ISOLATION AND ANTIBIOGRAM OF *ESCHERICHIA COLI, STAPHYLOCOCCUS* AND *STREPTOCOCCUS*SPECIES FROMMASTITIC COWS IN ZARIA, NIGERIA

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DECLARATION

I declare that this thesis entitled "**Isolation and Antibiogram of *Escherichia coli,Staphylococcus*and *Streptococcus* species from Mastitic cows in Zaria, Nigeria**" was carried out by me in the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahamdu Bello University, Zariaunder the supervision of Dr. M.A. Raji,Dr M. Bello and Dr. Sani Adamu. The information derived from the literature has been duly acknowledged in the text and a list of reference provided;no part of this thesis has previously been presented for the award of degree or diploma at any University.

Kuburat Bola RAJI _____ _____

Signature Date

CERTIFICATION

This thesis entitled "ISOLATION AND ANTIBIOGRAM OF *ESCHERICHIA COLI,STAPHYLOCOCCUS* AND *STREPTOCOCCUS* SPECIES FROM MASTITIC COWS IN ZARIA, NIGERIA" by **Kuburat Bola RAJI**, meets the regulation governing the award of **Master of Science degree** in Veterinary Microbiology of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to Almighty Allah whose sovereignty extends throughout the world and also to my father Alhaji A.B. Raji for his labour, encouragement,prayer, support and provisions both in cash and kind in the course of my M.Sc. programme. May the Almighty Allah reward himabundantly.

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ABSTRACT

This study was conducted to isolate and identify mastitis causing organisms;*E. coli,*Staphylococciand Streptococci from mastitic cows in Zaria andto determine their antimicrobial susceptibility to commonly used antimicrobial agents and the role of some potential risk factors and management practices on the occurrence of mastitis in cows.Milk samples froma total of 472 quarters'of125 white Fulani lactating cow herds were collectedin order to determine clinical mastitis (shown as slight swelling of affected quarters and changes in milk appearance) and subclinical mastitis(detected by CMT, for increase in somatic cell counts). The milk samples collected were cultured and isolated using different media for bacterial identification and confirmed by conventional biochemical test and microbact. The following bacteria were isolated from quarter milk samples at the respective rates;*Staphylococcus* species (*Staphylococcus aureus* 10.87%, *Staphylococcusintermedius* 13.04% *Staphylococcus haemolyticus* 8.70%, *Staphylococcus chromogen* 8.69%, *Staphylococcushyicus*3.3%and *Staphylococcus xylosus*32.60%), *Streptococcus* species (*Streptococcus uberis,*4.35%and *Streptococcus bovis* 1.08%) and *E coli*(17.40%).Isolationrates of clinical mastitis in herd, cow and quarter levels were 44%, 5.6% and 5.9%, respectively. Subclincal mastitis was found to be 66%, 11.2% and 11.86%, at herd, cow and quarter levels respectively. Among the pathogens isolated from mastitic milk, coagulase negative *Staphylococcus* species were the most frequently encountered (66.30%); theothers were *Staphylococcus aureus*(10.87%),*Streptococcus* species (5.35%) and *Escherichia. coli*(17.40%) The bacteria isolated exhibited multiple drug resistance. *Staphylococcus aureus* exhibited 90% resistance to ampicillin and 100% to penicillin. *Staphylococcus xylosus* showed 80 % and 90%resistanceto ampicillin and

penicillin.*E coli*exhibited 100%, 62.5% 56.4% resistantto ampicillin, tetracycline and gentamicin respectively.*Streptococcus uberis* showed 75% resistance to tetracycline and 25% resistance to gentamicin. Among the risk factors considered, lesion on teat, stage of lactation and parity number had no statistical significance associations($p>0.05$) to prevalence of subclinical mastitis. In conclusion, the bacteria lsolated were the most probable causesof the mastitis seen and they exhibited multiple and different antimicrobial resistance patterns.

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LIST OF ABBREVIETION

CNS Coagulase Negative *Staphylococcus*

Cfu Colony Forming Unit

CMT California Mastitis Test

CLSI Clinical and Laboratory Standard Institute

DNA Deoxyribonuclease Acid

EMB Eosin Methylene Blue

IMI Intramammary Infection

LPs Bacteria Lipopolysaccharide

MLST Multilocus Sequencing Typing

MSA Mannitol Salt Agar

MSCRAM Microbial Surface Components Recognizing Adhesive Matrix Modules

NAGase N-acetyl-β-D- glucosaminidase

NMC National Mastitis Council

ngs Un Groupable

PMNs Polymorphonuclear Neutrophil

PBP2 Penicillin –Binding Protein

PCR Polymerase Chain Reaction

PFGE Pulse Field Gel Electrophoresis

RNA Ribonuclease Acid

SCC Somatic Cell Count

SCCmec Staphylococcal Cassette Chromosome Mec

TSST-1 Toxic Shock Syndrome Toxin -1

TSI Triple Sugar Isron

CHAPTER ONE

INTRODUCTION

1.1 Back ground of the Study

Mastitis is an inflammation of the udder resulting in an enlarged quarter or quarters with a physical, chemical and bacteriological changes in milk and pathological changes in the udder (Blowey, 1999; Radostits *et al* 2000; Sharma, 2007). Mastitis is probably the most important and influential single health disorder affecting milk production in dairy farms. It is the disease thataffects the dairy industry globally and causes significant economic losses. In the United State of America, the resulting loss due to mastitis in dairy industry is about of $ 2 billion annually (Gruet *et al*., 2001; Hoblet*et al*., 1991). The severe economic losses are due to reduced milkyield; milk quality and early culling of severely affected animals. Also, colossal amounts are expended in antibiotic treatment of mastitis and veterinary services are wasted and economic losses are incurreddue to mortality of young animals (Gruet *et al*., 2001; Hoblet *et al*., 1991).

Mastitis can either be infectious, caused as a response to infection with microbial organisms or non-infectious, as a result of physical injury to the mammary gland (Blowey, 1999). The inflammatory response results in an increase in the blood proteins and white blood cells in the mammary tissue, which can then pass into the milk and milk products. Mastitis aims to destroy the irritant, repair the damaged mammary tissue and return the udder to its normal function. However, such inflammtion, as often experienced, results in reduction in milk output (Mustafa, 2003).The severity of the inflammation can be classified into subclinical, clinical and chronic forms, and its degree is dependent on

certain risk factors (Viguier*et al.*,2000). Only a small proportion of udder infections result in clinical mastitis, while the vast majority of cases exists as sub-clinical mastitis(Mustafa, 2003). The causes of infectionhave been established andbacterial species are the most common causes of mastitis, although, incidences of viral, algal and fungal - related mastitis have also been reported (Watts, 1988; Smith and Hogan, 2001). Majority of bacterial infections of the mammary glands are due to*Staphylococci, Streptococci* and gram-negative bacteria such as *E coli,*and *Klebsiella*(Bradley, 2002a), which occur usually when bacteria penetrate through the teat duct into the interior of the gland (Schalm *et al.*, 1971).

Mastitis is also classified as either contagious or environmental mastitis (Smith and Hogan, 1993; Blowey and Edmondson, 1995). The former is caused by organisms such as *Staphylococcus aureus* and *Streptococcus agalactiae* that are adapted to survive within the host, particularly in the mammary gland (Bramley and Dodd, 1984;Smith and Hogan, 1993). Environmental mastitis is caused by organisms such as *E coli, Streptococcus uberis* thatdo not usually live within the host but enter the teat canal when the cow comes into contact with a contaminated environment. (Blowey, 1999; Kudi *et al*., 2009)) Theyare common causes of clinical mastitis, with the infection occurring either between milkings such as teat contact with pathogenic material or during the udder preparation before milking(Edmondson, 2001). Dodd (1987) stated that 'Contamination with the teat end of a susceptible quarter with a pathogen is the first stage in the infection processes.

Streptococci were the first bacterial organisms to be incriminated as the cause of contagious mastitis right before the era of antibiotics. *Streptococcus agalactiae* (Lancefield group B), *Streptococcus dysgalactiae* (Lancefield group C) and *Streptococcus*

uberis are the main streptococci species involved in mastitis. *Streptococcus agalactiae* is the major cause of chronic mastitis, and is most prevalent in herds (Keefe, 1997).It can survive for long period only within the mammary gland and the effect of infection on udder is devastating. It could result in fibrosis and agalactiae (Keefe, 1997). Environmental Streptococci, *Streptococcus dysgalactiae*and *Streptococcus uberis*cause both subclinical and clinical mastitis (Wilesmith *et al*., 1986; Erskine *et al*., 1987).

There are fourty (40) different species of *Staphylococcus,* which are divided into coagulase positive and coagulase negative staphylococci (CNS) based on the ability to coagulate plasma. Among these, *Staphylococcus aureus*is by far the most pathogenic. It causes chronic and deep infections of the mammary gland that are extremely difficult to cure (Miles *et al*., 1992). Elsewhere, It was reported to be the most frequent and predominant cause of both clinical and subclinical mastitis (Kupur *et al.,* 1992; Myllys, 1998).

Coagulase negative staphylococci(CNS)were traditionally considered minor pathogens or non- pathogenic as infections with these organisms mostly remaining subclinical (Taponen *et al.,* 2006). However, they may cause persistent infection that can damage the udder. Some authors have reported a high percentage of clinical cases of mastitis evoked by CNS (Bradley and Green, 2001). *Escherichia coli*produce endotoxin, which is responsible for many of the observed inflammatory mammary and systemic changes. Cases of coliform mastitis are usually peracute, acute or chronic in nature and may cause death (Golodetz and White, 1983; Shpigel *et al*., 1997). Chronic clinical mastitis is characterized by periodic subacute to acute flare-ups that may persist for months (Bradley, 2002). Certain factors influence the prevalence of mastitis; suchas age, parity numbers and

stage of lactactions where most of the new infections occur during the early parts of the dry period (little milk production) and in the first 2 months of lactation (Erskine, 2001).

In most countries, surveys of dairy herds indicate that there is relatively high incidence of clinical mastitis and, many farms have high prevalence of subclinical mastitis too (Myllys, 1998). Prevalence with infection of mastitis pathogens is about 50% in cows and quarter infection rate of about 25% (Radostitis *et al*., 2000)

In Nigeria, Umoh*et al.,* (1990a) reported a prevalence rate of 30% and 3.2% for *Staphylococcus aureus* from settled and traditionally nomadic herds, receptively, in Zaria. In another study by Amosun*et al.* (2010)an incidence rate of 55.4% for *Streptococcus uberis*,24.6% *Streptococcus agalactiae* and 12.3%*Streptococcus dysgalactiae*was reportedinIbadan. Prevalence rate of 25.7% *Staphylococcus aureus*, 15.4% *Streptococcus agalactiae* and 14.1%. *Esherichiacoli* in bovine mastitis had also been reported by Kivaria*et al*. (2006), in Tanzania.

Treatment of mastitis in cows using antibiotic therapy remains an important control strategy during lactation and dry periods. Indeed, the disease is the most frequent reason for the use of antimicrobial agents in dairy farms (Erskine, 2000). Despite the progress made to control mastitis and improving general health of the udder over the past decades , mastitis still remains a major problem in dairy industry (Pyorala,2002). This has ledto a growing concern among dairy producers about the economic values of expensive but rather ineffective antibiotic therapy.There is also a general concernabout the possible human health risk due toconsumption of antibiotic residues in milk withsubsequent emergence of antibioticresistant strains of bacteria that may enter the food chain (Pyorala,2002).

1.2Statement of Research Problems

Mastitis has proved to be one of the most difficult cattle diseases to deal with (Bradley, 2002). The dynamic nature of mastitis, constant infection and variable susceptibility of cows to re-infection make it a major challenge (Sandholm*et al*., 1990). Despite intensive research over the past decades, bovine mastitis remains prevalent among cattle (Pyorala, 2002). The implementation of specific control programmes resulted in the eradication of *Streptococcus agalactiae* and substantially reduced other major mastitis pathogens in well managed herds. However, incidence of acute clinical and sub- clinical mastitis still persists and both have been shown to adversely affect subsequent production (Schrick *et al*., 2001) and in severe cases can lead to poisoning and premature death (Blowey,1999)

Mastitis therapy is commonly unsuccessful owing to several factors such as pathological changes that occur in the udder parenchyma as a result of inflammatory reaction, mastitogenic bacteria related factors, pharmacokinetic properties of antimicrobial drugs and resistance of bacteria, (Roberson, 1999; Gruet *et al.,* 200l; Lockhart *et al.,* 2007). In Nigeria, the overall impact of bovine mastitis and factors that are responsible for low milk production in dairy industry, are not fully known due to lack ofmonitoring and surveillance systems (Raji, 2007). However, among the problems identified are cattle grazing on poor grass qualities, low in protein and indigestible roughage, poor housing facilities and environment in which the cattle are kept.The dairy industry (National dairy herd) is not well developed and lack effective policy.The significant economic losses incurred from clinical and sub-clinical mastitis in the study area have not been quantified or cost estimated in terms of financial implication such as discarded milk, quality

penalties, treatment and reduction in output. Data on the characteristics of bovine mastitis causing organisms in the study area are still very limited (Umoh *et al*., 1990a). A better understanding of mastitis causing organisms from mastitis cow and their antibiogram will help explore better therapeutic approaches.

1.3 Justification of the Study

This study was necessitated by the fact that adversely affects the quality and quantity of milk produced (Reneau 1986; Reneau and Packard, 1991).Milk is one of the most important foods for human beings, recognized as a complete diet due to its essential components (Javaid *et al.,* 2009). It has also contributed significantly to poverty alleviation in Nigeria (Ameh *et al.,* 1999). Also, milk is known toprovide a regular source of household income, food and self employement particularly to women (Ameh *et al.*, 1999). Unfortunately, dairy farmers continue to experience sub-optimal performance in their animals.In the study area, milk is mostly consumed raw or unpasteurized. This has serious public health implications (health hazard) as the extensive use of antibiotics in treatments and control has possible health implication for human due to increased risk of antibioticresistant strains of bacteria entering the food chain ((Ungemach, 1999). This is becauseMastitis is dynamic in nature and microorganisms are constantly evolving, which when coupled with the antibiotic resistance developed by the bacteria poses a major challenge. Moreover, there is no national information, reports, monitoring or surveillance systems (Raji, 2007) that reflect the economic impact and public health significance of mastitis in Nigeria. The monitoring will not only generate data of importance and provide information on mastitis causing organisms, but also the trend in development of antibiotic

resistance, that might establish interventions regarding antimicrobial use. Though several studies had documented the occurrence of bacterial species (*E. coli*,*Staphylococcus* and *Streptococcus*) and antimicrobial susceptibilities of mastitis pathogens in the study area, this study will provide information on the current status of the bacteria that occur in mastitic cows and their antimicrobial susceptibility profiles inZaria, Nigeria.

1.4 Aim of the Study

This study wasaimed at isolating and identifying mastitis-causing microorganisms; such as *E.coli*,*Staphylococcus* and*Streptococcus* species from mastitisquarter's milk and determining their susceptibility profile to commonly used antimicrobial agents as well as examinning some risk factors to mastitis.

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1.5 Objective

1. To isolate and identify mastitis-causing microorganisms; *Escherichiacoli*, *Staphylococcus*and *Streptococcus* species from bovine mastitis using the conventional biochemical and microbact 12S identification system methods.
2. To determine the antimicrobial susceptibility profile of the isolates from bovine mastitic milk in Zaria
3. Toevaluate the management practices and selectedpotentialriskfactorsassociatedwith prevalence of bovinemastitis using structured questionnaires.

1.4 Research Question

a. Are bacterial agents especially *Staphylococcus*, *Streptococcus* and *Escherichia coli* responsible for bovine mastitis in Zaria?
b. Are the isolated bacterial agents of bovine mastitis in Zaria susceptible to commonly used antibiotics?
c. Do management practices and other risk factors influence the occurrence of the isolated bacteria as agents of bovinemastitis?

CHAPTER TWO

LITERATURE REVIEW

2.1 Mastitis

Mastitis is inflammation of the parenchyma of the mammary gland due to different causes. It results in physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue (Blowey, 1999; Radostits*et al*., 2000; Sharma, 2007).

2.2 Aetiological Agents of Mastitis.

The frequent aetiological agents of mastitis arewide spectrum of bacterial species However, viruses, mycoplasma, yeast and algae have been implicated as causes of mastitis (Watts, 1988; Smith and Hogan, 2001). Majority of infections of the mammary glands are caused by *Staphylococci; Staphylococcus aureus, and* coagulase negative staphylococci, *Streptococci; Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae*and *Streptococcus bovis* and gram-negative bacteria such as*E.coli* and*Klebsiella Pneumoiae*. (Bradley, 2002a).Other less frequent organisms includes *Klebsiella oxytoca*, *Pasteurella* spp., *Proteus* spp., *Corynebacterium bovis,Norcadia*, *Mycoplasm*a spp., and *Yeast*. The Enterobacteriacae were the commonest causes responsible for 40.9% of all mastitis, and *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae* accounted for only 10% of clinical cases (Dogan *et al*., 2006). Staphylocci are the bacteria most commonly isolated from subclinical mastitis. (Tenhagen *et al*., 2006).

2.2.1Classification of mastitis causing pathogens

Mastitis pathogens have been classified as either contagious or environmental pathogens (Blowey and Edmondson, 1995). In essence, the contagious are adapted to survive within the host, in particular within the mammary gland. These bacteria multiply in the udder, teat canal and are capable of establishing subclinical infections which manifest as an elevation in the somatic cell count (Leukocytes, predominately neutrophils and epithelia cells) of milk from affected quarters and are typically spread from cow to cow at or around the time of milking (Radostits *et al*., 1994). In contrast, the environmental pathogens are best described as opportunistic invaders of the mammary gland and are found in the cows environment; typically invade, multiply, engender host immune response and are rapidly eliminated from udder by the defense mechanism of the cow.

Mastitis is classified as either contagious or environmental mastitis (Smith and Hogan, 1993; Blowey and Edmondson, 1995). Contagious mastitis is caused by organisms such as *Staphylococcus aureus* and *Streptococcus agalactiae* that live on the skin of the teat and inside the udder. Contagious mastitis can be transmitted from cow to another during milking. In contrast, environmental mastitis is caused by organisms such as *E coli*, *Streptococcus uberis* that do not usually live upon the skin but enter the teat canal when the cow comes into contact with a contaminated environment and are common causes of clinical mastitis, with the infection occurring either between milkings such as teat contact with pathogenicmaterial (feces in bedding materials) or during the udder preparation (Edmondson, 2001).

