DETECTION AND PCR CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN GENESFROM MASTITIC COW MILK AMONG SOME DAIRY HERDS IN ZARIA, KADUNA STATE

BY

SHAFA'ATU MUHAMMAD, NURUDEEN

DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

DECEMBER, 2016

DETECTION AND PCR CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN GENESFROM MASTITIC COW MILK AMONG SOME DAIRY HERDS IN ZARIA, KADUNA STATE

BY

Shafa'atu Muhammad NURUDEEN, B.Sc. (Crescent University, Abeokuta) 2011

(MSC/SCI/46546/2012-2013)

ATHESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,

AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD

OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES,

AHMADU BELLO UNIVERSITY,

ZARIA, NIGERIA

DECEMBER, 2016

DECLARATION

I hereby declare that the work in this dissertation titled 'DETECTION AND PCR CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN GENES FROM MASTITIC COW MILK AMONG SOME DAIRY HERDSS IN ZARIA' has been performed by me in the Department of Microbiology under the supervision of Prof. I. O. Abdullahi and Prof. C. M. Z. Whong.

The information derived from the literature has been duly acknowledged and a list of references provided. No part of this dissertation was presented for another degree or diploma elsewhere.

Nurudeen, Shafa'atu Muhammad _____ _____
Signature Date

CERTIFICATION

This dissertation titled 'DETECTION AND PCR CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS ENTEROTOXIN GENES FROM MASTITIC COW MILK AMONG SOME DAIRY HERDS IN ZARIA, KADUNA STATE, NIGERIA," meets the regulations governing the award of Master of Science in Microbiology ofAhmadu Bello University, Zaria, and is approved for its contribution to knowledge andliterary presentation.

Prof. I.O Abdullahi Chairman, Supervisory Team, Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria	_____ Signature	_____ Date
Prof. C.M.Z. Whong Member, Supervisory Team, Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria	_____ Signature	_____ Date
Prof. I.O Abdullahi Head, Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria	_____ Signature	_____ Date
Prof. K. Bala Dean, School of Postgraduate Studies Ahmadu Bello University, Zaria	_____ Signature	_____ Date

DEDICATION

This Research is dedicated to Almighty Allah for His mercy without Him nothing can be done and to my parents, Alhaji and Hajia N. A Muhammad who have been supporting me morally and financially to attain the certain stage of education. I appreciate you for recognizing your parental role in my life. In sha Allah you will live longer to reap the fruit of your labour (Amin).

ACKNOWLEDGEMENTS

My sincere appreciation goes to Almighty Allah for making this Research a successful one.

I would like to express my sincere gratitude and indebtedness to my supervisors Prof. I. O. Abdullahi and Prof. C. M. Z. Whong for their critical reading of the manuscript, immerse contributions, encouragement and advices, valuable suggestion, corrections, support and superb direction towards the success of this Research.

I am thankful to Dr. E. E. Ella who helped to supply the kits and primers for my work.

I am thankful to Dr. A. B. Suleiman for his advices and encouragement.

My sincere thanks to Mal. A. Shitu and Mal. Sani, who provided me with all the necessary assistance in the laboratory and also Mal. Y. Y. Palla who provided me with all the necessary assistance during the molecular aspect of this Research.

Also my sincere appreciation to the dairy herds in all the sample location for their support and sincere co operation.

My sincere thanks and appreciation to my husband, Usman Dembo for his infinite patience, support and encouragement during the work.

Finally, I thank my parent who provided me with a sound educational foundation and supported me throughout my studies.

ABSTRACT

The study is aimed to detect and PCR characterize *S. aureus* enterotoxin gene from mastitic cow milk within Zaria metropolis. Four hundred and three quarter milk samples were collected from 104 lactating cows in 17 dairy hreds which included Palladan, Shika, Hanwa, Wusasa and Bomo. The physicochemical characteristics (Temperature, PH, and Specific gravity and Titratable acidity) and proximate composition (Moisture content, Protein content, Fat content and Ash content of the milk were determined. The prevalence of subclinical mastitis using California mastitis test (CMT), bacteriological analysis using standard methods, antibacterial susceptibility and identification of the gene encoding staphylococcal enterotoxin A and B using PCR technique were carried out. The Temperature, PH, Specific gravity and Titratable acidity of the milk ranged from 23-37^{0}C, 4.10 – 6.97, 1.023 – 1.032 and 0.14 – 0.23 respectively. The Moisture, Ash, Fat, and Protein content of the milk ranged from 86.99 – 87.67%, 0.66 – 0.69%, 3.22 – 3.81% and 3.13 – 3.45%. The prevalence of subclinical mastitis based on CMT reaction was (23.3%), 54 *S.aureus* were isolated from mastitic milk. The mean standard deviation ± of the total bacterial count from all the locations ranged from 6.22 ± 0.10 - 6.43 ± 0.12 and for the staphylococcal count from 2.98 ± 0.13 - 3.24 ± 0.11. The antibacterial susceptibility results shows that all the 54 *S. aureus* isolates were sensitive to vancomycin, norfloxacin and erythromycin (100%), 88.9% were sensitive to gentamicin and ciprofloxacin while 7.4% were sensitive to penicillin and methicillin. Out of the 15 isolates tested for enterotoxin A and B, 2 harboured gene for SEA and 5 isolates harboured gene for SEB but none of the isolates harboured both gene for SEA and SEB. The study concludes that further attention is needed to improve the hygienic and safety of milk and dairy farmers should be educated on the need to control mastitis, as it may decrease milk production and reduce the quality of the milk.

