PREVALENCE OF *Staphylococccus aureus* ISOLATED FROM BOVINE MASTITIS MILK IN ZARIA

BY

OKUNADE, EUNICE ADENIKE(B.Sc. ABU 2004)
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DECLARATION

I declare that the work in the dissertation entitled 'PREVALENCE OF *Staphylococccus aureus* ISOLATED FROM BOVINE MASTITIS MILK IN ZARIA' has been performed by me in the Department of Microbiology under the supervision of Prof. (Mrs) V.J. Umoh, Dr. C.M.Z. Whong and Dr. J. Kabir. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was presented for another degree or diploma in any University.

Okunade, Eunice Adenike _____ _____

Signature Date

CERTIFICATION

This dissertation entitled 'PREVALENCE OF *Staphylococccus aureus* ISOLATED FROM BOVINE MASTITIS MILK IN ZARIA' by Okunade Eunice Adenike meets the regulations governing the award of the degree or Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

_____ Date _____
Prof. (Mrs) V.J. Umoh
Major, Supervisory committee

_____ Date _____
Dr. C.M.Z. Whong
Member, Supervisory committee

_____ Date_____
Dr. J. Kabir
Member, Supervisory committee

_____ Date _____
Dr. S. O. Olonitola
HOD.Dept of Microbiology

_____ Date _____
Prof. S.A. Nkom
Dean, Postgraduate School

DEDICATION

TO THE UNSEEN HAND

THAT BOUND MY MUSCLES

LINKED MY BONES

PROGRAMMED ME IN CHROMOSOME

THEN BUILT A DAM ACROSS MY WILL

LETTING OUT A PENT-UP POWER

THAT HAS HELPED ME LIGHT MY PART

THROUGH MUCH DARKNESS TO GREATNESS

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ABSTRACT

This study investigates the prevalence of *S. aureus* isolated from bovine mastitic milk in Zaria. The samples (n =350 quarters) were randomly collected from 91 lactating cows in 20 herds in Sabon-pegi, Shika, Sabon gari, Wusasa, Nuba, Bomo and Hanwa and 20% of cows in each herds were sampled. The physicochemical quality, prevalence of subclinical mastitis using California Mastitis Test (CMT) and bacteriological quality of the milk samples were determined using standard methods. All the milk samples (100%) had no clots, were colourless and consistent.The specific gravity and pH of the milk samples had ranges of 1.023-1.033 and 3.11-6.99 respectively. The prevalence of subclinical mastitis was 75 (21.4%). The prevalence of *S. aureus* and coagulase negative staphylococci (CNS) in subclinical mastitis were 11 (14.7%) and 16 (21.3%) respectively, and in 350 quarter milk samples were 28 (8%) and 41 (11.7%) respectively. There was a statistical significant difference ($P < 0.05$) in *S. aureus* isolated in the quarter milk samples that were CMT positive than those that were CMT negative. There was also significant association ($P < 0.05$) between the CMT positive milk samples and specific gravity, and between *S. aureus* isolated and specific gravity of milk. The means ± standard deviation of bacterial isolates of the bulk milk samples and their products ranged from 1.02±1.40 $\log_{10}$ cfu/ml - 2.05±1.05 $\log_{10}$cfu/ml and 1.13±1.77 $\log_{10}$cfu/ml – 1.40±1.62 $\log_{10}$cfu/ml respectively and were within the acceptable microbial limit of $<10^2$ cfu/ml for *S. aureus* and $\leq 5.0 \times 10^2$ cfu/ml for bacterial growth. Out of the 17 bulk milk sampled, 1 (5.9%) of the bulk milk sample had antibiotic residues. *S. aureus* isolates in this study showed high susceptibility (≥80% each) to all the antimicrobial agents [amoxicillin (25µg), augumentin (30µg), chloramphenicol (30µg), cloxacillin (5µg), cotrimazole (25µg),

erythromycin (5µg), gentamycin (10µg) and tetracyclin (10µg)] used. The prevalence of mastitis was high as to cause a substantial economic loss to farmers. In conclusion, microbiological and epidemiological surveillance is necessary for effective treatment and control of the disease. Hence, prevention and control of ssmastitis needs to be instituted.

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Chapter 1

1.0 INTRODUCTION

Milk is defined as that fresh, clean and normal mammary secretion obtained by milking cows. Although several types of milk are known (e.g. human milk, donkey milk, goat milk etc), cow milk is of primary concern in this study. Milk constitutes a complete food, as it contains proteins, fats, lactose and other sugars, minerals and vitamins together with natural enzymes derived from milk microorganism (Olugbemi, 2002).

Mastitis is a common disease in dairy herds in many different countries and has been reported as one of the disease condition prevalent in dairy farms in Nigeria (Ameh *et al*., 1999). Mastitis is an inflammation of the mammary gland of dairy cows accompanied by physical, chemical, pathological and bacteriological changes in milk and glandular tissues (Hurley and Morrin, 1997 and Oliveirra *et al.,* 2000). It is characterized with cardinal signs of reddening, swelling and warm to touch and pains ons palpation. Mastitis causes important economic losses ranging from low milk yield, reduced milk quality to higher production cost (Miller *et al*., 1993, Wilson *et al*., 2004).

Mastitis may be caused by a large variety of bacteria pathogens and other microbes entering the gland through the teat duct (Shitandi, *et al*., 2004). The primary cause of mastitis in cattle, goats and sheep are well recognized group of microorganisms *Streptococcus* spp. *Staphylococcus* spp. *Pasteurella* spp; and Coliforms (*Escherichia coli*, *Enterobacter* spp; and *Klebsiella* spp.)

One of the most common types of chronic mastitis is caused by *Staphylococcus aureus* (Jones *et al*., 1998). *Staphylococcus aureus* is a major pathogen of bovine mastitis worldwide. Despite implementing intensive control measure, it is difficult to eradicate,

the intramammary infection caused by this pathogen persists and cause substantial source of economic loss (Guller *et al*., 2005). It has been reported by Lafi *et al*. (1994) that *Staphylococcus aureus* occurred predominantly in both clinical and sub clinical bovine mastitis.

The predominance of *Staphylococcus aureus*- mastitis in cow has been reported (Olugbemi 2002, Cenci-Goga *et al*., 2003). Adesiyun *et al*. (1987) also reported that *Staphylococcus aureus* is a common cause of bovine mastitis in Zaria. It has been reported that *Staphylococcus aureus* are ubiquitous in the environment and that many mammary gland is considered to be the primary reservoir from which bacteria spread to uninfected quarters during milking (Blood and Radostits, 1989 and Adesiyun, 1995).

Olugbemi (2002) reported an isolation rate of 45.5% for *Staphylococcus aureus* in bovine mastitis in NAPRI Shika while Ameh *et al*. (1999) reported that *Staphylococcus aureus* (34.6%) occurred more frequently in mastitis milk in Maiduguri, Borno State. A prevalence of 34.2% for *Staphylococcus aureus* was reported from small holder dairy herds in Kenya (Shitandi *et al*., 2004).

1.1 Statement of problems

Staphylococcus aureus is ubiquitous in nature and this occurs widely on human and animal surfaces and foldings. It is therefore easy for the organism to be found in the vesicles of the breast thereby gaining entrance to milk and milk products. Its status as a true pathogen is of public health significance. It produces toxins that destroy cell membrane and can directly damage milk-producing tissues which can lead to bovine mastitis (Jones *et al*., 1998). The high prevalence of intramammary *Staphylococcus aureus* infection indicates the potential of a food hazard problem.

1.2 Justification

Staphylococcus aureus in market milk may present a degree of risk to the consumers because of the organism's capacity to produce enterotoxins, which can lead to food poisoning. Carlos (1990) drew attention to the great public health significance of this organism in milk. The prevalence of *Staphylococcus aureus* in bovine mastitis has not been studied in any depth in Zaria, Nigeria. Therefore there is a need to study the prevalence of *Staphylococcus aureus* in bovine mastitic milk and suggest possible control measures.

1.3 Aim and objectives

1. To assess the quality of milk produced by diary herds in Zaria.
2. To determine the prevalence of mastitis in dairy herds in Zaria.
3. To determine the prevalence of *Staphylococcus aureus* in the milk of dairy herds, and their pattern of susceptibility to commonly used antibiotics.
4. To determine the antimicrobial residues in milk

1.4 Research questions

1. Ho: *Staphylococcus aureus* is not present in bovine mastitis milk

   Ha: *Staphylococcus aureus* is present in bovine mastitis milk
2. Ho: There is no association between the physicochemical quality of the milk and bovine mastitis.

   Ha: There is association between the physicochemical quality of milk and bovine mastitis.

3. Ho: There is no association between occurrence of *Staphylococcus aureus* and mastitis milk.

   Ha: There is association between *Staphylococcus aureus* and mastitis milk.

Chapter 2

2.0 LITERATURE REVIEW

2.1 Mastitis

The term Mastitis is from the Greek word Mastos, for 'breast' and titis, for 'inflammation of'. Mastitis is defined as an inflammation of the mammary gland or udder. The response to injury to the udder is called Inflammation. Mastitis is the reaction of milk-secreting tissues to injury produced by physical force, chemicals introduced into the gland or most commonly from bacteria and their toxins. Mastitis can occur in all mammals, including humans (Hurley and Morrin, 1997 and Oliveirra *et al.,* 2000). Many types of microbes can cause the infection and can be transmitted through both environmental sources (for example, contaminated water, soil, bedding) and from contagious sources (from other infected cows). The microorganism can enter the udder and multiply. The microorganism can enter milk that passes through the udder during milking process. Mastitis in cows (or other dairy animals) can be a source of disease causing bacteria (pathogens) and spoilage organisms in milk.

2.2 Milk and its products

Cow milk, a fresh, clean and normal mammary secretion is a good source of animal proteins, fats, vitamins and minerals to the human body. In addition, nutritionally less useful substances like enzymes are also present in normal milk. Some of the enzymes are used as indices in screening or quality control tests. In many homes in different geographical regions of the world, milk and milk products are fed to infants and form a major component of the diets of adults. Milk is obtained primarily from ruminant

domestic animals especially cows and goats, and it is processed either locally or in factories to derive products like powdered milk, yoghurt, 'nono', cheese.

The nutrient composition of raw milk is excellent and is thus favourable for bacteria growth (Ruuw and Bert, 2004). Results of extensive investigations over many years proved that both human and animal diseases are sometimes spread by milk and milk products. Fermented milk products such as yoghurt and 'nono' contain fermentative non-pathogenic bacteria.

Yoghurt is a cultured dairy product produced by lactic fermentation of milk (Hui, 1992). It is produced by the controlled fermentation of milk by two species of bacteria: *Lactobacillus bulgaris* and *Streptococcus thermophilus*. Any form of milk may be used to make yoghurt, but modern production is dominated by cow milk (Davis, 1974). Yoghurt has practically the same food value as the basic milk product (Ruuw and Bert, 2004). Yoghurt is derived from the Turkish word "Yoghurt mak" which means, "to blend", a reference to how yoghurt is made. Yoghurt was first commercially produced and sold in the United States in 1929 by American Immigrants. Yoghurt is acidic due to lactic acid produced which is effective in inhibiting the growth of bacteria (Pardeson, 1979). It is a perishable drink if not kept in cool place (Ihekoronye and Ngoddy, 1885).

'Nono' is a locally fermented cow milk which when mixed with a cereal dough (fura) forms a major part of the staple food of a good proportion of the populace in northern Nigeria where it is taken as reference drink and weaning food. (Ajala *et al*, 1991).

'Nono' is similar to yoghurt and is usually prepared by Fulani women. It is prepared from 'kindirmo' where by fat is removed from it and large volume of water

(Ajala *et al*, 1991). Eka and Ohaba (1977), have since shown that "nono" a fermented milk sold by Fulani milk vendors was highly contaminated with microorganisms. Also, Umoh in 1989 reported that about 99% of 'fura da nono' a cereal food made from fermented milk were contaminated with staphylococci. The low pH (acidic) of nono being 4.03 – 4.8, as reported by Kornacki and Marth (1982) may limit the survival and growth of pathogens at ambient temperature. However being a product of uncontrolled fermentation, human handling during processing of nono can be a source of bacterial contamination as well as water used for its processing which may also be contaminated. The major source of water used by Fulani milk maids for processing nono is from running streams (Olugbemi, 2002). Fermentation may occur to allow multiplication of pathogens. The obvious need for milk hygiene and thorough milk inspection before human consumption can therefore not be over emphasized, since in certain disease affecting the udder of the host, the milk could act as a vehicle for disease causing agent to be transmitted to the consumer (Connor, 1995).

Mastitis can be very detrimental to a dairy farm's profitability in terms of lost production (Houben *et al*., 1993, Wilson *et al.*, 2004) and treatment cost (Hoblet *et al*., 1991; Miller *et al*., 1993).

