixty samples were collected from 8 major markets from the two towns.Thirty fiveyoghurt and thirty five "nono" were collected from each of the markets.Representative of the entire sample types were collected based on availability in the market.

3.4 Sample Collection/Transportation

Samples of yoghurt and nono were collected based on their availability in the market. Adequate quantities of each product were collected in the manner offered to prospective customers and placed aseptically into a polythene bag and sample bottles and transported to the bacterial zoonosis laboratory of the department of veterinary public and preventive medicine, Ahmandu Bello University on ice in a coleman box.All samples were processed in the laboratory immediately or kept in the refrigerator at 4°C until processed within 24 hours.Samples were collected between December 2012 and May 2013.

3.5 Materials Used

3.5.1 Media

All Media used except otherwise stated were products of Oxoid® Ltd, Basingstoke,UK.The materials include Baird Parker agar, Modified Trypticase Soy Broth, Mueller Hinton Agar, Nutrient agar, Nutrient broth, Egg yolk Tellurite, Blood agar base, Mannitol Salt agar, DNase medium,Urease agar base and peptone water.

Blood were collected from sheep, rabbit, horse and human, for the blood agar preparation.

3.5.2 Sugars

Glucose, lactose, mannitol, xylose and maltose.

3.5.3 Antimicrobial disks and concentrations

Methicillin (MET,10µg), Oxacillin(OX,1µg), Penicillin(P,10IU), Sulphamethoxazole / Trimethoprim (SXT,25µg), Gentamicin (CN,10 µg), Ciprofloxacin (CIP,5µg), Erythromycin (E,15µg), Chloramphenicol (C, 30µg), Tetracycline (TE, 10µg), Ampicillin (AMP 10µg), Amikacin (AK 30µg), Cefixime (CFM 5µg), Vancomycin (VA 30µg), were obtained from Oxoid® Ltd, Basingstoke, UK. Oxacillin Resistant *Staphylococcus aureus* Basal Medium (ORSAB) was also used to presumptively determine methicillin resistant strains.

3.5.4 Test kits

Microbact 12S identification kits (Oxoid® Ltd, Basingstoke, UK), Penicillin-binding protein kits (Pbp2a) and betalactamase detection kits

3.5.5 Materials for molecular detection

DNA extraction kit (ZYMO®, Inqaba biotech, South Africa), Taq polymerase (Promega), Master mix, Primers *mec* A1 5’ – AAAATCGATGGTAAAGGTTGGC-3’and *mec* A2 5’ AGTTCTGCAGTACCGGATTTGC- 3’ (Inqaba, South Africa).

3.5.6 Other materials used

Disposable petridishes, conical flasks (250mls, 500mls), bijou bottles, test tubes, foil paper, masking tapes, permanent marker, hydrogen peroxide(3%), rabbit plasma, normal saline, safranin, glass slides, hand gloves, Pasteur loop, sterile sample bottles, microcentrifuge tubes.

3.6 Isolation and Characterization of *Staphylococcus Aureus* from Yoghurt and Fermented Milk "nono"

3.6.1 Isolation procedure for *Staphylococcus aureus*

Modified Trypticase broth was prepared according to manufacturer's instruction.Sodium chloride salt was added (Nacl) at 70mg/ml.About 10ml of each sample was aseptically added to 90ml of sterile Modified Trypticase Soy broth, mixed thoroughly and incubated at 37°c for 18 to 24 hoursfor enrichment.

Baird Parker medium with egg yolk tellurite was prepared according to manufacturer's instruction.A sterile wire loop was used to pick a loopful of the enrichment medium and aseptically inoculated onto the prepared Baird Parker agar plate by streaking out using the loop in order to obtain discrete colonies.The plates were incubated at 37°c for 24 hours.Presumptive colonies of *Staphylococcus aureus* species appearing as small,smooth,black shiny convex colonies with narrow white margins on Baird Parker agar were picked and inoculated onto nutrient agar slants,incubated at 37°c for 24hours and stored at 4°c pending further characterization.

3.6.2 Identification of *Staphylococcus*

Small (1-1.5mm diameter), smooth, black and shiny convex colonies with narrow white margin surrounded by zone of clearing (2-5mm) as described by Baird- Parker (1962), were picked and Gram stained.

3.6.3 Conventional biochemical tests for *Staphylococcus*

All isolates cultured on nutrient agar medium were subjected to the following conventional biochemical tests for identification of *Staphylococcus* species: gram staining, catalase, coagulase, sugar fermentation, haemolysis on 5% sheep, rabbit, horse and human blood, pigmentation on Mannitol salt agar and DNase activity (Barrow and Feltham, 1995).

3.6.3.1 Catalase test

Isolates suspected to belong to the genus *Staphylococcus* were tested for their ability to produce the enzyme catalase. One drop of 3% hydrogen peroxide was placed on a slide and a colony of the suspected isolate was picked with a wire loop and suspended in the hydrogen peroxide.A positive reaction was indicated by formation of bubbles indicating the production of oxygen and water.

3.6.3.2 Pigmentation test on mannitol salt agar

The isolates were tested for pigment formation on MSA.The medium was prepared according to manufacturer's instructions.The suspected organisms were streaked on the agar and incubated at 37°c for 24hours.*S.aureus* is characterized by formation of yellowish colonies on this medium.

3.6.3.3 Coagulase test

Suspected isolates were tested for coagulase activity using rabbit plasma by the tube method.To prepare rabbit plasma,rabbit blood was collected aseptically into 10%EDTA**.** This was dispensed in sterile tubes and centrifuged at 100g (MINOR bench centrifuge) for 5mins.The plasma was then collected and used for coagulase test.A 1:10 dilution of the plasma was madein normal saline and 0.5ml was dispensed into sterile bottles and 0.5ml of an 18 to 24hours tryptone soy broth culture of the test isolate was added.The tubes were covered with cotton plugs and then incubated at 37°c.The tubes were examined after 1,4 and 6 hours for coagulation.Those that were negative after 6hours were left at room temperature overnight and re-examined.A positive reaction was indicated by a definite clot formation recorded atthe hour the clot was observed.

3.6.3.4 Sugar fermentation tests

To 1.5g of Andrade peptone water, 1g of each sugar was added and distilled water added to make up to 100ml. After boiling to dissolve, 3ml of the mixture was dispensed into bijou bottles and autoclaved at 115°C for 20minutes. This was allowed to cool and then inoculated with a loopful of the test organism from a prepared trypticase soy brothculture and incubated at 37°c for 24 – 48hours.A tube containing the mixture without the test organism was used as negative control.A change in colour from pale pink to yellow was indicative of fermentation.The following sugars were tested: glucose, mannitol, xylose, lactose, and maltose

3.6.3.5 Haemolysis

The suspected *Staphylococcus* isolates were grown on 5% sheep, rabbit, horse and human blood. Blood agar base was prepared according to manufacturer’s

recommendation, boiled to dissolve, autoclaved and allowed to cool. 20 ml of blood from each specie was added to the prepared blood agar base (400ml). This was then poured onto sterile petridishes and allowed to solidify.The test organism was picked from nutrient agar slants and used to streak the blood agar and then incubated at 37°c for 24hours. Isolates with complete and clear zone of haemolysis were classified beta haemolytic while those with a partial zone of haemolysis (greenish) as alpha haemolytic and isolates without zone of clearing were non- haemolytic(delta and gamma haemolysis).

3.6.3.6 DNase test

Isolates suspected to be *S.aureus* were inoculated on DNase (Oxoid®) test agar and incubated at 37°c for 24hours.The plate was flooded with 1N-HCL; a positive result showed a clear zone around the colonies and the surrounding medium while a negative test result was seen as opaque.