TABLE OF CONTENT

Cover Page……....i

Fly Leaf……ii

Title Page…….....iii

Declaration…….…….....……..iv

Certification……...…….....v

Dedication…….....vi

Acknowledgement……..……...vii

Abstract…….……...…….....viii

Table of Contents…….…….……..……....ix

List of Tables……..…….....xiv

List of Figures……..…….....xv

List of Plates…….xvi

List of Appendices……...….....xvii

CHAPTER ONE

1.0 INTRODUCTION…….....1

1.1 Statement of Problem.....3

1.2 Justification.....3

1.3 Aims and Objectives…….....4

1.3.1 Aim……...4

1.3.2 Specific objectives…….....4

1.4 Research Questions.....5

CHAPTER TWO

2.0 LITERATURE REVIEW.....6

2.1 Mastitis.....6

2.2 Milk and Milk Products.....7

2.2.1 Effects on milk composition.....8

2.3 Types of Mastitis…….....8

2.3.1 Modes of Transmission…….…11

2.4 Causative Agents of Mastitis…….…..10

2.5 *Staphylococcus aureus* as a Major Pathogen of Bovine Mastitis……11

2.5.1 Identification and Morphology…….....12

2.5.2 Infection cause by *Staphylococcus aureus* ……...…….12

2.5.3 Transmission of *Stapyhlococcus aureus* infection…….13

2.5.4 Factors predisposing cow to mastitis…….13

2.6 Diagnosis of *S. aureus* Mastitis……15

2.6.1 California Mastitis Test……..15

2.7 Control of *S. aureus* Mastitis…….…..16

2.8 Prevention of Recurrent *S. aureus* Infection…….23

2.9 Antimicrobial Susceptibility of *Staphylococcus aureus*……....25

2.10 Staphylococcal Enterotoxins……...26

2.10.1 Staphylococcal food poisoning……..…26

2.10.2 *Staphylococcus aureus* enterotoxins……..27

2.10.3 Staphylococcal Enterotoxins and Food Poisoning Outbreaks…….....28

CHAPTER THREE

3.0 MATERIALS AND METHODS……30

3.1 Sampling Plan…….....30

3.1.1 Sample size……30

3.1.2 Study area ……..…….....30

3.1.3 Collection of samples …….…….…..30

3.2 Assessment of Physicochemical Quality of Milk……...…….…...33

3.2.1 Physical inspection of the milk……..33

3.2.2 Temperature……...33

3.2.3 PH……...33

3.2.4 Titratable acidity……....33

3.2.5 Specific gravity…..……....34

3.3 Proximate Composition of Milk……..34

3.3.1 Moisture content……34

3.3.2 Ash content……..……..34

3.3.3 Fat content……..35

3.3.4 Protein content……..……35

3.4 California Mastitis Test (CMT)……..36

3.5 Bacteriological Analysis of Milk…….36

3.5.1 Total bacteria Count…….....37

3.5.2 Total staphylococcal count…….....37

3.5.3 Identification of *Staphylococcus aureus*……..…….……37

3.6 Confirmation of *S. Aureus* using Microgen Staph-Id Identification……...39

3.7 Antibacterial Susceptibility Test……..…….....39

3.8 Molecular Characterization Using Polymerase Chain Reaction (PCR)…….40

3.8.1 DNA extraction……..40

3.8.2 PCR amplification ……..…….....40

3.9 PCR Mastermix……40

3.10 Agarose gel electrophoresis of PCR products……...40

3.11 Data Analysis……41

CHAPTER FOUR

4.0 RESULTS……42

4.1 Location of Dairy Herds with number of Cows and Quarters……42

4.2 Milk collected at Udder Quarters of Cows..……..42

4.3 Results of Physicochemical Quality of Milk…….....42

4.3.1 Physical inspection of milk……....42

4.3.2 Temperature of milk……...43

4.3.3 PH of milk……..43

4.3.4 Titratable acidity of milk……43

4.3.5 Specific gravity of milk……..49

4.3.6 Mean scores of physicochemical quality of milk according to CMT……49

4.4 Results of Proximate Composition of Milk……....49

4.5 Total Bacterial Counts in Milk……..…….53

4.6 Total Staphylococcal Counts in Milk…….....53

4.7 California Mastitis Test on Milk……...……..53

4.8 CMT Reaction based on Anatomic Site of Udder…….53

4.9 Isolation and Identification of *S. aureus*……58

4.10 Results of CMT Scores in Comparison with Frequency of Isolation of *S. aureus* from Milk Sample……58

4.11 Antibiotic Sensitivity Test…….....58

4.12 Detection of Enterotoxin Genes (A and B) by PCR……..58

CHAPTER FIVE

5.0 DISCUSSION……..64

CHAPTER SIX

6.1 CONCLUSION AND RECOMMENDATION…….72

6.1 Conclusion…….72

6.2 Recommendations……73

REFERENCES……..……..74

APPENDICES……..……88

LIST OF TABLES

Table	Title	Page
4.1	Location of Dairy Herds with number of Cows and Quarters	44
4.2	Frequency Distribution of Milk Collected from Udder Quarter of Cow	45
4.3	Frequency Distribution of Temperature of Milk from Dairy Herd Location	46
4.4	Frequency Distribution of pH of Milk from Dairy Herd Location.	47
4.5	Frequency distribution of Titratable Acidity of Milk from Dairy Herds Location	48
4.6	Frequency distribution of Specific gravity of Milk from Dairy Herd Location	50
4.7	Mean scores of Physicochemical Quality of Milk according to CMT	51
4.8	Proximate Composition of Milk from Dairy Herd location in Zaria	52
4.9	Mean of Total Bacterial Count Isolated from Milk Samples in Relation to Dairy Herd Locations	54
4.10	Mean of Total Staphylococcal Count Isolated from Milk Samples in Relation to Dairy Herd Locations	55
4.11	Frequency distribution of California mastitis test in Relation to Dairy Herd Location	56
4.12	The number of udder with respect to their anatomic sites and CMT reaction	57
4.13	Biochemical characterization of 81 gram positive cocci isolate from milk samples	59