2.3 Mastitis causing pathogens

Biology ofStaphylococci*; Staphylococcusaureus* and other speciesof *Staphylococcus*.

Rosenbach was given the credit for the genus name *Staphylococcus* in 1884. *Staphylococcus* is coined from Greek and Latin words (Greek staphyle – bunch of grapes; and (LatincocciSpherical *bacterium.*)*Staphylococcus* belongs tokingdom – bacteria; phylum Firmicutes; (lat: firmus strong, cutis skin, referring to the cell wall). Class, Bacilli; Order bacillales; Family Staphylococcaceae and genus*Staphylococcus* (Kuroda *et al.*, 2001).

The Staphylococci are a group of gram positive, spherical cocci that are widely distributed in the environment and on the integuments of man and animals (Burnett, 1976). They average 0.8μm in diameter. They seldom form chainsbutoccursingly and in pairs, tetrads, short chain andirregular aggregations or grape – like clusters, because the cells divide in three perpendicular manner on solid medium. Staphylococci are non-motile and do not form spores. They are facultative anaerobic, but most seem to prefer an aerobic condition and only very few are strictly anaerobic (Burnett, 1976).

Staphylococci are catalase-positive, oxidase-negative, can grow at a temperature range of 15 to 45 degrees celcus and at Nacl concentrations as high as 15 per cent. They are divided into coagulase positive and coagulase negative, on the bases of their ability to coagulate plasma.Nearlyall the strains of *S. aureus* produce the enzyme coagulase (Madigan and Martinko, 2005). Eleven capsular polysaccharide serotypes have been described for *S. aureus* (Sompolinsky *et al*., 1985) alone and the most common in clinical isolates are types 5 and 8. The main capsular components are N-aceylaminouronic acids

and N-acetylfucosamine. The genome of *Staphylococcus aureus* is circular and consists ofapproximately 2.8 mega base pairs long with approximately 2,600 open reading frames, comprising 84.5% of the genome (Gill *et al.,* 2005). It has a G+.C content of about 33% and a single plasmid ofabout 25,000 base pairs in length with G+C content of 28% (Gill *et al.,* 2005) and for *Staphylococcus aureus*,in addition to the normal complement of housekeeping genes, the chromosome contains many accessory genetic elements that are not necessary for growth under laboratory conditions (Gill *et al.,* 2005). *Staphylococcus epidermidis*which has two genomes sequenced in the species is about2.5mega-base pairs long with 1,681 open framed and its G+C content is 32%. *Staphylococcus haemolyticus is* about 2.68 mega+base pair long with a G+C content of 32 %.

2.3.1 Contamination of teat orifice and entry of*Staphylococcus aureus*into the mammay gland.

Staphylococcus aureus is recognized as a major contagious pathogen that causes subclinical intra-mammary infectionsthat remain persistence (Anderson, 1983)causeof clinical mastitiswithmoderate to seriouslocal and systemicsigns (Kupur *et al.,* 1992). As a well-armedpathogen, its success, andability to cause infection lie in its extensive virulence factors. The main reservoirs of *S. aureus* are the infected quarters, and the skin of the udder and teats. The presence of lesions on the teat skin such as chaps and cracks, allows persistent colonization of mammary gland. Once the teat orifice is contaminatedby *S aureus*, bacteria can persist and multiply and enter the teat canal.

2.3.2 Colonization of mammary gland by *S aureus*

Studies have shown that it is possible to induce experimental *S. aureus* intramammary infection by intrammary infusion of a low number of bacteria (<100cfu) through the teat canal (Postle and Pankey, 1978). These observations demonstrate that milk can support the growth of *S. aureus,* because it canhydrolyse casein and ferment lactose. The natural defence mechanisms of the mammary gland against infection are inefficient to prevent survival of the bacteria. Bacterial surface properties play a major role in the host-bacterium relationship (Smith, 1977). During infection, surface compnents participate in bacterial adhesion to host mammary tissues and resistance to phagocytosis by milk cells.

2.3.3 *Staphylococcus aureus*virulence factors

S. aureus has a diverse arsenal of components and products that contributes to the pathogenesis of infection. These components and products have overlapping roles and can act either alone or in concert (Mongodin *et al*., 2002). Staphylococci are known to produce microbial surface components recognizing adhesive matrix modules (MSCRAMMs), which comprise the main adhesives of the organism and include collagen-binding protein, fibronectin binding proteins, fibrinogen binding protein, elastin binding protein, clumping factor, and the matrix adhesion factor(Patti *et al*., 1994). As many as twelve other surface proteins may contain membrane anchor domains and potentially qualify as MSCRAMMs.Various virulence factors, including production of haemolysins, leucocidins, exfoliative toxins, enterotoxins, toxic-shock syndrome toxin, and slime and biofilms formation were found in *S. aureus* strains isolated from bovine mastitis

(Cucarella *et al*., 2004). Their roles in virulence have been postulated but not confirmed with regard to infections of domestic animals (Bramley *et al.,* 1979). The first step in the colonization of the mammary gland by *S. aureus* seems to be adhesion to epithelial cells. The in-vitro adhesion of *S. aureus* to bovine mammary epithelial cells has been demonstrated (Opdebeeck*et al.,* 1988). And this phenomenon varies with the strain and with origin of the cells. The attachment of *S. aureus* to epithelial cells of lactiferous ducts has been confirmed invivo by microscopic examination of sections of experimentally infected mammary glands (Gudding *et al.,* 1984). The mechanism of *S. aureus* adhesion to mammary epithelial cells is not well understood. However, two types of interactions could be involved; non-specificphysiochemical interactions and specific interactions between bacteria cell wall associated receptors and host components (Sutra and Poutrel, 1994).*S. aureus*have a high surface hydrophobicity and this surface characteristic could favour the fixation of bacteria toextracellular proteins suchascollagenand fibronectin.

2.3.4 Role of extracellular enzymes and coagulase in *S aureus* mastitis

S. aureus produces numerous extracellular enzymes (Anderson, 1977) including hyaluronidase, phosphatase, nuclease, lipase, catalase, staphylokinase and proteases, which have been implicated in the pathogenesis of bovine intra mammary infection. (IMI) The ability of *S. aureus* to multiply rapidly in milk is probably a major component of its virulence. Enzymes could allow *S. aureus* to use milk substrates for their metabolism and consequently, to adapt to milk and grow (Anderson, 1976).The role of free coagulase in the virulence of *S.aureus* is uncertain. However, since it is produced by majority of strains and has biological function as a prothrombin activator, it is considered a probable

virulence factor. The cell wall bound cluming factor, another fibrinogen binding protein, shares a significant sequence similarity with coagulase, and its role in adherence is clearer than that of coagulase (Sibooet *al*., 2001).

2.3.5 Role of toxins in *S. aureus*mastitis

S. aureus can produce four different haemolytic toxins: α, β, γ and δ toxins. Two (α and β) toxins appear to play a major role in the virulence of *S. aureus.* Alpha toxin produced between 20% and 50% (Sutra and Pourtel, 1994), is a cytolysin that binds to cell membranes and forms hexametric pores leading to cell death as a consequence of rapid egress of cytoplasmic components (Bhakdi and Tranum, 1991).

2.3.6 Expression of antiphagocytic factors by *S. aureus*

The ability of *S. aureus* to avoid opsonins present in normal serum is an important factor in the success of infection. Antibodies that recognize cell surface components such as teichoic acid, peptidoglycan and surfa associated proteins are present in most sera. *S. aureus* expresses surface associated anti-opsonic proteins and a polysaccharide capsule that can both interfere with the deposition of antibodies and complement formation by classical and alternate pathways, or with their access to neutrophil complement receptor and fe receptor. Therefore, efficient phagocytosis by neutrophils that requires recognition of bound complement and antibody is compromised (Foster, 2005).

2.3.7 Source of infection and infected intramammary gland with *Saureus*

The primary and most important reservoir of *S. aureus* is the infected mammary gland, milk of lactating cows, body sites of prepartum heifers, and environmental sites.(Roberson *et al.,* 1998a;Roberson *et al.,* 1998b). The most common source of *S. aureus* in dairies was intramammary infection (IMI) in the lactating cows, heifers. Nearly one-third of new cases of *S. aureus* IMI in lactating herds were due to heifers that were re-infected with *S. aureus* (Roberson *et al.,* 1994), and new herd addition (whether cow or heifer) represents another source of contagious mastitis pathogen. *S. aureus*IMI of ruminants is usually initiated either bycolonization of the teat canal with bacteria derived from the epidermis or by an influx of contaminated milk entering the gland during milking. Bacteria multiply in the milk and gain access to the upper part of the gland where they adhere to the ductular and alveolar epithelium (Frost, 1975). Adherent bacteria trigger macrophage activation and neutrophil migration from the blood into the milk resulting in an increase in the somatic cell count (SCC), impairment of the host immune system, and epithelial cell damage. As a result bacteria gain access to the basal sub-epithelial cell layers, bind fibrinogen and other host receptor proteins and establish an infection which often becomes chronic (Foster and Hook, 1998). It is likely that *S. aureus* has the capacity to survive intracellular which may facilitate immune and antibiotic avoidance (Dziewanowaska *et al*., 1999). The production of cytolytic toxins such as α-, β- and a number of different leukotoxins may contribute to the severity of infection and high levels of expression may results in clinical mastitis (Barrio *et al*., 2006).

2.3.8 Body site colonization

It has been known that *S. aureus* may colonize the body sites of mammals, including humans. Matos (1988) found *S. aureus* in 40% of 70 lesions sampled on teats and udders of lactating animals, demonstrating that this organism readily colonizes wounds. The second most common source of *S. aureus* is from heifer colostrum or body site; although most body size colonization appear to be transient (Roberson *et al*., 1994; Roberson *et al*., 1998a; Roberson *et al*.,1998b). Whether it be social contact or suckling, it is likely that the heifer calf carrying *S. aureus* could play a role in the epidemiology of *S. aureus* IMI in heifers because she is a source of *S. aureus* that could be constantly transferred to herd mates. Likewise adult body site carriers of *S. aureus* may represent the reservoir both for themselves and other cows during the dry period (Matos,1988).

2.3.9 Environmental sources/reservoirs

In dairy herd workers, the hands may be considered the most important site of *S. aureus* because the hands are in intimate contact with the udder. Based on biotyping or phage typing patterns, there have been several reports of human strains of *S. aureus* isolated from dairy cow IMI. Hence human carriers may be regarded as important factors in the spread of diseases (Matos, 1988).

2.4 Coagulates Negative *Staphylococcus*

Coagulates negativeStaphylococci(CNS)havetraditionallybeen considered to be opportunistic skin organisms that can cause mastitis as opportunitis bacteria (Devriese and Dekeyser, 1980). They are considered minor pathogens when compared with major pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*. They seem to lack the ability to cause severe mastitis. Generally, they areassociatedwith subclinical and occasional bouts of clinical mastitis. Usually the clinical signs are mild (Taponen *et al*., 2006), however, they may persist in the mammary gland and moderately increase somatic cell count and decrease milk quality, which has resulted in underestimation of the occurrence of CNS mastitis, which can also damage the udder tissue thus decrease milk production. CNS mastitis is generally expected to beeliminated spontaneouslyand is commonly left without antimicrobial treatmenttherapy (McDougall, 1998). CNS mastitisis especially common in heifers and in cows during first lactation (Myllys, 1995a).The CNS group consists of about 40 different *Staphylococcus* species. Thesedifferent CNS species are not necessarily a uniform group. Some CNS may be more contagious in nature than others and some may be more virulent than others. (Myllys, 1995a).

The reservoirs of mastitis causing CNS remain unclear, although a number of CNS species have been isolated from different bovine body and extra mammary sites, (White *et al*., 1989) such as body sites of cows, heifers and calves, The CNS have been isolated from udder secretions and milk, and from the cows' environment (Devriese and De Keyser, 1980; White *et al.,* 1989; Matos *et al.,*1991; Matthews *et al.,* 1992). A wide range of CNS species have been isolated and identified, most often, using methods based on the phenotype. *S. chromogenes*, *S. epidermidis*, *S. intermedius*, *S. warneri*, *S. haemolyticus*, *S.*

sciuri and *S. xylosus* were isolated in several studies from milk samples, teat canals, teat skin or skin on other body sites (Devriese and De Keyser, 1980; Matthews *et al.,* 1992; Chaffer *et al.,* 1999).

2.4.1 Virulence factors of coagulase negative *staphylococcus*

Possible virulence factors of coagulase negative *Staphylococcus* have been investigated both by measuring phenotypic expression of substances assumed to be associated with virulence and by screening of genes encoding these.Only few studies have focused on the search for such virulence factors from CNS isolated from mastitis. Adherence and internalization of CNS into mammary epithelial cells was studied in cell cultures (Hyvonen*et al*., 2007); were shown to be able to adhere to bovine mammary cells. The adhesive capacity of various CNS was almost equal to the adhesive capacity of *S. aureus*, but the invasive capacity of *S. aureus* was stronger than that of CNS strains. Cytotoxic activity in cell cultures of CNS and *S. aureus* isolated from mastitis, is possibly caused by a metalloprotease was reported by Zhang and Maddox (2000).Kuroishi *et al.* (2003) found that a high percentage of both *S. aureus* and CNS from bovine subclinical, chronic or acute mastitis produced staphylococcal enterotoxins and/or toxic shock syndrome toxin-I. Somewhat surprisingly, production of different staphylococcal enterotoxins and toxic shock syndrome toxins- were as common in CNS isolates as in *S. aureus* isolates, and as common in isolates originating from subclinical mastitis as in those from chronic or acute mastitis (Kuroishi *et al*., 2003).

2.5 Streptococcus

*Streptococcus*is a member of theStreptococaceae family. It affects both human and animal. It was first described by Rosenbach 1884. They are gram positive cocci that grow in chain or pairs; they are small (1-3mm in diameter),smooth translucent and convex, gray or almost whiteon blood agar (Spellerberg and Brandt 2007) .They are catalase negative, oxidase negative , show forms of various haemolysis. Thegenome content is 1.8-2.3 mega-bases in side encode 1,700-2,300 protein.Streptococci species are classified on the basis of their haemolytic properties.α-, β and gammahaemolytic.β-haemolytic were further characterized via Lancefield serotyping, which describes specific carbohydrate presence on the bacteria cell wall called Lancefield group. There are 20 described serotypes named Lancefield group A-V (excluding I and J). Of most important are the α-haemolytic and that of the β-haemolytic Lancefield groups A, B, C and D. In bovine mastitis, Lancefield group B (*Streptococcus agalactiae*), Lancefield group C (*Streptococcus dysgalactiae*), *Streptococcus uberis*and noneenterococci group D (*streptococcus bovis*, *Streptococcus equinus*) are most important. *Streptococcus* species are isolated frequently from bovine mammary glands(Freney *et al.*, 1992; Baron *et al*., 1994;Facklam, 2002).*Streptococcus agalactiae,Streptococcusdysgalactiae* and*Streptococcus uberis* have been reported as the three most common aetiological agentsof mastitis (Calvinho and Oliver, 1998; Khan *et al.*, 2003;Amosun *et al*., 2010).Other*Streptococcus*species suchas *Streptococcus epidemicus* and *Streptococcus bovis*have been implicated in bovine mastitis, although they are relatively infrequent (Lammler,1991; Khan *et al*., 2003; Amosun *et al*., 2010).

2.5.1 *Streptococcus agalactiae*

This is highly contagious pathogens of bovine mammary glands(McDonald, 1977) anda majorpathogen that causes subclinical mastitis in dairy herds. It has been widely reported as an important pathogen of animal and man(Schucat and Wenger, 1994; Keefe,1997; Ko*et al*., 2001).This organism primarily infects the cisterns and the ductal system of the mammary gland and produces irritants causing inflammation of gland, which is mostly subclinical with occasional clinical symptoms (Myllys *et al.,* 1995b). According to the later, accumulation of bacterial waste products intensify the inflammatory response, which results in destruction of milk producing tissue, which causes reduced milk yield or agalactiae. It rarely causes severe illness; however, it causes extensive scarring of a quarter and renders it unproductive in subsequent lactation (Myllys *et al.,* 199b).

2.6 Environmental Pathogens Causing Mastitis

Theseareheterogeneousgroup of bacterial genera and species referred to as the environmental mastitis pathogensandtheythriveon high temperature, high humidity, and organic bedding materials and simply love overcrowded poorly ventilated facilities (Smith *et al.*, 1985). They are amajor contributor to cases of clinical mastitis. These organisms are present in the environment of cows and can directly infect cow or contaminate product placed on the teats (teat dips) or infused into mammary gland (Smith *et al*., 1985; SmithandHogan, 1995).The environmental pathogens mostly encountered are species of Streptococci other than*Streptococcus agalactiae* andgram negative bacteria, primarily the coliformsbacteria. Examples of environmental streptococcal species are*Streptococcus uberis, Streptococcusdysgalactiae, Streptococcus equines, and Enterococcus faecaclis.* Gram negative bacterial includes*Escherichiacoli, Klebsiella* species, *Pseudomonas*

species, *Proteus* species, and *Citobacter*species (Smith and Hogan, 1993).Incidence of mastitis caused by environmental pathogens is greater during the initial 2weeks of dry period and this mastitis is associated with milk cessation. When manurebedding and other contaminate are not removed at each milking, this increases the bacteria load at the teat end.Without milkremoval, the gland enlarges and the teat ends dilates, allowing environmental pathogens easier access into the mammary gland.

Infections caused by environmental pathogens are mostly of short duration incomparison to contagious pathogens, and result in clinical mastitis and less likely cause herd problems that is usually associated with subclinical mastitis such as high bulk milk somatic cell count (Smith, 1983;Harmon, 1994).

Environmental streptococcal intramammary infections aregenerally presentfor less than 30 days, while the coliforminfections are of very short duration with over half of these infections lasting less than 10days (Smith *et al*., 1985).

The major reservoirs of environmental pathogens are the cow environment and those factors in the environment that result in the contamination of teat ends and which contribute in a major way to the number of environmental pathogens in intra-mammary infection in a dairy herd(Smith *et al.,* 1985). Sources of environmental pathogens include manure, dirt, mud, pools of standing water, feed stuffs and bedding material (Hillerton, 1996). Bedding materials are a significant source of environmental pathogens in dairy herd and are in frequent and prolonged contact with teat ends (Hogan *et al*., 1989).Many intra-mammary infections arecaused by the environmental pathogens that originate in the dry period (Eberhart, 1986; Smith*et al*., 1985) and the prevalenceare generally higher during the dry period than during the lactation.