2.3 Forms of mastitis

Mastitis occurs in different forms based on the clinical syndrome and symptom which is categorized depending on the severity of the inflammatory response. The symptoms include redness, swelling, heat, pain and loss of function, including decreased milk production, change in milk composition and change in physiochemical properties

(Radostits and Blood 2000). The syndromes include per acute, acute, sub acute and chronic mastitis (Radostits and Blood, 2000).

Sub clinical mastitis: It is the most common form of mastitis. There is no swelling of the udder and no observable abnormalities in the milk. However, special screening tests, such as California Mastitis Test (CMT), Wisconsin Mastitis Test (WMT) and the catalase test are utilized to show changes in the milk. This type of mastitis is referred to as "hidden". It is detected on an estimation of somatic cell counts.

Clinical Mastitis can be severe. Mild clinical mastitis involves abnormality in the milk such as flakes, clots, and a watery or other unusual appearance. A hot or sensitive udder may be slight or absent, however there may be signs of swelling.

Severe clinical mastitis: Involves a hot, hand sensitive udder that is quite painful to the cow. The onset is sudden and the cow may become ill showing signs of fever (28^0-29^0C), rapid pulse, depression, weakness and loss of appetite. When the whole system of the cow is affected, the condition is referred to as acute systemic mastitis or bluebag.

Chronic Mastitis: A persistent udder infection exists most of the time in the sub clinical form and occasionally can develop into the clinical form before returning to the sub clinical. The results are hard lumps in the udder from the "walling off" of bacteria and the forming of connective tissue.

2.4 Aetiologic agents of mastitis

Mastitis begins with the penetration of pathogenic bacteria through the teat canal into the interior of the gland. Survival of the invading bacteria is enhanced by a favorable internal environment of the mammary gland, which results in their multiplication. The products of these bacteria metabolism and growth may irritate the delicate mammary

tissue and induce an inflammatory reaction, manifesting as obvious clinical signs (Radostits and Blood, 2000). Mastitis in dairy cows may be caused by a large number of bacteria pathogen (Grohn *et al*., 2004).

Microorganisms that caused mastitis include:

(a) Gram-positive catalase positive cocci: They are the most frequently isolated organisms from bovine mammary gland from the family, Micrococcaceae, the genus Staphylococcus contains twenty three species and fourteen of which have been isolated from bovine mammary gland (Kloos and Schlerifer, 1975). *Staphylococcus aureus* is the major pathogen of mastitis worldwide (Guller *et al*., 2005).

(b) Gram-positive catalase negative cocci: *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberus* have been reported as the three commonly isolated species of this group.

(c) Gram-positive aerobic and facultative anaerobic bacilli *Corynebacterium bovis* has been considered a minor mammary gland pathogen; others include *Corynebacterium pyogenes*, and *Corynebacterium ulcerans*. *Listeria monocytogens* are infrequently isolated as a cause of bovine mastitis.

(d) Gram-negative facultative anaerobic bacilli: *Enterobacteria* are most often isolated from intramammary infections. Most frequently isolated of this family are *Escherichia coli*, *Enterobacter aerogenans*, *Enterobacter cloacae*, *Klebsiella* sp, *Citrobacter* sp. *Salmonella typhi*, *Salmonella cholerasius* and *Salmonella enteritidis.*

(e) Gram negative aerobic and micro aerophilic bacilli: They include the *Pseudomonas*, *Brucella Campylobacter* and have been isolated from bovine milk.

(f) Mycoplasma: have been isolated from infected udder and the most frequently isolated are the *Mycoplasma bovigenitallium* and *Mycoplasma californicum*.

(g) Yeasts and Fungi: Bovine mastitis due to yeast infection is usually associated with contaminated antibiotic preparation and infusion equipment. Infections by yeast and fungi are usually mild.

2.5 *Staphylococcus aureus* as a major cause of bovine mastitis

S. aureus was discovered in Aberdeen, Scotland in 1880 by the Surgoen Sir Alexander Ogston and first used Staphylococcin in 1881 as a common name to identify the round organism in pus. In 1884, Rosenblach introduced the generic name *Staphylococcus aureus* to identify the cluster forming cocci. It belongs to the family micrococcacea (Ogston, 1984).

2.5.1 Morphology and Identification

Staphylococcus aureus (Literally means "Golden cluster seeds") is a spherical bacterium which appears as grape-like clusters when viewed through a microscope. It is gram-positive non-spore forming, non-motile microorganism that grows readily under aerobic and microaerophillic conditions. They form yellow, large colony on media and often are haemolytic on blood agar. The golden yellow appearance is etymological root of the bacteria's name: ''aureus'' means "golden" in Latin.

Staphylococcus aureus is catalase positive and thus able to convert hydrogen peroxide to water and oxygen. This is what makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. *Staphylococcus aureus* is primarily coagulase positive while most other species are coagulase negative.

The morphology of *Staphylococcus aureus* colonies on media are usually raised round with smooth edges with glistering appearance. The media usually changes from pink to yellowish while *Staphylococcus aureus* colonies usually appear as yellow to deep golden yellow colonies on the medium after 24 hours of incubation at 37^{O}C. It grows at a temperature range of 15- 45^{O} C and sodium chloride (NaCl) concentration as high as 15%. They grow readily on most bacteriological media with Mannitol Salt Agar, and Baird Parker Agar being the media of choice.

2.5.2 Damage caused by staphylococcal Mastitis

The combined effect of intracellular factors and toxins as well as invasive properties of the strain determines the pathogenic capacity of a given strain of *Staphylococcus aureus*. The defense mechanism determines the likelihood of development of staphylococcal infection (Shopsin *et al*., 2000, Li *et al*., 2005).

Staphylococcus aureus bacteria produce toxins that destroy cell membranes and can directly damage milk-producing tissue. White blood cells (leukocytes) are attracted to the area of inflammation where they attempt to fight off the infection. Initially, the bacteria damage the tissue lining the teat and gland cisterns within the quarter. The bacteria, then move up into the duct system and establish deep-seated pockets of infection in the milk secreting cells (alveoli). This is followed by walling-off of bacteria by scar tissue and the formation of abscesses, which is responsible for poor response to antibiotic treatment (Jones *et al*., 1998 and Radostits and Blood, 2000). Alveolar and duct cells may be destroyed and combine with leukocytes and clog the milk ducts that drain the alveolar areas, contributing to further scar tissue formation (Jones *et al*., 1998). The ducts may reopen and release *Staphylococcus aureus* to other areas of the mammary gland. Further

abscess formation occurs. Eventually they become quite large and can be detected as lumps within the udder.

Clinically infected quarter often has moderate swelling and visible signs of garget or chunks of milk, especially in fore strippings. Acute *Staphylococcus aureus* infections generally develop late in the lactation or just prior to calving. However, the clinical symptoms (udder swelling or hardness, changes in appearance of milk) do not show up until calving or early in the next lactation. It becomes difficult to successfully treat an infection because drugs are not able to penetrate to all infection sites and because the bacteria live inside the white blood cells. *Staphylococcus aureus* produce an enzyme that inactivates most penicillin based treatment, resulting in ineffective antibiotics.

2.5.3 Transmission of Staphylococcus aureus infection

The major reservoirs of the organisms are infected udder teat canals, and teat lesions, but these bacteria also have been found on teat skin, muzzles, nostrils and vagina. The bacteria are spread to infected quarters by teat cup liner, milkers' hands, wash cloths and flies. They do not persist on healthy teat skin but readily colonize teat canals if there are lesions near the teat where they multiply and readily enter the udder. Infected heifers at calving may represent the most important reservoir to uninfected herd mates.

2.5.4 Predisposing Factors of Bovine Mastitis

Some factors of predisposition include:

(a) Management factors: Faulty management factors such as bad feeding, bruising, chilling, excessive milking and such other conditions which expose the udder to infections (Smith *et al*., 1985). These may however

depend on the immune status of the animal and the pathogenecity of the infective microorganisms.

(b) Udder and teat canal abnormalities: Pendulous udder due to relaxation or rupture of the udder and teat, and also the dilation of the teat canal due to over-exploitation or sucking habits of calves and to blockages of teat canal increases the chances of mastitis (Addo *et al.*, 1980 and Smith *et al*., 1985). The organism multiplies in infected lesions or colonizes teat canals and can readily enter the udder (Jones *et al*., 1998). It was observed that mastitis seems to increase as the teat canals become wider as the animal ages (Blood and Radostits, 1989).

(c) Age and stage of lactation: Mastitis is more prevalent in older than in young, and also more at the 1st to the 3rd stage of lactation. (Olugbemi 2002). High milk producers are more susceptible than low producers. The dilation of the teat canal, the rate of suckling by young ones, and increased activity of the glandular tissue is responsible for this, thus, increased chances of colonization of mammary tissues by causative agents of mastitis, in this case *Staphylococcus aureus*.

(d) Pathogenecity and invasive ability of causative organisms: Bacteria agent should be able to survive in the cow‘s immediate environment and resist environmental influences which include cleaning and disinfections procedures (Blood and Radostits, 1989), and adhere to the mammary epithelium to set up a mastitis reaction.

(e) Trauma and Skin lesions: Trauma to the mammary gland can result due to friction on the udder from sucking calves, tear, or cuts from sharp objects and rupture of the suspensory lingament (Radostits and Blood, 2000). Viral infections such as cowpox can predispose udder to mastitis through the activity of the secondary invaders.

2.5.5 Prevalence

Surveys of the incidence of mastitis, irrespective of cause, show comparable figures of about 40% morbidity among dairy cows, in most countries.

The predominant position of *Streptococcus agalactiae* as a cause of bovine mastitis has been usurped by *Staphylococcus aureus* especially in areas where the treatment of mastitis with penicillin has been practiced intensively and where machine milking has replaced hand milking (Olugbemi, 2000).

Surveys of the incidence of the disease show varying results. In the United Kingdom, a survey showed that 8.4% of ewe deaths and up to 34% of lamb deaths were due to mastitis (Blood and Radostits, 1989). The prevalence of mastitis in Kenya was assessed by the result of physical evaluation of the mammary gland by palpation, and by bacteriological evaluation of milk secretion. It was found to be 19.6% (n=49) while the prevalence of major pathogens isolated was 34.2% for *Staphylococcus aureus*, 21.1% for CNS, 16.4% for *streptococcus sp.* and 7.3% for *E. coli* (Shitandi *et al.*, 2004).

In an abattoir study of bovine mastitis in indigenous breeds of cattle in Nigeria, the overall incidence of bovine mastitis using macroscopic examination by visual inspection and palpation of udder was 12.30% (Olufemi, 1977).

Olufemi (1977) also reported that the predominant breed was white Fulani accounting for 54.60% of total animal examined, Sokoto Gudali 23.24% while Rahaji (Red bororo) was 8.75% of the total animal examined. The incidence of bovine mastitis in Rahaji (22.22%) was higher than in white Fulani (10.65%). Majority of the affected animals were aged cows (70.18%) i.e. age above 7years while others were matured cows (Age between 3 to 7 years). No mastitis was detected in young cows (Age below 3years).

A predominance of *Staphylococcus aureus* mastitis in cows has been reported (Olugbemi 2002, Cenci-Goga *et al*., 2003). Adesiyun *et al*. (1987) also reported that *Staphylococcus aureus* is a common cause of bovine mastitis in Zaria. It has been reported that *Staphylococcus aureus* are ubiquitous in the environment and that many mammary gland is considered to be the primary reservoir from which bacteria spread to uninfected quarters during milking (Blood an Radostits, 1989 and Adesiyun, 1995). Olugbemi (2002) reported that prevalence of mastitis in NAPRI, Shika was 33.89% and an isolation rate of 45.5% for *Staphylococcus aureus* in bovine mastitis milk. Ameh *et al*. (1999) reported that *Staphylococcus aureus* (34.6%) occurred more frequently in mastitic milk in Maiduguri, Borno State.

2.6 Diagnosis of *Staphylococcus aureus* Mastitis

One of the best ways to detect mastitis is to use the California Mastitis Test (CMT) using a four Compartment paddle using CMT reagents. It is a measure of inflammation within a quarter and can be scored on the basis of gel formation.

Other methods include the physical examination of the udder and milk for clinical signs, and bacteriological test on milk to determine the kinds of microbes present.

Milk that is watery, thick or ropy means an active case of mastitis has progressed enough to be recognized as clinical mastitis. However such clinical cases make up only 20% of mastitis in dairy herds (Robert *et al*., 2006). Infected quarters usually produce normal appearing milk and infections may persist for weeks before the abnormal milk or soreness of the udder is noticed.