4.14	Results of CMT Scores in Comparison with Frequency of Isolation of *S. aureus* from Milk Samples	60
4.15	Antibiotic Sensitivity of 54 Isolates of *S. aureus*.	61

LIST OF FIGURE

Figure	Title	Page
1	Map of Zaria……..	...32

LISTS OF PLATES

Plate **Title** **Page**

1 Amplicons of SEA gene of *S.aureus* with size of 102bp…….....62

2 Amplicons of SEB gene of *S.aureus* with size of 164bp…….....63

LIST OF APPENDICES

Appendix	Title	Page
I	Nucleotide sequence and predicted size of PCR products	88

CHAPTER ONE

1.0 INTRODUCTION

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 (Ruuw and Berts, 2004).

Mastitis is the inflammation of mammary gland and is a complex and costly disease in dairy herds (Husain *etal*., 2012; Atasever, 2012). Mastitis may have a variety of causes; Bacterial being the predominant cause of mastitis among dairy cattle (Wellenberg *etal*., 2002).

Mastitis manifestation can be of clinical or subclinical (Eriskine, 2001). Sub clinical mastitis are those in which no visible appearance of changes in the milk or udder, but milk production decreases, bacteria are present in the secretion and composition is altered (Eriskine, 2001). Clinical cases of mastitis are characterized by the presence of one or more of symptoms such as abnormal milk, udder swelling and systemic signs including elevated temperature, lethargy and anorexia (Eriskine, 2001).

Mastitis may be caused by a large variety of bacterial 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 type of chronic mastitis is caused by *Staphylococcus aureus* (Jones *et al*., 1998). *Staphylococcus aureus* is a major pathogen of bovine mastitisworldwide. It has been reported by Lafi *et al*. (1994) that *Staphylococcus aureus* occurred predominantly in both clinical and sub clinical bovinemastitis.

Mastitis caused by *Staphylococcus aureus* is recognized worldwide in dairy cows as subclinical and clinical intramammary infection (Akineden *et al*., 2001). The infection is spread at milking time when *S. aureus* contaminated milk from an infected gland comes in contact with an uninfected gland, and the bacteria penetrate the teat canal. Moreover Ebliny *et al*. (2001) reported that *S. aureus* causes infection of longer duration.

The number of bacteria present in a milk sample is of importance. Milk from cows infected with mastitis generally has higher total bacteria and somatic cell 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 yield more than 200 colonies of bacteria per ml of milk, with fewer counts suggesting the invasion phase before the infection is established (Blood and Radostits, 1989).

S. aureus is a Gram-positve, non spore forming spherical bacterium that belongs to the Staphylococcus genus. *S. aureus* produces staphylococcal enterotoxins (SE) and is responsible for almost all staphylococcal food poisoning (Montville and Mattews 2008; FDA 2012).

Staphylococcal food poisoning is an intoxication that is caused by the ingestion of food containing pre-formed SE (Argudin *et al*., 2010). There are several different types of SE; enterotoxin A is most commonly associated with staphylococcal food poisoning. Enterotoxins

D, E and H, and to a lesser extent B, G and I, have also been associated with staphylococcal food poisoning (Seo and Bohach 2007; Pinchuk *et al*., 2010). Also Argudin *et al* (2010) reported that *S. aureus* enterotoxins have been divided into 5 serological 'classical types' (SEA,SEB,SEC,SED,SEE), and among them SEA is considered as the main cause of SFP (Staphylococcal food poisoning) outbreaks in the United State, Japan, France, and UK.

1.1 Statement of Problem

Staphylococcus aureus is one of the important causative agent of mastitis all over the world (Cabral *et al*., 2004), causing both clinical and subclinical form of mastitis in cattle (Pradeep *et al*., 2003). *S. aureus* bacteria produce toxins that destroy cell membrane and can directly damage milk producing tissues which can lead to bovine mastitis (Jones *et al*., 1998).

S. aureus mastitis is a serious problem in dairy production and infected animals may contaminate bulk milk. Additionally, human handlers, milking equipment, the environment, and udder and teat skin of dairy animals may be other possible sources of bulk milk contamination. From a food safety perspective, this is a concern because enterotoxigenic *S. aureus* may be a risk of SFP (Staphylococcal food poisoning) after consumption of raw milk products (Jorgensen *et al*., 2005).

1.2 Justification

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 e.t.c. The nutrient composition of raw milk is excellent and is thus favorable for bacteria

growth (Ruuw and Berts, 2004). Results of extensive investigations over many years have proved that both human and animal diseases are sometimes spread by milk and milk products.

The bacterial contamination of milk from affected cows renders it unfit for human consumption and provides a mechanism of spread of diseases.