2.6.1 *Escherichia coli*

Escherichiacoli(*E. coli*)was first described in 1885by TheodorEscherich.Escherich, a Bavarian paediatrician performed studies on the intestinal flora of infants and has discovered a normal microbial inhabitant in healthy individuals, which he named *bacterium coli commune*. In 1919, the *bacterium* was renamed *E.coli* (Kaper, 2005) in his honour.

E. coli is a member of the Enterobacteriaceae family.It is gram-negative cylindrical rod, non- sporeforming. They are relatively small 2 to 3µ by 0.4 to 0.6µ. They are aerobic or facultativeanaerobic and are able to ferment lactose. They are catalase positive and oxidase negative. The guanine plus cytosine content (G+C) of DNA is 39 – 59 mole percent (Balows *et al*.,1992). On agar plate its coloniesappear grey to brown and range in size from 3 to 5mm in diameter; circular, convex, entireglisteningand mucoid. Less than15% of *E. coli* are haemolytic. On selective and differential media such as MacConkey agar, *E .coli*appears as pink colonies surrounded by a pink zone of precipitated bile salts (Harmon *et al*., 1990). Bacterial lipopolysaccharide (LPS), also termed endotoxin is considered to be the primary virulence factor of*E.coli*and isresponsible formost pathophysiological reactions in mastitis (Burvenich *et al*., 2003; Hogan and Smith, 2003).

*E. coli*is partof the normal intestinal flora of humans and animals. It is the most common facultative anaerobic bacterial species in the gut and is constantly excreted in the faecessin to the environment. Pathogenic *E. coli* can cause intestinal and extra intestinal infections in mammalian and avian hosts (Cullor, 1996).

E. coli is an opportunistic *bacterium* causing mastitis (Nemeth *et al.,* 1994). Bovine mastitis resembles urinary tract infection and in both, is ascending and caused by bacteria from the environment of cow. The source of mastitis-causing *E. coli* strains may be found in the intestinal flora of the affected cow (Linton and Robinson, 1984), other cows or calves. Teat-endhyperkeratosisand a dirty udder significantly increase the risk of clinical *E. coli* mastitis (Breen *et al*., 2009).Clinical mastitis in *E.coli* can range from mild with only local sign to severe disease with systemic sign. In severe cases the outcome can be acute tissue damage and complete loss of milk production or even the death of the diseased cow (Golodetz and white, 1983; Shpigel*et al*., 1997).The major groups of virulence factors of *E. coli* comprise adhesions, which help the bacteria to adhere to and colonize mucosal surfaces, and toxins, which are proteins with the ability to disturb or modify the normal function of the host cell and to help the bacteria to cross the epithelial barrier and to invade the tissue (China and Goffaux, 1999; Kaper *et al*., 2004). Other factors, like siderophores, which help bacteria to chelate iron, and factors that confer serum resistance, also contribute tothe bacterial virulence (Hirsh *et al*., 1993, Hogan and Smith, 2003).

2.6.2 *Streptococcus uberis*

Streptococcus uberis is a versatile environmental pathogen responsible for high proportion of clinical, but mostly subclinicalmastitis in lactating cows, heifers, multi-parious cow and is the predominant organism isolated from mammary glands during the non-lactatingperiod (Bramley, 1984; Bramley and Dodd, 1984; Oliver, 1988).It can cause persistencecolonization without an elevation in somatic cellcount. The most important reservoirs appear to be the skin, udder surface teat and it hasability to survive in severaldiverse environments (Sweeney, 1964; Schalm*et al*., 1971; King, 1981).Virulence factors associated with the *Streptococcus uberis*arehyaluronic acid, capsule hyaluronidase, neuraminidase, streptokinase, lactoferrin – binding proteins, amino acidand opine binding protein. (Fang and pyorala, 1996). The organism has been isolated frommanysites on cows, including the tonsil, genital tract, rumen, rectum, coat (Kruze and Bramley, 1982).

2.6.3 *Streptococcus dysgalactiae*

Streptococcus dysgalactiae belongs to Lancefield serological group C (Garvie*et al.,* 1983) environmental pathogen. *Streptococcus dysgalactiae*had been isolated from infectedmammary glands and teat injuries (Schalm *et al.,* 1971) and was transmitted primarily during the milking process, between milking, during the dry period and prior to parturition in the first lactation heifer (Bramley andDodd., 1984). Isolation fromextra-mammary reservoirs such as cattle tonsils, mouth and vaginahave been reported (Schalm *et al*., 1971; Cruz Colque*et al.,* 1993). The occurrence of *S dysgalactiae*inIMI during the non- lactating period in herds with no previous history of infection, suggested that the organism also behaved as an environmental pathogen.(Bramley and Dodd, 1984)

In spite of its highprevalence, little is known about factors that contribute to its virulence.In past decade, severalcell-associated and extracellular factors of*Streptococcusdysgalactiae*havebeen identified; yet, the relative importance of these factors in the transmission and pathogenesis of mastitis cause by theorganism is not understood (Calvino *et al.,* 1998). *Streptococcus dysgalactiae*can interact with severalplasma and extracellular host-derived proteins such as immunoglobulin G, albumin, fibronectin, fibrinogen, collagen, vitronectin, plasminogen, and α_2-macroglobulin(Calvino and Oliver, 1998). These interactions are mediated by bacterial surface proteins.*Streptococcus dysgalactiae* also producehyaluronidase and fibrinolysin, which may be involved in promoting dissemination of the organism in the host tissue.

2.7Pathogenesis of Mastitis

The primary cause of mastitis is a wide spectrum of bacterial strains but also incidences of viral, algal and fungal-related mastitis were reported (Pyorala, 2003).The teat canals are normally tightly close by sphincter muscles, thus preventing the entry of pathogens. It is lined with keratin, which is a waxy material derived from stratified squamous epithelium that obstructs the migration of bacteria and also contains antimicrobial agents that help in combating the infection. However, the efficiency of this keratin is limited (Craven and Williams, 1985). This is because fluid accumulation within the mammary gland as parturition approaches, results in increased intramammary pressure (Paulrud, 2005) and the mammary gland becomes vulnerable to infection caused by the dilation of the teat canal and leakage of mammary secretions (Sordillo and Streicher ,2002). Additionally,

during milking, the keratin is flushed out and there is distension of the canal (Rainard and Riollet, 2006).

The bacteria, once gain access elude the cellular and humoral defence mechanisms of the udder (Sordillo and Streicher, 2002). If they are not eliminated, they start multiplying in the mammary gland. They liberate toxins and induce leukocytes and epithelial cells to release chemo attractants including cytokines such as tumor necrosis factor –α (TNF-α), interleukin (IL)-8, IL-1, oxygen radicals and acute phase proteins (APPs) (e.g haptoglobin, serum amyloid) which further attract circulating immune effector cell, mainly polymorphonuclear neutrophils (PMNs) to the sites of infection (Giri *et al*., 1984; Paape *etal.,* 2003; Zhao and Lacasse, 2008). PMNs act by engulfing and destroying the invading bacteria via oxygen-dependent and oxygen-independent systems. Theycontain intracellular granules that store bactericidal peptides, proteins, enzymes (such as myeloperoxidase) (Bank and Ansorge, 2001). The released oxidants destroy the bacteria and some of the mammary gland epithelia cells, resulting in decreased milk production and release of enzymes, such as N-acetyl-b-D- glucosaminidase (NAGase) and lactate Dehydrogenase (LDH) The dead and sloughed off mammary epithelia cells also in addition to the dead leukocytes, are secreted in to the milk, resulting in high milk,somatic cell count (SCC) (Kelly *et al*, (2006) and as the infection persists, internal swelling within the mammary epithelium can occur. The mammary gland alveoli become damaged and start losing anatomical integrity. The blood-milk barrier is breached, causing extracellular fluid components, such as, chloride, sodium, hydrogen, potassium and hydroxide ions, to enter the gland and mix with the milk (Zhao and Lacasse, 2008).

2.7.1 Pathology

The pathological changes of the mammary gland during infection have been investigated by microscopis examination of section of mammary gland and different histological lesions have been reported (Gudding *et al.,* 1984). There is ulceration and erosion of lactiferous sinus and ductular epitheli. There is infiltltration of the parenchyma conjunctive tissue by macrophages and PMNs which cross the mmammary epithelium in to lactiferous sinus and glandular alveoli. These lesions of the alveolar secretory epithelial cells are of variable severity, from a simple decrease of secretory activity to a total lysis and also shrinkage of alveoli, proliferation of conjunctive tissue and accumulation of cellular fragments in the glandular alveoli (Gudding *et al.,* 1984).

2.7.2 Compositional changes in milk during mastitis

Compositional changes in milk during mastitis reflect inflammatory changes in the udder. The status of the udder can be monitored by the indicators of inflammation. The first detectable change is the appearance of plasma protein in milk as a consequence of increased permeability between the blood and milk compartments. Neutrophil Leukocytes migrate in to the milk due to various chemotactic stimuli. Electrolytes, such as sodium and chloride, also leak in to milk and the pH of milk approaches that of blood.The synthesizing capacity of the gland is thus suppressed. Pathological effects on the udder tissue vary with the type of the invading organism (Schalm, 1977). .

2.7.3Clinical signs of mastitis in cow

Clinical signs of mastitis include abnormalities of milk such as discoloration, blood clots, flakes, abnormalities of the size, consistency of mammary gland and a systemic reaction. Clinical forms of mastitis are classified on basis of severity of inflammation and the degree of severity is dependent on the nature of causative pathogen, stage of lactation and age of the animal. Inflammatory response to mastitic infections also lead to welfare issues within the herd with inflammations of udder causing undue pain and discomfort for animal, whilst severe cases can lead to poisoning and premature death (Blowey,1999). Forms of clinical cases of mastitis are subclinical mastitis, clinical mastitis and chronic mastitis.Clinical cases of mastitis are characterized by one or more of the following: clots and/or blood in milk, udder swelling and systemic signs including an elevated temperature, lethargy and anorexia (Erskine, 2001). Subclinical cases show no visible changes in the appearance of milk or the udder but milk production decreases.The milk composition is altered and is characterized by high somatic cell count (Erskine, 2001). Chronic mastitis cases are a rare form of the disease but result in persistent inflammation with little change in milk and often characterized by indurations of glandular tissue. .

2.8Diagnosis of Mastitis.

Diagnosis of mastitis depend largely on the detection of clinical signs, abnormalities of milk and other mammary gland abnormalities and indirect test such as California mastitis test(CMT), NAGase(N-acetyl-β-D-glucosaminidase). Direct test cell count are valuable stool for screening for subclinical mastitis .However, it can only detect the presence of inflammatory changes without a visible abnormalities. Bacteriological examination is the most reliable method of diagnosis for determining particular causative mastitis pathogen

in order to have effective treatment. Culture and isolation of organism using specific identification medium(Quinn *et al.*,1994), using colony morphology, gram staining, variety of conventional biochemical test such as (catalase, coagulase, sugar fermentation, DNase, Voges-Proskauer test (Acetoin production), nitrate test, esculin test, CAMP test, Hippurate hydrolysis test, motility, TSI, citrate test, urease test etc.) are used for confirmatory identification, to gives phenotypic characteristics of bacteria. Others test such as microbact 12S, microbact 24E, API Staphidentification systems, Lancefield Antigen immunoassays (Lancefield antigen grouping kit) can be used for futher confirmation. They are reliable and rapid in identification.

2.8.1 Newer laboratory identifications method in the diagnosis of mastitis pathogen

These involve bacteriological culture and isolation of bacteria with specific media. Using colony morphology and various conventional biochemical reactions to identify specific bacteria species. Identifiedbacteria species can further be confirmed with commercial biochemical or nucleic Acid Test systems. The commercial kit and automated systems can identify a number of bacterial species with accuracy of 70% to > 90% with relative speed and simplicity (Kool and Bannerman, 1994; Pfaller and Herwaldt, 1988). Examples of commercial kit are Microbact 12S, API STAPH, VITEK (bioMerieux), Microbact 24E, Strep and the MIDI Sherlock identification systems, microbial identification systems that automates microbial identification by combining cellular fatty acid analysis with computerised high-resolution gas chromatography(MIDI, Newark, Del.). The advent of molecular methods, based on molecular genetics such as Polymerase Chain Reaction (PCR) technique has enhanced disease diagnostic capacities to a point that identification

of causative agents is less obscured(Deplano *et al*.,2000).Various molecular techniques can give specific information on molecular characterization of particular organism. Example is nucleic acid amplification by using polymerase chain reaction which is a method where specific segment of either DNA or RNA are amplified into a thousand copies and are analyzed. The two main options for the target are Detection of bacteria harboring a given gene (a gene coding for a given function) anddetection of taxonomic entity.Through usingsuch molecular technique to detect molecular characterization of an organism, the following are known:-

- Genomic – genome sequencing (identification of organism, the virulence marker of an organism.
- Gene characterization

  Hybridization: Detection of DNA fragment

  Per cent of Guanine and cytosine
- Whole genome sequence. This is the most specific and informative method of identification and typing. The ability to sequence the entire genomic of pathogen has resulted in the new comparative of genomic and such approach is applicable to detect genes, virulence factor and detect if specific isolated strain contain plasmid. Examples of molecular technics in use includes the followings; Pulse-field Gel Electrophoresis (PFGE), Spa typing,Multilocus Sequence Typing (MLST), Plasmid profiling, Rep-PCR and DNA microarray(Tenover *etal.*, 1994; Shopsin *et al*., 2000.)

2.9 Antibiotic Resistance

The discovery of antimicrobial agents has been a critical element of the therapeutic armamentariumof modern medicine, however, the emergence of antibiotic resistant microorganisms had beenwidely reported(Saxena *et al*., 2003) andwere no longer restricted to the hospital environment (nosocomial) as it was in the past. In past decade, antibiotic resistant bacteria have become a major concern in different parts of the world. In Zaria, Nigeria, multiple- antibiotic resistance had been reported(Olayinka *et al*., 2004; Suleiman, 2011;Umaru *et al*., 2012.).

Antimicrobial efficacy requires that the antimicrobial interferes with an essential bacterium at a molecular level. Some organisms are resistant to certain antimicrobials because of their innate metabolic characteristics, Mycoplasma,for instance,do not have cell wall, thus, cell wall- active antimicrobials do not affect them (Courvalin,1994). This is otherwise called intrinsic resistance, while other organisms may be susceptible to antimicrobials by nature but, however may develop mechanisms to protect themselves. This type of resistance is called an acquired resistance mechanism that is always apparent (Alekshun and Levy, 2007). Acquired mechanisms involve mutation of genes targeted by the antibiotic and the transfer of resistance determinant borne on plasmids, bacteriophages transposon, and other mobile genetic materials (Alekshun and Levy, 2000).This exchange is accomplished through the process of transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons) andtransformation (incorporation in to chromosome of chromosomal DNA,plasmid, and other DNAs from dying organisms.(Levy and Marshall, 2004).Mechanismsconferring antimicrobial resistance include membrane permeabilityalteration, active antimicrobial extrusions efflux,

antimicrobial target alteration, metabolic bypass and enzymatic modification of antimicrobial agent (Livermore, 2003).Membrane permeability alteration may prohibit influx of certain antimicrobialse.g. Aminoglycosides.Where target alterations result in lowered affinity for antimicrobial e.g.β-lactamase and macrolides. Enzymatic modification of antimicrobials may inhibit membrane permeability or ability to alter with the target of action e.g. β-lactamase and chloramphenicol (Forbes *et al*., 1998).It has been suggested that antibiotic therapy is a selective force in the development of antibiotic resistance by pathogenic bacteria which might be due to the practice of dosing animal with sub-therapeutic concentrations of the antibiotics (Amabile-Cuevas *et al*., 1995; Costa *et al*., 2000).

Staphylococcalresistance to Penicillin 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 penicillin such as Methicillin, Oxacillin, Cloxacillin and flucloxacillin are able to resist degradation by staphylococcal penicillinase. (Brumfitt and Hamilton-miller, 1989) The mechanism of resistance to methicillin is mediated via the mec operon part of the staphylococcal cassette chromosome *mec* (SCC*mec)*. Resistance is conferred by the *mec*A gene, which codes for an altered Penicillin-binding protein (PBP2aor PBP2) that has a lower affinity for binding β-lactams (Penicillin, Cephalosporin and Carbapenems).This allows for resistance to all the β-lactam antibiotics and obviates their clinical uses (Forbes *et al*., 1998; Fuda *et al.*, 2004). Aminoglycosides such as kanamycin, Gentamicin etc, were once effective against staphylococcal infections until the organism evolved a new mechanism to alter their actions. This occurs via protonated

amine and /or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosome (Carter *et al*., 2000)

***Escherichia coli*resistance to Tetracycline**

Antibiotics that were effective at treating and controlling infections caused by *E coli* are no longer effective as the *bacterium* has acquired resistance to these agents (e.g. Tetracycline). Resistance to tetracycline is as a results of the uptake of new genes (Chopra and Roberts, 2001).There are 23 efflux genes (which code for energy dependent efflux of tetracycline), 11 ribosomal protection genes which code for proteins that protect bacteria ribosomes), 3 genes that code for enzymes that modify and inactivate tetracycline and 1 gene that has an unknown mechanism (Schwarz et *al*., 2006).

Currently, only the first two mechanisms are important in bacteria of veterinary importance. The efflux resistance genes,*tet*A, *tet*B, *tet*C, *tet*D and *tet*H, are the most widelyspread in gram negative bacteria and are located on the transposons (Lawley *et al*., 2000) and plasmids (Schwarz *et al*.,2006).The *tet*B gene confers resistance to both tetracycline and minocycline but not to the new glycyclines.

2.9.1 Treatment of Mastitis

Treatment of mastitis using antimicrobial therapy remains important tool in controlling mastitis during lactation and dry period. Antimicrobial susceptibility testing can guide in selecting most appropriate antimicrobial agent (Owen*et al*., 1997). However, the efficacy

of therapeutic, preventive measures in the treatment of bovine mastitis with antimicrobial drugs depends on the nature of the causative etiological agent, clinical manifestation or phase of the mastitis, antibiotics susceptibility of etiological agent, efficiency of immunological system, proper use of drug under consideration and sanitation procedure are paramount in the treatment of mastitis (Owen*et al*., 1997). The decision to treat a particular case systemically by parental injection or locally by intramammmary infusion depends on the forms of mastitis involves and this is crucial. The selections of antimicrobial agent for treatment of mastitis depend on the pharmacokinetic of the drugs used, knowledge about the diffusion in to mammary tissue, the degree of binding of a drug in to mammary tissue and secretions (Saran and Leitner, 2000), their ability to pass through the lipid phase of milk and the degree of ionization. The interactions between the antibiotics and immunological factors could leads either to augment the antibacteria effect on the target tissue or diminishing it. Thus appropriate elimination of bacteria requires both the effectiveness of an antimicrobial drug against bacteria and a well-functioningdefence system of the animal (Saran and Leitner, 2000).