2.6.1 *Principle of California Mastitis Test (CMT)*

The animal body defends itself against injury and infection. When infection occurs, white blood cells or leukocytes gather to engulf bacteria and stop the spread of infection. If an injury is sterile, few leukocytes are present, but if infection sets in the white blood cells congregate in great numbers.

Therefore, high leukocyte counts in milk strongly indicate mastitis-causing bacteria are present. The California Mastitis Test (CMT) reagent when added to milk, react with a part of the leukocytes, to form a gel. The greater the mastitis infection, the more leukocytes present and the more gel-like substance that forms.

Table 1 showing how leukocyte count in milk influences the appearance of the test and affects the California Mastitis Test (CMT) score.

Leukocyte count per ml	Test Appearance	CMT Score
Below 200,000	Mixture liquid, no precipitate	Negative or no reaction (Healthy quarters)
150,000 – 500,000	Slight precipitate, tend to disappear with paddle movement	T (trace) or + (Subclinical)
400,000-1,500,000	Distinct precipitate but does not gel with paddle movement	1 (weak positive) or ++ (Subclinical)
800,000-5,000,000	Distinct gel formation	2 Distinct positive or +++ (Subclinical)
Over 5,000,000	Strong gel formation that adhere to paddle. Forms distinct central peak.	3 (strong positive) or ++++ (clinical)

Adapted from California Mastitis Test (CMT) Fact Sheet. International Livestock Institute,Rural Dairy Technology. Addis Abbas, Ethopia (2003).

The purple colour that results form the test is generally more intense in samples from infected quarters. This is because such samples have a basic or alkaline pH. The deep

colour indicates abnormal composition of milk which means the mammary gland is infected.

Limitation of the test

Not all inflammation is caused by bacteria. However, reactions to California Mastitis Test (CMT) are usually weak when caused by bacteria. Several types of bacteria may infect the udder, and the California Mastitis Test (CMT) does not identify the type. The California Mastitis Test (CMT) does not indicate whether treatment will be effective.

2.6.2 Diagnostic test

Unlike the screening which only gives an indication of an ongoing mastitis, the diagnostic test goes a step further to identify the specific organisms responsible for the infection (Adesiyun *et al*., 1987).

The number of bacteria present in a milk sample is of importance. Milk from cows infected with mastitis generally has higher total bacteria counts and somatic cells counts than milk from uninfected cows. Therefore bacteria counts and somatic cell count are used by dairy farmers and milk processors as indicators of milk quality. There are regulatory standards for microbial numbers (total bacteria count) as well as quality control. Infected gland usually yields more than 200 colonies of bacteria per ml of milk, with fewer counts suggests the invasion phase before the infection is established (Blood and Radostits, 1989).

Culturing of milk samples is a standard method of examining mastitis. It may be carried out on individual quarter samples or composite samples including milk from all four quarters (Blood and Radostits, 1989).

2.7 Control of *Staphylococcus aureus* mastitis

When penicillin was introduced in the 1949's, it was assumed that with such effective treatments mastitis would soon be eliminated (Blowey, 1985). This showed not to be the case. Many control methods which is applicable to other diseases are not relevant in mastitis. Eradication of mastitis will not be possible because sources of infections are quite numerous.

The most effective control is to prevent new infections by minimizing or eliminating conditions that contribute to the exposure of teat ends through spread of infections from cow to cow and conditions which allow bacteria to contaminate and penetrate the teat canals.

(a) Hygiene procedures: Rubber gloves should be worn by milkier. Teat disinfection is a good method of preventing bovine mastitis in a herd and this is achieved by teat dipping. If the teats are very dirty, they should be washed with sanitizing solution, using a low volume of water. The teat should be pre-dip with a tested disinfection and allowed to have a contact for about 30 seconds. A separate cloth towel should be used to dry the teats and scrub the teats for 20 seconds (Rasmussen *et al*., 1992). The towel must not be used on more than one cow; teat ends should be examined for chaps, cracks or lesions that harbor mastitis. An effective post-milking teat dip must be used and the teat dip left in the dip cup must be discarded at the end of each milking and the cups rinsed with water after every milking and drying (Jones *et al*., 1998).

(b) Milking of first Lactation Cows: Uninfected First Lactation Cows should be milked before older cows carrying sub clinical mastitis infections, if possible if

the milking herd can be grouped, a separate group of uninfected first lactation cows is recommended, which can be milked first and fed differently. If the first lactation cows cannot be separated from infected or high SCC cows, cows with clinical mastitis, *Staphylococcus aureus* infection and those receiving antibiotics should be milked last or milked with separate milkers or milking unit equipped with back flush to avoid spread of infection via contaminated teat cup liners. (Jones *et al*., 1998). Segregation of *Staphylococcus aureus* infected cows has been proven to significantly reduce the prevalence of *Staphylococcus aureus* mastitis and bulk tank somatic cell counts (Wilson *et al*., 1995).

(c) Antibiotic Treatment of *Staphylococcus aureus* Cows

i) Treatment during Lactation: Treatment may not control this disease but it may shorten the duration of the infection. Treatment effectiveness decreases as cows become older. Sol *et al*. (1997) reported that cures were only 34% when 89 cows in 10 Dutch herds were treated for sub clinical *Staphylococcus aureus* mastitis. The result showed that probability of cure would be low in older cows with high somatic cell count (SCC), infected in hind quarters during early and mid lactation. *Staphylococcus aureus* infections were found in 36% of clinical mastitis cases in Finish's herds (Pyorala and Pyorala, 1997) and only 39% of these responded to treatment. Successful treatment is greater during lactation if detected and treated early and response is lower when treating chronic infection. Martin *et al*. (2004) reported that bacteriological cure rates with anti-bacteria therapy during the dry

lactation are generally poor (certainly worse than during the dry period). He emphasized that research indicates that a cure can be expected in approximately 20-60% of cases. Prolonged antibiotic treatment can be used and are generally reported to give improved bacteriological cure rate than conventional regimes. Tissue damage can be minimized if treated during early stage of infection.

ii) Dry Cow Treatment: Jones *et al*. (1998) reported that administration of specially formulated dry cow treatment will help prevent new *Staphylococcus aureus* infection during the dry period and also will eliminate many existing infections present at dry off. Dry treatment is more effective in eliminating infections than lactating treatment. During the first 2 weeks and the last 7-10 days of dry period, cows are susceptible to becoming infected. When cows are not treated, spontaneous cure have been very low. Dry cow antibiotic treatment is very cost effective (Kirk *et al*., 1997). When drying off a cow, all quarters should be treated with a commercial dry cow product. To dry off, cows must be milked out completely, teats dipped in post-milking teat dip and Blotted dry after 30seconds contact time. Teats should be scrubbled with alcohol pads before particularly inserting tube into teat. After treatment, it should be teat dipped again.

iii) Treatment of pregnant heifers: New infections can be found in many heifers, either at calving or in early lactation. As many as one third of these infections may be caused by *Staphylococcus aureus*. Often these

Staphylococcus aureus infections, if untreated become clinical and recur throughout the first lactation and into the second lactation. Several newly tested procedures can be used on heifers before they calve. Pregnant heifer should not be grouped with dry cows.

Dry cow therapy: Louisiana studies have examined the feasibility of giving antibiotics therapy to heifers (Nickerson *et al*., 1995). A dry cow product containing penicillin and dihyrostreptomycin was administered at the first, second or third trimester of pregnancy in 35 bred heifers from four herds. Although prevalence of infection and somatic cell count (SCC) was reduced by treatment in all three groups of heifers, heifers dry-treated during the second trimester of pregnancy demonstrated greater reduction in mastitis and SCC at calving. It is recommended that heifers can be treated with dry cow treatment at 60days before expected calving date.

Lactation therapy: In Tennessee, a lactation cow antibiotic treatment continuing either cloxacillin or cephapirin was administered to heifers at 7 to 10 days before expected calving (Oliver *et al*., 1992). Mastitis infections were reduced. Cephapirin was reported to give better treatment result but also resulted in some antibiotic residue in milk at 3 days after calving. The residue was not present when heifers were treated at 14 days before expected calving. (Jones *et al*., 1998). It can be recommended that heifers can be treated with lactating cow mastitis treatment at 14 sdays before expected calving.

2.8 Prevention of new *Staphylococcus aureus* infection

To prevent spread of infection, *Staphylococcus aureus* infected cows must be isolated from other cows. This may be the most important preventive practice. (Jones *et al.*, 1998). Infected cows should be marked with unique, visible identification, such as special coloured leg band or neck chains. Possible means of isolation include:

(1) Avoid milking cows and heifers with the same teat cup/ claw unit used in milking cows with mastitis.

(2) Segregate *Staphylococcus aureus* infected cows into one group and milk last. Alternatively, infected cows can be sorted out before each milking and restrained in an isolation pen until all other cows have been milked.

(3) If heifers or cows are purchased, they should be segregated until milk samples can be cultured and their mastitis pathogen status can be determined.

(4) Culling of infected cows, especially those with other problem. Often it is not economically feasible to cull a Staphylococcus infected cow that produces 80-90 lb milk. However, any cow that has had clinical mastitis in the same quarter for three or more occasions or any cow whose milk has been withheld from shipment for more than 28 days during the current lactation should be considered for culling from the herd. It is important to keep mastitis records, including cows and quarters treated and treatment used.

(5) Biting flies traumatize the teat end. Flies also carry a number of mastitis causing organisms that can colonize these teat lesions. Elimination of fly breeding sites is one aspect of fly control. Flies breed in decaying feeds or manure that has accumulated, including exercise yards, calf pens, and box

stalls. Other options include back nibbers, feed additives and ear or tail tags. In one trial where tail tags contained insecticides, only 1 of 100 heifers had a mastitis infection compared to 18 of 100 untagged heifers (Jones *et al*., 1998).

2.9 Antimicrobial susceptibility of *Staphylococcus aureus*

B-Lactam antibiotics are among the most widely used antimicrobial agent for the treatment of bovine mastitis. Many studies from different countries have shown that among *Staphylococcus aureus* strains isolated from bovine mastitis cases, the highest resistance was observed against penicillin, a β-lactam antibiotic, and resistance to other antimicrobial was usually low (Aaestrup *et al*., 1995, Erskine *et al*., 2002, 2004; Vintov *et al*., 2003; Sabour *et al*., 2004 and Guller *et al*., 2005).

Guller *et al*. 2005 reported that in accordance with prevail studies, resistance frequency in his study on was highest (63.3%) against β -lactam antibiotics, penicillin and ampicillin.

However, the frequency of penicillin resistance varies among countries. This may be linked to many factors such as the difference in animal production systems and national policies for the use of antimicrobial drugs in each country. Vintov *et al*., (2003) found penicillin resistance with an average of 32.4% among *Staphylococcus aureus* isolated from bovine mastitis in 9 European countries and the United States. Penicillin resistance was high among the isolates from Ireland (71.4%), the United Kingdom (67.3%) and the United States (50%) where as it was low among the isolates from Scandinavian countries, with 2% in Norway.

Watts and Salmon (1997) also found that penicillin and Ampicilin were most affected by β -lactamase activity, out of the five β -lactam compounds tested. In Nigeria,

Olugbemi (2002) reported the highest resistance (81.1%) of ampicilin to *Staphylococcus aureus* isolates of NAPRI cow milk.

Staphylococcus aureus has developed multidrug resistance in many regions of the world (World Health Organization 2002) although reported prevalence rate indicates that wide variation exists regionally and even from herd to herd (Waage *et al*., 2002). Shitandi and Sternesjoo (2004) reported that in an Argentina study, penicillin resistance was reported to be 30% (Gentilini *et al*., 2000). In Finland more than 50% of the *Staphylococcus aureus* isolates are penicillin resistance (Pitkala *et al*., 2004), which is significantly higher than data reported from other Nordic countries, for examples 30% in Demark, 5% in Norway (NORM/NORM-VET, 2000) and 7% (*Staphylococcus aureus* isolates from acute clinical mastitis) in Sweden (SVARM, 2003). Moroni *et al*., (2006) reported that ampicillin and amoxicillin had very poor invitro activity and 100 and 98.5% of the isolates of *Staphylococcus aureus* were resistant to these respective antimicrobial agents. On the other hand, he reported that cloxacillin and amoxicillin + clavulanate (augumentin) were both highly effective.

Studies have also shown that penicillin-clavulanate, penicillin-novobiocin, erythromycin, pirlimycin demonstrated good activity against *Staphylococcus aureus* strains (De Oliveira *et al*., 2000). All the *Staphylococcus aureus* strains tested in the study of Guller *et al*., (2005) were found susceptible to Oxacillin.

Results of other studies also indicate that methicillin resistance, which is important for human isolates, is not a problem in bovine isolates (De Oliveria *et al*., 2000; Gentilini *et al*., 2000; Calvinho *et al*., 2002; Erskine *et al*., 2004).