Mastitis agents like *S. aureus* in 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. Therefore there is a need to detect and study *Staphylococcus aureus* mastitic milk and suggest possible control measures.

1.3 Aim and Objectives

1.3.1 Aim

The study is therefore aimed to detect and PCR characterization of *Staphylococcus aureus* enterotoxin genes from mastitic cow milk among some dairy herds in Zaria.

1.3.2 Specific objectives

The specific objectives of this research were to:

1. Determine the physicochemical and proximate composition of milk obtain from some dairy herds.
2. Determine Total bacterial count and Staphylococcus count of the milk sample.
3. Isolate and characterize *Staphylococcus aureus* present in the milk.

4. Determine the pattern of susceptibility of *S. aureus* to antibiotics

5. Detect the gene encoding Staphylococcal enterotoxin A and B using PCR.

1.4 Research Questions.

1. Ho: Enterotoxigenic *S.aureus* is not present in bovine mastitic milk.

   Ha: Enterotoxigenic *S.aureus* is present in bovine mastitic milk.

CHAPTER TWO

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 (Oliveirra *et al.*, 2000).

Mastitis 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).

Mastitis is a complex and multi factorial disease the occurrence of which depends on variables related to the animal, environment and pathogen (Radostits *etal*., 2007). Among the pathogens, bacterial agent are the most common one, the greatest share of which resides widely distributed in the environment of dairy cows, hence a common threat to the mammary gland (Bradley, 2002).

Mastitis can cause devastating effects to farmers because of the serious economic losses and the danger that the bacterial contamination of milk from affected cows may render it unsuitable for human consumption (Quinn *etal*., 1999). Mastitis occurs worldwide among dairy animals and it has been described to have an extreme zoonotic and economic impact (Al-Majali *etal*., 2008).Mastitis is generally considered the most costly diseased of dairy animal. 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 *etal*., 1999). Mastitis can be very detrimental to a dairy farm's profitability in terms of lost production (Houben *etal*., 1993, Wilson *etal*., 2004) and treatment cost (Hoblet *etal*., 1991; Miller *etal*., 1993). In Nigeria, Ameh *et al*. (1999), reported 34.6% *S. aureus* recovery rate from bovine mastitic milk in Maiduguri. Also a prevalence rates of 30.2% *S.aureus* from mastitic milk in plateau state was reported by Suleiman *et al*. (2012).

2.2 Milk and Milk Product

Milk is a food of outstanding interest and has been taken by humans since the earliest prehistoric times and still forms the basis of most nation economics (Egwaikhide *et al*., 2014) It is designed by nature to be a complete food for young animals and of high nutritional values. The principal constituents of milk include fat, protein, total solid, lactose, ash and in addition to this, it contains several hundred minor constituents which include milk fat, vitamins, metal ion and flavor compounds, which have a major impact on the nutritional, technological and sensory properties of milk and dairy products (Egwaikhide *et al*., 2014). According to Byron *et al*., (1994), the average composition of milk are (i) Water (87.20%), (ii) Protein (3.50%), (iii) fats (3.70%), (iv) Milk sugar or lactose (4.90%), (v) Ash (0.70%) and (vi) Dry matter (12.80%).

As an agricultural product, milk is extracted from mammals during or soon after pregnancy and is used as food for humans. Worldwide, dairy farms produced about 730 million tones of milk in 2011 (Food outlook-Global market analysis 2012), from 260 million dairy cows (FAO 2014). New Zealand, the European union' s 28 member states, Australia, and the united States are the world's largest exporters of milk and milk products. China and Russia are the world's largest

importers of milk and milk products (Blasko, 2011). In Africa, milk is produced in most agricultural system. It is either sold fresh, consumed as fermented milk or manufactured into products such as butter and cheese (O'Connor 1995). In developing countries, most milk is produced by a large number of small holders with small quantity of milk (Barabas 1994).

Milk is processed into a variety of dairy products such as cream, butter, yoghurt, kefir, ice cream, and cheese. Modern industrial processes use milk to produce casein, whey protein, lactose, condensed milk, powdered milk, and many other food-additives and industrial products.

2.2.1 Effect on Milk Composition

Mastitis can cause a decline in potassium and lactoferrin. It also results in decreased casein, the major protein in milk. As most calcium in milk is associated with casein, the disruption of casein synthesis contributes to lowered calcium in milk. The milk protein continues to undergo further deterioration during processing and storage (Jones and Bailey 2010). Milk from cows with mastitis also has a higher somatic cell count (Kandasamy *et al*., 2011).

2.3 Types of Mastitis

Bovine mastitis is a single most common disease syndrome of adult dairy cows recognized mainly as clinical and sub-clinical types worldwide, which has great economic impact on dairy industry with complex multifactorial etiology (Nooruddin *et al.*, 1997).

The Clinical Mastitis (CM) is accompanied by physical, chemical, pathological and bacteriological changes in milk and glandular tissue (Samad, 2008).

Clinical cases of mastitis are characterized by the presence of one or more of symptoms such as abnormal milk, udder swelling and systemic signs including elevated temperature, lethargy and anorexia (Eriskine, 2001). Clinical Mastitis can be mild, severe, and/ or chronic. Mild clinical mastitis involves abnormality in the milk such as flakes, clots, and a watery or other unusual appearances. 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, 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. In 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 (Wellenberg *etal*., 2002).