2.10 Economic Losses from Mastitis.

Mastitis assumes a major economic importance in dairy cow and is the most costly disease in dairy herds. It decreased productivity as it causes temporal or permanent loss in milk production and increases cost of production. It causes poor milk quality, for example reduction in milk fat content, resulting in dairy product with less organoleptic properties. It also result to losses due to discarding of milk after antibiotics treatment, additional

treatment costs examples, drug and veterinary services and also cause premature culling of potentially profitable cows or reduced productive-life of cow (Gruet *et al*., 2001).

2.11 Effect of Mastitis on Human

There is a risk- danger with cases of mastitis; this is due to fact that bacteria contamination of milk from affected cows renders it unfit for human consumption and provides mechanism of spread of diseases like tuberculosis, sore throat, Q-fever, brucellosis etc. Consumption of these contaminated milk and its products are of public health significance. Unpasteurised mastitic milk can be source of foodborne-pathogen (Umoh *et al*., 1990b; Loir *et al*, 2003; Oliver *et al*., 2005). It can cause sporadic disease outbreak and also be a vehicle for many Zoonotic diseases to further emphasize on the inherent danger associated with such products, because some organism do have the capability to produce enterotoxins and a toxic shock syndrome toxin (TSST-1) and verocytoxigenic; example of such food borne intoxication are those of*Staphylococcus aureus*and*E. coli* origin(Joffe and Barrnovics, 2006).

Staphylococcus aureus is a common cause of foodborne illness. Some strains of *S. aureus* cause staphylococcal food poisoning through production of heat-labile staphylococcal toxins. All individuals are believed to be susceptible to this type of bacterial intoxication. However, the intensity of the symptoms varies and is related to the amount of contaminated food ingested and susceptibility of the individual to the toxin. (ICMSF, 1998; Loir *et al*., 2003) Staphylococcal food poisoning symptoms ranges from 30 minutes to seven hours (the mean is two to four hours) after consumption of foods containing preformed toxins. Nausea, vomiting, abdominal cramps, prostration and diarrhoea are

common symptoms. (ICMSF, 1998) Typical foods associated with *S. aureus* outbreaks contaminated milk and it products that are mishandled during processing or preparation or not properlypasteurised. Time and temperature control to prevent the growth of the organisms is the primary control measure of *S. aureus*. Personal hygiene is also important to help prevent product contamination from food handlers. It is important to note that heating after toxin is formed is not an effective control method (ICMSF, 1998).

2 .12 Management Factors

Management factors such as housing facilities, hygienic condition of herds, milking procedures, milk management, feeding programmes, treatment regimes, sanitation and others that influence udder health are important in the control of mastitis. (Jones *et al*., 1998; Halasa *et al*., 2007). These factors, when not strictly enforced become risk factors that predispose infections within the glands. The importance of these factors in determining the prevalence of mastitis was reported by Kivaria *et al. (*2004). Predisposing factors includes poor milking hygiene, faulty milking machine, poor milking management, constitutional factors, teat injuries, teat sore, and population of pathogens on the cow's skin, epithelia and in its environments. The most effective ways to prevention of mastitis is to adhere strictly to means of eliminating conditions or factors associated with occurrence of mastitis and is best achieved by good hygiene procedures; maintaining a clean, dry environment for lactating, non-lactating and breeder heifers.(Kivaria *et al.*, 2004). Premilking and postmilking teat disinfection, milker should always wear gloves,

dirt should be brushed off teat with use of a dry, single-use towel and water should not be used as it can introduce pathogen that are very difficult to cure. A commercially available predip should be applied with dipper; teat end should be examined for evidence of chapping, cracks or lesions, which may harbour mastitis-causing bacteria. Milking infected cow last, proper sanitation of the farms and properly functioning of milking equipment is essential in preventing infections.House should be well structured to allow for good ventilation and ease of cleaning .The bedding materials used should bean extremely important management priority.

2.13 Mastitis Control and Prevention

The five- point plan for mastitis control has been the gold standard for control strategies for many years (Giesecke, 1998) and has been successful in reducing the incidence of mastitis. The strategy addresses areas where the risk of infection is greatest and promotes the use of treatment at specific times. The five points listed by Giesecke (1998) include:

1. Milking hygiene especially disinfection and dryingteat before putting on milking machine cups and dipping all teat after milking
2. Proper maintance and hygiene of the milking parlour, housing, milking procedures and adequate milking equipment
3. Culling of chronically mastitic cows
4. Antibiotic dry-cow therapy
5. Prompt treatment of clinical mastitis during dry period and lactation.

The National Mastitis Council (NMC), a non-profit organization based in the USA recommended a similar strategy but strongly suggested that other management practices

also form part of the control programme. These include good record keeping of clinical mastitis cases and treatment times, outcome of treatments and various other records of somatic cell counts (SCC) to monitor the incidence of subclinical mastitis. Planning and regular reviewing of the strategy is also recommended and adequate communication between the farmer, veterinarian and staff is necessary so that the strategies can he implemented practically (NMC, 2007)

2.14 Situation of Bovine Mastitis in Nigeria

Information onprevious studies on bovine mastitis are scanty and far between, however, in Ibadan, Adetosoye*et al*. (1986) conducted a bacteriological survey of milk samples from cows with mastitis and the antimicrobial susceptibility of *S. aureus* isolated from the study. Umoh, *et al*. (1990a)in Zaria reported a prevalence of 3.2% *S. aureus* in traditional herds and 30% in settled herds of cows with mastitis. In Maiduguri, a survey by Ameh *et al*. (1999) revealed a prevalence of 34.6% *Staphylococcus aureus*, 6.7% *E.coli* from lactating and dry cows. Ajuwape *et al*. (2004) reported a 50% incidence rate of *Staphylococcus epidermidis* associated to caprine mastitis in Ibadan while investigating the bacteriology and haematology of clinical mastitis in goats. Amosun *et al.* (2010) in Ibadan reported a 55.4% incidence rate of *Streptococcus uberi*s and 12.3% of *Streptococcus dysgalactiae*.In Zaria, Umaru*et al*. (2012) reported a prevalence of 12.6%for *Staphylococcus aureus*from fresh and fermented milk in Zaria and Kaduna.While in another study bySuleiman, (2011) a prevalence rate of 30.9% for *Staphylococcus aureus* from mastitic cows was reported in Jos.

2.15Methods used in Diagnosis of mastitis in Nigeria

The methods used in diagnosis of mastitis in Nigeria, involve the isolation and conventional biochemical test which is can be further confirmed by microbact or commercial kit. The advent of molecular methods, based on molecular genetics such as polymerase chain reaction (PCR) technique has enhanced disease diagnostic capacities to a point that identification of causative agents is less obscured, however, its use is still limited probably due to scarcity of laboratory equipment facilities in the country and is still expensive to run.

CHAPTER THREE

MATERIALS AND METHODS

Materials

This includes, measuring cylinder, conical flask, Petri dish, test tubes, bijour, bottles, autoclave, incubator, oven, fridge, microscope, centrifuge, CMT paddle, towel, cotton, pipette, glass slide, thumb forceps, pipettes, water bath, ice-pack, cotton. Others materials are antibiotic sensitivity discs (Oxoid, UK), distill water, Normal saline, Needle and syringe, Indicator brommothymol blue and hydrogen peroxide

Media

The media used in this study include; Blood Agar(Oxoid,UK) MacConker Agar (Oxoid,UK) Eosin Methylene Blue agar (Oxoid, UK) Dnase Agar(Oxoid,UK), Mannitol Salt Agar (Oxoid,UK), Brain Heart Agar (Merck, Germany), Nitrate Agar (Oxoid,UK) Baird Pairker Agar (Oxoid,UK) Sugars (Mannitol, Lactose, Sucrose, Maltose, Xylose, Arabinose, Raffinose, Salicin Sorbitol) Nutrient Agar (Oxoid,UK), CMT (California

Mastitis Test) reagent (Oxid,UK), TSI Agar (Oxoid,UK), Citrate Agar (Oxoid,UK), SIM Media (Oxoid,UK), Muller Hinton Agar. (Oxoid, UK), Microbact – Microbact 12s identification system (Oxoid, UK), and Streptococcal grouping kit (Oxoid, UK).

3.1 Study Area

The study was conducted in three Local Government Areas in Zaria, Kaduna State, within latitudes $11^{0}7^{'}$ to 11^{0} $12^{'}$N and longitude $07^{0}41^{'}$ E. The Local Government Areas were Zaria, Sabon Gari and Giwa (Ministry of Economic Development, 1996). Zaria, which is located in the North Guinea Savannah Zone of Nigeria, has an annual ambient temperature, ranging between 18^{o}C and 31.8 ^{0}C. The harmattan season (the cold-dry period of the year) in Zaria, lasts from November to February, while the hot-dry season lasts from March to May and the rainy season from June to October (Ayo *et al*., 2011). The 3 local Government Areas were selected because they have high populations of cattle in Zaria. Also, villages and herds in these local Government Areas supply a major peri-urban dairy to National Animal Production and Research Institute (NAPRI) milk processing plant and other individual bodies that use milk in the production of yogurt and other milk products.

3.2 Cattle Herds.

In each Local Government, simple random sampling technique was used to select one village out of 5 villages. The study animals were lactating White Fulani cows, herded during the day time and in traditional (left on open field without housing facilities) enclosures overnight. All the herds practice hand milking and feeding was mainly by

grazing on field and no other source of supplement was provided. The calves were separated from their dams during the night. In all the herds, milking was usually carried outvery early in the morning once in a day.

3.3 Sample Plan/procedure and Sample Size.

Sampling was conducted after simple random sampling, using balloting, to select villages to be covered in each Local Govrnment Areas. The villages identified were considered as cluster and in each cluster, three (3) herds of cattle were selected by simple random type of sampling by balloting, where at least 10-20 lactating quarters, were sampled per herd. However, where there were more than 20 lactating cows in a herd, then simple random sampling was used again to identify cows to be sample. The villages sampled in each local Government Areas were Gambari, Hayin liman, Nasawa / Sabofege. A sample size of 322 quarter milk samples based on the prevalence rate of 30% reported by Umoh *et al.* (1990a) was used. However, the sample size was increased to 472 to increase precision and the sample size was determined by this equation below as described by Thrushfield (2007)

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

Where n=sample size

Z=standard normal deviation (1.96)

P=prevalence of 30%,

Q= 1-p

d= the desir absolute degree of precision level 5% or 0.05

$$n = \frac{1.96^2 \times 0.30(1 - 0.30)}{0.05^2}$$

= 322

3.4. Clinical Sign

Clinical mastitis was diagnosed at the quarter level, based on visible and palpable signs of inflammation (warm and swollen quarter, and pain upon palpation, discolouration of milk, watery milk, blood clots in milk and flakes and elevated body temperature).

3.5 Farm lnspection and Questionnaire

Management practices studied in herds were based on risk indicators associated with exposure to pathogens (housing facilities, hygiene condition of herds, milking procedures and treament) Halasa *et al.*(2007) and hygienic practices of milking personnel. Also, to find similarity between source of mastitis-causing organisms and occurrence of mastitis, thus swab sample from the teat skin, udders and hands of milking personnel were collected. Structured questionnaires (pre-tested) were administered. Information regarding cow, which evaluate the effect of potential risk factors on the prevalence/occurrence of

mastitis were assessed from the Questionnaires. Risk factors considered were stage of lactation (early, middle and last stage of lactation), quarter location rear and front, and presence /absence of tick or lesion on the udder skin or teat and parity number. Information generated from the administration of questionnaires were analysed

3.6 Milk Sample Collection

In this study, 472 quarter milk samples were collected and examined. The lactating cows were subjected to physical and clinical examination for the diagnosis of mastitis. Clinical mastitis was diagnosed on basis of visible signs of inflammation; swelling, heat, and had pain on upon palpation of the udder and or hard and fibrotic udder. Also visible changes in milk, like watery milk, milk discoloration, blood clots and flakes were noted. While cows with subclinical mastitis which shows no visible changes in the appearance of milk and /or the udder, however manifest as an elevation in the somatic cell count (SCC) and was detected by indirect test, California mastitis test (CMT). Farm observations which include milkers and farm hygiene (sanitary condition, milking procedure/ udder-hyigene practices including washing of hand before milking, use of separate towel for each lactating cow and drying of udder after washing) was observed and judged either as good or bad.

3.7 Samples from Extra-Mammary Site

Prior to milk collection, samples from extra-mammary sites; teat, udder and hands of the milking personnel were swabbed with sterile swab stick and placed inside a container filled with 5 ml of Tryptone soya broth for microbial analysis.

3.8 California Mastitis Test (CMT)

The udder, teats and adjacent flank areas were thoroughly washed and dried with single service sanitary paper towel. The teats were disinfected with 70% alcohol before sampling. Milk sample from each apparently normal quarter was tested for sub-clinical mastitis using the California mastitis test (Oxoid UK) as described by Quinn*et al. (*1994), while clinical mastitis was detected using clinical signs (blood clots and flakes in milk). The udder was massaged thoroughly, the first three milking streams, from each quarter were discarded. Approximately about 3 mls of milk was collected from each quarter into CMT paddle, labelled A-D (for Rear- left and right, Front- right and left, respectively). Equal amounts of CMT reagent (Alkyl aryl sulphonate) were added to each of the four compartments of the CMT paddle. The paddle was then rotated horizontally to allow proper mixing of milk with the reagent and the mixture was observed for evidences of colour change, viscocity and thickness of the gel formed. The test results were interpreted based on the thickness of the gel formed and scored as negative (O), trace, (T) + (weak positive), ++ (distinct positive), +++ (strong positive). In this study, CMT scores of 0 were excluded, because they were assessed as having originated from cows free of subclinical mastitis, While CMT result of ≥+ 1 were classified as having evidence of subclinical mastitis (Quinn *et al.,* 1994; Assefa *et al.,* 2006). Quarters with CMT scores of ≥ 1 or above were judged as positive. Cows were considered positive for subclinical mastitis when at least one quarter turned out to be positive for CMT.

About 10 ml of milk sample were collected in sterile universal bottle directly from each quarterthat was positive for mastitis. Milk samples were transported in an ice box to the department of Veterinary diagnostic laboratory, Faculty of Veterinary Medicine Ahamdu

Bello University, Zaria for bacteriological analysis. Milk sampled was also subjected to another test to further confirm the accurate number of cells (somatic cell count) /ml of milk and was determined using haemocytometer according to method described by (Barbara, 1980). One hundred and seventy (170), one hundred and twelve (112) and one hundred and ninety (190) samples were examined from the three (3) local Govrnment Areas respectively.

3.9 Bacteria Culture

Bacterial organisms were isolated from milk samples in lactating cattle according to the protocols of the National Mastitis Council (Harmon *et al*., 1990; Hogan *et al.,* 1999).A loop-full of each milk sample collected was cultured on MacConkey agar and on 10% blood agar and incubated at 37^{o}C for 24 hours. Similarly, 27 swab samples from extra mammary sites like udder, teat and hands of the milking personnel (unwashed prior to sampling) were swabbed and cultured on blood and macConkey agars.

3.9.1 Bacteria isolation and identification

Bacterial growths on each medium were subjected to bacteriological identification according to method described by Barrow and Feitham (1993). Colonies on each cultured medium were examined noted for size, shape, colour, consistency and presence or absence of haemolysis on blood agar. Each isolate was Gram stained. Bacteria that tested gram positive were subjected to conventional biochemical tests such as catalase,oxidase,

coagulase, DNase, mannitol salt agar(pigment production), nitrate, Voges- Proskaurer (Acetion production), sugar fermentation, esculin hydrolysis and carbohydrate utilization tests for confirmatory identification based on standard procedure described by Cowan (1974). Those that were Gram negative were further sub-cultured on EMB (Eosin Methylene Blue) and those that produced typical greenish metallic sheen were identified as *Escherichia coli*

3.9.2Catalase test.

Isolates suspected to be Gram positive were tested for their ability to produce catalase enzyme. A drop of 3% hydrogen peroxide was put on a clean slide and a colony of suspected isolate was picked with sterile wire loop and suspended in the hydrogen peroxide. A positive test was indicated by formation of bubbles indicating the production of oxygen and water.(Cruickshank*et al.*, 1975)

3.10 Conventional BiochemicalTest

All agar-based media were prepared according to the manufacturer's instructions. Colonies that showed typical greenish metallic sheen were picked and streaked on Simmon citrate agar, urea agar and butt of triple sugar iron werestabbed and the slants were streaked. The isolates were also inoculated into5 ml of indole and M.R-V.P broth and were incubated at 37^{o}C for 24hours. All 16 isolates identified as*Escherichia coli* gave

acid over acid reaction in TSI agar with gas production, citrate negative, and urea negative and were motile with distinct brush like line along line of stabbing.

To theindole broth, 2 mlof kovac's reagent was added and then shaken gently. Apositive reaction showed a red ring colour in the surface of layer within 10 minutes.About 1 ml of MR-VPbroth was transferred to a small serological tube then, 2 drops of Methyl red was added. A red coloration signified a positive methyl red test. To the rest of the broth in the original tube, 5 drops of 40% potassium hydroxide were added followed by 5 drops of 5% alpha-Naphthol and shaken, therewas no change in the colour of the broth, indicating a negative reaction.Isolates that were Gram positive but catalase negative, suspected as Streptococci, were further subjected to Esculin hydrolysis and Carbohydrate utilization tests.

3.10.1Esculinhydrolysis for *Streptococcus*

The esculin agar (Oxoid UK) was prepared, according to the manufacturers' instructions, and inoculated with suspected organism and incubated at 37^{o}C for 24hours. It was then examined; a blacking in and around the bacterial growth were indicative of positivereaction (Cruikshank *et al*., 1975; Barrow and Feitham, 1993).