3.6.3.7 Urease test

Isolates suspected to belong to the specie *Staphylococcus* were tested for their ability to utilize urea.Urea agar slants were prepared according to manufacturer's instruction.The suspected isolates were stab-inoculated onto the slants and incubated at 30°c for 24 hours.A change in colour from orange to pink indicated a positive reaction.

3.6.4 Identification of isolates based on conventional biochemical tests

Isolates were identified as *S.aureus* on the basis of colonial morphology when streaked onto Baird-Parker, gram positive cocci in clusters, catalase positive, coagulase production in rabbit plasma, haemolysis on blood agar of various species, fermentation

of mannitol and glucose and non fermentation of xylose, DNase production,and yellow pigmentation on mannitol salt agar.

3.6.5 Microbact® testing of *Staphylococcus*

The Microbact® 12S test systems are standardised micro-substrate systems designed to emulate conventional biochemical substrates used for identification of *Staphylococcus* species.Reactions occurring after incubation are seen as easily interpreted colour changes.Organism identification is based on pH change and substrate utilization.Once each reaction was read and recorded on the microbact codereport form,each block of three reactions was converted into a numeric value to obtain a 4 digit of the Microbact code together with the numeric code obtained from the last three blocks to form a five digit numberor octal code which is then fed into a computer aided identification package for interpretation. The results show the likely *Staphylococcus* species and the extent to which it was in percentage.The substrates for Microbact 12S are maltose, mannitol, mannose, sucrose, trehalose, N-acetyl glucosamine, arginine, urease, beta-glucosidase, alkaline phosphatase, beta-glucoronidase and beta-galactosidase.The other tests giving a numerical value to form the fifth digit number added to the identification kit are latex/coagulase, DNase and pigment.

3.7 Determination of the Susceptibility of S*taphylococcus aureus* to Commonly Used Antimicrobial Agents.

3.7.1 Disk diffusion tests for *S.aureus*

The Kirby-Bauer method was used for the antimicrobial susceptibility testing on Mueller Hinton agar (Bauer *et al*., 1996) using 13 antimicrobial agents and interpreted

according to the recommendations of Clinical and Laboratory Standards Institute (CLSI, 2006).

Three well isolated colonies from Baird Parker agar were picked with a wire loop and transferred into a tube containing 5ml of trypticase soy broth. The broth was incubated at 35°C for 18hours. To standardize inoculum density or turbidity, the culture broth was adjusted to match that of McFarland (0.5%) by adding sterile saline (CLSI, 2006). This matched broth suspension was used to flood the prepared(2ml) Mueller Hinton agar plates, swirled, the remnant drained out and the plates allowed to dry.

The discs were applied with the aid of a dispensing apparatus (Oxoid).The plates were then inverted and placed in an incubator at 35°C.Each plate was then examined after 18hours of incubation and 24hours for vancomycin and oxacillin (CLSI, 2006).

Zones of inhibition were measured to the nearest millimetre using a ruler.The sizes of the zones of inhibition were interpreted by comparing with the breakpoints as described by Clinical and Laboratory Standard Institute (CLSI,2006).The number of methicillin resistant isolates was expressed as a percentage out of the total number of isolates examined as well as for other antibiotics.

Multiple antimicrobial resistance was also determined (MAR) by dividing the number of antibiotic each isolate was resistant to by the total number of antibiotic included in the panel.

3.7.2 ORSAB test (Oxacillin Resistance *Staphylococcus aureus* broth)

This medium helps in identifying *S.aureus* isolates that are presumptively resistant to methicillin.The medium incorporates an optimised salt level, lithium chloride and polymyxinB to suppress competing flora, aniline blue indicator for mannitol

fermentation and oxacillin at the level of 2mg/l.Presumptive MRSA(oxacillin resistant strains)colonies appear as bright blue.The ORSAB was prepared according to manufacturer's instructions. The suspected organisms were streaked and incubated at 37°c for 24 hours before interpretation.

3.8 Detection of *Mec* A in Isolates Using Polymerase Chain Reaction

3.8.1 Protocol for DNA extraction of *S.aureus* isolates

Three colonies were picked from baird parker agar, and inoculated into 5ml peptone water.All the tubes containing the organism were first placed in water bath at 60°c for 30mins.DNA extraction was carried out according to steps in the instruction manual of the ZYMO research kit (ZR, Pretoria, S.A.Inqaba Biotec) but 5µl of lysotaphin was added to facilitate lysis of the bacteria and enhance DNA extraction.

3.8.2 Protocol of PCR for amplification of mecA gene

DNA of all the 17 *S. aureus* isolates were subjected to amplification by polymerase chain reaction(PCR) for the presence of *mec* A gene using primers, *mec*A1 5' AAAATCGATGGTAAAGTTGGC-3'(forward) and *mec*A2 5'AGTTCTGCAGTACCGGATTTGC-3' (reverse) yielding a product of 533bp (Lee, 2003). PCR was performed in a 50µl of reaction mixture using 14µl of nuclease free water, 7µl of DNA template, 2µl of each of forward primer, 2µl of reverse primer and 25µl of the mastermix. The master mix consisted of PCR buffer which contained 200µm concentration of each deoxynucleoside triphosphate (dNTP) (Promega Madison Wis), 10mM Tris –Hcl (pH 8.3), 50mM Kcl, 2.5mM of $Mgcl_2$, 2.5 units of Taq polymerase. DNA amplification was carried out in 40 cycles of amplification with an initial denaturation of 95°c for 5mins, 94°c for 30secs denaturation, 55 - 70°c annealing

and extension at 72°c for 30secs with a final extension of 72°c for 5mins.This was carried out in a thermal cycler.PCR products were analysed in a 1.5% agarose gel.10µl of the PCR product wasmixed with a drop of the loading dye (G- stain) andloaded into the wells of the gel and electrophoresed for 45mins at 100volts. The bands were viewed under a UV transilluminator (UVtec, Sigma, Germany) in order to visualize the PCR products with an expected band size of 533bp.

3.8.3 Protocol for determination of penicillin-binding protein (Pbp2a) among MRSA isolates (Oxoid)

Overnight culture of isolates on Mannitol salt agar was used for the test. Four drops of extraction reagent 1 was put into the micro centrifugetube and a sterile loop to pick the colonies and emulsified in the tube. The tubes were then heated at 95°C in a water bath for 3minutes.This was allowed to cool to room temperature and a drop of extraction agent 2 added. This was then centrifuged at 1500 rpm for 5mins to obtain a supernatant.A drop of the supernatant was added to the round circles on the test papers and a drop of latex reagent added, the test card then rotated gradually to check for agglutination. Presence of clumps or granules against a cloudy background was recorded as positive while a homogenous suspension was recorded as negative.

3.8.4 Protocol for the determination of β-lactamase enzymes among MRSA isolates (Oxoid)

The can holding the β -lactamase impregnated sticks were removed from the freezer and allowed to reach room temperature. The coloured end of the β -lactamase stick was used to pick a mass of well isolated colony. This test requires moisture and one or two drops of distilled water was used to moisten the coloured end of the stick. The sticks were

then examined for 5minutes, 15minutes and one hour for colour change. A positive reaction is shown by the development of a pink-red while no colour change is observed with organisms that do not produce lactamase.

3.9 Statistical Analysis

Prevalence of *Staphylococcu* sand MRSA were calculated. Chi square was used to determine the level of association between positive isolates, location and sample type. P-value less than 0.05 was taken to be statistically significant.