Subclinical mastitis is the most common form of mastitis. It is 15-40 times more common than clinical mastitis. There is no gross inflammation of the udder, no gross changes in the milk. There is decreased production and decreased milk quality. The subclinical form of mastitis in dairy cows is important because this form is (a) 15 to 40 times more prevalent than the clinical form, (b) it usually precedes the clinical form, (c) it is of long duration, (d) it is difficult to detect, (e) It reduces milk production, and (f) it adversely affects milk quality (Seegers *et al.,* 2003).

Sub-clinical mastitis in the mammary gland is detectable only by determining high somatic cell count (SCC) in milk or by bacterial culture (Quinn *etal*. 1994). Subclinical mastitis is the dominant form of mastitis affecting dairy cattle yet often goes undetected or is left untreated (Bramley and Dodd, 1984; Oliver *etal*., 2004).

2.3.1 Mode of transmission

Contagious mastitis organisms often involve the transmission of infection from a carrier to a susceptible animal primarily during the milking process. Reduction in the number of new infections is a major goal of any contagious disease control program. This is accomplished by means of optimizing milking procedures and hygiene and post milking teat dipping. Eliminating existing infections is accomplished with treatment during lactation and/or dry off or by means of culling. Separation of infected animals from susceptible animals, particularly during the milking process is also an effective means of reducing exposure to infection. Most contagious organisms cause new infections early in lactation (Becker, 1989).

Environmental mastitis organisms are opportunistic and can infect the mammary gland when the environment is contaminated with these organisms to the point where they can gain access to the teat cistern (Smith *etal*, 1985). There is a wide range of microorganisms that can invade and infect the udder; however, coliforms and environmental streptococci are the most important. Severity and duration of infection is dependent upon the type and strain of organism involved as well as a number of host factors that may include metabolic and nutritional status, existing disease, age and stage of lactation. Sources of environmental exposure include manure, bedding, water, dirt, mud and feed. Adequate use of clean, dry bedding is crucial to minimizing exposure to environmental mastitis. This is particularly critical during the dry and transitional period. Frequent cleaning and application of new bedding to the maternity are has also been identified as a means of preventing new environmental mastitis infections. Milking procedures, properly functioning milking equipment and pre and post milking hygiene can also influence the number of new infections.

2.4 Causative Agents of Mastitis

Mastitis in dairy cows may be caused by a large number of bacterial pathogen (Grohn *etal*., 2004). These includes *Staphylococcusaureus, Streptococcusagalactiae*, *Streptococcusdysgalactiae, Corynebacteriumbovis, Corynebacteriumulcerans*, *Listeriamonocytogenes, Escherichiacoli, Enterobactercloacae, Klebsiella* sp, *Citrobacter* sp. *Salmonellatyphi, Salmonellacholerasius* and *Salmonellaenteritidis*.*Pseudomonas* sp, *Brucella* sp, *Campylobacter* sp, *Mycoplasmabovigenitallium* and *Mycoplasmacalifornicum.*

Coliforms have been reported to be among the most pathogenic among the mastitis causing agents. It causes chronic, clinical or subclinical mastitis associated with a reductionof milk yield and difficulty to treat (Sol *etal*., 2000). *Staphylococcus aureus* is the major pathogen of mastitis worldwide (Guller *et al*., 2005).

2.5 *Staphylococcusaureus* as a Major Pathogen of Bovine Mastitis

Staphylococcus aureus is from the Greek and Latin words (Greek. Staphyle bunch of grapes; and Latin.*Coccus*= spherical bacterium, *aureus* golden) or golden staph, is the most common specie of *Staphylococcus* bacteria causing infection in humans and animals (Ryan and Ray, 2004).

S. aureus was discovered in Aberdeen, Scotland in 1880 by the Surgeon 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 Identification and morphology

S. aureus is Gram-positive non-spore forming, non-motile microorganism that grows readily under aerobic and microaerophillic conditions.

S. aureus appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates (Ryan and Ray, 2004). *S. aureus* reproduces asexually by binary fission. The two daughter cells do not fully separate and remain attached to one another, so the cells are observed in clusters.

S. aureus is catalase-positive. Catalase converts hydrogen peroxide (H_2O_2) to water and oxygen. Catalase-activity tests are sometimes used to distinguish staphylococci from enterococci and streptococci. Previously, *S. aureus* was differentiated from other staphylococci by the coagulase test. However, not all *S. aureus* strains are coagulase-positive (Ryan and Ray, 2004) and incorrect species identification can impact against effective treatment and control measures (Matthews, 1997).

2.5.2 Infection cause by *Staphylococcus aureus*

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).

The infection process occurs as follows:

Once the bacteria enter the teat canal, the first infection stage is adhesion. The bacteria attach themselves by releasing "sticky" proteins that glue themselves to cells inside the teat.

Once attached, the bacteria can begin to multiply without being eliminated during milking. The *S. aureus* bacteria produce a toxin that destroys cells and damages milk-producing tissue. The bacteria begin their assault by damaging the tissues lining the teat and gland cisterns, which leads to formation of scar tissue in those areas.

These bacteria are very highly adapted to survival inside the mammary gland. The *S. aureus* bacteria are able to form a "biofilm"—a slime cover that shields them from certain types of protective cells of the immune system and antibiotics. They also have the ability to live inside the white blood cells of cows, which also protects them from the expected effect of antibiotic therapy.

The bacteria continues to move up into the milk-secreting cells to establish deep-seated pockets of infection. Once there, they permanently damage the alveolar and ductal cells, reducing milk yield. The cow's immune system sends cells to form a wall (called an "abscess") around the infected area of the gland.