Overuse and misuse of antibacterial agents have been incriminated as the major selective forces encouraging the development of resistance in bacteria (WHO, 2002). Several factors other than antimicrobial usage can influence the overall susceptibility pattern of mastitis pathogens. Scar tissue in the udders of cattle chronically infected by *Staphylococcus aureus* often prevents the penetration of antimicrobial agent (De Oliveria *et al*., 2000).

Chapter 3

3.0 METHODOLOGY

3.1 Animal sampling

3.1.1 Sampling location and selection of herds

The method of Mahajan (1997) was used to estimate the sample size. Twenty dairy herds managed under semi intensive to free range system were sampled in 7 predetermined regions in Zaria namely Bomo, Hanwa, Nuba, Sabon Gari, Sabon Pegi, Shika and Wusasa during the periods of March to May, 2007. The consent of the herds men were sought prior to sample collection. Twenty percent of cows from each herd were sampled. Quarter milk samples were collected from herdsmen that were willing to allow milk samples to be collected.

3.1.2 Sampling procedures

Prior to quarter sampling, the teat ends were cleaned with absorbent cotton wool moistened in 70% alcohol. Approximately 5ml of fore milk was collected into sterile universal bottles. The sample bottles were properly labeled and transported in ice to the laboratory for analysis.

3.2 Assessment of physicochemical parameters of milk

The milk samples were checked for clots, discoloration or consistency. The temperature, pH and specific gravity of the milk samples were also determined with parts of the milk. Temperature was determined using a clean thermometer, pH, using a pH meter (HANNA HI 991000) and specific gravity using a lactometer.

3.3 California Mastitis Test

Fore milk was drawn from each quarter of the udder into separate cups of a four-cup CMT paddle. The paddle was tilted to equalize milk quantities in the cup at about half teaspoon, each half teaspoon of the test reagent was added to each cup, the paddle was rotated to mix, changes in colour and gel formation within 10 to 15 seconds after mixing was observed. Reactions were graded according to California Mastitis Test (CMT) Fact Sheet, International Livestock Institute, Rural Dairy Technology. Addis Abbas, Ethopia.

3.4 Bacteriological analysis of milk

3.4.1 Bacterial counts of bulk milk and dairy products

Immediately after delivery of the milk sample to the laboratory, the milk samples were inoculated on Mannitol Salt Agar (MSA) (Oxoid UK) plates. For the bulk milk and milk product (nono, yoghurt, kindirimo), a ten fold dilution was conducted aseptically. A dilution of $10^{-1,}$ 10^{-2} and 10^{-3} were surfaced plate on MSA in duplicate plate for staphylococcal count. All the plates were incubated at 35±1^{o}C for 24 hours, after which colonies from bulk milk and milk product were counted.

3.4.2 Enumeration and Identification of Staphylococcus aureus

For quarter milk samples, a sterile wire loop was used to streak the milk samples on MSA plate and was incubated at 35±1^{o}C for 24 hours. Cultures were examined for morphological, staining and cultural characteristics. Pure cultures that were gram positive cocci were stored at 4^{o}C for further biochemical characterization.

3.4.2.1 Gram Staining

A fixed smear of the bacteria isolate was made on a clean glass slide using a sterilized wire loop. Crystal violet solution was added to the fixed smear for one minute and rinsed with clean water. Gram's iodine was added for thirty seconds and was thereafter decolourized with acetone and rinsed immediately with water. Neutral red was added for one minute and rinsed with water. The slide was allowed to dry and was then viewed under the microscope.

3.4.2.2 Coagulase test

The slide coagulase test method was used. A loopful of normal saline was placed on a clean glass slide and emulsified with bacteria to give a homogenous suspension. A drop of oxalated rabbit plasma was added and mixed for five seconds. Positive coagulase was indicated by agglutination which did not re-emulsify.

3.4.2.3 Catalase test

A loopful of bacterial broth was placed on a clean glass slide. A loopful of hydrogen peroxide was added. A positive catalase test was indicated by an effervescence caused by liberation of oxygen.

3.4.2.4 Haemolysis

Pure culture was streaked on blood agar (Oxoid UK) to observe the ability of the organism to produce haemolysin. Positive heamolysis was indicated by clear zone.

3.4.2.5 DNase

Pure culture was streaked on DNase agar (Oxoid UK). After 24 hours of incubation, 10% hydrochloric acid was used to flood the surface of the agar. A positive result was indicated by a clear zone.

3.5 Detrmination of antimicrobial drug residues in bulk milk samples

A microbial commercial inhibition test kit DOLVOTEST® SP MINI (Eagleville, USA) was used to test milk samples for the presence of antimicrobial drug residues. The required number of ampoules were cut off and marked with the milk identification code. A hole was carefully punched in the alumilium foil of the ampoule using the blunt end of a syringe. One nutrient Tablet was placed in each ampoule using a plastic forceps and 0.1ml of each milk sample to be tested was pipette into the corresponding labeled ampoules. The ampoules were incubated at the same time in a pre warmed water bath (Grant Instruments (Cambridge) Ltd, England), at $64\pm1^{O}C$ for 3hours. After the incubation the colour change was determined using the colour chart provided in the test kit. The set up was further incubated for another 3 hours and the colour change was observed.

3.6 Antimicrobial susceptibility test

This was carried out as described by the methods of Bauer- Kirby as standardized by the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2002a). Each isolate was standardized using McFarland standard No. 0.05 and inoculated on Mueller Hinton Agar (Oxoid, Basongstoke, UK) plate. Gram positive multiple antibiotic disc (Abtek, London) consisting of amoxicillin (25µg), augumentin (30µg), chloramphenicol (30µg), cloxacillin (5µg), cotrimazole (25µg), erythromycin (5µg), gentamycin (10µg) and tetracyclin (10µg) was placed on the surface of the medium using a sterile forcep. The plate was incubated at $37^{O}C$ for 24 hours, after which the diameter of each zone of inhibition was measured using a graduated ruler. A controlled strain (*Staphylococcus aureus* ATCC 29213) was also used. Antibiotic susceptibility was reported as either

sensitive or resistant according to the standard diameter of zones of inhibition adapted from CLSI, (2002a)

Chapter 4

4.0 RESULTS

A total of 91cows (350 quarters, 20 dairy herds) were examined of which 14 cows had lost a quarter each due to genetic defects. Majority of the herds sampled 5(25%) were from Bomo, although the greater percentage 23(25.3%) of cows sampled and quarters 87(24.9%) were from Sabon pegi. Out of all the locations visited, only one herd in Nuba (kakiye village) had no defects in the quarters (Table 2). Out of the 350 quarters sampled, 75 quarters had milk that tested positive for CMT and a total of 69 *Staphylococcus* were isolated of which 28 were biochemically identified to be *Staphylococcus aureus*. Likewise, the highest percent 13(31.7) of CNS was observed in Sabon pegi with the least percent 1(2.4%) in Shika (NAPRI dairy herd).

4.1 Result of physicochemical characteristics

All the milk samples had no clots, were colourless and consistent. Table 3 shows the frequency distribution of bacteria isolates based on physicochemical properties of milk. Sixteen *S. aureus* isolates were from milk sample with specific gravity ranging from 1.023-1.027, while the remaining *S.aureus* (n=12) were from samples with specific gravity ranging from 1.028-1.032 (standard for good quality milk). Twelve isolates of CNS were from milk sample with specific gravity ranging from 1.023-1.027. Sixteen *S. aureus* isolates and 12 CNS were from milk samples with specific gravity ranging from 1.023-1.027 while 12 *S. aureus* and 29 CNS were from milk samples with specific gravity ranging from 1.028-1.032. Fifty three samples with specific gravity range of 1.023-1.027 and 227 samples with range of 1.029-1.032 had no microorganism isolated from them. Only 1 sample had specific gravity of 1.033 and no microorganism isolated

from it. Isolates of *Staphylococcus aureus* were more prevalent in milk with specific gravity below 1.028 and are statistically associated ($x^2 = 17.76$, $P<0.05$) (Appendix I).

Out of the twenty-eight *Staphylococcus aureus* isolated, 14 were from milk samples with pH below 6.4, 12 from samples with pH between 6.4 and 6.7(standard for good quality milk), and 2 from samples with pH above 6.7. Out of the 41 CNS obtained, 8 were from milk samples with pH below 6.4, 23 from those with pH between 6.4 and 6.7, and 10 from samples with pH above 6.7. A slightly higher percentage (50%) of *Staphylococcus aureus* was isolated from milk samples with pH range below 6.4. There was no statistical association ($x^2=1.2$, P□0.05) between the presence of *Staphylococcus aureus* and pH of milk samples (Appendix I).

Table 4 shows the CMT scores of milk sampled according to their physiochemical properties. Out of the 275 milk samples that were negative for CMT, 33 had specific gravity with range below the acceptable range of 28-32.0, 241 samples had specific gravity above the range. Out of the 75 samples that were CMT positive, 48 had specific gravity with range below the acceptable range. Higher percentage (62.7%) of CMT positive milk samples had specific gravity below 1.028 and the result of statistical analysis shows a significance association ($x^2= 81.03$, $P<0.05$) between CMT reaction and specific gravity of milk (Appendix I).

Eighty-one samples with pH below 6.4, 154 samples with pH between 6.4-6.7 and 40 samples with pH above 6.7 were CMT negative. Thirteen samples with pH below 6.4, 38 samples with pH between 6.4-6.7, and 24 samples with pH above 6.7 were CMT positive. A slightly higher percentage (51%) of CMT positive milk samples had pH

range of 6.4-6.7 and there was no significant association (x^2= 0.47, P□0.05) between CMT reaction and pH of milk (Appendix I).

The temperature of all the milk samples ranged from 23 - 37^{O}C. All the bacterial isolates were from samples with temperature range of 29-34 ^{O}C, of which all the *S. aureus* isolates were from samples with the temperature range of 30-32 ^{O}C. All the samples that were CMT positive had temperature range of 27-34 ^{O}C.

4.2 Result of CMT reaction and evaluation of quarters for *S. aureus*

Out of the 75 (21.4%) quarter milk samples that were CMT- positive, only 11 (14.7%) contained isolates of S. *aureus* and 16 (21.3%) contained coagulase negative staphylococci (CNS). The prevalence of *S. aureus* and CNS in the 350 milk samples was 28 (8%) and 41 (11.7%) respectively. Based on the analysis carried out on the data obtained in the course of the study, the prevalence of *Staphylococcus aureus* was higher in CMT positive milk (14.7%) than in CMT negative milk (6.18%), and there was a statistical significant difference (x^2= 4.66, P<0.05) in isolates of *S. aureus* in milk that are CMT positive than in those that are CMT negative (Table 5 and Appendix I).

Table 6 shows the frequency of bacterial isolates and CMT reaction based on anatomic sites of the udder. The number of quarters are right hind, 88(25.1%); left hind, 89(25.4%); right fore, 85(24.3%); left fore, 88(25.1%). Out of the right hind, left hind, right fore and left fore hind udders, 19(21.59%), 19(21,59%), 21(24.7%) and 16(18.18%) were positive for CMT respectively. Six *Staphylococcus aureus and* 9 *Staphylococci* were isolated from the right hind udder, 10 *Staphylococcus aureus* and 11 *Staphylococci* from left hind udder, 6 *Staphylococcus aureus* and 10 *Staphylococcus sp.* from right fore udder, and 6 *Staphylococcus aureus* and 11 *Staphylococci* from the left fore udder.

Although the right fore udder had higher prevalence of positive CMT result, there was no statistical significant association (x^2 =1.1, P>0.05) between anatomic site of the udder and CMT reaction. However, *Staphylococcus aureus* was more prevalent (35.71%) in the milk samples of the left hind udder but there was no statistical difference (x^2= 1.7, P>0.05) between the *Staphylococcus aureus* isolated and the anatomic site of the udders (Appendix I).

4.3 Result of bacterial counts of bulk milk and dairy products

Table 7 shows the total *Staphylococcus aureus* count of bulk milk and product samples obtained from different locations. Bulk milk from sabon pegi had a mean of 2.05±1.05 cfu/ml, Hanwa, 1.80±2.50 cfu/ml, Sabo, 1.95±2.80 cfu/ml, Bomo, 1.02±1.40 cfu/ml, Nuba, 1.37±1.19 cfu/ml, Shika, 2.40 cfu/ml and Wusasa 1.07±1.85 cfu/ml.

Milk product (nono) obtained from Sabon pegi, Nuba and Wusasa had means of 1.40±1.62 cfu/ml,1.30±1.15 cfu/ml, and 1.13±1.77 cfu/ml respectively. Nono obtained from the other herds had no growth. Yoghurt obtained from Sabon pegi and Shika had no growth or isolation of microorganism.