CHAPTER FOUR

RESULTS

4.1 Occurrence of *Staphylococcus* Species

The distribution of samples collected from Kaduna and Zaria is presented in Table 4.1.A total of 75 isolates of *Staphylococcus* spp were identified based on conventional biochemical tests giving a prevalence rate of 13.39%.Microbact® 12 S identification kit system revealed a total of 17 *S.aureus* species yielding a prevalence of 3.04 % (Table 4.1). The overall prevalence rate of *S.aureus* was higher in Kaduna (3.21) than in Zaria (2.86%) (Table 4.1).

There was no statistically significant difference (P □ 0.05) between location and occurrence of *Staphylococcus* spp, *S. aureus* and MRSA, although the strength of association between MRSA and these locations were high (OR□ 1), it was not significant (Table 4.1).

In Kaduna, Station market had the highest prevalence of 18.6%, 5.71%, 5.71% respectively for *Staphylococcus* species, *S.aureus* and MRSA (Table 4.2). The total prevalence of *Staphylococcus* in yoghurt and nono in all the markets in Kaduna were 12.14%. There was a statistically significant association between the occurrence of *Staphylococcus* and markets in Kaduna (P□ 0.05) (Table 4.2).

In Kaduna, the prevalence of *Staphylococcus* in nono was 17.14% while in yoghurt it was 7.14%; while for *S.aureus* and MRSA, prevalences were 5.71% and 0.71% in nono and yoghurt respectively. There was no statistically significant association between the occurrence of these organisms and location (Tables 4.3).

Table 4.1 Prevalence of *Staphylococcus* species and MRSA in Nono and Yoghurt in Kaduna and Zaria.

Location	Samples size	No (%) Staph	No (%) *S.aureus*	No(%) MRSA
Kaduna	280	34(12.14)	9(3.21)	9(3.21)
Zaria	280	41(14.64)	8(2.86)	8(2.86)
Total	560	75(13.39)	17(3.04)	17(3.04)

Staph χ= 0.7544 p= 0.09 OR= 0.8057 C.I = 0.494 – 1.313

MRSA χ = 0.0607 p =0.8054 OR = 1.129 C.I = 0.4292 – 2.971

Table 4.2 Prevalence of *Staphylococcus* and MRSA in Kaduna

Location	size	Staph(%)	*S.aureus*(%)	MRSA(%)	OR	CI at 95%
Barnarwa	70	7(10)	1 (1.43%)	1(1.43%)		
Central	70	11(15.7)	3(4.28%)	3(4.28%)	3.09	0.3133-30.47
Station	70	13(18.6)	4 (5.71%)	4(5.71%)	4.18	0.4552-38.41
Kawo	70	3 (4.3)	1(1.43%)	1(1.43%)	1.00	0.0613-16.32
Total	280	34(12.14)	9(3.21%)	9(3.21%)		

Staph χ^2 =7.901 p= 0.0481 P□ 0.05

MRSA χ^2 =3.10 p=0.3765 P□ 0.05

Table 4.3 Prevalence of *Staphylococcus* and MRSA in Nono and Yoghurt in Kaduna

Location	Sample size	No (%) Staph	No (%) *S.aureus*	NO (%) MRSA	OR	95% C.I
		NonoYoghurt	NonoYoghurt	NonoYoghurt		Nono
Barnarwa	35	4(11.4)3(8.6)	1(2.86) 0(0)	1(2.86) 0(0)		
Central	35	10(28.57)1(2.9)	3(8.57) 0(0)	3(8.57) 0(0)	3.188	0.3149-32.26
Station	35	7(20)6(17.14)	3(8.57)1(2.85)	3(8.57)(2.85)	3.188	0.3149-32.26
Kawo	35	3(8.6) 0 (0)	1(2.86) 0(0)	1(2.86) 0(0)	1.00	0.06-16.66
Total	140	24(17.14)[a]10(7.)[b]	8(5.71)[a]1(0.71)[b]	8(5.71)[a]1(0.71)[b]		

a- Staph $\chi^2 = 6.034$ $p = 0.1099$

a - MRSA $\chi^2 = 2.121$ $p = 0.5476$

Nono from Kaduna had the highest contamination rate of *S.aureus* and MRSA (5.71%) as compared to nono from Zaria (2.86%) (Table 4.3 and 4.5)

In Zaria, Zaria city had the highest prevalence of *Staphylococcus*(27.14%) while Samaru market gave a prevalence of (5.71%) for both *S.aureus* and MRSA. The total prevalenceof *Staphylococcus* was 14.64% for nono and yoghurt in all the four locations in Zaria.There was a statistically significant association between the occurrence of *Staphylococcus* and the markets in Zaria (Table 4.4).

Out of these, nono had a prevalence of 13.57%. There was also a statistically significant association between the occurence of *Staphylococcus* in nono in Zaria (P□ 0.05) (Table 4.5) while prevalence of *Staphylococcus* in yoghurt was 15.71% and it was not statistically significant (P□ 0.05).

Nono had the highest isolation rate of *Staphylococcus* species 43(15.36%) and that of yoghurt was 32(11.43%) The isolation rate of *S.aureus* and MRSA were higher in nono 12(4.29%) than in yoghurt 5(1.79%) (Table 4.6). The results further showed that 12(4.29%) of the MRSA were from nono while 5(1.79%) were from yoghurt .There was no significant difference in the occurrence of MRSA in nono and yoghurt (P□ 0.05), although there was a high strength of association between MRSA and its occurrence in nono and yoghurt (OR□ 1) (Table 4.6).There were 17 antimicrobial resistance patterns (Table 4.7).

Table 4.4 Prevalence of *Staphylococcus* and MRSA in Zaria

Location	Samples size	No (%) Staph	No(%) *S.aureus*	No(%) MRSA
Samaru	70	8(11.4)	4(5.71)	4(5.71)
Sabo	70	7(10)	3(4.28)	3(4.28)
Tudun/Wada	70	7(10)	0(0)	0(0)
Zaria City	70	19(27.14)	1(1.43)	1(1.43)
Total	280	41(14.64)	8(2.86)	8(2.86)

Staph $\chi^2 = 11.74$ $p = 0.0083$

TABLE 4.5 Prevalence of *Staphylococcus* and MRSA in Nono and Yoghurt in Zaria

Location	Sample size	No (%) Staph		No (%) *S. aureus*		No (%) MRSA	
		Nono	Yoghurt	Nono	Yoghurt	Nono	Yoghurt
Samaru	35	3(8.57)	5(14.26)	2(5.71)	2(5.71)	2(5.71)	2(5.71)
Sabo	35	1(2.86)	6(17.14)	1(2.86)	2(5.71)	1(2.86)	2(5.71)
Tudun/W	35	1(2.86)	6(17.14)	0(0)	0(0)	0(0)	0(0)
Zaria city	35	14(40)	5(14.26)	1(2.86)	0(0)	1(2.86)	0(0)
Total	140	19(13.57)[a]	22(15.71)[b]	4(2.86)[a]	4(2.86)[b]	4(2.86)[a]	4(2.86)[b]

a- Staph χ^2 = 28.44 p= 0.0001

b- Staph χ^2 = 0.2157 p= 0.9750

TABLE 4.6 Prevalence of *Staphylococcus* and MRSA based on Sample Type

Sample type	Sample size	No(%)staph	No(%) *S.aureu*)	No(%)MRSA
Nono	280	43(15.36)	12(4.29)	12(4.29)
Yoghurt	280	32(11.43)	5 (1.79)	5(1.79)
Total	560	75(13.39)	17(3.04)	17(3.04)

Staph χ^2 = 1.546 p = 0.2137 OR = 1.295 CI = 0.8611 – 1.947

MRSA χ^2 = 2.973 p = 0.0847 OR = 2.463 CI = 0.8558 – 7.087

Other species of *Staphylococcus* isolated were as follows: *S.intermedius* (10.67%), *S.hyicus* (49.33%) *S.epidermidis* (6.67%), *S.chromogenes* (8.0%), *S. simulans* (1.3%) , *S.xylosus* (1.3%).