This wall slows or stops the bacteria from further spreading, although the organisms continue to survive and remain protected from antibiotics inside the abscess. Abscesses can become quite large and are detectable as lumps that can be felt in the udder.

2.5.3 Transmission of *Stapyhlococcus aureus* infection

The bacteria survive in the mammary gland, teat canals, and teat lesions of infected cows. They thrive in scabby or injured teat ends caused by overmilking, chapping, frostbite, biting flies, or

improper pulsation or vacuum settings. The infection is spread during milking when *Staph. aureus*contaminated milk from an infected gland comes in contact with an uninfected gland and the bacteria enter the teat canal. This spread may occur through contaminated udder wash rags, common towels, teat cup liners, milkers' hands, flies, and cross-suckling. Improperly functioning equipment, especially irregular vacuum fluctuations, can force bacteria up into the teat canal and begin a new infection.

2.5.4 Factors predisposing cow to mastitis

Several factors have conspired to ensure that mastitis has not been completely controlled to date. Firstly, in the dairy industry emphasis is often placed on breeding cows for increased milk production (Burton *et al*., 2001). A negative correlation exists between milk production capacity and resistance to mastitis; this is attributed, in part, to the increased metabolic stresses associated with the synthesis and secretion of milk (Pyöräla, 2002). The nutritional status of the cow is also an important factor in determining susceptibility to infections: deficiencies of selenium, copper, zinc or vitamin E levels can predispose the cow to infection (Pyöräla, 2002). Milking machines are often the cause of problems, as they can induce changes in the width of the teat duct, increasing the risk of colonisation of the teat duct and new intramammary infections (Pyöräla, 2002). Invasion by environmental pathogens such as *Strep.uberis*and *E. coli* almost certainly occurs at or soon after milking or if there is any teat damage. Surprisingly, even though animal husbandry practices have improved greatly, there has been no reduction in the number of environmental infections. Probably the greatest problem in treating mastitis is the plethora of pathogens that can cause mastitis - if protection or immunity is enhanced against one pathogen, it doesn't protect against another (Sordillo and Streicher, 2002).

2.6 Diagnosis of *S. aureus* mastitis

Diagnosis of *Staphylococcus aureus* infections helps in developing preventive strategies and in determining where deficiencies in mastitis control procedures may occur. Identification is based on bacterial culture of a properly collected milk sample. Bulk tank cultures can also be used to alert the producer to the presence of the organism and the need to examine individual problem cows. The best method to detect *Staphylococcus aureus* mastitis is to (1) identify all high SCC cows (more than 200,000 cells/ml), (2) perform the California Mastitis Test (CMT) on those cows to determine which quarter or quarters are affected, and (3) culture the milk from those quarters. It is extremely important to identify infections early in order to prevent spread and to increase the chance of cure.

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).

2.6.1 California mastitis test

The California Mastitis Test (CMT) is a simple, inexpensive way of detecting unseen infections. The CMT is a cowside test that gives valuable, rapid result. The CMT test estimates the SCC content of the milk. It is base d on the reagent which dissolves cell membranes and nuclei. Consequently, DNA is released and it forms a transient gel with the reagent. The more the DNA in the sample, the higher the viscosity of the gel.

Mastitis milk is slightly more alkaline than normal milk, and the gel turns purple in the presence of bromcresol purple. Therefore , one has actually two indicator principle within the same test, DNA-quantification and pH-analysis (Sandholm *et al*., 1995a).

2.7 Control of *S. aureus* Mastitis

The most effective ways to prevent new infections are to eliminate conditions that expose teat ends to bacteria and reduce the possibility of spread from cow to cow – many of which are discussed below.

1. Hygienic procedures

a. Milkers should always wear gloves and change them frequently, especially when dirty or after stripping an infected animal. It is recommended to forestrip five squirts of milk from each quarter and check for abnormal milk or flakes.

b. Dirt should be brushed off teats with the use of a dry, single-use towel. Water should not be used as part of any milking procedures, even if a sanitizing solution is added. Sanitizers do not maintain activity throughout a milking, and water can introduce pathogens that are very difficult to cure.

c. A commercially available predip should be applied with a dipper or cup and given 30 seconds of contact time. Sprayers can be used, but proper coverage is difficult to attain, especially on the teats furthest from the milker. Foamers are also commonly used, but caution must be taken to ensure that adequate parts per million of the active ingredient reach the teat end and teat skin.

d. A separate paper or cloth towel should be used to dry teats and scrub teats five times or for 20 seconds (Rasmussen *et al*., 1992). Towels must not be used on more than one cow.

e. Milking units should be attached 90 seconds after first tactile stimulation (stripping or wiping, whichever comes first).

f. Teat ends should be examined for evidence of chapping, cracks, or lesions, which may harbor mastitis-causing bacteria.

g. An effective postmilking teat dip should be applied after milking, ensuring that the entire barrel of each teat is covered. At the end of each milking any teat dip left in the dip cup should be discarded and cups should be rinsed with water and allowed to dry.

2. Milk *S. aureus* infected cows separately and last

a. Cows with clinical mastitis, *S. aureus* infections, or those that have been treated with antibiotics should be milked last, or milked with separate milkers. Segregation of *S. aureus* infected cows has proven to significantly reduce the prevalence of *S. aureus* mastitis and bulk-tank SCC (Wilson *et al*., 1995). In many instances, if the animals cannot be culled, they must be separated to prevent the spread of contagious pathogens and a rapid and negative impact on bulk-tank SCC. In the odd case in which a separate group absolutely cannot be made, it is best to identify these animals with a leg band to ensure proper care in the parlor, such as changing gloves after prepping.

b. Uninfected, first-lactation cows should be milked before older cows carrying subclinical mastitis infections. Since heifers may be infected at calving and because cure rates are

highest in younger animals, aseptic milk samples should be collected and cultured shortly after calving.