3.10.2Carbohydrate utilization for *Streptococcus*

Carbohydrate utilization test (Cruickshank*et al.*, 1975) was conducted in peptone water as containing lactose, maltose, mannitol, raffinose, salicin, sorbitol and sucrose. All carbohydrates were prepared according to the manufacturers' instructions and were

inoculated with each isolate to be tested and incubated at 37^{o}C for 24 hours. Positive tests were indicated by a change from blue colour to yellow using bromothymol blue as indicator. Isolates that were Gram positive and catalase positive were subjected to following tests:

3.10.3 Coagulase test.

Suspected isolates were tested for coagulase activity using rabbit plasma by tube method. To prepare, 2ml of rabbit blood was aseptically collected in to 0.3ml 10%EDTA. It was dispensed into sterile tube and centrifuge at 1000RPM for 5minutes.The plasma was then collected. The test was carried out by addition of 0.1ml of 24-hour tryptone soya broth culture of the test isolate to 0.5ml of 10^{-1} dilution of plasma and then incubated at 37^{o}C. The tubes were examined after 1, 2, 3 and 6 hours for coagulation. Those that were negative after 6 hours were left at room temperature overnight and re-examined. Positive tests were indicated by definite clot formation. (Baer *et al*., 1976; Cheesbrough, 2002)

3.10.4 Plasma slide agglutination test

A colony of each isolate was emulsified in a drop of saline on a microscope glass slide until a thick suspension was produced. The bacterial suspension was stirred with a straight

wire loop that has been dipped into rabbit plasma. A positive result was indicated by macroscopic clumping, within 5 seconds (Cowan, 1974).

3.10.5 DNase (deoxyribonuclease) activity

All the suspected*Staphylococcus* isolates werestreaked on the surface DNase test agar (Oxoid) and incubated at 37^{o}C for 24 hours. After incubation, the plates were flooded with IN HCL. A positive result was indicated by a clear zone or halo around the growth (Cowan, 1974;Cruickshank*et al.*, 1975)

3.10.6 Voges – proskauer (acetion production)

Two to three fresh colonies were inoculated into glucose phosphate medium(Oxiod) with a loop and vortexed, and then incubated at 37^{o}C for 24hours. To the broth suspension 4 drops of 5%α-naphthol solution and 2drops of 40% potassium hydroxide solution were added and thoroughly shaken, then examined after 15minutes, 30minutes and1 hour. A positive reaction was indicated by a strong red colour (Cowan, 1974).

3.10.7Nitrate reduction test

Each isolate was inoculated into nitrate broth (Oxoid) and incubated at 37^{o}C for 24 hours; tothe broth,3 drops of nitrate I and nitrate II were added. A colour change from straw to red indicated that nitrate was present (positive) and has been reduced. (Cowan, 1974).

3.10.8Pigmentation test on mannitol salt agar (MSA)

Suspected *Staphylococcus* isolates were tested for pigment formation on MSA. The media were prepared according to the manufacturer's instructions. Each isolate was streaked on the MSA (Oxoid) and incubated at 37^{o}C for 24hours. In *S.aureus,* fermentation of the mannitol was characterized by formation of yellowish colonies on the media and for other species it showed pinkish or whitish colonies. (Crickshank, 1975; Cheesbrough, 2002)

3.10.9Sugarfermentation.

These tests were carried out on all suspected*Staphylococcus* isolates (Chessbrough, 2002) To 90ml of peptone water, 10ml of 2%bromothymol blue indicator was added and sterilized at 121^{o}C for 15 minute. Ten gram of appropriate sugar dissolved in 90ml of water and steamed for 30minutes, were added to the sterile peptone water. From these, 5ml was dispensed into sterile tubes and 2 pure colonies of each isolates from fresh culture were inoculated into the prepared sugar solution and incubated at 37^{o}C for 24 hours. A change in colour from blue to yellow was indicative of fermentation. The isolates were subjected to the various sugar tests such as mannitol, sucrose, lactose, xylose, arabinose and raffinose tests.

3.10.10Microbact 12s staphylococcal identification system.

Seventy-one (71) *Staphylococcus* isolates were identified by conventional biochemical tests. Tenwere*Staphylococcus aureus* and 2 isolateseach of *Staphylococcus intermedius*, *Staphylococcus hyicus*, *Staphylococcushaemolyticus*, *Staphylococcus chromogens*, and*Staphylococcus xylosus,* wereselected at random and were further confirmed with Microbact Staphylococcal 12S identification system test. (Oxoid, UK)

Twenty isolates were tested according to the instructions of the kit's manufacturers' (Oxoid Ltd., Basingstoke, and U.K). Briefly, four colonies werepicked and emulsifiedin 3ml of staphylococcalsuspending medium to a homogenous suspension using a sterile bacteriological loop. Using a sterile Pasteur pipette, 100µl of the bacterial suspension was added to the wells of each test strip, to well no.7 was overlay with mineral oil and incubated at 37^{o}C for 24hours. After the incubation, a drop of fast blue reagent was added to well no.12 containing beta-galactosidase and observed for colour change. The results, as shown in the wellswere read and recorded onto the microbact® Organism ID Report form provided in the kit. Interpretation of result was aided by a colour chart provided in the kit. Once results had been entered on the report form; each block of three reactions was converted into a numeric value. The three numbers were added together to obtain 5 digits of the Microbact® code, which was then entered into the computer aided soft- ware for identification.The results of the percentage fiqure shown against the organism named is the percentage share of the probability for that organism as part of the total probability for all choice.

3.10.11Serotyping of *Streptococcus* species by Streptococcalgrouping kit

Serological grouping of isolates was performed with a commercial latex agglutination kit (Oxoid UK) for identification of streptococci groups A,B,C,D,F and G.The 5*Streptococcus* groups were tested usingthe broth method described by the manufacturers.A drop was dispensed from each latex reagent in to the six circular rings on the reaction card. Pasteur pipette was used to add a drop of extract (5 pure colonies emulsified in the *Streptococcus* extraction enzyme, incubated for 10 minute at 37^{o}C in

water bath then allowed to cool to room temperature) to each of the six rings. Mixing sticks provided was used to spread the mixture over the entire area of the ring using a separate stick for each ring. Visible agglutination which occurred within one minute was considered positive.

3.10.12 Antimicrobialsusceptibilitytesting

The susceptibility of 92 isolates to 11 antimicrobial agents was determined using Kirby-Bauer disk diffusion method (Bauer*et al*., 1966). The antimicrobial agents and concentrations used werePenicillin g (10 iu), gentamicin (10µg), trimethoprim-sulfamethazole(1.25µg),chloramphenicol(30µg),vancomycin(30µg),tetracyclin(30µ), ciprofloxacin(5µg), ceftriaxone(30µg) and cefoxitin (30µg),amoxicillin/clavulnaic acid (10µg), ampicillin (10µg).Three colonies ofeachisolatetobetested were picked andsuspended in normal saline and the turbidity of the suspension was adusted to match 0.5 McFarland standard. The diluted bacterial suspension was transferred into Mueller-Hinton agar (Merck, Darmstadt, Germany) plates using sterile cotton swabs.For*Streptococcus*species 10% blood agar was added to the plating media. The respective plates were seeded uniformly by rubbing the swab against the entire agar surface. Each antimicrobial impregnated disk was applied into the surface of the inoculated plate using a sterile forceps. The plates were incubated at 37^{o}C for 18 hours. After the incubation period the diameters of the antibiotic inhibition zones were measured to the nearest millimeter (mm) using a meter rule. The interpretation of the growth inhibition zones and classification of isolates as susceptible, intermediate and resistant followed the guidelines of the Clinical and Laboratory Standards Institute

(CLSI,2011).Those isolates classified as intermediate were considered as susceptible for the purpose of overall interpretation of antibiogram.

3.11 Data Analysis

Data obtained from the study were summarized into percentages and tables. Prevalence was calculated using the formula; below;

$$\frac{\text{No of positive sample}}{\text{Total no of samples}} \times 100$$

Chi-square or fisher's exact test and odd's ratio where necessary were used to test for association between the presence of mastitis and variables or risk factors such as the number of parity, stage of lactation, quarters location and present of teat lesion. Values of $P < 0.05$ were considered significant.The analysis was done using Graph pad prism version 5.0

CHAPTER FOUR

RESUITS

4.1 Detection of Clinical and Subclinical Mastitis

Out ofFour hundred and seventy two (472) samples tested for clinical mastitis and subclinical mastitis. Twenty eight (5.9%) samples showed evidence of clinical mastitis with distribution of 8, 4, and 16 sample positive from Zaria, Sabon gari and Giwa Local Government Areas respectively. California mastitis test (CMT) was conducted on the remaining four hundred and forty four (444) quarter milk sample for presence of subclinical mastitis, out of which 56, 11.8% of the entire 472 samples showed evidence of

subclinical mastitis with a distribution of 20, 20, and 16 positivefrom Zaria, Sabon gari and Giwa Local Government Areas respectively. Three hundred and eighty-eight (388) were CMT negative.Positive samples were scored 1+, 2+ and 3+ as positive. Over all CMT result observed in 28% were 1+, 34% were 2+ and 38% were 3+ (Tables 4.1&4.2) and there was significant difference between the number of positive samples across each of the Local Government Areas However, there is no significant difference between the isolation of bacteria in milk and the location sampled. (Table 4.3)

4.1: Results of California mastitis test on quarter milk samples from three Local Government Areas in Zaria.

CMT score	Standard somatic cell count /ml	No.% of positive milk samples examined using CMT
1+	400 000-1500 000	16(28)
2+	800 000-4 000 000	19(34)
3+	>5 000 000	21(38)
Total		**56(11.8)**

4.2: Distribution of quarter milk sample Califonia mastitistest scoresfromthree Local Government Areasin Zaria

Location LGA	CMT Results-Scores			No of quarter milk samples	No. of Mastitic Milk Test positive to CMT
	+	+ +	+++		
Zaria	5	7	8	170	20
Sabon Gari	8	9	3	112	20
Giwa	3	3	10	190	16
Total	16(28%)	19(34%)	21(38%)	472	56

X^2 = 6.003df=2 p=0.0497 p<0.05

Table 4.3: Distribution of Mastitis-Positive Quarter Milk Samples among the three Local Government Areas in Zaria

Location	No. of quarter milk samples examined	Clinical mastitis	Subclinical mastitis	No of samples positive (%)
Zaria	170	2(8)	5(20)	28(16.47)
Sabon Gari	112	1(4)	5(20)	24(21.43)
Giwa	190	4(16)	4(16)	32(16.84)
Total	472	28	56	84(17.80)

$X^2 = 1.33$ df = 2P = 0.5136

4.2 Prevalence of Mastitis at Herd, Cowand Quarter'slevel

Out of the eighty four (17.8%) samples analyzed and found to be positive for mastitis, 4(44%) herds, 7(5.6%) cows and 28(5.9%) quarters had clinical mastitis, while 6(66%) herds, 14(11.2%) cows and 56(11.8%) quarters had subclinical mastitis. (Table 4.4)

Tables 4.4: Prevalence of Clinical and Subclinical Mastitis at Herd, Cow, and Quarter's Level in Lactating Cows from the Three Local Government, in Zaria.

Observation level	**Clinical mastitis**			**Subclinical mastitis**		
	No.	+ve cases No. (%)	+ve cultures No. (%)	No.	CMT +ve No. (%)	+ve culture No. (%)
Herd level	9	4(44.4)	4 (44)	9	6(66)	6(66)
Cow level	125	7(5.6)	7 (5.6)	125	14(11.2)	14(11.2)
Quarter level	472	28(5.9)	28 (5.9)	472	56(11.8)	56(11.8)

4.3 Bacteria lsolated from Mastitic Milk

All the28 clinical mastitis samples and cultured, were positive for bacteria growth and 56 subclinical mastitis samples were also positive for 64 bacteriagrowthsgiving a total of 92 bacterial isolates. They were identified by conventional biochemical test. The 92 isolates consisted of 71(77.17%) *Staphylococcus* species, 16(17.40%) *E.coli* and 5(5.43%) *Streptococcus* species (Tables 4.5,4.6,&4.7) The seventy-one *Staphylococcus* species consisted of 10 *Staphylococcus aureus* ,12 *Staphylococcus intermedius,*8 *Staphylococcus chromogens,*3*Staphylococcus hyicus,* 8 *Staphylococcus haemolyticus*and 30 *Staphylococcus xylosus.* These were further confirmed using microbact 12S identification system. The followings were percentage probabilities for each of the *Staphylococcus* species.*S. aureus* were (64.89%, 64.68%, 99.85%, 99.99%, 99.99%, 99.99%, 84.60%, 80.11%, 64.04% and 84.60 %.) The Percentage probabilities for.*S. haemolyticus* were 53.29% and 53.29% while S.*intermedius* were-88.2%, and 99.8%. Others, include S. *xylosus*- 96.93% and 96.70%, S. *hyicus* were 53.62% and 99.58% while*S. chromogen*were70% and 70%. TheFive *Streptococcus*isolates were identified,as 4 *Streptococcus uberis* and 1 *Streptococcus bovis*. Serological typing confirmed the *Streptococcus bovis* to belong to Lancefield group D, while the remaining 4 isolates (*Streptococcus uberis*) were un-typeable by the Lancefield serological test and 16 *Escherichia coli* were isolated.Followings are Prevalence rate of the bacteria isolated.*Staphylococcus aureus*2.1*%, Staphylococcusintermedius* 2.5*%, Staphylococcus chromogens*1.69*%, Staphylococcushyicus* 0.6*%, Staphylococcushaemolyticus*1.69*%, Staphylococcus xylosus* 6.35*%, E. coli* 3.4%,*Streptococcus uberis* 0.85% *and Streptococcus bovis* 0.2 *%*

4.5. Occurance of Bacteria Isolates in Bovine Mastitis Quarter Milk Samples in Zaria

Bacteria organism	**No. of isolates (%)**
Staphylococcus species	71(77.17)
E. coli	16(17.40)
Streptococcus species	5(5.43)

Table 4.6: Bacteria Isolates from Clinical and Subclinical Bovine Mastitic Milk Samples in Zaria. Using Conventional Biochemical Tests

No	Type of isolate	No.(Percentage) fromClinical Mastitis	No.(Percentage) from Subclinical Mastitis	Total (%)
1	*S. aureus*	1(3.57)	9 (14.06)	10 (10.87)
2	*S. hyicus*	0(0)	3 (4.69)	3(3.3)
3	*S. chromogens*	1(3.57)	7(10.94)	8(8.70)
4	*S. intermedius*	2(7.14)	10(15.63)	12(13.04)
5	*S. haemolytic*	2(7.14)	6(9.37)	8(8.69)
6	*S. xylosus*	6(21.43)	24(37.5)	30(32.60)
7	*E. coli*	15(53.57)	1(1.56)	16(17.40)
8	*Streptococcus uberis*	1(3.57)	3(4.69)	4(4.35)
9	*Streptococcus bovis*	0(0)	1(1.56)	1(1.08)
	Total	28(100)	64(100)	92(100)

Lengend**:** Biochemical tests include; Nitrate reduction test, pigmentation test, sugar fermentation DNase activity, Voges-Proskauer test, Coagulase test,carbohydratutilization Esculin hydrolysis, indole, motility, Triple sugar Iron, MR-VP Citrate and urea test

Table 4.7: Occurrence of Different Species of Bacteria Isolate from Bovine Mastitis Sample in the Three Local GovernmentAreas Zaria

Location	Clinical mastitis	Subclinical Mastitis	Type of isolate			*Staph aureus*	*Staph zylosus*	*Staph intermedius*	*Staph hyicus*	*Staph chromogen*	*Staph haemolycus*	*E. coli*	*Strep ubreris*	*Strep bovis*
			Staph	*E. coli*	strep									
Zaria LG	8	20	23	4	3	1	17	1	-	1	3	4	2	1
Sabon gari LG	4	20	19	2	1	-	9	-	3	6	1	2	1	-
Giwa LG	16	16	29	10	1	9	4	11	-	1	4	10	1	-
Total	28	56	71	16	5	10	30	12	3	8	8	16	4	1

4.4 Bacteria Isolated from Exramammary Sites

Nine samples each was collected from extra-mammary site (udder, teat and hand of milking personnel) from selected cow in each herds from the three Local Governments Areas in Zaria and results presented in (Table 4.8) were cultured andidentified. When suspected gram+ve cocci organism were subjected to catalase test, they showed both negative and positive catalase reactions suggestive of presence of Staphylococci and Streptococci. The suspected gram negative rods were identified as *E coli* based on the green metallic sheen on the eosin methylene blue agar. All the swabbed samples collected (9 udder, 9 teat and 9milker's hands) from all the nine herds were positive for *Staphylococcus, E coli,* non-lactose fermented gram negative and *Streptococcus.* Bacteria isolated from extramammary site and hands of milking personnel were grouped; Environmental pathogens (*E coli*, *Streptococcus*species) and minor/ major pathogens (*Staphylococcus* species).

Tables 4.8: Bacteria Isolates from Extra-Mammary Sites in Cow and Hand of Milking Personnel from Different Locations in Zaria

Location	**Samples source/origin**	**Number of sample collected**	**Number of sample positive**	***Type of bacteria isolates***
Zaria city	**Teat**	3	3	*Staph, E.coli,*non-lactose fermenter, *Strep*
	Udder	3	3	***Staph, E.coli, Strep.***
	Hand	3	3	***Staph, E.coli.***
Sabon-Gari	**Teat**	3	3	***Staph, E.coli, Strep***
	Udder	3	3	***Staph, E.coli, non-lactose fermenter***
	Hand	3	3	***Staph.***
Giwa	**Teat**	3	3	***Staph, E.coli, Strep, non lactose fermenter***
	Udder	3	3	***Staph, E.coli,***
	Hand	3	***3***	***Staph.***

4.5 Risk Factors Associated with Prevalence of Mastitis

Out of the 125 cows tested 64 were in the early stage of lactation while 61 were in the middle stage of lactation, eight (12.5%) and 6 (9.84%) cow in the early and middle stage of lactation respectively were positive for subclinical mastitis. Also, 89, 30, and 6 of the 125 cows had 1-3, 4-5 and >5 parity respectively out of which 9 (10.11%), 3 (10.11%) and 2(33.33%) of the different number of parities respectively were positive for subclinical mastitis (Table 4.9). A total of 472 that is (237 front and 235 rear) quarters were examined from 125 cows during the studies. Twenty seven (11.3%) front quarters examined had subclinical mastitis while 29 (12.34%) of rear quarters also had subclinical mastitis and only 33.33% out of the 6 teats that had lesion were positive for subclinical mastitis (Tables 4.9). The risk factors analysis carried out indicated that there was no significant association ($p>0.05$) between the stage of lactation, parity number as well as quarter location and positivity for subclinical mastitis (Table 4.9)

Tables 4.9: Risk Factors Associated with the Prevalence of Subclinical Mastitis at Cow level from Mastitic Cow in Zaria.