4.2 Susceptibility of Isolates to Antimicrobial Agents

All the 75 *Staphylococcal* isolates were tested for susceptibility to 13 antimicrobial agents and the isolates showed the following percentage resistances: Oxacillin (97.3%) Sulphamethoxazole/Trimethoprim (69.3%), Methicillin (97.3%) Ampicillin (78.6%) Amikacin (45.3%) Ciprofloxacin (61.3%) Chloramphenicol (73.3%), Erythromycin (78.7%), Cefixime (49.3%), Penicillin (97.3%), Tetracycline (62.7%), Gentamicin (78.7) and Vancomycin (53.3%) (Figure 4.1). All 17 *S.aureus* were resistant to more than one of the 13 antimicrobials tested including the beta lactam drugs. A hundred percent resistance to methicillin, penicillin and oxacillin were recorded. Relatively low levels of resistance were observed for cefixime, tetracycline and vancomycin (Figure 4.2).

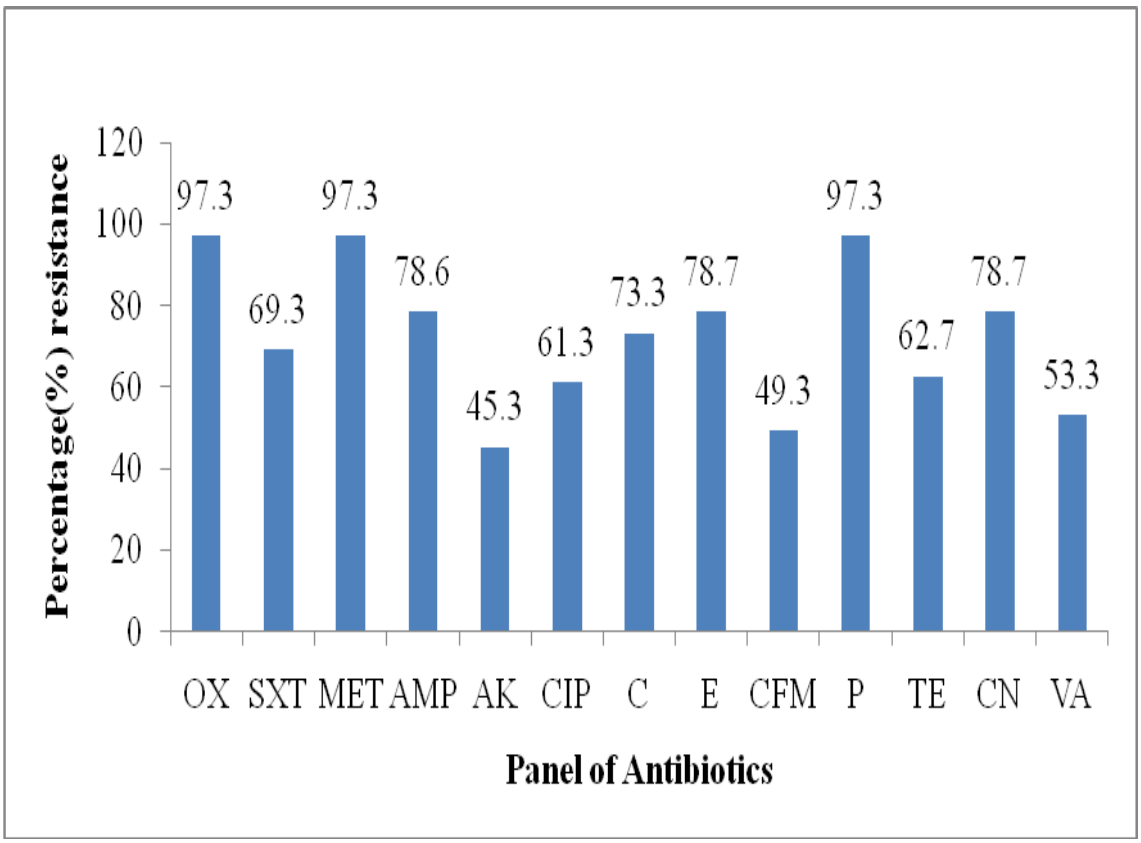


Figure 4.1 Percentage Resistance of Staphylococci species isolated from Nono and Yoghurt in Kaduna and Zaria to antimicrobial agents

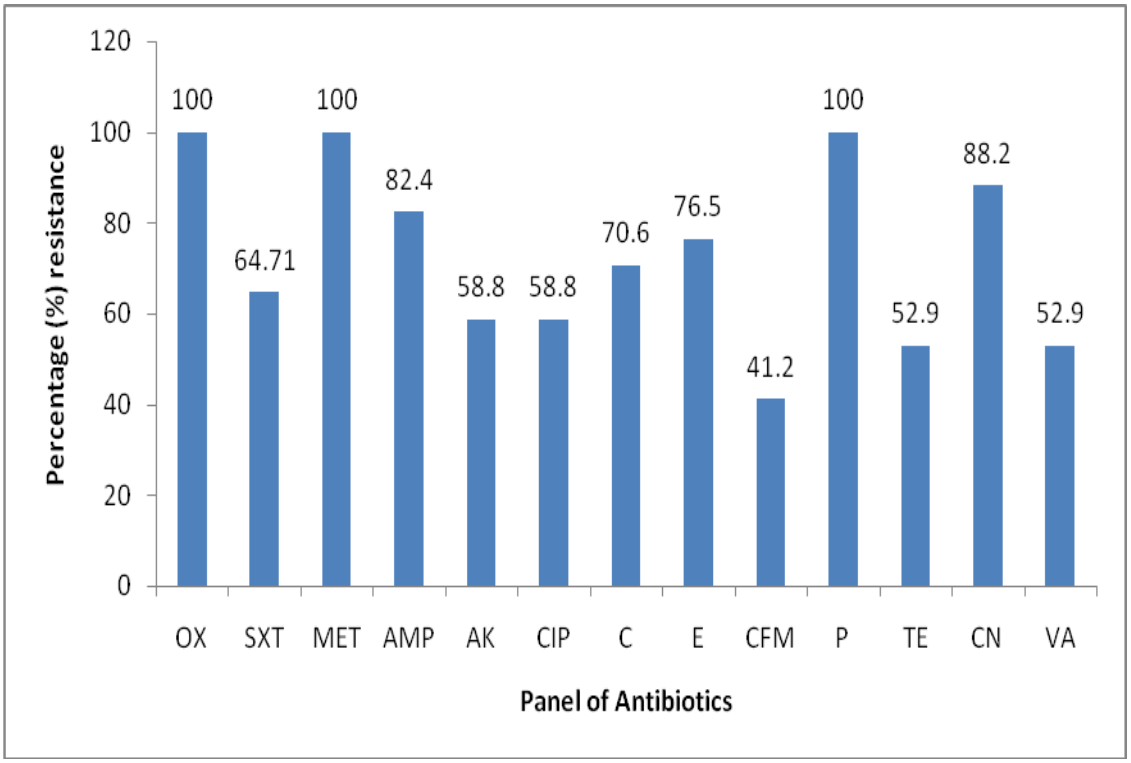


Figure 4.2 Percentage Resistance of *Staphylococcus aureus* and MRSA from Nono and Yoghurt in Kadunaand Zaria to antimicrobial agents

Out of 75 isolates tested using ORSAB, 52(69.3%) were found to be presumptively methicillin resistant,while Kirby-Bauer method revealed that 97.3% were actually resistant to methicillin.Twelve out of the 17 *S.aureus* confirmed using microbact 12S were methicillin resistant by ORSAB agar but were all found to be resistant by the disk diffusion method.