4.4 Result of determination of antimicrobial drug residues in bulk milk samples

Table 8 shows the result of drug residues in milk. Out of all the 17 bulk milk samples obtained for this experiment, only one (5.9%) tested positive. After 3 hours incubation of samples in the water bath at 64±1^{o}C, all the samples turned completely yellow except sample HW6 (bulk milk obtained from herd 6 in Hanwa) which remained purple. After additional 3 hours, HW6 was partially yellow. Hence only one of the bulk milk from Hanwa tested positive.

4.5 Result of antimicrobial susceptibility testing

Antimicrobial susceptibility test result of 20 *Staphylococcus aureus strains* are shown in Table 9. A total of 14(70%) *Staphylococcus aureus* strains were susceptible to all antimicrobial agents tested. Four (20%) strains were resistant to cotrimazole, 2(10%) to cloxacillin. Only one (5%) each was resistant to amoxycillin, chloramphenicol and augumentin. No resistance was detected for erythromycin and gentamycin.

Details of antimicrobial resistant pattern of *Staphylococcus aureus* strains are shown in Table 4.8. One (5%) isolate each showed multidrug resistance to amoxycillin/tetracyclin, cloxacillin/cotrimazole, and cloxacillin/cotrimazole/augumentin. Two isolates (10%) were resistant to only cotrimazole and 1(5%) to chloraphenicol, 14 (70%) was resistant to none of the antibiotics.

Table 2. Frequency of bacteria isolates with reference to the dairy herds

Location	nH	nC	nQ	nQL	nSA	nCNS	Total BI
Bomo	5	21	82	2	0	8	8
Hanwa	2	10	37	3	0	2	2
Nuba	3	11	44	0	0	5	5
Sabo	2	10	39	1	0	8	8
Sabonpegi	4	23	87	5	25	13	38
Shika	1	5	18	2	2	1	3
Wusasa	3	11	43	1	1	4	5
Total	20	91	350	14	28	41	69

KEY:

nH- No. of herds

nC- No. of cows

nQ- No. of quarters sampled

nSA- No. of *Staphylococcus aureus* isolate

nCNS- No. of CNS

Total BI- Total bacterial isolates

Table 3 Frequency of bacteria isolate according to physiochemical properties of milk

	Specific gravity			pH		
Isolate	1	2	3	1	2	3
S.aureus	16	12	0	14	12	2
CNS	12	29	0	8	23	10
No growth	53	227	1	72	157	52
Total	81	268	1	94	192	64

KEY

Specific gravity range used
Below 1.028 – 1 1.028 – 1.032 – 2 Above 1.032 – 3

pH range used
Below 6.4 – 1 6.4-6.7 – 2 Above 6.4 – 3

Table 4 CMT scores of milk according to physiochemical characteristics of milk

	Specific gravity			pH		
CMT	1	2	3	1	2	3
-	33	241	1	81	154	40
+	48	27	0	13	38	24
Total	81	268	1	94	192	64

KEY:

- Negative

+ Positive

Specific gravity range used
Below 1.028 – 1 1.028 – 1.032 – 2 Above 1.032 – 3

pH range used
Below 6.4 – 1 6.4-6.7 – 2 Above 6.4 – 3

Table 5 Frequency of bacteria isolates from CMT positive and CMT negative milk.

Isolates	CMT + n (%)	CMT – n (%)	Total n (%)
S.aureus	11(14.7)	17(6.18)	28(8.0)
CNS	16(21.3)	25(9.1)	41(11.7)
No growth	48(64.0)	233(84.7)	281(80.3)
Total	75(21.42)	275(78.58)	350(100)

Table 6 The number of udder with respect to their anatomic sites and CMT reaction.

	CMT reaction		Bacterial isolates			Total
Udder	+	-	*S.aureus*	CNS	No growth	
FR	21	64	6	10	69	85
FL	16	72	6	11	71	88
HR	19	69	6	9	73	88
HL	19	70	10	11	68	89
Total	75	275	28	41	281	350

KEY:

HR- right hind

HL-left hind

FR-right fore

FL-left fore

Table 7 Total *S. aureus* count of bulk milk samples and its products

Location	N	Mean ± standard deviation of log_{10} of counts per ml		
		Bulk milk log_{10} (cfu/ml)	Nono log_{10} (cfu/ml)	Yoghurt log_{10} (cfu/ml)
Bomo	5	1.02±1.40	-	*
Hanwa	2	1.80±2.50	-	*
Nuba	3	1.37±1.19	1.30±1.15	*
Sabo	2	1.95±2.80	-	*
Sabon pegi	4	2.05±1.05	1.40±1.62	-
Shika	1	2.40	-	-
Wusasa	3	1.07±1.85	1.13±1.77	*

Key:

-No growth of bacteria

*No sample was obtained

Table 8 Antimicrobial drug residues in bulk milk

	Bulk milk		
Location	+	-	Total
Sabon-pegi	0	4	4
Hanwa	1	1	2
Sabo	0	2	2
Bomo	0	5	5
Shika	0	1	1
Wusasa	0	3	3
Total	1	16	17

+ Screening positive (presence of antibiotics)

- Screening negative (absence of antibiotics)

TABLE 9 Antimicrobial sensitivity/resistance of 20 isolates of *S. aureus*.

Antimicrobial drug	Sensitive strain (%)	Resistant strain (%)
Amoxycillin	19(95%)	1(5%)
Augumentin	19(95%)	1(5%)
Chloramphenicol	19(95%)	1(5%)
Cloxacillin	18(90%)	2(10%)
Cotrimazole	16(80%)	4(20%)
Erythromycin	20(100%)	0
Gentamycin	20(100%)	0
Tetracyclin	19(95%)	1(5%)

Table 10 Multiple antimicrobial resistance pattern of the 20 isolates of *S. aureus*

Resistance pattern	Isolates	%
Cotrimazole	2	10
Chloramphenicol	1	5
Amoxicillin & tetracycline	1	5
Cloxacillin & cotrimazole	1	5
Cloxacillin, cotrimazole & augumentin	1	5
Sensitive to all antibiotics	14	70
Resistant to all antibiotics	0	0

CHAPTER 5

5.0 DISCUSSION

The findings in this research revealed that out of the 350 quarter samples assayed, 75 quarter samples were positive by the CMT indicating a prevalence of 21.4%. In a similar study, Umoh *et al.* (1990a) had a 31% CMT positive samples from settled herds around Zaria, which was slightly higher than the 21.4% obtained in this study. The slight difference between this study and Umoh *et al.* (1990a) could be in part attributed to the higher number of Fulani herds in this study that practice free range and hand milking method. In this research, cows were hand milked which could exert little or no force on the udder tissues, but in settled herds, machine used for milking may exert direct trauma on the teat making it more susceptible to bacterial invasion and mastitis as found in the study of settled herds by Umoh *et al*. (1990a).

Mastitis is usually caused by a large number of bacteria (Grohn *et al.,* 2004). Guller *et al.* (2005) reported that *S. aureus* is a major pathogen of mastitis world wide. In this study, *Staphylococcus spp.* was isolated from 27 milk quarters of the 75 CMT positive quarters representing 36%. Out of this number, 11 were *S. aureus* i.e. 14.7%. This did not agree with the work of Umoh *et al.* (1990a) who isolated staphylococci in all the CMT positive quarters from settled herds but agrees with the same work that isolated staphylococci in 58 (62.4%) CMT-positive samples in nomadic herds. The reason for this difference could be due to confinement of the cows in settled herds which predisposes them to greater source of infection such as the dirty and wet beddings, vectors like flies, suckling by young cows for longer periods of time, unlike in this study where cows move from one place to another in search of green pasture thereby reducing the risk of

infection. However Olugbemi (2002) reported an isolation rate of 45.5% for *S. aureus* in subclinical mastitis. In this study, *S. aureus* isolation of 14.7% agrees with the work of Pitkala *et al.* (2004) and Myllys *et al.* (1999) who reported an isolation rate of 10.2% and 11.4% for *S. aureus* respectively.

On the other hand, 275 quarter samples were CMT negative representing 78.58%. Out of this number, *Staphylococcus spp* was isolated from 42 samples representing 15.28% out of which *S. aureus* accounted for 6.18%. The isolation of staphylococci from CMT negative milk samples in this present study was in agreement with previous observations, which indicates that mastitis-causing bacterial pathogens can be isolated from clinically normal ruminant mammary glands (Ameh *et al.,* 1993; Egwu *et al*., 1994). However, *S. aureus* was isolated more from CMT positive milk than in CMT negative milk sample ($P<0.05$). This agrees with the works of Olugbemi (2002) and Pitkala *et al.* (2004) who reported a higher isolation rate for *S. aureus* in subclinical mastitis milk. The higher isolation of *S. aureus* may be because the organism is normally found in milk and can persist well in mammary gland causing a low grade infection. Also, in disease situation, there is decrease in epithelial immunity which allows commensals to increase in number.

In relation to the distribution of pathogens associated with mastitis, several workers have established the presence of staphylococci (Wilson *et al*., 1997; Schalbaum, 1999; Poelerends *et al*., 2001). *S. aureus* was the most common cause of sub clinical mastitis in the Netherlands and Switzerland (Schallbaum, 1999 and Poelarends *et al*., 2001) as well as Kenya in the reports of Shitandi *et al.* (2004). The finding was similar to reports of earlier investigations in other regions of Kenya (Munyua *et al*., 1989, Odongo

and Ambani, 1989; Omore *et al*., 1996) and Tanzania (Shekimweri *et al*., 1998) indicative of the frequency of *S. aureus* mastitis in the small dairy herds.

Although subclinical mastitis and *S. aureus* was slightly higher in terms of absolute frequencies, in the right fore udder and the left hind udder respectively, there was no significant difference (P□0.05) in the number of quarters affected and isolates of *S. aureus* in relation to their anatomical position. The insignificant difference may be due to rapid spread of infection from infected quarters to uninfected quarters during milking or suckling. This is in agreement with the work of Ameh *et al.* (1999) and Shitandi *et al*. (2004) who reported that the number of cases found in fore- and hindquarters showed no statistical difference. However, Kalra and Dhanda (1964) found in their survey that hindquarters were more frequently affected with mastitis, and that this difference was statistically significant.

It has been reported that *S. aureus* are ubiquitous in the environment and that many mammary gland is considered to be the primary reservoir from which bacteria spread to uninfected quarters during milking and suckling (Blood and Radostits, 1989; Adesiyun, 1995; Jerry, 1999). This study therefore did not establish any relationship between subclinical mastitis and anatomic site of the udder; it is not unlikely that under Nigerian diary production system, mastitis or infections could easily spread from quarter to other quarters through milking (milkier' hands or milking equipment) and suckling by the young cows. Fly control may help reduce transmission of *S. aureus* to prepartum heifers. The study of Palmer *et al*., (1941) suggested that flies may have been responsible for the spread of *S. aureus,* as the vectors were observed on the teats of heifers at pastures.

In this study, a very high percentage of CMT positive milk samples had the specific gravity range of 1.028-1.0.032 which is in agreement with an earlier report that states this range as the acceptable standard (www.fao.org/AG/aGa/AGAP/ Dairyman/ Dairy/Viu/p/htm). In this study, 36% of the mastitis milk had the specific gravity within this range; thus, higher percentage (64%) of mastitis milk had specific gravity below this acceptable range. This agrees with the work of Ahmad *et al.* (2005) that the severity of mastitis is significantly associated with lowered specific gravity of milk. Haggag et al. (1991) also pointed out that specific gravity decreases with severity of mastitis and may be attributed to increase in chloride and decrease in lactose contents as specific gravity is positively correlated with lactose and negatively correlated with chloride contents. Specific gravity is a measure of the quality of a milk sample; a lower specific gravity indicates an adulteration of milk and low quality of milk.

Fresh milk has a pH 6.7 and therefore slightly acidic. Fresh cow milk has a pH of between 6.7 and 6.5. Values higher than 6.7 denote mastitic milk and values below pH 6.5 denote the presence of colostrums or bacteria deterioration (www.indiaagronet /DIARYCONTENT/Milk% 20properties.htm). pH lower than 6.5 therefore indicates that considerable acid development has taken place. This is normally due to bacterial activity (www.ilri.org/inforserv/webpub/Fulldocs/ILCA_ manual4/ Milk chemistry.htm).

In this study, a higher percentage 154 (56%) of CMT negative milk samples had an acceptable pH range between 6.4- 6.7. This indicates the freshness of the milk samples which had not permit the growth of microorganism. A slightly higher percentage 38 (51%) of CMT positive milk samples had an acceptable pH range. Out of the remaining 37 (49%) CMT positive milk that had pH range that are not within the acceptable range,

24 (64.8%) had pH above the acceptable range. The possible reason for this may be because the high pH (alkaline) may support the growth of *S. aureus,* an aetiologic agent of mastitis. There was no significant association (P□0.05) between CMT reaction and pH. This agrees with the work of Ahmad *et al.* (2005) who indicated that pH increased due to mastitis but disagree with the same work that established a statistical significant difference in pH due to severity of mastitis.