Risk factors Tested	Total number for subclinical N	Number positive mastitis (%)	*p-value*	OR(95%Cl)
Stage of Lactation				
Early(1-120)days	**64**	**8(12.50)**	**0.637**	**0.76(0.25-2.35)**
Middle(121-240)days	**61**	**6(9.84)**		
Parity				
1-3	**89**	**9(10.11)**	**0.212**	**4.4(0.711-27.77**
4-5	**30**	**3(10.11)**		**4.50(0.57-35.84)**
>5	**6**	**2(33.33)**		
Quarter Location				
Front	**237**	**27(11.39)**	**0.7501**	**0.91(0.52-1.60)**
Rear	**235**	**29(12.34)**		
Presence of teat lesion				
Present	**6**	**2(33.33)**		
Absent	**466**	**0**		

4.6 Farm Management

The results of farm management practices are presented in (Table 4.10). There were more than two labourers in each herd with over ten years of experienceindairypractice none of the herds was found to be keeping records and grazing of animals was done outdoors for all the herds. Generally, hygiene waspoorforthe entire herds studied.

Table 4.10: Management Practices in the Nine Dairy Herds in the Three Local Government Areas in Zaria.

Variables	No. of Herds	%
General Management		
Herds Size		
1-5	0	0
6-10	1	11
n>11	8	89
No. of Labourers		
n<2	0	0
n>2	9	100
Experience in dairy practice		
0-5 years	0	0
6-10 years	0	0
11-15 years	3	33
>16 years	6	67
Grazing		
In-doors	0	0
Out- door	9	100
Manure Disposal		
Good	0	0
Poor	9	100
Record Keeping		
No	9	100
Yes	0	0

4.7 Maintenance conditions within Herds

The results of maintenance condition are presented in (Table 4.11). All the herds were kept in the open as it is typical of Fulani herds. Nine (100%) of the herd owners tethered their animals where they were kept. Overall, hygiene was poor. This study revealed that there were general poor sanitary conditions in all the herds.

Table4.11: Housing Condition in Nine Dairy farms inthe Three Local Government Areas in Zaria.

Variables	No. of Herds	
Housing		
Concrete	0	0
Earth	9	100
Bedding		
Yes	0	0
No	9	100
Sleeping Area of Cows		
Same as feeding	0	0
Separate Areas	0	0
None	9	100
Animal Tethered		
Yes	7	78
No	2	22
Cleaning Procedure		
Sanitary Condition		
Good	0	0
Poor	9	100
Presence of flies		
Yes	7	78
No	2	2

4.8 Cow Management and Milking Procedures/Hygiene

All the herdsdidnot observed dry cow therapy. In 8(89%) of the herds, the cows were restrained before milking. In all the herds, hand washing with soap before milking was not practiced and all 9 herds (100%) practiced stripping type of milking. Udder preparation by washing the teats before milkingwasnot done inall the (100%) herds. The use of single towel for drying the teats was not observed inall of the herds. Milk was used as a lubricant in 9(100%) of the herds. The herdsmen do not carry out screening for subclinical mastitis in all the herds (Table 4.12).

Table 4.12: General Practice observed in the Nine Dairy Herds

Variables	No. of Herds	
Sources of Water		
Tap	0	0
Bore-hole	0	0
Stream	9	100
Management ofCows		
Dry Cow Therapy		
Yes	0	0
No	9	100
Milking Procedures		
Cow restraint for milking		
Yes	8	89
No	1	11
Hand washing		
With soap	0	0
Without Soap	9	100
Milking Techniques		
Four Fingers squeezing	0	
Stripping	9	100
Udder Preparation		
Washing only the teat	0	0
Washing the whole Udder	0	0
Udder cloth		
Single Towel	0	0
Separate Towel	0	0
Type of teat lubricant		
Cooking oil/Jelly	0	0
Milk	6	66.6
No application for lubricant	3	33.3
Feeding after milking		
Yes	9	100
No	0	0

4.9 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing on the isolates to 11 different antimicrobial agents was carried out and the result is as shown in (Tables 4.13 & 4.14).*Staphylococcus aureus* showed high resistant topenicillin (100%), ampicillin (90%), amoxicillin/ clavulanic acid (30%).vancomycin.(20%), ciprofloxacin (30%), Sulphamethoxazole+trimethoprim.(20%) butlow resistance to tetracycline (10%), chloramphenicol (10%) and ceftriaxone (10%).*Staphylococcus intermedius* showed high resistance to ampicillin (100%)penicillin (100%), tetracycline (33.4%), however one isolates was sensitive to the entired antimicrobial agent tested. Likewise one isolates from *S hyicus,* was sensitiveto all the antimicrobials used but the remaining two *S hyicus* displayedantimicrobial resistance.*Staphylococcus xylosus* showed high resistant (90%) to penicillin, ampicillin (80%), amoxicillin – clavulanic acid (13.4%), tetracycline (36.7%) and Sulphamethoxazole + trimethoprim (33.4%). However,*Staphylococcus xylosus*showed low resistant to vancomycin (6.7%) and ciprofloxacin (6.7).*Streptococcus uberis* showed highresistance to tetracycline (75%) and gentamycin (25%). While *Streptococcus bovis*showed high resistance to tetracycline(100%) only. The sixteen isolatesof *E.coli*showed variableresistanceto all the antimicrobial agents; sulphamethoxazole/trimethoprim (68.75%), ampicillin (100%), tetracycline (62.5%), chloramphenicol (75%) and gentamycin (56.25%).

All the 90 bacterial isolates testedexhibited multiple antibiotic resistance to the 11 antibiotics tested as is shown in (Table 4.14).

Altogether,40 isolates showed multiple drug resistance patterns against the tested antimicrobial agents. Out of which 25*Staphylococcus*species showed multiple

drugresistace pattern and AMP, PEN has the higest frequeny (35%) and MAR index value of 2.27. *Escherichia .coli*showed 13 multiple drug resistances pattern to the antimicrobial agentsand SXT, AMP, CIP, AMC, TE, C, and CN (18.7%) were the most commonly encountered with MAR index value of 0.3 while *Streptococcus uberis* showed2 multple drug resistance patterns.

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Table 4.13: Multiple Drug Resistance Patterns of Bacteria Isolate from Bovine Mastitis Milk Tested against 11 Antimicrobial agents.

Susceptibity Pattern	No. of Isolate	lsolates %	MAR
Staphylococcus			
PE	4	5.6	0.36
AMP, PE	25	35.2	2.27
AMP	4	5.6	0.36
AMP, PE, SXT	6	8.4	0.54
AMP, PE, AMC, CIP	5	7.0	0.45
AMP PE, CIP	3	4.2	0.27
AMP, PE, TE, SXT	2	2.8	0.18
AMP, PE, VAN, CEP	1	1.4	0.09
AMP, PE, VAN TE, CH, SXT	1	1.4	0.09
AMP, PE TE	2	2.8	0.18
PE, TE	2	2.8	0.18
AMP, PE, AMC	1	1.4	0.09
AMP, PE, CH	1	1.4	0.09
AMP, PE, TE, CH, VAN	1	1.4	0.09
PE, VAN, SXT	1	1.4	0.09
AMP, PE, CIP, CEP	1	1.4	0.09
AMP, PE, TE, VAN, SXT	1	1.4	0.09
AMP, PE, CIP, SXT	1	1.4	0.09
TE	1	1.4	0.09
AMP, PE, CEP	1	1.4	0.09
AMP, PE, CH, SXT	1	1.4	0.09
AMP, PE, TE, VAN, CH, CN, SXT	1	1.4	0.09
AMP, PE, TE, CH CN, SXT	1	1.4	0.09
PE, TE, CH, SXT	1	1.4	0.09
Streptococcus			
TE	2	40	4
TE, CN	1	20	2
E.coli			
SXT, AMP, CIP, AMC, PE, TE, CH, CN	3	18.7	0.33
SXT, AMP, AMC, PE, TE, CH	2	12.5	0.25
SXT, AMP, AMC, PE, CN	1	6.25	0.12
SXT, AMP, AMC, PE, CH	1	6.25	0.12
SXT, AMP, AMC, PE	1	6.25	0.12
AMP, CIP, AMC, PE, CN	1	6.25	0.12
CIP, AMC, PE, CH, CN	1	6.25	0.12
AMP, AMC, PE, CH	1	6.25	0.12
AMP, AMC, PE TE, CH, CN	1	6.25	0.12
AMP, AMC, PE, TE, CH	1	6.25	0.12
SXT, AMP, AMC, PE, TE, CH, CN	1	6.25	0.12
SXT, AMP, AMC, PS TE, CH, CEP	1	6.25	0.12
SXT, AMP, AMC, CIP, PE, TE, CH, CEP, CN	1	6.25	0.12
Total	90		

n=Number of isolates

CHAPTER FIVE

5.0 Discussion

The 5.9% prevalence of clinical mastitis recorded in this study is comparable with that 5.3 % reported by Bishi (1998) in Ethiopia. However, it is higher than the 2.1% prevalence rate reported by Ameh *et al*. (1999) in a study carried out in Maiduguri. The difference in the prevalence rates of clinical mastitis in these two geographical locations of Nigeria may be attributable to differences in management practices, especially with regard to farm, udder and milking hygiene practices observed by the herdsmen.

The 12% prevalence of subclinical mastitis recorded in this studywas similar to that 12.6% reported earlier by Umaru *et al.* (2012) from a study in the same area. It also tallies with that 14.7% in the report of Strastskova *et al*. (2009) from a study conducted on bulk tank milk in Czech Republic. However, it appeared comparatively lower than the 30% rates reported by Umoh *et al*. (1990a) in this same location and30.9% reported by Suleiman (2011) in Jos. It is important to note that these latter authors conducted their work on settled farms that use machines to milk dairy cow. Umoh *et al*. (1990a) attributed the high prevalence of subclincal mastitis, such as observed in their study, to possible damage to the teats of the udders by the milking machine, which rendered them more vulnerable to bacterial infections. The low prevalence recorded in this study relative to those in the reports of Umoh *et al*. (1990a) and Suleiman (2011) might, thus, be chiefly due to difference in the milking procedures in the two farm management systems. Also, it is probable that the low prevalence was due, in part, to abuse use of antibioticsby the herdsmen on milking cows, a practice that has become pervasive among nomads. The fact that the present study was conducted mainly in the dry season of the year , the period

known to record low prevalence of organisms and also the period during which the pH of milk tends to be low, which inhibits the growth of most tmicroorganismmight have contributed to the low prevalence.

The finding in this study that the coagulase negative *Staphylococcus (*CNS) were the predominant bacterial pathogens isolated (66.30%) from subclinical mastitis cases tallies with reports on studies conducted in other several countries (Tenhagen *et al*., 2006; Lim *etal*., 2007). The overall prevalence of CNS for both clinical and subclinical mastitis(2.3% and 10.59%) recorded in this study compares very well with that of Bishi (1998). The high prevalence of CNS in this study may probably be due to the fact that CNS are normal flora of the skin of cows and teat and teat canal, which increases the probability of infection (Matos *et al*., 1991; Tenhagen *et al.,* 2006). The 2.1% prevalence rate of *Staphylococcus aureus*, which is the most important isolate recorded in this study was quite lower than those in previous reports (Umoh *etal*., 1990a; Sori *et al*., 2005; Sulieman, 2011). This may be attributed to differences in the farm management systems under which the cows were kept. Although low prevalence was recorded, its isolation has a serious public health implication such as Staphylococcal foodborne intoxication and spread of zoonotic diseases especially when the milk is not preparely pasterurised. Ameh*et al*.(1993) and Lafi *et al*.(1994) had similarly reported occurrence of both *Staphylococcus aureus* and CNSas predominant isolates in subclinical mastitis. Subclinical mastitis, though frequently goes unnoticed, had been reported to impair milk production. The finding in the study that certain percentage of the cows sampled had subclinical mastitis may propably compound the low milk production capacity of Bunaji breed of cattle on which this study was carried out.Similarly, the isolation of *E coli* and

Streptococcus species, which are environmental pathogens and some of the major causes of clinical mastitis in cow, is also a cause for concern.

The contamination of the environment and milkers' hand with these microbes, the unhygienic milking procedure and milking from mastitic quarters along with other normal healthy quarters, could serve as source of wider spread of these pathogens. This study has also documented a wide range of antibiotic resistance patterns exhibited by the different bacterial isolates from the mastitic milk sampled. At least each bacterium showed resistance to 2-4 of the antimicrobials tested. The observation, in this study, that *Staphylococcus aureus* exhibited multi-drug resistance to the rate earlier reported by Suleiman (2011) and Umaru *et al*. (2012). The extensive antimicrobial usage and abuse in dairy industry has no doubt necessitated bacteria pathogens to acquire various resistance genes and thus become more resistant to such agents (Otalu *et al*., 2011). The observation in this study that *Staphylococcus* species, and indeed most of the isolates, were resistant to almost all the commonly used antimicrobial agents (ampicillin, penicillin, tetracycline, amoxicillin/clavulanic acid) may imply that continued use of such antimicrobials especially in regards to penicillin and ampicillin may not yield the desired therapeutic result in cases of clinical of mastitis.

The only antimicrobial agents to which almost all the *Staphylococcus* species were found to be susceptible to were gentamicin and cefoxitin, which is in line with the reports of Sulieman, (2011). The finding that all the isolates showed resistance to penicillin was not unique to this study as similar report had been made, previously by (Otalu *et al*.(2011), Suleiman,(2011) and Umaru *et al*.(2012),where by (100%, 100%, and 100%)where found to be resistant to penicillin respectively. The multi-antimicrobial resistance (at least to 2

to 4 antimicrobials) exhibited by almost all the isolates in this study may be suggestive of the long term effect of irrational use of antibiotics by the herdsmen in treatment of infections and may also reflect the failure in observing strict drug control and legislations with regards to sale and use of antimicrobials in Nigeria (Geidam *et al*., 2012).

Several authors had reported similar pattern of resistance to antimicrobial agents in previous studies (Taiwo*et al*., 2008; Otalu *et al*., 2011; Suleimam, 2011; Umaru*et al*., 2012). *E coli* showed variable resistance to all the antimicrobial agents tested, while *Streptococcus uberis* and *Streptococcus bovis* were resistant to tetracycline and gentamicin, respectively.

The demonstration of multiple resistances to antimicrobials by *Staphylococcus* species, *Streptococcus* species and *E coli* could be a cause for concern in trying to institute a successful antibiotic therapy. Irrational use, abuse, sub-therapeutic use of antimicrobials therapeutic and prophylactic measures are often the factors responsible for development of multiple drug resistance (Amabile-cuevas *et al.*, 1995; Costa *et al*., 2000). The resistance of mastitis-causing pathogens to antimicrobial agents has two pertinent implications; first that it could lead to incomplete cure rates in treatment of cases of clinical mastitis (Owen*et al*., 1997); secondly there is the development of the potential for transmission of antimicrobial-resistant bacteria to humans via the food chain (Ungemach,1999). The exceptionally high susceptibility to gentamicin as observed in this study may be due to infrequent use of this drug in the livestock industry as a consequence of its high cost. Despite the fact that tetracycline is the most commonly used antibiotic, surprisingly, the resistance to this drug was low in regards to *Staphylococcus*

species, which could suggest its rational use in the study area. In a previous study conducted, by Umoh *et al.* (1990b) tetracycline resistance was observed to be 6.9%. In this study it was found that vancomycin was the most reliable therapeutic agent when used against infections that caused by methicillin resistant *Staphylococcus aureus*. However, there have been reports of vancomycin and *Staphylococcus* resistant strains from USA, France, Korea and South Africa (Chang *et al.*, 2003). According to them, emergence of vancomycin resistant in *Staphylococcus aureus* could be a global issue. However, 20% resistance to vancomycin observed in this study was previously reported in Zaria (Umaru *et al*., 2012).

This study identified a similarity between the bacterial isolates from the extramammary sites and those recovered from the milk sampled, which could suggest cow's environment constitutes a potential source of mammary infections. Thus, the likelihood of a cow becoming infected with the mastitis-causing pathogens depends on presence and pathogenicity of the microorganism, and the management practices that must create the opportunity for the cow to come in contact with pathogens (Sear and McCarthy, 2003). The teat-end exposure to such environmental mastitis-causing organisms is a major risk factor of infection. The cow's environment such as linings and beddings are often contaminated with faeces and urine, which increase the bacterial population at teat-end and there by contributing to increase in the incidence of mastitis. Work carried out by Blowey (1999) and a study by Kudi *et al*.(2009) established the existence of a relationship between bacteria population within the bedding materials and incidence of mastitis due to infections. Susceptibily of teat-end to mastitis pathogens is most probable

during the period of milking or just after as the teat-end remains partially open and thus, allows for entry of bacteria into the teat canal. Therefore, regular changing of bedding, good milking hygiene, clean and dry environment could reduce bacteria population and consequently, the likelihood of infection with mastitis pathogens. It has been shown that the transfer of pathogens onto susceptible teat ends can be reduced by allowing the animal's defense mechanisms to tackle such infections more effectively (Kudi *et al*., 2009).

Findings in this study revealed no significant ($p>0.05$) association between the risk factors considered (such as quarters affected, presence of teat lesion, number of parity, stage of lactation) and prevalence of mastitis, which run contrary to findings in previous reports (Sori *et al*., 2005). The difference may be due to the low number of animals examined in the present study. However, the prevalence was higher at early stage of lactation and as the parity number increases.

Farm attributes observed during farm inspections that could influence the occurrence of mastitis include environmental hygiene as observed in this study where by the herds examined were found to operate under poor-hygiene environments which may render animals prone to infection with mastitis -causing pathogens, as none of the farmers were found to employ CMT or other screening test to estimate the somatic cell count for detecting subclinical mastitis. The farmers did not have knowledge of subclinical mastitis in their lactating cows so no effortsweremade totreatorcontrolinfections. Generally, there was a poor udder-hygiene practice as all the farm attendants failed to wash their hands,

udder and teats and none use towels to dry each teat just before milking. Such practice could potentially lead to possible contamination of the quarter.

The prevalence of mastitis recorded in this study may be attributed to the improper milking hygiene and poor environmental conditions. Contamination of milkers' hand, clothes, failure to wash the whole udder has been reported to increase incidence of mastitis-causing pathogens and also serves as a risk factor for occurrence of mastitis (Blowey and Edmonson, 2001; Karimuribo, 2002). The unhygienic milking practices adopted by the pastoralists as observed in this studymay be due to the lack of knowledge on the causes of clinical and subclinical mastitis and their economic implications on milk production (Ameh *et al*., 1999). In Nigeria, it has been established that intra mammary infections have negative effects on milk quality and milk production.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

- In this study 15.4% *Staphylococcus* species, 1.1% *Streptococcus* species and 3.3% *Escherichiacoli*were isolated from bovine mastitic milk in Zaria.