4.3 Detection OF *mec A* gene by Polymerase Chain Reaction (PCR)

None of the 17 *S. aureus* was found to be positive for *mec* by PCR.

4.4 Detection of Pbp2a and Beta-Lactamase

The results for detection of Pbp2a in 17 MRSA isolates showed that 11(64.71) % were positive,4 of the isolates were from yoghurt and 7 from nono while 13(76.47%) were betalactamase positive out of which 4 of the isolates were from yoghurt and 9 from nono.

TABLE 4.7 Multiple Drug Resistance Profiles and MAR Index of 17 MRSA Isolates

Isolates No	Antimicrobial profile	MAR Index
KDNB6	OX, SXT, MET, CIP, C, E, P, CN, VA	0.69
KDNCI	OX,SXT,MET,AMP,AK,CIP,C,CFM,P,TE,CN	0.85
KDNC24	OX, SXT, MET, AMP, C, E, P, TE	0.62
KDNC30	OX,SXT,MET,AMP,AK,CIP,C,E,P,TE,CN,VA	0.92
KDNS1	OX, MET, AMP, AK, CFM, P, TE, CN, VA	0.69
KDNS4	OX,MET,AMP,AK,CIP,C,E,CFM,P,CN,VA	0.85
KDNS5	OX, MET, CIP, C, E, P, CN	0.54
KDYS20	OX, MET, C, E, P, CN, VA	0.54
KDNK9	OX,SXT,MET,AMP,AK,CIP,C,E,P,TE,CN,VA	0 .92
ZRNSA2	OX, MET, AMP, E, P, TE	0.46
ZRNSA4	OX, SXT, MET, AMP, CIP, C, E, P, TE, CN	0.77
ZRYSA6	OX, SXT, MET, AK, CIP, E, CFM, P, CN	0.69
ZRYSA19	OX, MET, AMP, AK, C, CFM, P, CN	0.62
ZRNSB31	OX, MET, AMP, E, P, CN, VA	0.54
ZRYSB3	OX,SXT,MET,AMP,AK,CIP,C,E,P,TE,CN,VA	0.92
ZRYSB35	OX, SXT, MET, AMP, AK, CFM, P, TE, CN, VA	0.77
ZRNZC7	OX, SXT, MET, AMP, CIP, C, E, CFM, P, CN	0.77

TABLE 4.8 Haemolytic Patterns of *Staphylococcus aureus and MRSA* isolates

RBC	No (%) of isolates with different Haemolytic patterns*			
	α	β	γ	δ
Horse	4(23.5)	6(35.3)	1(5.9)	6(35.3)
Human	2(11.8)	2(11.8)	13(76.5)	0 (0)
Rabbit	8(47)	9 (52.9)	0 (0)	0(0)
Sheep	6 (35.3)	11(64.7)	0 (0)	0(0)
Total	20	28	14	6

Numbers in Parenthesis are in percentages

*α, β, γ and δ haemolysis

CHAPTER FIVE

DISCUSSION

The overall prevalence of *Staphylococcus* specie, *S.aureus* and MRSA from both nono and yoghurt, identified by conventional biochemical tests and microbact identification system were13.39% and 3.04% respectively in this study. The prevalence of *S.aureus* was higher in Kaduna (3.21%) than Zaria (2.86%) but showed no statistically significant difference (P□ 0.05).This is low compared to the 37-43% and 81.3% reported by Umoh (1989) and Umoh *et al*. (1990b) respectively in studies carried out in the same area. It is also low compared to 12.6%, 15.9 and 14.7% reported by Ghali (2009), Zouharova and Rysanet (2009) and Strastpova *et al* (2000) in Nigeria, Turkey and Czech Republic respectively. Although, cognizance should be taken of the other *Staphylococcus* species that were isolated which could have contributed to the low prevalence of *S.aureus* in this study.This difference may be due to the fact that fermentation of these products could have created an acidic condition that denatures bacteria hence affecting their growth. Samples were collected in this study during the dry period of December to April in which prevalence of certain organisms was low and Ph of milk tended to be low (Umoh *et al*., 1990b).The prevalence of MRSA (3.04%) in this study is in agreement with the 4.8% obtained by Ghali (2009), but lower than 29.3% obtained in China by Lee (2003).This could be due to larger sample size and the type of samples in the various studies.

The prevalence of *S.aureus* and MRSA was higher in Kaduna (3.21%) than in Zaria (2.86%) but there was no statistically significant association between the occurrence of *Staphylococcus* and MRSA (p□ 0.05) and locations.There was however, statistically significant difference in the occurrence of *Staphylococcus* among markets in Kaduna

and Zaria, ($p< 0.05$).This may have been due to the larger number of isolates obtained from some of the location as compared to others.The prevalence of *Staphylococcus* in nono in Zaria was also statistically significant ($p \square 0.05$) with respect to the markets where they were obtained. Prevalence of MRSA and *S.aureus* was higher in nono (4.29%) than in yoghurt (1.79%) but not statistically significant ($p \square 0.05$).Prevalence of *Staphylococcus* was higher in nono (15.36%) than yoghurt (11.43%) but not statistically significant ($p \square 0.05$) (Table 4.6) .The lower prevalence of these organismsin yoghurt compared to"nono" could be due to better hygienic practices during production, distribution, packaging, storage and the need by the manufacturers to protect a brand name.

The MRSA isolates from this study werefound to be resistant to most of the antibiotics tested; 9(52.9%) and 7 (41.2%) of the 17 MRSA isolates were resistant to vancomycin, tetracycline and cefixime respectively indicating that cefixime may be more effective since a lower percentage of isolates showed resistance to it. All isolates displayed multidrug resistance and the susceptibility of some of the isolates to sulphamethoxazole/trimethoprim maybe an indication of community associated MRSA. Mixed fermentation has been reported to occur in nono as fermentation is not controlled and different organisms are present at a time, as a result, transfer of resistance between organisms can occur and this may have accounted for the high resistance seen in nono as compared to yoghurt where only one known bacteria culture is used during fermentation.The percentage resistance to methicillin and vancomycin is alarming and has been widely reported internationally (Fridkin, 2001; Hiramatsu *et al*., 1997) even in Nigeria (Ikeh, 2003; Olayinka *et al*., 2005). In another study carried out in a long-term

care facility in California, isolates of MRSA USA 300 clone were shown to be highly resistant to vancomycin, linezolid and daptomycin (Tattevin *et al*., 2009).

The resistance was reported to be due to the acquisition of resistance determining genes, like *mec*A, *mec*C, *ica* gene cluster, *bla*Z (methicillin resistance) and *van* A, B, C responsible for vancomycin resistance (Hiramatsu *et al*., 1997; Kim *et al*., 2000) or as a result of the thickening of the cell wall as reported by some authors (Kim *et al*., 2000; Denis *et al*., 2009). These genes were however not determined in this work with the exception of *mec* A. Isolation of vancomycin resistant *Staphylococcus aureus* (VRSA) from USA, France and South Africa has been reported (Chang *et al*., 2003b).