3. Milking equipment

a. *Staphylococcus aureus* infections can occur during milking when organisms penetrate the teat canal. Irregular vacuum fluctuations caused by liner slips, flooded lines, etc., may cause a backflow of milk against the teat end. With sufficient force, bacteria can be propelled up into the teat canal and teat cistern. Therefore, properly functioning equipment is essential in preventing new infections.

b. Conditions that are associated with high-impact force against the teat end – including liner slips, excessive temporary vacuum losses, low vacuum reserve, inefficient vacuum regulation, an abrupt milking unit removal – should be minimized. Teat cups should not be removed from the cow until the vacuum has been shut off. Research has shown that slipping teat cup liners may cause 10 to 15 percent of new mastitis infections. Liner slippage early in milking often results from a low vacuum level, blocked air vents, or restrictions in the short milk tube. Liner slippage late in milking is commonly caused by poor cluster alignment, uneven weight distribution in the cluster, or poor liner condition. Incomplete milking can be caused by poor type or condition of liner, a mismatch between claw inlet and short milk tube, clusters that are too light, clusters that do not hang evenly under the udder, or high milking- vacuum levels (Halleron, 1997).

c. Regular, preventive maintenance is essential for milk quality and mastitis prevention. Vacuum controllers (regulators), pulsators, and air filters need to be cleaned monthly. All rubber components must be changed according to the manufacturer's instructions.

d. Rubber that is cracked, flattened, or otherwise deteriorated should be replaced even if the recommended life span of the product has not been reached. The milking system should be evaluated every three months or 500 hours of operation, to include the following tests: vacuum reserve, vacuum level, vacuum recovery time, vacuum regulator response, pulsator graphs, and stray voltage. Many of these tests need to be conducted during milking time and not between milkings.

4. Antibiotic treatment of mastitis

Antibiotic treatment will not control this disease but it may, in certain cases, shorten the duration of the infection. Treatment effectiveness decreases as the cow becomes older and even as the first lactation progresses. Cure rates were 34 percent when 89 cows in 10 Dutch herds were treated for subclinical *S. aureus* mastitis (Sol *et al*., 1997). The results showed that the probability of cure was lower in older cows with high SCC and in cows infected in hindquarters during early and midlactation. *S. aureus* infections were found in 36 percent of clinical mastitis cases in Finnish herds (Pyorala and Pyorala 1997). Of these, 39 percent responded to treatment. Successful treatment during lactation is greater if detected and treated early, whereas the response is lower when treating chronic infections. Use of a strip cup or similar device is strongly recommended for detecting abnormal milk. New clinical infections should be treated promptly and appropriately, especially in first-lactation cows. Tissue damage can be minimized if animals are treated during the early stages of infection. As always, consult a veterinarian regarding off-label treatment options. The use of DHI program SCC records in addition to visual observation of forestripped milk and milk culture results will indicate effectiveness of treatment.

Many researchers have looked at the efficacy of pirlimycin treatment both in heifers prior to calving and in all animals as an extended therapy treatment during lactation. According to the manufacturer, pirlimycin is one of the most effective compounds against *S. aureus* because its chemical nature allows it to penetrate mammary tissues. In heifers, a single tube of pirlimycin treatment in each quarter six to 12 days prior to calving significantly reduced *S. aureus* infections at calving (Roy *et al*., 2007). Furthermore, mastitis data presented to the FDA suggests that two tubes, administered 24 hours apart to infected quarters of cows during lactation, resulted in a cure rate of 36.6 percent, whereas only 1.1 percent of nontreated controls recovered spontaneously. In field cases, the rate of cows cured during lactation increased to 49.4 percent. However, trials using the same treatment scheme at Louisiana State University and Iowa State University found cure rates of only 12 percent or less for chronically infected *S. aureus* cows during lactation.

Single-quarter, extended therapy with repeated label doses of pirlimycin has been examined as a means of providing drug levels beyond the expected life of the leukocytes that naturally fight off this infection. This protocol has been widely adopted for new intramammary infections with *S. aureus*, as it increases cure rates. Four-quarter extended treatment with repeated label doses will provide adequate therapeutic concentrations for many *S. aureus* bacteria. A cure rate of 50 percent at four weeks after treatment was found in more than 100 treated cows (Belschner *et al*., 1996). Whether these cure rates justify the additional expenses and effort, not to mention the potential risk of extra-label use and antibiotic residue, is unknown.

5. Dry cow therapy

Dry cow therapy (DCT) is more effective in eliminating infections than lactating treatment. However, DCT is not effective if the infections have become chronic by the end of lactation. When cows are not given DCT, spontaneous cures have been very low. DCT is cost-effective (Kirk *et al*., 1997). When a cow is dried off, it is recommended to treat all quarters with a commercially available DCT.