- *Staphylococcus aureus* resistance to antibiotics tested ranged from 10-100% and *Streptococcus uberis*resistance range from 25-75%. While for *E. coli* resistance ranged from 6.25-100%. These bacteria isolates were resistant to commonly used antimicrobial agents as follows; 35% for *Staphylococcus* species, 18.7% for *E.coli*and40% *Streptopcoccus* species. Also, some of the bacteria showed MAR index value > 0.2, indicating that the bacteria isolated originate from a source of high bacteria contamination where several antibiotic are used.
- Poor environmental hygiene practices and farm management practices observed in this studymay have rendered the animalssusceptible to infections with mastitis pathogens.
- Although there was no stastically significant association between the factors considered, it was observed that age, stage of lactation,and parity number may have influencdthe prevalence of mastitis in the dairy herd

6.2Recommendations

Based on this study, it is recommended that:

- Isolation and identification of mastitis pathogens is crucial in mastitis management. Therefore, routine mastitis detection test (screening test) should be carried out to facilitate successful treatment.

- Its important to conduct antibiotic susceptibility test on mastitis pathogens so that most effective antibiotics can be selected for the treatment of affected cow

- Government, should legislate and enforce laws that will limit the prescription and dispensing of antimicrobial drugs only to the qualied professional

- Milk should be pasteurized adequately before consumption

- Others etiological agents of mastitis like fungi, mycoplasma should be investigated

- Further work should be done, like genotypic characterization of these bacterial species.

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DEPARTMENT OF VETERINARY MICROBIOLOGY
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QUESTIONNAIRE

This questionnaire is part of research that is aim at investigating bovine mastitic milk.

Please kindly fill and tick (✓) where appropriate

Type of farms single [ ] mixed [ ]

Type of management intensive [ ] extensive [ ] semi intensive [ ]

Medical record, kept [ ] on kept [ ]

Consult veterinary Yes [ ] No [ ]

How often animal treated, routinely [ ] when sick [ ]

No of cow on the farm [ ] no of lactating cow on farm [ ]

Udder examination before

Milking, never [ ] always [ ] some time [ ]

Method of udder examination 1. Massage [ ] 2. Fore milking [ ] 3. I and 2 [ ]

Udder wash before milking Yes [ ] No [ ]

Source of water stream [ ] borehole [ ] Tap [ ]

Washing water containing disinfectant Yes [ ] No [ ]

Cow are confirmed or stable, Yes [ ] No [ ]

Average least lactation, < 8 month [ ] 8-10 month [ ]

Is there milking palour Yes [ ] No [ ]

Floor barn floor station of milking palour earth [ ] concrete [ ]

- Use of towel, single [ ] double [ ]
- Type of breeds

- Average age of herd size
- Maximum herd size
- Stage of lactation early [ ], middle [ ], late [ ]
- Parity 1-2, 3-5, 5 > 6

  Quarter location front [ ], rear [ ]

  Presence or absent of tick Yes [ ] No [ ]

  Management of cows dry cow therapy Yes [ ] No [ ]

  Length of dry cow penned 60 days [ ] < 60 days [ ] > 60 days [ ]

  Hand washing with soap [ ] without soap [ ]

  Screening for subclinical mastitis Yes [ ] No [ ]

Appendix I: Questionaire

Biochemical test reaction of *Staphylococcus* species isolated from bovine mastitic milk sample in Zaria

Staph. Aureus	**Haemolysis**	**Coagulase**	**Slide agglutination**	**DNA Test**	**Mns**	**Nitrate**	**Acetoin production**	**Mannitol**	**Sucrose**	**Lactose**	**Maltose**	**Xylose**	**arabinose**
5gy	βH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
5gw	αH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
5h	βH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
5i	βH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
7b	βH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
10b	αH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
13b	αH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
8a	βH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
8by	βH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
8cy	βH	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve

Biochemical test reaction of *Staphylococcus* species isolated from bovine mastitic milk sample in Zaria

Staph. Intermedius	Haemolysis	Coagulase	Slide agglutination	DNA Test	Mns	Nitrate	Acetoin production	Mannitol	Sucrose	Lactose	Maltose	Xylose	arabinose
10a	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
7a	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
14a	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
7c	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
5e	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
5c	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
8bw	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
8cw	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
5f	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
6c	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
6a	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
6b	-ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve

Staph. Chromogen	**Haemolysis**	**Coagulase**	**Slide agglutination**	**DNA Test**	**Mns**	**Nitrate**	**Acetoin production**	**Mannitol**	**Sucrose**	**Lactose**	**Maltose**	**Xylose**	**arabinose**
3e	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
3f	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
3g	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
4a	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
3b	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
3c	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
4a	-ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve
13e	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve

Staph. Haemolyticus	**Haemolysis**	**Coagulase**	**Slide agglutination**	**DNA Test**	**Mns**	**Nitrate**	**Acetoin production**	**Mannitol**	**Sucrose**	**Lactose**	**Maltose**	**Xylose**	**arabinose**
15a	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
5a	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
5c	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
5d	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
6d	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
16h	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
16i	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
13h	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve

Staph. Hyicus	Haemolysis	Coagulase	Slide agglutination	-st	Mns	Nitrate	Acetoin production	Mannitol	Sucrose	Lactose	Maltose	Xylose	arabinose
3a	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
3d	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
3f	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve

Staph. xylosus	Haemolysis	Coagulase	Slide agglutination	DNA Test	Mns	Nitrate	Acetoin production	Mannitol	Sucrose	Lactose	Maltose	Xylose	arabinose
1a	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
2a	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
2b	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
2c	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
5a1	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
4b	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
4c	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
4d	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
4e	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
4f	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
10d	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
15b	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
15c	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
12a	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
12b	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
13a	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
13c	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
13d	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
13f	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve

13g	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
11d	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
11e	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
14c	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
14d	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
14e	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
16a	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
16b	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
16e	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
16k	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
14a1	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve

Appendix II: conventional biochemical test reaction of *Staphylococcus* species

Biochemical test reaction of *Streptococcus* species isolated from bovine mastitic milk sample in Zaria.

Organism	Haemolysis	Esculin Hydrolysis	Lactose	Mannitol	Raffinose	Sorbitol	Sucrose	salicin	Lancifield
Non-groupable									
Streptococcus uberis	α	Positive	Positive	positive	Negative	positive	positive	positve	Ngs
Groupable									
Streptococcus bovis	α	Positive	Positive	positive	Positive	negative	positive	positive	D

ngs=ungrouped

Appendix III: conventional biochemical test reaction of *Streptococcus* species

Biochemical test reaction of *E coli* isolated from bovine mastitic milk sample in Zaria.

E.coli	TSI	Citrate	Urease	SIM	MR	VP	Indole
8de	acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
10e	acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
11e	acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
12ce	acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
1a	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
4ge	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
14be	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
14fe	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
9ae	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
11ae	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
11be	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
11ce	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
10f	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
16e	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
16Le	Acid/acid+gas	-ve	-ve	+ve	+ve	-ve	+ve
16kge	Acid/acid+gass	-ve	-ve	+ve	+ve	-ve	+ve

Appendix IV: conventional biochemical test reaction of *E.coli*.

Table 4.9: Results of *Staphylococcus* Specie from Microbact Test

	Maltose	Mannitol	Mannose	Sucrose	Trehalose	N-Acetyl glusamine	Argine	Urease	Beta glucasidase	Alkaline phosphatase	Beta – glucoron	Beta galactosi	Coagulase	Dnase	Pigment
S-aureas	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve
	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve
	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve
	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve
	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve
	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve
	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
S.xylosus															
	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
S. intermedus															
	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve

	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
S.haemolyticus															
	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
S.hyicus															
	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
S.Chromogen															
	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve

Table 4.14: Multiple Resistance Pattern of the 92 Bacteria Isolate from Bovine Mastitis Milk tested against 11 Antimicrobial agents

ISOLATES	Resistance PATTERN	FREQUENCY	MAR
Staphylococcus aureus			
	PEN	1	0.09
	AMP,PEN	3	0.27
	AMP,PEN,SXT	1	0.09
	AMP,PEN,AMC,CIP	3	0.27
	AMP,PEN,VAN CEP	1	0.09
	AMP,PEN,VAN,TE,CH,SXT	1	0.09
Staphylococcus intermedius	AMP,PEN,TE,SXT	1	0.09
	AMP,PEN	5	0.45
	AMP,PEN,AMC	1	0.09
	AMP,TE,CH	1	0.09
	AMP,PEN,AMC,CIP	1	0.09
	AMP,PEN,TE,CH	1	0.09
	AMP,PEN,TE,CH,VAN	1	0.09
Staphylococcus chromogens	AMP,PEN	3	0.27
	AMP,PEN,CIP	1	0.09
	AMP,PEN,TE	1	0.09
	PEN,VAN,SXT	1	0.09
	AMP,PEN,CIP,CEP	1	0.09
	AMP,PEN,TE,VAN,SXT	1	0.09
Staphylococcus hyicus	AMP	1	0.09
	AMP,PEN	1	0.09
Staphylococcus haemolyticus	AMP	1	0.09
	PEN	1	0.09
	AMP,PEN	4	0.36
	AMP,PEN,CIP,SXT	1	0.09
Staphylococcus xylosus	AMP	2	0.18
	PEN	2	0.18
	TE	1	0.09
	AMP,PEN	9	0.81
	AMP,PEN,AMC,CIP	1	0.09
	PEN,TE	2	0.18
	AMP,PEN,TE	1	0.09
	AMP,PEN,SXT	4	0.36
	AMP,PEN,CIP	2	0.18
	AMP,PEN,CEP	1	0.09
	AMP,PEN,CH,SXT	1	0.09
	AMP,PEN,TE,SXT	1	0.09
	AMP,PEN,TE,VAN,CH,CN,SXT	1	0.09
	AMP,PEN,TE,CH,CN,SXT	1	0.09
	PEN,TE,CH,SXT	1	0.09

Streptococcus uberis	TE	2	0.2
	TE,CN	1	0.1
Streptococcus bovis	TE	1	0.1
Escherichia coli			
	SXT,AMP,AMC,PEN,CN	1	0.12
	SXT,AMP,AMC,PEN,CH	1	0.12
	SXT,AMP,AMC PEN,TE,CH	2	0.25
	SXT,AMP,AMC,PEN	1	0.12
	AMP,CIP,AMC,PEN,CN	1	0.12
	CIP,AMC,PEN,CH,CN	1	0.12
	AMP,AMC,PEN,CH	1	0.12
	AMP,AMC,PEN,TE,CHL,CN	1	0.12
	SXT,AMP,CIP,AMC,PEN,TE,CHL,CN	3	0.3
	AMP, AMC, PEN, TE, CH.	1	0.12
	SXT,AMP,AMC,PEN,TE,CH,CN	1	0.12
	SXT, AMP, AMC, PEN, TE,CH, CEP.	1	0.12
	SXT,AMP,AMC, CIP, PEN,TE,C,CEP CN	1	0.12

SXT =Sulphamethoxazole+Trimethoprim, AMP=Ampicillin; AMC=Amoxicillin/Clavulnaic acid; PEN=Penicillin; CIP= Ciprofloxacin; VA= Vancomycin; TE= Tetracycline; C =Chloramphenicol; CN= Gentamicin; Cn= Ceftriaxone

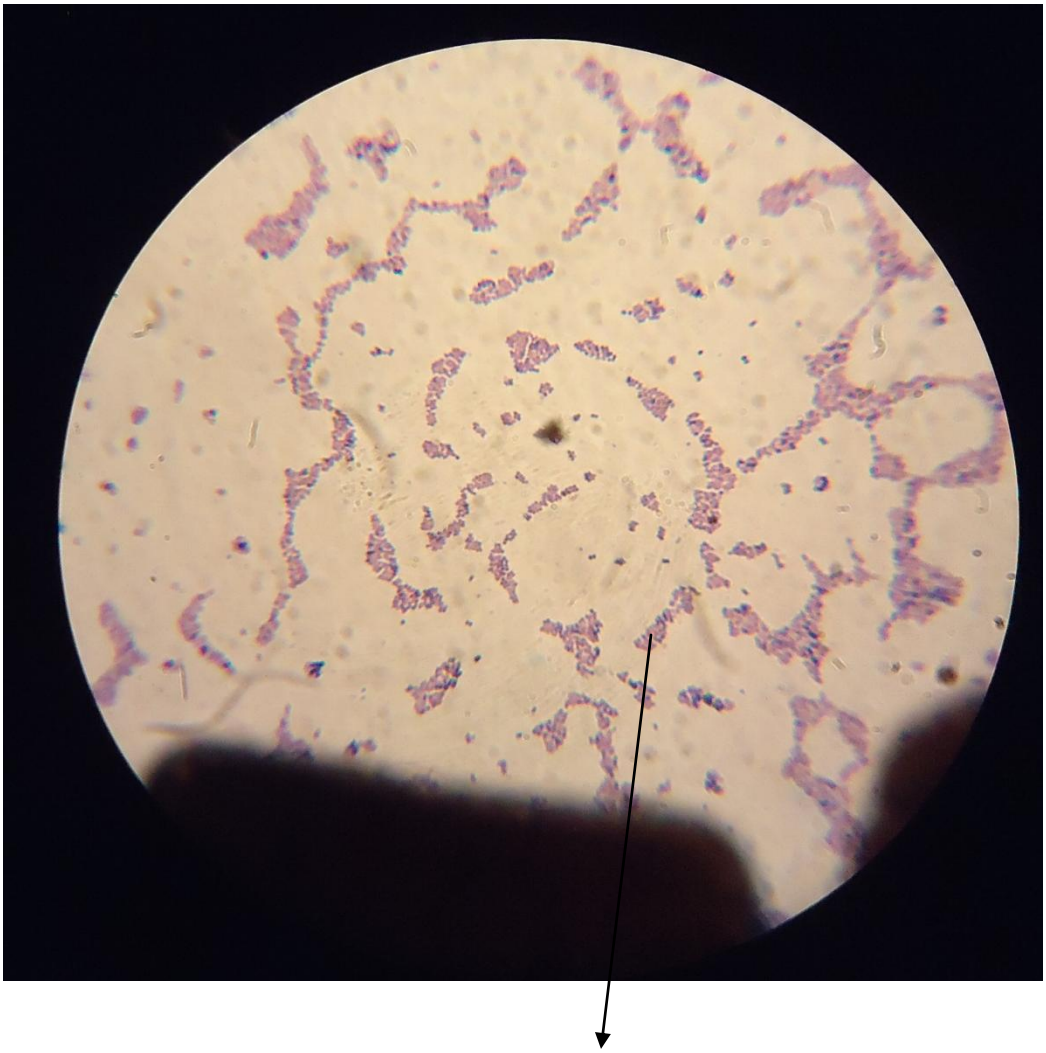
Appendix V: multiple resistance patherns of the 92 bacteria isolate from bovine mastititc milk tested against 11 antimicrobial agents

Table 4.13: In-Vitro Susceptibility Testing Results of n Bacteria Isolates from Bovine Mastitis Milk to Antimicrobial agents Tested

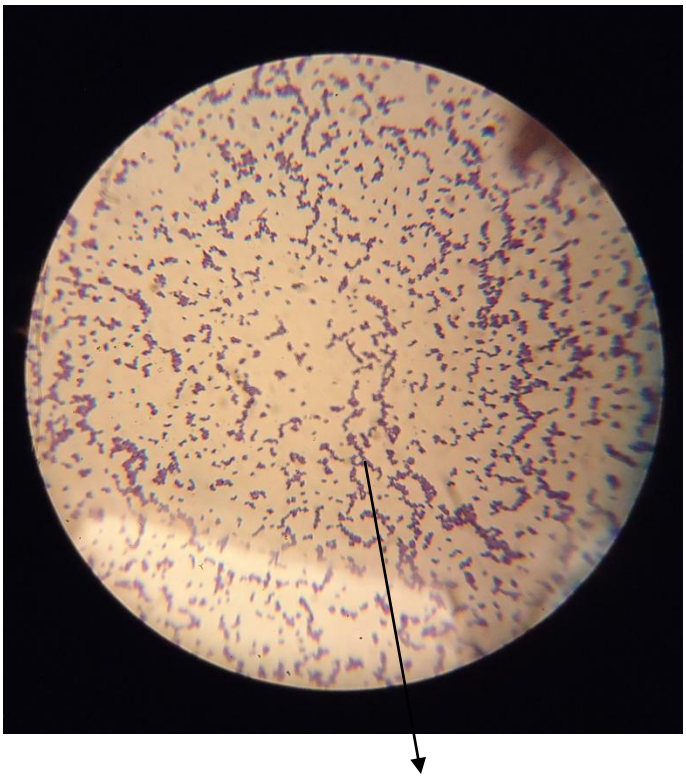
Isolates	**No tested**	**(%)of isolates resistant to individual antimicrobial agents**										
		SXT	AMP	AMC	PEN	CIP	VAN	TE	CHL	CN	CET	CEP
Staph. aureus	10	2(20)	1(90)	3(30)	10(100)	3(30)	2(20)	1(10)	1(10)	0	1(10)	0
Staph. Xylosus	30	9(30.0)	24(80)	1(3.3)	26(86.6)	3(10)	1(3.3)	8(26.6)	4(13.3)	2(6.6)	1(3.3)	0
Staph. intermedius	11	1(9.0)	11(100)	1(9.0)	11(100)	1(9.0)	1(9.0)	3(27.3)	4(36.4)	0	0	0
Staph. hyicus	2	0	50	0	50	0	0	0	0	0	0	0
Staph. haemolyticus	8	2(25)	7(87.5)	0	8(100)	1(12.5)	0	0	0	0	0	0
Staph. chromogens	8	2(25)	7(87.5)	0	8(100)	2(25)	2(25)	2(25)	1(12.50	0	1(12.5)	0
Strep. Uberis	4	0	0	0	0	0	0	3(75)	0	1(25)	0	-
Strep. Bovis	1	0	0	0	0	0	0	100	0	0	0	-
E. coli	16	11(62.5)	16(100)	16(100)	-	7(43.75)	-	11(62.5)	12(75)	9(56.25)	1(6.25)	-

No= number of isolate test against; SXT =Sulphamethoxazole+Trimethoprim, AMP=Ampicillin;