The resistance patterns observed in this study is in agreement with Olonitola *et al*.(2007) where 70% of the MRSA were resistant to ampicillin, Onanuga *et al* .(2006) where 100% of the MRSA were resistant to penicillin and Raghunath *et al*.(1981) where 68% were resistant to tetracycline and 48.2% were resistant to chloramphenicol.The high rate of resistance of the isolates to gentamicin(88.2%) in this study could be due to its frequent use in the treatment of viral diseases such as canine parvoviral enteritis in dogs,bacterial and fungal ear and eye infections in both man and animals as drops,paste and injections (www.shelter.com ; www.vetunicen.edu.ar).

Raghunath *et a*l; (1981) showed that some isolates of *Staphylococcus aureus* were resistant to over 80% of the antibiotics in the panel tested which agrees with this study and this high resistance could be due to invitro cross infection between isolates.

The low prevalence of *S.aureus* and MRSA in nono and yoghurt maybe due to pasteurization and fermentation to which the products are subjected(Umoh, 1989; Ghali, 2009).Unsatisfactory hygiene or sanitation have accounted for a significant occurrence

of MRSA as compared to countries in Europe and USA. Also despite fermentation and pasteurization, the enterotoxin of *S. aureus*can remain in contaminated milk even after processing (Novak *et al*., 2000).

A number of studies have also suggested the transfer of *S.aureus* between humans and cattle through milk and its products (Lee, 2003).There has been reports ofoccurrence of MRSA in over 50% Nigerians(Onanuga *et al*., 2006a,2006b;Olonitola *et al* .,2007) suggesting that MRSA isolates may have originated from humans, therefore increasing chances of food contamination.This may also explain the high percentage of resistance by the isolates to antibiotics (ciprofloxacin,vancomycin and sulphamethoxazole-trimethoprim).These drugs are frequently used in human medicine. Also "nono" sellers live close to their animals indicating a possible explanation for the inter transmission of *Staphylococcus* and also explains the high resistance of the organisms to most antibiotics.

Staphylococcus has a high ability to change over time and because of this MRSA will continue to be a problem in the future(Olowe *et al*., 2007).In Africa, MRSA is relatively high in Nigeria, Kenya and Cameroon (20-76%)and below 10% in Tunisia and Algeria(Nwankwo *et al*.,2010). Documented studies have shown the prevalence of MRSA infection in humans in Nigeria to range from 12.5% in Jos (Ikeh, 2003), 28.6% in Kano (Nwankwo *et al*., 2010) and 52.5% in Ilorin (Fadeyi *et al*., 2010).

The absence of *mec*A gene among isolates tested in this study suggests that other factors maybe responsible for resistance. It may also be necessary to combine detection of *mec*A gene with detecton of Pbp2a, homologues of *mec* A such as *mec*C, mobile elements, tranpososons and phages which can harbour other genes responsible for resistance. Primers used in this study were specific for the amplification of *mec*A gene.

However, in one study in Zaria, Nigeria, Olonitola *et al*. (2007) observed that all the MRSA obtained from nasal swabs of healthy individuals were *mec* A negative but β-lactamase producing and concluded that resistance in MRSA maybe due to the presence of β-lactamase.Other studies have shown the discovery of a strain of *S.aureus* (LGA 251) isolated from bulk milk phenotypically resistant to methicillin but negative for *mec* A gene(Garcia-Alvarez*et al*., 2011).In that study a divergent *mec*A homologue(*mec*ALGA251) was discovered in the SCC *mec* element designated type X1-SCC. In another study, out of 100 isolates of methicillin resistant *Staphylococcus aureus*, only 5 (5.0%) of the isolates were confirmed as MRSA by the detection of *mec*A gene while 95% were negative for the *mec* A gene (Kumurya, 2013).Also low level of *mec*A gene was shown by Griethuysen *et al*. (2005) where they found 14.4% MRSA isolates lost their *mec*A gene. Loss of the *mec*A gene in such a large percentage of MRSA isolates have also been reported by Hurlimann *et al*. (1992).

Although routine culture and antimicrobial susceptibility will identify *S.aureus* isolates with novel *mec* A homologue as methicillin resistant,present confirmatory methods will not identify them as MRSA.Thus,new diagnostics guidelines for the detection of MRSA strains of *S.aureus* should consider test for such homologues as *mec*A LGA251(Garcia-Alvarez *et al* ., 2011).

Furthermore, detection of penicillin-binding protein (Pbp2a) and betalactamse were carried out to determine other factors that may be responsible for resistance among the MRSA isolates; 11 (64.71%) and 13(76.47%) of the 17 isolates tested expressed Pbp2a and betalactamase respectively.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

A cross- sectional study was carried out to determine the occurrence of *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* in yoghurt and "nono" and in Kaduna and Zaria. Overall prevalence of *Staphylococcus* species was 75 (13.39%)using conventional biochemical tests,while 17(3.04%) were confirmed to be *Staphylococcus aureus* and MRSA by the Microbact 12S identification system and antibiotic susceptibility tesying respectively.(69.3%) out of the 75 staphylococci isolates were positive for methicillin resistance when tested with Oxacillin resistant *Staphylococcus aureus* basal medium(ORSAB) while 12 (70.5%) out of the 17 *Staphylococcus aureus* isolates were positive on ORSAB. All the 75 staphylococcal isolates showed high level of resistant to more than one of the antibiotics tested including the beta lactams such as methicillin ,oxacillin and penicillin (97.3% each) with the least resistance to amikacin (45.3%).All the 17 *Staphylococcus aureus* and MRSA isolates were resistant to methicillin,oxacillin and penicillin (100%) while the least resistance by the isolates was to cefixime(41.2%).Amikacin and cefixime are drugs of choice considering that fewer isolates showed resistant to them. None of the 17 MRSA harboured the methicillin resistance gene *mec* A gene by PCR, but 11(64.71%) and 13(76.47%) were positive for Pbp2a and betalactamase respectively suggesting that resistance by strains of *S.aureus* in this study was by Pbp2a and beta-lactamase production. Consequently, findings from this study suggest that nono and yoghurt can serve as vehicles of

transmission of food borne diseases and are a food safety problem. The study also suggests that *Staphylococcus aureus* and MRSA is becoming increasingly resistant to drugs commonly used in humans and animal population and even to drugs previously thought to be effective against the organism.

6.2 Recommendations

From the results of this study, the following recommendations were made:

1. Nonoand yoghurt producers should be encouraged and educated on how to undertake proper fermentation and pasteurization of their productsto decrease the presence of *Staphylococcus aureus* and MRSA in the products. This can be achieved through workshops, seminars, fulani day, herd and farm visits by the relevant authorities.

2. Hygienic and healthy sanitary practices such as washing of utensils, hands, covering of food and cleanliness on the part of the seller should be maintained through the chain of production until it gets to the final consumer.

3. There is need to include different groups of antibiotics in the panel during susceptibility testing of MRSA to antimicrobials in order to arrive at the best choices of treatment of MRSA infections

4. New diagnostic guidelines for the detection of MRSA should consider tests for homologues of the *mec*A gene and other factors (such as production of resistance proteins and enzymes) in strains isolated from Nigeria.