On the other hand, a slightly higher percentage 35 (51%) of *Staphylococcus* spp. were from samples with an acceptable pH range, out of which 12(34%) were *S. aureus* , although a slightly higher percentage 16 (57%) of the *S. aureus* were from samples that had pH range that are not acceptable. Isolation of *Staphylococcus* spp from milk samples that had acceptable pH range could be due to introduction of staphylococci or CNS (a normal human flora) during milking and transportation of samples to the laboratory. Charjan *et al.* (2000) reported that milk pH could serve as the best indicator to assess the condition of udder health in dairy animals, and pointed that the probable reason of pH increase due to severity of mastitis may be attributed to the lowered acidity as has been found in mastitic milk. The lowered acidity in mastitic milk is due to reduction in lactose contents as the lactic acid formation is minimal in this case.

Bulk milk from two locations (Sabon pegi and Hanwa) had an average specific gravity lower than that reported by Cornor (1995). This perhaps indicates adulteration of the milk samples and could be due to higher bacterial metabolic activity as considerable number of isolates was from Sabon pegi (Table 2). Also since milk from these locations is usually sold for yoghurt production, it may be adulterated for maximum profit.

No growth was observed in the yoghurt samples, although there was growth in all the bulk milk samples from all the locations sampled, but they were within the acceptable microbial limit (*S. aureus* $<10^2$ cfu/ml, bacterial growth $\leq 5x10^2$ cfu/ml) as stated by Liston and Matches (1976), Nickerson and Sinckey(1994), Whong *et al*.,(2003), IDF, (1981) and NMC, (1999). Nono/ kindirimo from only three locations had growth of *S. aureus* but were within the acceptable microbial limit. This could be due to low pH level of yoghurt which does not encourage proliferation of bacteria.

Davis (1974) reported that pH of standard yoghurt ranges between 4.0-4.5 and at this pH, low bacterial contaminants are expected to be present. Although food borne outbreaks are rarely reported in Nigeria, the acid pH of 4.13-4.81 for nono as reported by Kornacki and Marth (1982) may limit the multiplication or survival of pathogens at ambient temperature. However, being product of uncontrolled fermented milk, fermentation failures may occur to allow multiplication.

Dairy foods are frequently contaminated with *Staphylococcus* (Umoh *et al*., 1990a) and cases of staphylococci contamination in milk and milk products have been recorded (Umoh *et al*., 1985). Milk from the udder of healthy cows contained few microorganisms that have gained entrance to the teat canal through the teat opening and are flushed out mechanically during milking. If however disease condition prevails, bacteria may be present in large numbers, although it must be emphasized that their entrance to milk is gained in most cases during and after milking (Dauda, 1999). From the time milk leaves the udder until it is dispersed into containers for processing, everything it comes in contact with is a potential source of microorganism. These include the air in the environment, the milking and storage vessels and the milking personnel. *S.*

aureus is found in the nasal passage, the throat and the hands, clothes and skin of 50% or more of healthy individuals(Kloos,1980) and this can be an indication of the dangers of contamination of milk and milk product through manual handling. Dairy cattle's rearing by Fulani is a family business operated at subsistence level without any regards to quality control. In this study, the hazardous level of 10^6cfu/ml of *Staphylococci* was not reached, but the presence of *S. aureus* in milk and milk products should be discouraged as their amount could increase if the milk product is improperly stored.

Antibiotics are used on many farms to treat infections such as mastitis. Cows under antibiotic treatment for mastitis may have antibiotic residues in their milk; therefore milk from treated cow should either be discarded or collected into a separate tank. Milk containing antibiotic residues is suitable for human consumption. The legal standard as defined by regulatory agencies such as the FDA requires that milk contain no detectable antibiotics when analyzed with approved test method. In this study, 1 (5.9%) of bulk milk from the different herds had residues of antibiotics. Antibiotic residues must have entered the milk supply when treated cows are milked early or when a cow retains antibiotic residues in her for an extraordinary length of time. In earlier studies (Shitandi and Sternesjoo, 2001) penicillin was the most commonly found type of antibiotic residues in milk. Antibiotic residues in milk may lead to severe allergic reactions in sensitive consumers, culture failure and subsequent loss of product, a change in consumer perception of milk being a pure, unadulterated, natural product. All of these concerns may result in major economic losses to the dairy industry.

In this study, *S. aureus* isolated from milk from dairy herds around Zaria showed high susceptibility to almost all antimicrobial agents tested. All the isolates were sensitive (100%) to erythromycin, a macrolide antimicrobial agent and gentamicin, an

aminoglycosides, whereas 95% were sensitive to amoxycillin, tetracycline, chloramphenicol and augumentin (amoxicillin and clavulanic acid). Cloxacillin and cotrimazole had 90% and 80% sensitivity respectively. The proportions of *S. aureus* that are resistant to amoxycillin, tetracycline, chloramphenicol and augumentin respectively are the same (5% each). Proportion resistant to cloxacillin and cotrimazole are 10% and 20% respectively which compares with the work of Umoh *et al.* (1990b). Although results for cloxacillin and amoxycilllin from other studies differed remarkably from our findings as no isolates were resistant to cloxacillin and 100% of the isolates were resistant to amoxycillin (Moroni *et al*., 2004). The result for gentamicin was slightly different from that obtained from the studies of Gentilini *et al*., (2002) as only 3.4% of *S. aureus* isolates was resistant to gentamicin.

The proportion of *S. aureus* isolates sensitive to chloramphenicol in this study is higher than that reported by Teixeira *et al*. (1995) (74%) and Akpede *et al*. (1995) (40.6%). Kurek and Niemczyk (1987) observed 55% sensitivity of *Staphylococcus sp.* isolates from mastitic milk. Proportion of isolates resistant to cotrimazole (trimethoprim-sulfamethioxazole) (20%) was higher than that obtained in Turkey (1.8%) (Guller *et al*., 2005) and findings for augumentin (95% of isolates sensitivity) was in agreement with that of Turkey (Guller *et al*., 2005). The high susceptibility of antimicrobial drugs used in this study suggests these drugs have not been abused in treatment of cows in Zaria.

5.1 CONCLUSION

Majority of the milk samples collected from 20 herds in Zaria had physicochemical characteristics within acceptable levels based on milk physical appearance, pH and specific gravity. Up to 75 (21.4%) of the 350 milk quarters tested showed positive CMT

reaction, *S. aureus* was isolated from 11(14.7%) of the positive quarters. Overall *S. aureus* was isolated from 28(8.0) out of the 350 quarters. Milk around Zaria was of good physicochemical characteristics but showed high level of subclinical mastitis. Most of the isolates (90-100%) were susceptible to commonly used antimicrobial agents used clinically, there was low level occurrence of antibacterial residues detected in milk in dairy herds in Zaria..

5.2 RECOMMENDATION

I recommend milk samples from dairy herds for consumption but dairy herdsmen should be educated by the government on the need for control of mastitis as it reduces milk yield and increases cost of production. The best method of control of infection is prevention of spread of infection. Possible means of prevention include:

(1) Milking the infected cows at the end of milking to reduce the possibility of spread to other cows.

(2) Good in principle on the grouping, housing, feeding routines on the farm.

(3) Avoiding the use of common towels or rags between teats.

(4) Use of clean, disposable latex gloves and disinfectant regularly during milking to prevent dirty cracked hand contact with the teat.

(5) Culling infected cows, especially those with other problems.

(6) Boiling of fresh milk (pasteurization) helps to reduce rate of infection

I also recommend that further study should be done to determine if there is any association between bacterial isolates of bovine mastitis and bacterial infection in cow milkier or milk handler.

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Appendix I Table of association

	x^2 observed	df	A	X^2 expected	Decision
CMT and pH	0.47	1	0.05	3.84	Accept H_0
CMT and *S. aureus*	4.6	1	0.05	3.84	Reject H_0
CMT and specific gravity	84.9	1	0.05	3.84	Reject H_0
CMT and udder Position	1.097	3	0.05	7.81	Accept H_0
S. aureus and position of udder	1.702	3	0.05	7.81	Accept H_0
S. aureus and pH	1.2	1	0.05	3.84	Accept H_0
S. aureus and specific gravity	26.251	1	0.05	3.84	Reject H_0

Accept H_0 means there is no association.

Reject H_0 means there is association.

Appendix II: Physiochemical characteristics of milk and identification of isolates.

S/N	BR	UDD	SG	TP	CMT	PH	CM	GR	CO	CAT	HA	DN	IN
1	1	1	2	2	1	3	0	0	0	0	0	0	0
2	1	2	2	2	1	3	0	0	0	0	0	0	0
3	1	3	1	2	2	3	2	1	1	1	1	1	1
4	1	4	1	2	2	3	2	1	1	1	2	1	1
5	1	1	2	3	1	3	0	0	0	0	0	0	0
6	1	2	2	2	1	1	0	0	0	0	0	0	0
7	1	3	2	2	1	1	0	0	0	0	0	0	0
8	1	4	2	2	1	1	0	0	0	0	0	0	0
9	1	1	2	2	1	1	0	0	0	0	0	0	0
10	1	2	2	3	1	1	0	0	0	0	0	0	0
11	1	3	2	2	1	1	0	0	0	0	0	0	0
12	1	4	2	2	1	1	2	1	1	1	1	1	1
13	1	1	1	2	2	1	0	0	0	0	0	0	0
14	1	2	1	2	2	1	2	1	1	1	2	1	1
15	1	3	1	2	2	1	0	0	0	0	0	0	0
16	1	4	1	2	2	1	2	1	2	1	2	1	2
18	1	1	1	2	2	1	0	0	0	0	0	0	0
19	1	3	2	2	1	1	0	0	0	0	0	0	0
20	1	2	2	2	1	1	0	0	0	0	0	0	0
21	1	4	2	2	1	1	0	0	0	0	0	0	0
22	1	1	1	2	2	1	0	0	0	0	0	0	0
23	1	3	2	2	1	1	0	0	0	0	0	0	0
24	1	2	1	2	1	1	0	0	0	0	0	0	0
25	1	4	2	2	1	1	0	0	0	0	0	0	0
26	1	3	1	2	1	1	1	1	1	1	2	1	1
27	1	1	2	2	1	1	1	1	2	1	1	1	2
28	1	4	1	2	1	1	2	1	1	1	1	1	1
29	1	2	2	2	1	1	1	2	2	1	2	2	2
30	1	1	1	2	1	1	0	0	0	0	0	0	0
31	1	2	1	2	2	1	1	1	1	1	2	1	1
32	1	3	2	2	1	1	1	12	2	1	2	2	2
33	1	4	2	2	1	1	1	12	2	1	2	2	2
34	1	3	2	2	1	1	2	4	1	1	2	1	1
35	1	4	2	2	1	1	2	2	1	1	2	1	1
36	1	1	2	2	1	1	0	0	0	0	0	0	0
37	1	2	2	2	1	3	0	0	0	0	0	0	0
38	1	1	2	2	1	1	0	0	0	0	0	0	0
39	1	2	2	2	1	1	0	0	0	0	0	0	0
40	1	3	2	2	1	1	0	0	0	0	0	0	0
41	1	4	2	2	1	1	0	0	0	0	0	0	0
42	1	2	1	2	1	1	2	5	1	1	2	1	1
43	1	4	1	2	2	1	0	0	0	0	0	0	0
44	1	1	1	2	1	1	2	1	1	1	2	1	1