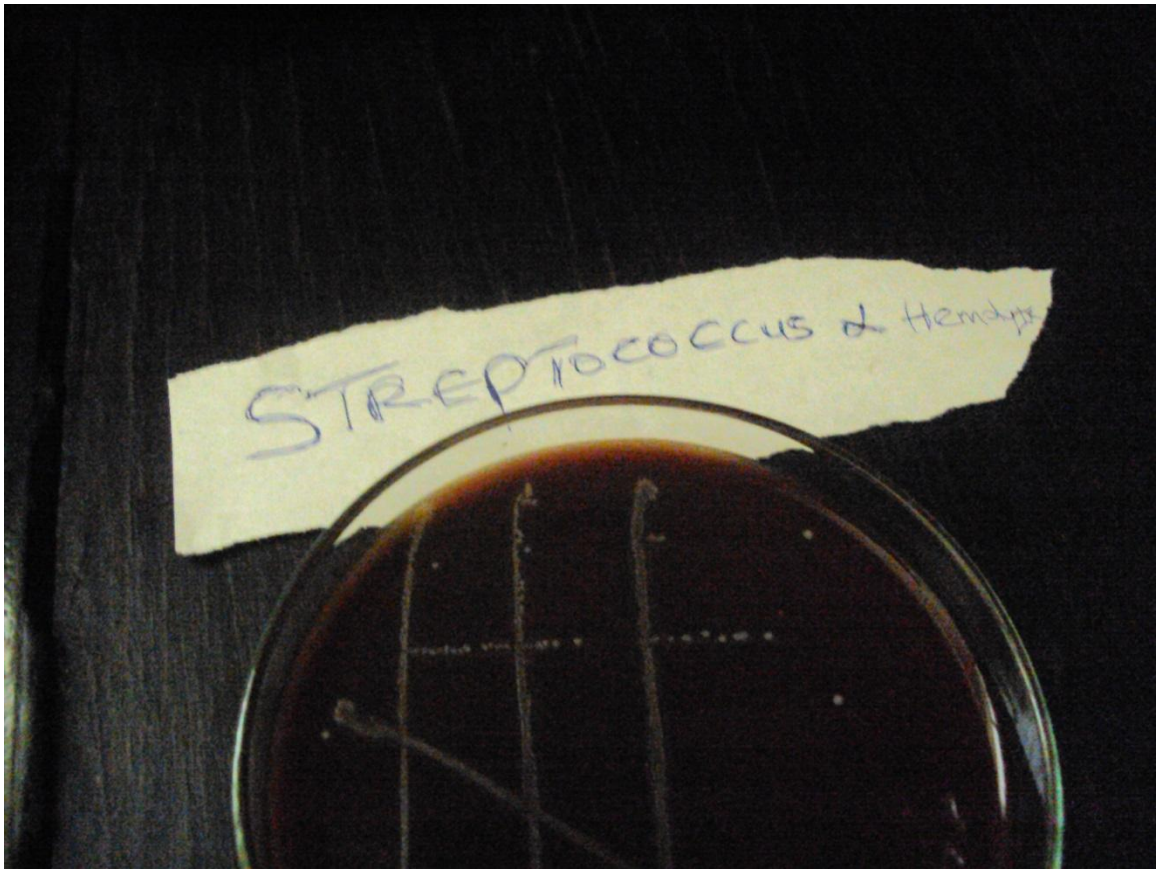
AMC=Amoxicillin/Clavulanic acid; PEN=Penicillin; CIP= Ciprofloxacin; VA= Vancomycin; TE= Tetracycline; C =Chloramphenicol; CN= Gentamicin; CE= Ceftriaxone; CEP=Cefoxitin.



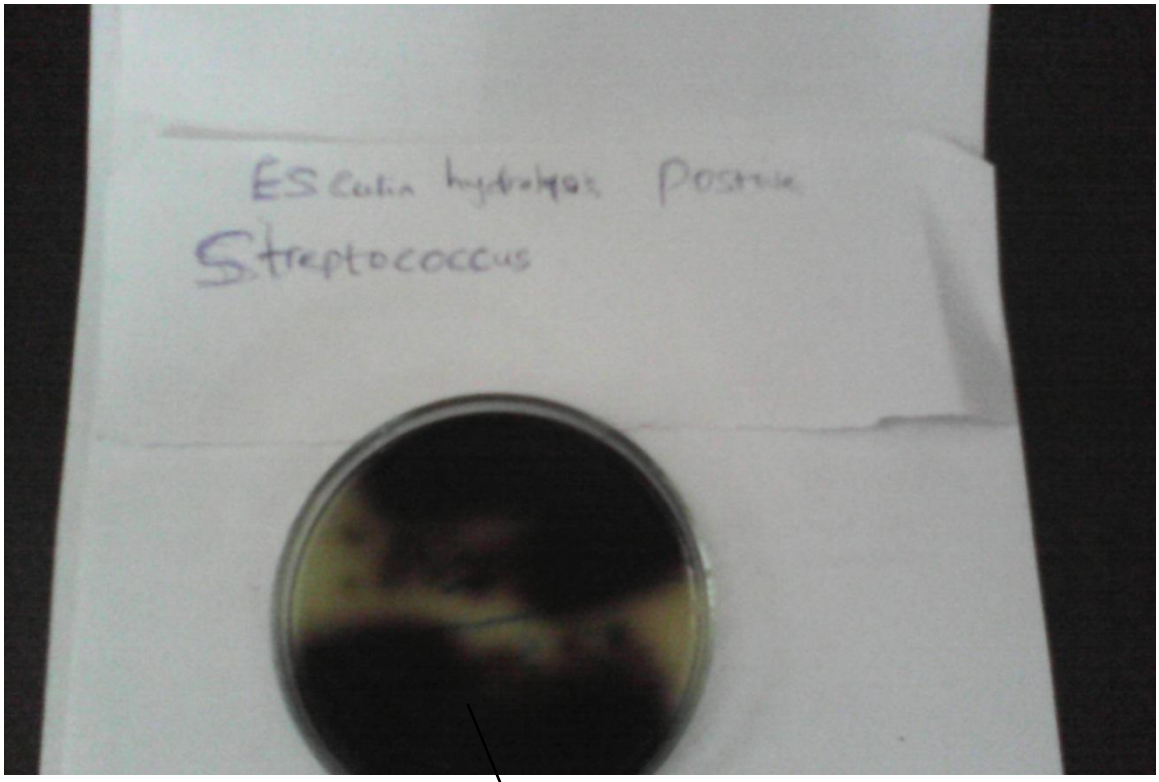
Appendix VI: Microscope view of grap-like cocci of *Staphylococcus* species X100 magnification



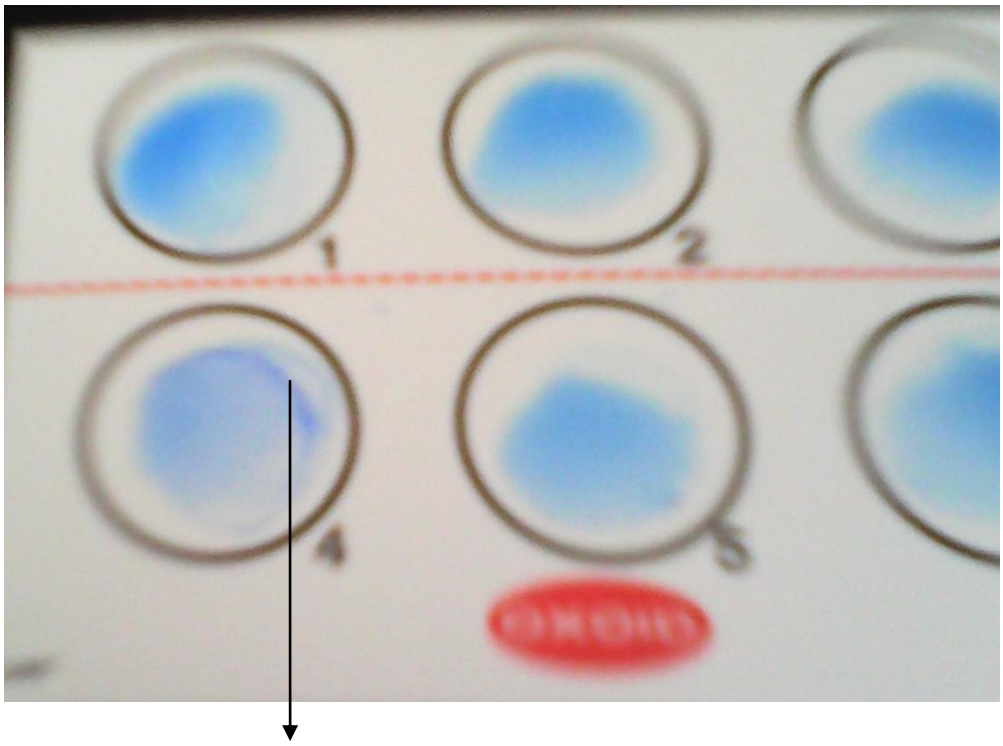
Appendix: VII. Microscopic view of chain-like cocci of *Streptococcus* species X 100 mannification



Appendix Vlll. *Streptococcus* colonies on blood agar plate

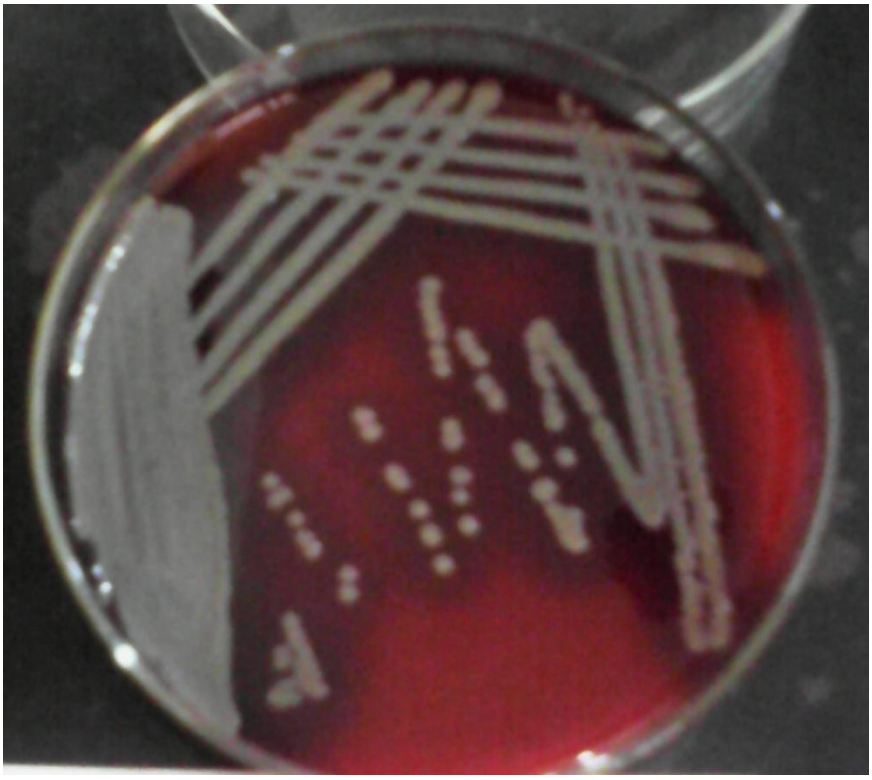


Appendix lX. Hydrolysis of esculin by *Streptococcus* species. Blacking of bacteria colonies

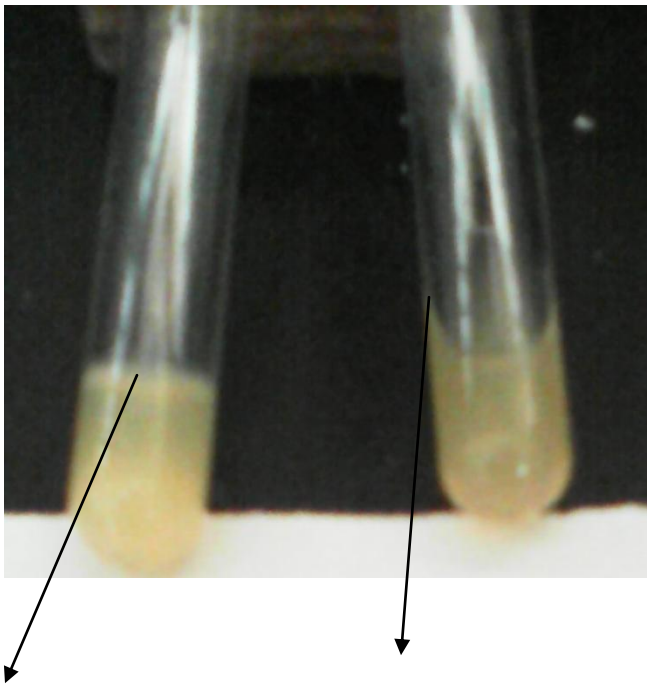


Appendix X. *Streptococcus* serotyping, Positive test reaction; Showing aggulutination granules formation

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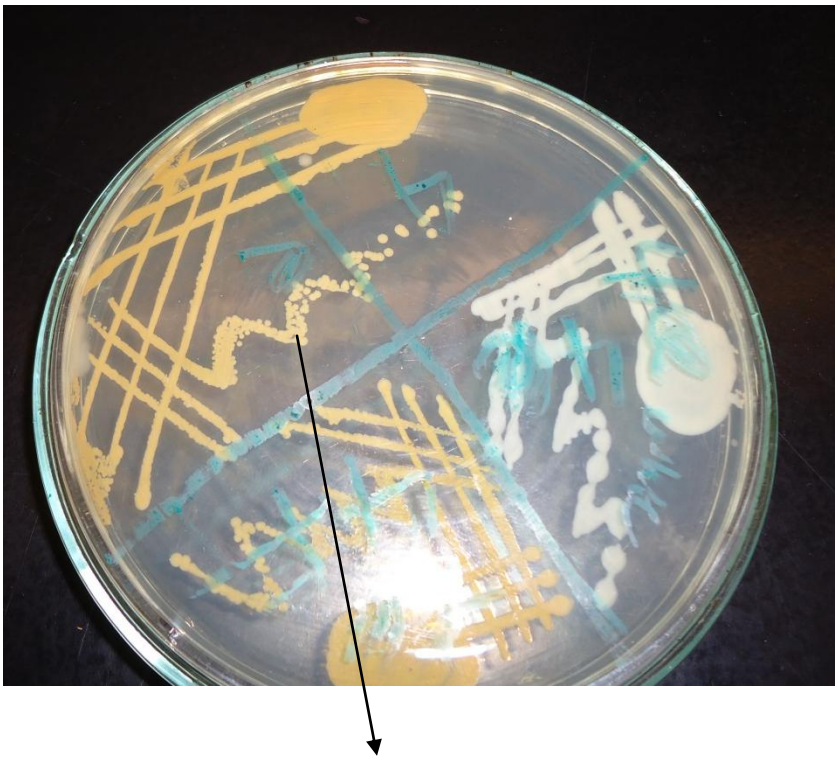


Appendix XI *Staphylococcus.*β haemolysis on blood agar



(a) Coagulase positive **(b) Coagulase negative**

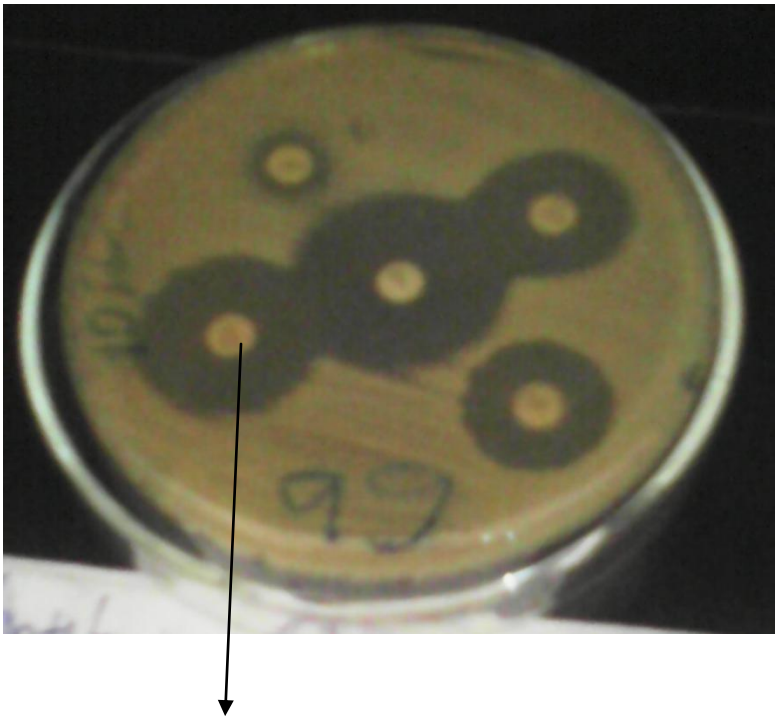
Appendix XIl. Coagulase test for *Staphylococcus* species a and b



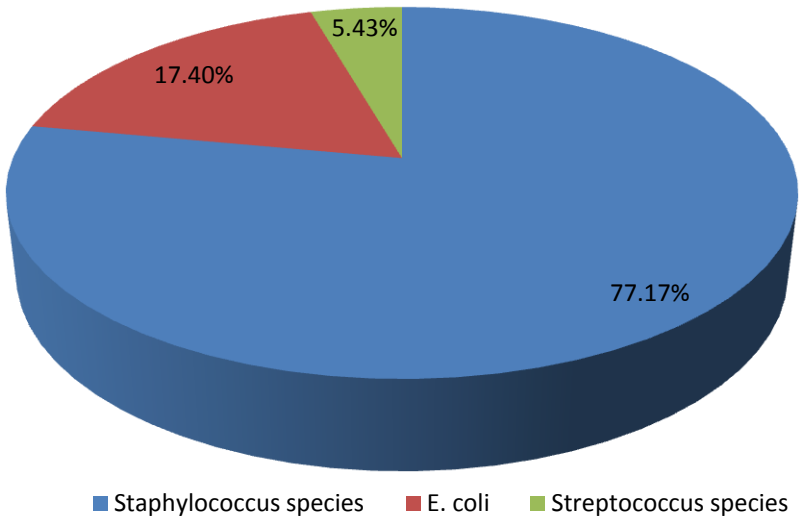
Appendix XIll. DNase activity of *Staphylococcus* Species positive test clearing round the colonies



Appendix XIV. Microbact test reaction after 24 hours incubation; sugars (mannitol, mannose, maltose, trehalose, sucrose and N-acetyl glucosamine, Arginine utilization, urease test, Beta-glucosidase, alkaline phosphatase, Beta-glucuronidase, Beta-galactosidase



Appendix XV.Antibiotic sensitivity test. Using different antibiotics (AMP, AMC, CH, TE, CN, VAN, PEN, SXT, CEF and CEP)



Appendix XVI.**Occurance of Bacteria Isolates in Bovine Mastitis Quarter Milk Samples in Zaria**