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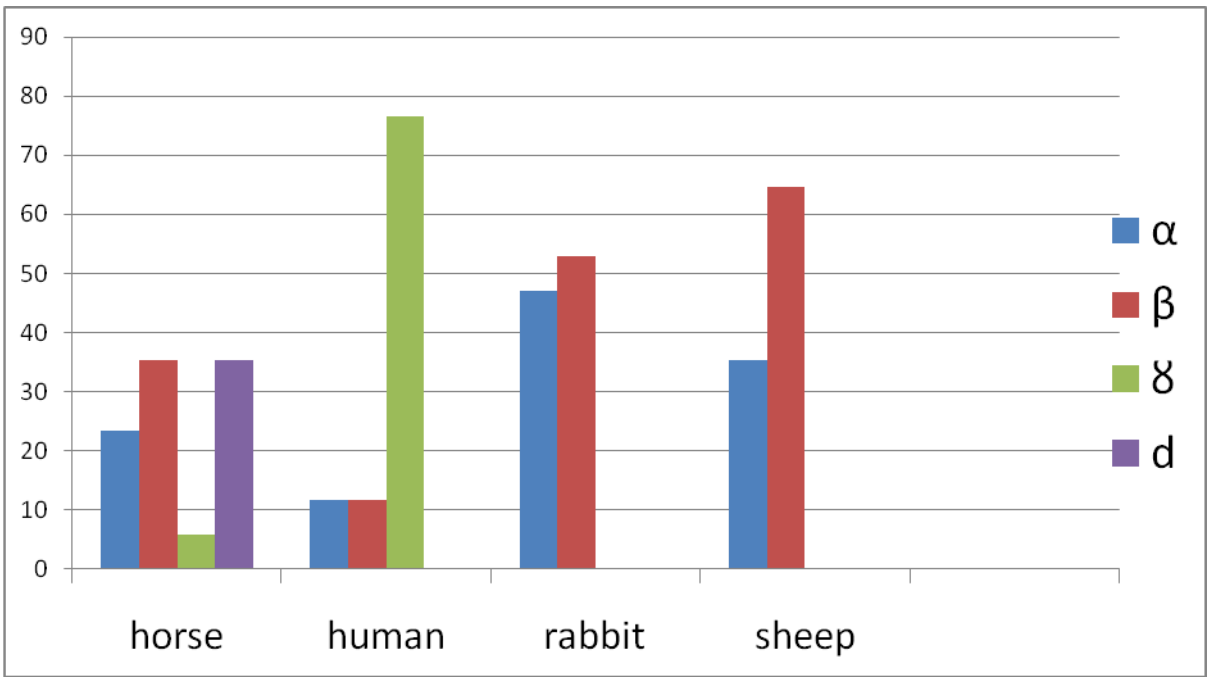
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APPENDICES

Appendix I: Haemolytic patterns for *Staphylococcus aureus* and MRSA based on source of red blood cells



Appendix II: Summary of result for the sugar fermentations, hemotlyic patterns and gowth on ORSAB

SAMPLE NO	GLUCOSE	MALTOSE	MANNITOL	UREASE	XYLOSE	HORSE	RABBIT	SHEEP	HUMAN	ORSAB
KDNB2	+	+	+	-	-	B	B	β	□	-
KDNB6	+	+	+	+	+	B	B	β	□	+
KDNB7	+	+	-	+	-	D	A	β	□	-
KDNB11	+	+	+	+	-	A	B	β	B	+
KDYB10	+	+	-	+	-	A	β	β	A	+
KDYB11	+	+	+	+	-	B	β	β	□	+
KDYB18	+	+	-	+	-	D	β	β	B	-
KDNC1	+	-	-	+	-	B	β	β	□	+
KDNC3	+	+	-	+	-	D	α	β	□	+
KDNC4	_	_	+	_	-	D	β	α	□	+
KDNC6	+	+	-	+	-	D	α	β	□	-
KDNC7	+	+	-	+	-	A	β	β	B	+
KDNC16	+	+	-	+	-	D	β	α	□	-
KDNC24	+	+	+	+	-	D	α	α	B	-
KDNC30	+	-	-	-	-	D	β	α	□	+
KDNC31	+	-	-	+	-	D	β	α	□	+
KDYC5	+	+	-	+	-	A	β	β	□	+
KDNS1	+	-	+	+	-	B	α	β	□	+
KDNS4	+	+	+	+	-	D	β	α	□	+
KDNS5	+	+	+	+	-	D	α	β	□	+
KDNS8	+	+	+	+	-	D	β	β	□	+
KDNS17	-	+	+	+	-	D	β	β	□	+

KDNS30	+	+	-	+	-	D	α	β	□	+
KDNS35	+	+	+	+	-	A	β	β	□	+
KDYS4	+	+	+	+	-	D	β	β	□	+
KDYS6	-	+	+	+	-	A	α	α	□	+
KDYS10	-	-	+	+	-	B	α	β	□	-
KDYS20	-	+	+	+	-	B	α	α	□	+
KDYS31	+	+	-	+	+	D	α	β	□	-
KDYS33	+	-	+	+	+	D	β	β	□	-
KDNK9	+	+	-	+	-	D	α	β	□	-
KDNK28	+	+	+	-	-	A	β	β	□	+
KDNK31	-	-	-	+	+	D	α	β	□	-
ZRNSA2	+	+	+	+	-	D	α	β	□	+
ZRNSA4	+	+	+	+	-	B	α	β	□	-
ZRNSA5	+	+	+	+	-	B	α	β	□	+
ZRYSA6	+	+	+	+	-	A	β	α	A	+
ZRYSA19	-	-	+	+	-	A	B	β	□	+
ZRYSA29	+	-	+	+	-	D	B	α	□	+
ZRYSA31	+	-	+	+	-	D	B	α	□	-
ZRYSA33	+	-	+	+	+	B	B	β	□	+
ZRNSB31	+	+	+	+	-	A	B	α	□	-
ZRYSB3	+	-	+	+	-	B	B	β	□	-
ZRYSB13	+	-	+	+	-	A	B	α	□	-
ZRYSB14	+	-	-	+	-	D	B	β	□	-
ZRYSB17	-	-	-	+	-	D	A	α	A	+
ZRYSB18	+	-	-	+	-	A	A	β	B	+

ZRYSB35	+	-	+	+	-	A	B	β	□	+
ZRNT8	+	+	+	+	-	B	B	β	B	+
ZRYT1	-	+	+	+	-	D	B	β	□	+
ZRYT3	+	+	+	+	-	D	B	β	□	+
ZRYT4	+	+	+	+	-	B	B	α	□	+
ZRYT5	+	+	+	+	-	B	B	β	□	-
ZRYT19	+	+	+	+	-	B	A	α	□	+
ZRYT20	+	+	+	+	-	B	B	β	A	-
ZRYZC27	+	+	+	+	-	A	B	β	A	+

SAMPLE NO	GLUCOSE	MALTOSE	MANNITOL	UREASE	XYLOSE	HORSE	RABBIT	SHEEP	HUMAN	ORSAB
ZRYZC32	+	-	+	+	-	D	B	α	□	-
ZRYZC34	+	-	-	+	-	D	B	α	□	+
ZRYZC35	+	-	-	+	-	D	A	β	□	-
ZRNZC3	+	-	-	+	-	D	A	β	□	+
ZRNZC4	-	-	-	+	-	D	A	β	□	+
ZRNZC7	-	+	-	+	-	D	A	β	□	+
ZRNZC8	-	-	-	+	-	D	A	β	□	+
ZRNZC15	+	-	-	+	-	D	A	β	□	+
ZRNZC23	+	-	+	+	-	B	A	α	□	+
ZRNZC33	+	+	+	+	-	B	B	β	A	+
ZRNZC34						A	B	β	A	+
ZRNZC35	-	-	-	+	+	D	B	β	A	+
ZRNZC28	+	-	-	+	-	D	B	α	□	+
ZRNZC29	+	-	-	+	-	A	B	α	A	+