45	1	3	1	2	1	1	2	1	1	1	2	1	1
47	1	1	1	2	2	2	2	1	1	1	2	1	1
48	1	2	1	2	2	2	2	1	1	1	2	2	2
49	1	4	1	2	2	2	1	13	2	1	2	2	2
50	1	4	2	2	1	2	0	0	0	0	0	0	0
51	1	3	2	2	1	2	0	0	0	0	0	0	0
52	1	2	2	2	1	2	0	0	0	0	0	0	0
53	1	1	2	2	1	2	0	0	0	0	0	0	0
54	1	2	2	2	1	2	2	12	2	1	1	2	2
55	1	3	1	2	1	2	1	12	2	1	2	2	2
56	1	1	1	2	1	2	1	12	2	1	1	2	2
57	1	4	2	3	1	2	1	12	2	1	2	2	2
58	1	3	1	2	1	2	0	0	0	0	0	0	0
59	1	4	1	2	1	2	0	0	0	0	0	0	0
60	1	1	2	3	1	2	0	0	0	0	0	0	0
61	1	2	2	3	1	2	0	0	0	0	0	0	0
62	1	4	2	2	1	2	0	0	0	0	0	0	0
63	1	3	1	2	2	2	2	1	1	1	2	1	1
64	1	1	2	2	1	2	0	0	0	0	0	0	0
65	1	2	2	2	1	2	2	1	1	1	2	1	1
66	1	2	1	2	1	2	0	0	0	0	0	0	0
68	1	1	2	2	1	2	0	0	0	0	0	0	0
69	1	2	2	2	1	2	2	1	1	1	2	1	1
70	1	3	2	2	1	2	0	0	0	0	0	0	0
71	1	4	1	2	1	2	0	0	0	0	0	0	0
72	1	1	2	2	1	2	0	0	0	0	0	0	0
73	1	3	1	2	1	2	0	0	0	0	0	0	0
74	1	4	1	2	1	2	2	1	1	1	2	1	1
75	1	2	1	2	1	2	0	0	0	0	0	0	0
76	1	1	2	2	1	2	0	0	0	0	0	0	0
77	1	4	2	2	1	2	0	0	0	0	0	0	0
78	1	3	2	2	1	2	2	1	1	1	2	1	1
79	1	1	2	2	2	3	2	1	1	1	2	1	1
80	1	2	2	2	1	2	2	1	1	1	2	1	1
81	1	3	2	2	1	2	0	0	0	0	0	0	0
82	1	1	1	2	1	2	1	12	2	1	2	2	2
83	1	2	2	2	1	2	2	1	1	1	2	1	1
84	1	3	1	2	2	2	1	12	2	1	2	2	2
85	1	2	1	2	2	2	2	1	1	1	1	1	1
86	1	1	1	2	1	2	2	1	1	1	1	1	1
87	1	4	2	2	1	2	0	0	0	0	0	0	0
88	1	1	1	2	1	2	0	0	0	0	0	0	0
89	1	2	1	2	2	2	2	1	1	1	2	1	1
90	1	4	1	2	2	2	2	1	1	1	2	1	1
92	1	2	1	3	2	2	0	0	0	0	0	0	0
93	1	1	1	2	2	2	0	0	0	0	0	0	0

94	1	4	1	3	2	2	0	0	0	0	0	0	0
95	1	3	1	3	2	2	0	0	0	0	0	0	0
96	1	4	1	2	2	2	0	0	0	0	0	0	0
97	1	1	1	2	2	2	0	0	0	0	0	0	0
98	1	2	1	2	2	2	0	0	0	0	0	0	0
99	1	3	2	2	1	2	0	0	0	0	0	0	0
100	1	4	2	2	1	2	0	0	0	0	0	0	0
101	1	1	2	2	1	2	1	1	2	1	2	2	2
102	1	2	2	2	1	2	0	0	0	0	0	0	0
103	1	2	2	3	2	2	0	0	0	0	0	0	0
104	1	1	1	3	2	2	0	0	0	0	0	0	0
105	1	4	1	3	2	2	0	0	0	0	0	0	0
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107	1	2	1	2	2	2	0	0	0	0	0	0	0
108	1	1	1	2	2	2	0	0	0	0	0	0	0
109	1	4	1	2	2	2	0	0	0	0	0	0	0
110	1	3	1	2	2	2	0	0	0	0	0	0	0
111	1	2	1	2	2	2	0	0	0	0	0	0	0
112	1	4	1	2	2	2	0	0	0	0	0	0	0
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117	1	4	2	2	1	2	0	0	0	0	0	0	0
118	1	3	1	2	2	2	0	0	0	0	0	0	0
119	1	2	2	2	1	2	0	0	0	0	0	0	0
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121	1	4	1	2	2	2	0	0	0	0	0	0	0
122	1	3	2	2	2	2	0	0	0	0	0	0	0
123	1	4	2	2	1	2	0	0	0	0	0	0	0
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127	1	3	2	2	1	2	0	0	0	0	0	0	0
128	1	1	2	2	1	2	0	0	0	0	0	0	0
129	1	4	2	2	1	2	0	0	0	0	0	0	0
131	1	1	1	2	2	2	0	0	0	0	0	0	0
132	1	2	2	2	1	2	0	0	0	0	0	0	0
133	1	3	1	2	1	2	0	0	0	0	0	0	0
134	1	4	2	2	1	2	0	0	0	0	0	0	0
135	1	2	2	2	1	2	0	0	0	0	0	0	0
136	1	4	2	2	1	2	0	0	0	0	0	0	0
137	1	3	2	2	1	2	0	0	0	0	0	0	0
138	1	2	2	2	1	2	0	0	0	0	0	0	0
139	1	3	2	2	1	2	0	0	0	0	0	0	0
140	1	4	2	2	1	2	0	0	0	0	0	0	0
141	1	1	2	2	1	2	0	0	0	0	0	0	0

142	1	2	2	2	1	2	0	0	0	0	0	0	0
143	1	1	2	2	1	2	0	0	0	0	0	0	0
144	1	3	2	2	1	2	1	6	2	1	2	2	2
145	1	4	2	2	1	2	0	0	0	0	0	0	0
146	1	2	2	2	2	2	1	7	2	1	2	2	2
147	1	1	1	2	2	2	1	1	2	1	2	1	2
148	1	3	2	2	1	2	0	0	0	0	0	0	0
149	1	4	2	2	1	2	0	0	0	0	0	0	0
150	1	3	1	2	2	2	0	0	0	0	0	0	0
151	1	4	2	2	1	2	0	0	0	0	0	0	0
152	1	2	2	2	1	2	0	0	0	0	0	0	0
153	1	1	2	2	1	2	0	0	0	0	0	0	0
155	1	1	2	2	1	2	0	0	0	0	0	0	0
156	1	2	2	2	1	2	0	0	0	0	0	0	0
157	1	3	2	2	1	2	0	0	0	0	0	0	0
158	1	4	2	2	1	2	0	0	0	0	0	0	0
159	1	2	2	2	2	2	1	8	2	1	2	1	2
160	1	1	2	2	1	2	0	0	0	0	0	0	0
161	1	4	2	2	1	2	1	13	2	1	2	1	2
162	1	3	2	2	1	2	0	0	0	0	0	0	0
163	1	2	2	2	1	2	0	0	0	0	0	0	0
164	1	1	2	2	1	2	0	0	0	0	0	0	0
165	1	4	2	2	1	2	1	2	2	1	1	1	2
166	1	3	2	2	2	2	1	9	2	1	2	1	2
167	1	1	1	2	2	2	0	0	0	0	0	0	0
168	1	2	2	2	1	2	1	11	2	1	2	1	2
169	1	3	2	2	1	2	0	0	0	0	0	0	0
170	1	4	2	2	1	2	0	0	0	0	0	0	0
172	1	2	2	2	1	2	0	0	0	0	0	0	0
173	1	1	2	1	1	2	0	0	0	0	0	0	0
174	1	4	2	2	1	2	0	0	0	0	0	0	0
175	1	3	2	2	1	2	0	0	0	0	0	0	0
176	1	2	2	2	1	2	0	0	0	0	0	0	0
177	1	1	2	2	1	2	0	0	0	0	0	0	0
178	1	4	2	2	1	2	0	0	0	0	0	0	0
179	1	3	2	2	1	2	0	0	0	0	0	0	0
180	1	1	1	2	2	2	0	0	0	0	0	0	0
181	1	2	2	2	1	2	0	0	0	0	0	0	0
182	1	3	1	2	2	2	1	1	2	1	2	1	0
183	1	4	2	2	1	2	0	0	0	0	0	0	0
184	1	1	3	2	1	2	0	0	0	0	0	0	0
185	1	3	2	2	1	2	0	0	0	0	0	0	0
186	1	4	2	2	1	2	0	0	0	0	0	0	0
187	1	2	2	2	1	2	0	0	0	0	0	0	0
188	1	1	2	2	1	2	0	0	0	0	0	0	0

189	1	2	2	2	1	2	0	0	0	0	0	0	0
190	1	3	2	2	1	2	1	8	2	1	2	1	2
191	1	4	2	2	1	2	0	0	0	0	0	0	0
193	1	1	2	2	1	2	0	0	0	0	0	0	0
194	1	3	2	2	1	2	0	0	0	0	0	0	0
195	1	4	2	2	1	2	0	0	0	0	0	0	0
196	1	2	2	2	1	2	0	0	0	0	0	0	0
197	1	1	2	2	1	2	1	10	2	1	1	1	2
198	1	3	2	2	1	2	0	0	0	0	0	0	0
199	1	4	2	2	1	2	0	0	0	0	0	0	0
200	1	2	2	2	1	2	0	0	0	0	0	0	0
201	1	3	2	2	1	2	0	0	0	0	0	0	0
202	1	4	2	2	1	2	0	0	0	0	0	0	0
203	1	1	2	2	1	2	0	0	0	0	0	0	0
204	1	2	2	2	1	2	0	0	0	0	0	0	0
205	1	1	2	2	1	2	0	0	0	0	0	0	0
206	1	2	2	2	1	2	0	0	0	0	0	0	0
207	1	3	2	2	1	2	0	0	0	0	0	0	0
208	1	4	2	2	1	2	0	0	0	0	0	0	0
209	1	1	2	2	1	2	0	0	0	0	0	0	0
210	1	3	2	2	1	2	1	1	2	1	2	2	2
211	1	4	2	2	1	2	0	0	0	0	0	0	0
212	1	2	2	2	1	2	0	0	0	0	0	0	0
214	1	2	2	2	2	2	1	12	2	1	2	2	2
215	1	1	2	2	1	2	0	0	0	0	0	0	0
216	1	4	2	2	1	2	0	0	0	0	0	0	0
217	1	3	2	2	1	2	0	0	0	0	0	0	0
219	1	3	2	2	2	2	0	0	0	0	0	0	0
220	1	4	2	2	1	2	0	0	0	0	0	0	0
221	1	1	2	2	1	2	0	0	0	0	0	0	0
222	1	2	2	2	1	2	0	0	0	0	0	0	0
223	1	3	2	2	1	2	0	0	0	0	0	0	0
224	1	4	2	2	1	2	0	0	0	0	0	0	0
225	1	2	2	2	1	2	0	0	0	0	0	0	0
226	1	3	2	2	1	2	0	0	0	0	0	0	0
227	1	4	2	2	1	2	0	0	0	0	0	0	0
228	1	1	2	2	1	2	2	5	2	1	1	2	2
229	1	2	2	2	1	2	0	0	0	0	0	0	0
230	1	4	2	2	1	2	0	0	0	0	0	0	0
231	1	2	2	2	1	2	0	0	0	0	0	0	0
232	1	1	2	2	2	2	0	0	0	0	0	0	0
233	1	3	2	2	2	2	0	0	0	0	0	0	0
234	1	3	2	2	1	2	0	0	0	0	0	0	0
235	1	1	2	2	1	2	0	0	0	0	0	0	0
236	1	2	2	2	1	2	0	0	0	0	0	0	0
237	1	4	2	2	2	2	0	0	0	0	0	0	0

238	1	2	1	2	2	2	0	0	0	0	0	0	0
239	1	1	2	2	2	2	0	0	0	0	0	0	0
240	1	3	2	2	2	2	0	0	0	0	0	0	0
242	1	3	1	2	1	2	0	0	0	0	0	0	0
243	1	2	2	2	1	2	0	0	0	0	0	0	0
244	1	1	2	2	2	2	0	0	0	0	0	0	0
245	1	4	2	2	1	2	0	0	0	0	0	0	0
246	1	3	2	2	1	2	0	0	0	0	0	0	0
247	1	2	2	2	1	2	0	0	0	0	0	0	0
248	1	1	2	2	1	2	0	0	0	0	0	0	0
249	1	4	2	2	1	2	0	0	0	0	0	0	0
250	1	1	2	2	1	2	0	0	0	0	0	0	0
251	1	4	2	2	1	2	0	0	0	0	0	0	0
252	1	3	2	2	1	2	0	0	0	0	0	0	0
253	1	2	2	2	1	2	0	0	0	0	0	0	0
254	1	4	2	2	1	2	1	11	2	1	2	2	2
255	1	1	2	2	1	2	1	1	2	1	2	2	2
256	1	2	2	2	1	2	0	0	0	0	0	0	0
257	1	3	2	2	1	2	0	0	0	0	0	0	0
259	1	2	2	2	1	2	0	0	0	0	0	0	0
260	1	1	2	2	1	2	0	0	0	0	0	0	0
261	1	3	2	1	1	2	0	0	0	0	0	0	0
262	1	4	2	2	1	2	0	0	0	0	0	0	0
263	1	4	2	3	1	2	0	0	0	0	0	0	0
264	1	1	2	3	1	2	0	0	0	0	0	0	0
265	1	2	2	3	1	2	0	0	0	0	0	0	0
266	1	3	2	3	1	3	0	0	0	0	0	0	0
267	1	3	2	3	1	2	0	0	0	0	0	0	0
268	1	1	2	2	1	2	0	0	0	0	0	0	0
269	1	4	2	2	1	2	0	0	0	0	0	0	0
270	1	2	2	3	1	3	0	0	0	0	0	0	0
272	1	3	2	2	1	2	0	0	0	0	0	0	0
273	1	2	2	2	1	2	0	0	0	0	0	0	0
274	1	4	2	2	1	2	0	0	0	0	0	0	0
275	1	4	2	2	1	2	0	0	0	0	0	0	0
276	1	3	2	3	1	2	0	0	0	0	0	0	0
277	1	2	2	3	1	3	2	1	2	1	2	2	2
278	1	4	1	3	1	2	2	12	2	1	2	2	2
279	1	1	1	3	1	2	0	0	0	0	0	0	0
281	1	1	1	3	2	2	0	0	0	0	0	0	0
282	1	2	2	1	2	2	0	0	0	0	0	0	0
283	1	4	2	2	1	2	0	0	0	0	0	0	0
284	1	1	2	2	1	2	1	12	2	1	1	2	2
285	1	1	2	3	1	2	0	0	0	0	0	0	0
286	1	2	2	2	2	2	0	0	0	0	0	0	0
287	1	3	2	3	1	2	0	0	0	0	0	0	0