ZRNZC14	+	-	-	+	-	A	B	β	□	+
ZRNZC16	-	-	-	+	+	D	B	α	□	+
ZRNZC6	-	+	-	+	-	D	B	α	□	+

SAMPLE NO	OX 1µg (mm)	SXT 25µg	MET 10µg	AMP 10µg	AK 30µg	CIP 5µg	C 30 µg	E 15µg	CFM 5 µg	P 10 µg	TE 10µg	CN 10µg	VA 30µg	microbact
KDNB2	5	5	5	5	15	12	7	5	10	5	5	5	5	S.hyicus
KDNB6	5	5	5	31	21	11	5	5	26	5	7	5	11	S.aureus
KDNB7	5	6	5	21	11	14	5	5	19	5	9	18	5	S.chromoge
KDNB11	5	6	7	6	10	10	7	5	28	7	5	16	5	S.hyicus
KDYB10	5	5	5	5	18	14	9	5	22	10	5	5	5	S.intermedi
KDYB11	5	30	27	20	21	30	25	28	7	29	17	23	29	S.hyicus
KDYB18	5	5	5	5	13	12	5	5	25	5	8	5	5	S.hyicus
KDNC1	5	5	5	5	5	13	5	16	5	16	5	9	20	S.aureus
KDNC3	5	5	5	25	23	15	5	5	21	5	5	22	28	S.hyicus
KDNC4	5	5	5	29	11	14	10	5	27	9	11	5	5	S.hyicus
KDNC6	5	5	21	12	18	14	8	5	30	7	5	5	23	S.intermedius
KDNC7	5	20	24	11	13	20	19	17	5	23	24	13	34	S.intermedius

KDNC13	5	5	5	35	13	14	9	5	27	5	8	5	5	S.chromogenes
KDNC16	5	5	5	5	14	13	5	5	31	5	5	5	5	S.hyicus
KDNC24	5	5	5	5	16	28	11	8	31	11	5	19	24	S.aureus
KDNC30	5	5	5	5	9	11	5	5	25	5	5	5	5	S.aureus
KDNC31	5	25	5	5	21	20	20	21	5	5	5	11	23	S.hyicus
KDYC5	5	5	5	31	9	23	25	5	5	5	6	7	14	S.hyicus
KDNS1	5	21	5	5	5	15	19	17	5	14	5	5	5	S.aureus
KDNS4	5	21	7	5	5	5	5	11	5	10	12	5	7	S.aureus
KDNS5	5	15	5	34	9	11	5	5	17	13	29	5	21	S.aureus
KDNS8	5	5	5	5	10	12	5	5	27	5	5	5	5	S.hyicus
KDNS17	5	18	7	7	5	16	5	19	5	17	7	11	20	S.hyicus
KDNS30	5	24	7	8	5	5	18	16	5	18	20	8	5	S.intermedi
KDNS35	5	22	25	25	21	30	5	16	5	16	5	8	21	S.epidermidis
KDYS6	5	27	7	9	6	15	15	17	17	18	7	8	5	S.hyicus

KDYS10	5	5	12	38	14	12	8	5	27	9	8	10	22	S.hyicus
KDYS20	5	18	5	31	32	17	5	5	20	8	18	5	11	S.aureus
KDYS31	5	5	5	23	14	12	5	5	5	5	18	20	20	S.intermedi
KDYS33	5	5	5	38	17	12	7	5	27	5	8	8	5	S.chromogene
KDNK9	5	5	5	5	12	12	6	5	26	5	5	5	5	S.aureus
KDNK28	5	5	5	5	8	19	5	5	21	10	5	5	5	S.hyicus
KDNK31	5	26	5	21	14	18	18	18	25	29	23	16	31	S.intemedius
ZRNSA2	5	24	10	11	14	16	13	5	21	19	5	14	20	S.aureus
ZRNSA4	5	5	5	5	14	13	9	5	32	5	5	5	20	S.aureus
ZRNSA5	5	24	5	30	7	18	5	16	5	5	7	7	21	S.xylosus
ZRYSA6	5	5	5	30	5	5	16	12	5	5	5	13	21	S.aureus
ZRYSA19	5	24	7	9	5	19	5	18	5	16	7	8	21	S.aureus
ZRYSA29	5	24	7	8	5	18	17	20	5	16	5	9	22	S.hyicus
ZRYSA31	5	5	13	5	20	21	5	5	26	5	5	5	21	S.hyicus

ZRYSA33	5	5	5	5	16	13	7	5	29	7	5	5	20	S.hyicus
ZRNSB31	5	29	5	5	17	19	27	5	30	5	18	7	5	S.aureus
ZRYSB3	5	5	5	5	10	14	9	5	31	7	5	11	5	S.aureus
ZRYSB13	5	5	5	19	12	20	5	5	17	5	8	16	5	S.hyicus
ZRYSB14	5	5	5	5	12	12	9	5	29	5	5	5	5	S.hyicus
ZRYSB17	5	21	5	31	22	27	26	5	32	5	25	18	16	S.hyicus
ZRYSB18	5	5	5	8	31	19	5	5	31	5	10	5	12	S.hyicus
ZRYSB35	5	5	5	5	5	15	16	16	5	17	5	11	5	S.aureus
ZRNT8	5	5	5	5	20	11	26	5	8	5	5	13	21	S.hyicus
ZRYT1	5	21	9	8	5	20	22	19	5	19	5	11	30	S.hyicus
ZRYT4	5	5	5	5	13	11	5	5	21	5	5	5	5	S.chromoge
ZRYT5	5	5	5	5	13	13	8	5	28	7	5	5	21	S.intermedi
ZRYT19	5	23	5	30	7	19	18	18	21	13	9	8	22	S.chromoge
ZRYT20	5	5	5	5	9	11	6	5	28	5	5	5	5	S.intermedius

ZRYZC27	15	24	5	6	27	19	5	21	5	16	5	5	5	S.hyicus
ZRYZC29	5	23	24	5	11	15	18	12	31	9	18	10	7	S.epidermidis
ZRYZC32	5	14	5	5	14	13	9	9	15	8	5	22	5	S.hyicus
ZRYZC34	5	5	5	5	17	14	8	5	31	5	5	8	5	S.hyicus
ZRYZC35	5	5	5	5	13	13	8	5	30	7	5	5	23	S.epidermidis
ZRNZC3	5	5	26	5	22	11	7	5	5	5	8	22	21	S.hyicus
ZRNZC4	5	5	5	5	24	12	9	5	5	5	5	13	5	S.hyicus
ZRNZC7	5	5	5	5	23	13	10	5	5	8	16	6	22	S.aureus
ZRNZC8	5	5	5	30	22	11	11	5	5	5	10	5	25	S.epidermidis
ZRNZC15	5	5	5	10	21	11	25	5	5	5	10	8	5	S.hyicus
ZRNZC23	5	5	5	8	22	9	21	5	5	5	5	6	22	S.hyicus
ZRNZC33	5	5	5	7	35	19	5	5	5	5	16	20	21	S.hyicus
ZRNZC34	5	5	5	33	25	13	7	5	5	5	15	5	23	S.hyicus
ZRNZC35	5	5	5	28	24	12	7	5	5	5	13	12	21	S.epidermidis

ZRNZC28	5	5	5	11	22	10	21	5	7	5	8	11	10	S.hyicus
ZRNZC29	5	5	5	12	20	10	8	5	7	5	8	8	5	S.hyicus
ZRNZC14	5	5	5	9	20	14	12	5	10	5	5	22	5	S.simulans
ZRNZC16	5	5	5	10	14	13	11	5	9	5	9	5	5	S.hyicus
ZRNZC6	14	22	5	6	17	17	19	5	5	5	13	11	23	S.chromogenes

Numbers in red, green and black are resistant. intermediate and susceptible values by each of the isolates to each antibiotic.