288	1	4	2	3	1	2	0	0	0	0	0	0	0
289	1	4	2	2	1	2	0	0	0	0	0	0	0
290	1	2	1	3	1	2	0	0	0	0	0	0	0
291	1	1	2	3	1	2	0	0	0	0	0	0	0
292	1	3	2	3	1	2	0	0	0	0	0	0	0
293	1	1	2	2	1	2	0	0	0	0	0	0	0
294	1	2	2	3	1	2	0	0	0	0	0	0	0
295	1	4	2	2	1	2	0	0	0	0	0	0	0
296	1	3	2	3	1	2	0	0	0	0	0	0	0
297	1	3	2	2	2	2	0	0	0	0	0	0	0
298	1	1	2	2	2	2	0	0	0	0	0	0	0
299	1	2	2	2	2	2	2	1	2	1	2	2	2
300	1	4	2	2	2	2	2	8	2	1	2	2	2
301	1	2	2	2	1	2	0	0	0	0	0	0	0
302	1	3	2	2	1	2	0	0	0	0	0	0	0
303	1	1	2	2	1	2	0	0	0	0	0	0	0
304	1	4	2	2	1	2	0	0	0	0	0	0	0
306	1	1	2	2	1	2	0	0	0	0	0	0	0
307	1	3	2	2	1	2	0	0	0	0	0	0	0
308	1	2	2	2	1	2	0	0	0	0	0	0	0
309	1	4	2	2	1	2	0	0	0	0	0	0	0
310	1	1	2	2	1	2	2	2	1	1	2	1	1
311	1	3	2	2	1	2	2	6	2	1	2	2	2
312	1	2	2	2	1	2	0	0	0	0	0	0	0
313	1	4	2	2	1	2	0	0	0	0	0	0	0
314	1	4	1	2	1	2	0	0	0	0	0	0	0
315	1	3	2	2	1	2	0	0	0	0	0	0	0
316	1	2	2	2	1	2	0	0	0	0	0	0	0
317	1	1	2	2	2	2	0	0	0	0	0	0	0
318	1	2	2	2	1	2	0	0	0	0	0	0	0
319	1	1	2	3	1	2	0	0	0	0	0	0	0
320	1	4	2	2	1	2	0	0	0	0	0	0	0
321	1	3	2	2	1	2	0	0	0	0	0	0	0
322	1	1	2	2	1	2	0	0	0	0	0	0	0
323	1	2	2	2	1	2	2	3	1	1	1	1	1
325	1	1	2	2	1	2	0	0	0	0	0	0	0
326	1	4	2	2	1	2	0	0	0	0	0	0	0
327	1	3	2	2	1	2	0	0	0	0	0	0	0
328	1	2	2	2	1	2	0	0	0	0	0	0	0
329	1	3	2	2	2	2	0	0	0	0	0	0	0
330	1	4	2	2	2	2	0	0	0	0	0	0	0
331	1	1	2	2	1	2	0	0	0	0	0	0	0
332	1	2	2	2	2	2	0	0	0	0	0	0	0
334	1	3	2	2	1	2	0	0	0	0	0	0	0
335	1	1	2	2	1	2	0	0	0	0	0	0	0
336	1	4	2	2	1	2	0	0	0	0	0	0	0

337	1	2	2	2	1	2	0	0	0	0	0	0	0
338	1	1	2	1	1	2	0	0	0	0	0	0	0
339	1	2	1	2	1	2	0	0	0	0	0	0	0
340	1	3	2	2	1	2	0	0	0	0	0	0	0
341	1	4	2	2	1	2	0	0	0	0	0	0	0
342	1	4	2	2	1	3	0	0	0	0	0	0	0
343	1	1	2	2	1	2	0	0	0	0	0	0	0
344	1	3	2	2	1	2	0	0	0	0	0	0	0
346	1	2	2	2	1	2	0	0	0	0	0	0	0
347	1	3	2	2	1	2	0	0	0	0	0	0	0
348	1	4	2	2	1	2	0	0	0	0	0	0	0
349	1	1	2	2	1	2	0	0	0	0	0	0	0
350	1	1	2	2	1	2	0	0	0	0	0	0	0
351	1	3	1	2	1	2	0	0	0	0	0	0	0
352	1	4	1	2	2	2	2	1	2	1	1	2	2
353	1	2	1	2	1	2	0	0	0	0	0	0	0
354	1	2	2	2	1	2	0	0	0	0	0	0	0
355	1	1	2	2	1	2	0	0	0	0	0	0	0
356	1	4	2	2	1	2	0	0	0	0	0	0	0
357	1	3	2	2	1	2	0	0	0	0	0	0	0
358	1	3	2	2	1	2	0	0	0	0	0	0	0
359	1	1	1	2	2	2	0	0	0	0	0	0	0
360	1	2	2	2	1	2	0	0	0	0	0	0	0
361	1	4	1	2	2	2	1	3	2	1	2	2	2
362	1	1	1	2	1	2	0	0	0	0	0	0	0
363	1	2	1	2	1	2	0	0	0	0	0	0	0
364	1	3	2	2	2	2	1	6	2	1	2	2	2
365	1	4	2	2	1	3	0	0	0	0	0	0	0
366	1	3	1	2	1	2	0	0	0	0	0	0	0
367	1	4	2	2	1	2	0	0	0	0	0	0	0
368	1	1	2	2	2	2	2	1	1	1	1	1	1
369	1	2	2	2	1	2	2	2	1	1	2	2	2

KEY
Breed-BR
Bunaji – 1

Udder – UDD
Right hind (HR) -1 Right fore (FR)- 3
Left hind (HL) -2 Left fore (FL) – 4

Specific gravity- SG
Below 1.028 – 1 1.028 – 1.032 – 2 Above 1.032 – 3

Temperature – tp
23-27^{0}c – 1 28-32^{0}c – 2 33- 37^{0}c – 3

California Mastitis Test – CMT
Negative - 1 Positive - 2
ss
pH
Below 6.4 – 1 6.4 – 2 Above 6.4 – 3

Colonial morphology – CM
No growth – 1
Round raised white colony – 2
Round raised yellow colony - 3

Gram's reaction -GM
Gram positive cocci in clusters -1
Gram positive cocci in clustersand chain – 2
Gram positive cocci in clusters, chains and pairs – 3
Gram positive cocci in clusters, chains, pairs and singles – 4
Gram positive cocci in clusters and pairs – 5
Gram positive cocci in clusters and singles – 6
Gram positive cocci in clusters, singles and pairs – 7
Gram positive cocci in chains – 8
Gram positive cocci in chains and pairs - 9
Gram positive cocci in chains, pairs and singles - 10
Gram positive cocci in chains and singles – 11
Gram positive cocci in singles – 12
Gram positive cocci in singles and pairs - 13

Coagulase – CO, Catalase – CAT, Haemolysis – HA, DNase – DN
Nil – 0
Positive – 1
Negative – 2

Inference – IN
Nil – 0
Staphylococcus aureus - 1
Coagulase negative staphylococci (CNS) – 2

Appendix III: Physicochemical properties of bulk milk

S/no	Breed	Udder	Specific gravity	Temp	Cmt	Ph
17	1	BM1	1	2	2	3
46	1	BM2	1	2	2	1
67	1	BM3	1	2	2	2
91	1	BM4	1	2	2	3
114	1	BM5	1	3	2	2
130	1	BM6	1	2	2	2
154	1	BM7	2	1	2	2
171	1	BM8	2	2	2	2
192	1	BM9	2	2	1	2
213	1	BM10	2	2	1	2
218	1	BM11	2	2	1	2
241	1	BM12	2	2	2	2
258	1	BM13	2	2	1	2
271	1	BM14	2	2	1	2
280	1	BM15	2	3	1	2
305	1	BM16	2	2	2	2
324	1	BM17	2	2	1	2
333	1	BM18	2	2	1	2
345	1	BM19	2	2	1	3
370	1	BM20	2	2	1	2

KEY:
Bulk milk (Number of herd) - BM(1)
Others are as for appendix ii

Appendix IV: Antimicrobial sensitivity test – Kirby Bauer method

Slant no.	Amx	Ery	Tet	Cxc	Gen	Cot	Chl	Aug
28	S	S	S	S	S	S	S	S
31	S	S	S	S	S	R	S	S
35	S	S	S	S	S	S	S	S
42	S	S	S	S	S	S	S	S
44	S	S	S	S	S	S	S	S
47	S	S	S	S	S	S	S	S
63	S	S	S	S	S	S	S	S
65	S	S	S	S	S	S	S	S
69	S	S	S	S	S	S	S	S
74	S	S	S	S	S	R	S	S
78	S	S	S	S	S	S	R	S
80	S	S	S	R	S	R	S	R
83	S	S	S	S	S	S	S	S
85	S	S	S	S	S	S	S	S
86	R	S	R	S	S	S	S	S
89	S	S	S	S	S	S	S	S
90	S	S	S	S	S	S	S	S
310	S	S	S	R	S	R	S	S
323	S	S	S	S	S	S	S	S
368	S	S	S	S	S	S	S	S
S. aureus	S	S	S	S	S	S	S	S

KEY
Amx – amoxycillin(25ug)
Ery – erythromycin(5ug)
Tet – tetracyclin(10ug)
Cxc – cloxacillin(5ug)
Gen – gentamycin(10ug)
Cot – cotrimazole(25ug)
Chl – chloraphenicol(30ug)
Aug – augumentin(30ug)

Appendix V: Minimum Inhibitory Concentration(MIC) (ug)

Slant no.	Amx	Ery	Tet	Cxc	Gen	Cot	Chl	Aug
28	0.25	0.125	0.0675	0.0675	8	0.25	1	2
31	0.25	0.125	0.0675	4	2	8	16	4
35	0.125	0.25	0.125	0.2	0.125	0.25	16	4
42	0.125	0.125	0.0675	0.0675	8	0.125	1	1
44	0.125	2	0.0675	4	8	0.125	8	2
47	0.125	0.5	0.125	4	0.125	0.125	16	1
63	0.125	0.125	0.0675	4	8	0.25	0.25	4
65	0.125	0.25	0.0675	0.0675	8	0.5	0.0675	4
69	0.125	0.25	0.0675	0.5	8	0.25	0.25	4
74	0.125	0.125	4	0.0675	0.125	0.5	0.0675	4
78	0.25	0.125	0.0675	4	0.125	16	-	4
80	-	1	0.0675	-	0.125	8	2	8
83	0.125	0.125	0.0675	0.0675	0.125	0.25	0.0675	2
85	0.125	0.125	0.0675	4	4	0.25	16	4
86	16	0.125	32	4	8	0.25	16	4
89	0.25	2	0.125	0.125	0.125	0.25	0.125	4
90	0.25	0.125	0.0675	0.0675	8	0.5	16	4
310	0.125	0.125	0.125	16	0.125	32	2	4
323	0.25	0.125	0.0675	4	4	0.5	0.0675	4
368	0.25	0.0675	8	0.0675	0.25	1	0.25	4

KEY: The same as for appendix iv

Appendix VI: Standard diameter of zones of inhibition for diffusion method

Antibiotic disc	Potency(µg)	Sensitive (mm)	Resistance (mm)
Amoxycillin	25	≥29	≤28
Ertthromycin	5	≥23	≤ 13
Tetracyclin	10	≥19	≤14
Cloxacillin	5	≥13	≤10
Gentamycin	10	≥15	≤12
Cotrimazole	25	≥16	≤10
Chloramphenicol	30	≥18	≤12
Augumentin	30	≥20	≤19

Adapted from CLSI, 2002a. 2002b.