Steps:

a. Cows must be milked out completely, teats dipped in postmilking teat dip and blotted dry after 30 seconds contact time.

b. Teat ends should be scrubbed with alcohol pads before partially inserting the antibiotic tube into the teat (1/8 inch). Although internal teat sealants do not prevent the spread of *S. aureus* infections, the commercially available product does help to reduce new environmental infections. Internal teat sealants are especially useful during the first two weeks and the last seven to 10 days of the dry period when cows are most susceptible to mastitis pathogens. This increase in susceptibility is in part due to the stress associated with drying and the precalving period.

c. Teats should be dipped again after treatment, prior to turning the cows out into a clean, dry environment.

6. Pregnant heifers

New infections are commonly found in heifers, either at calving or in early lactation. Up to one-third of these infections are caused by *S. aureus.* Often these *S. aureus* infections, if untreated, become clinical and recur throughout the first lactation and into the second lactation. Furthermore, these infections increase the chance of contagious spread to other animals in the herd. Several management practice can be used on heifers prior to calving to eliminate infections before the start of lactation.

Administration of dry cow therapy to heifers has been evaluated in several Louisiana studies. A dry cow product containing penicillin and dihydrostreptomycin was administered during the first, second, or third trimester of pregnancy in 35 bred heifers from four herds. Although prevalence of infection and SCC were reduced by treatment in all three groups of heifers, heifers dry-treated during the second trimester of pregnancy demonstrated the greatest reduction in mastitis and SCC at calving (Nickerson *et al*., 1995). It is recommended that heifers be treated with dry cow treatment at 60 days before expected calving date. Teat ends should be properly cleaned and disinfected before and after treatment. It is important to check milk for the presence of antibiotic residue at three to five days after calving or before the milk is allowed in the bulk tank.

In Tennessee, a lactating cow antibiotic treatment containing either cloxacillin or cephapirin was administered to heifers at seven to 10 days before expected calving (Oliver *et al*., 1992). Cephapirin gave better treatment results than cloxacillin but resulted in antibiotic residue in milk at three days after calving. Treating heifers with cephapirin 14 days before expected calving eliminated the residue problem. It is therefore recommended that heifers be treated

with lactating cow mastitis treatment at 14 days before expected calving, using precautions indicated under the preceding section about dry cow therapy.

7. Precautions at calving

Many mastitis infections (not specifically *S. aureus*) originate in the peripartum period. A well-drained pasture is preferred as a calving area, with no access to ponds, swampy areas, or drainage ditches. A clover-grass sod is desired, in contrast to fescue or muddy, beaten-up lots. Lots and pastures should be managed to prevent muddy areas where cattle would lie down. Filthy, damp, or muddy pens, lots, or pastures continually expose the teat end to a barrage of bacteria. Pens should be well-bedded, clean, dry, and comfortable. Selenium-vitamin E supplementation or injections at two to three weeks before expected calving have been shown to reduce mastitis after calving. Vitamin E levels of 1,000 IU/day during the dry period and 500 IU/day during lactation are recommended by the National Research Council. Other minerals and vitamins shown to reduce the incidence of mastitis include vitamin A/ beta-carotene, copper, and zinc. By testing animals to identify micronutrient deficiencies, providing a balanced ration, avoiding poorly fermented silages, and including dietary supplementation of vitamin E and selenium, proper nutrition can be maintained to reduce incidence of mastitis.

2.8 Prevention of Recurrent *S. aureus* Infection

The best treatment for *S. aureus* mastitis is prevention. Recommendations to prevent spread of contagious mastitis pathogens include:

1. Do not milk cows and heifers with the same teat cup/claw unit used to milk mastitis-problem cows.
2. Segregate *S. aureus*-infected cows into one group and milk them last. Another alternative is to sort out infected cows before each milking and restrain them in an isolation pen until all other cows have been milked. In a short period of time, these cows will become trained to sort.
3. If heifers or cows are purchased, segregate them until milk samples can be cultured and their mastitis pathogen status can be determined. If possible, examine the DHI SCC of cows before agreeing to make the purchase.
4. Cull infected cows, especially those with additional problems. Often, it is not economically feasible to cull a *S. aureus*-positive cow that produces 80-90 pounds of 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. Place *S. aureus*-infected cows on a do-not-breed list. Cull them when their milk is no longer needed (e.g., to make base) or they have been in milk for 305 days.
6. 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 feed or manure that has accumulated in exercise yards, calf pens, and box stalls. Another option for control is use of back-rubbers, feed additives, and ear or tail tags with insecticide. In one trial the use of tail

tags containing insecticide resulted in only one of 100 tagged heifers with a mastitis infection, compared to 18 of 100 untagged heifers.

7. It is unclear whether waste milk from *S. aureus* infected cows is a problem. One study showed a positive association between feeding *S. aureus* infected colostrum and heifers calving with S. aureus mastitis (Roberson *et al*., 1998). Therefore, not feeding contaminated waste milk is a simple precaution that will most likely reduce a possible reservoir of bacteria.

8. Many *S. aureus* vaccines have been designed and tested over the years. One research study indicated little-to-no effect on new intramammary infections following vaccination with a commercial product. However, another investigation demonstrated a 61 percent reduction in *S. aureus* mastitis using the same product (Nickerson *et al*., 2008). Future research endeavors may focus on alternative vaccine options that will successfully prevent *S. aureus* mastitis.

2.9 Antimicrobial Susceptibility of *Staphylococcusaureus*

Antimicrobial resistance is influenced by the different practices of administering antimicrobial product (dosage, timing frequency, type and frequent change of types) (Aarestrup, 1999). Also, extended use and inappropriate use of antibiotics in agricultural stock farming and in the treatment of human diseases were identified as the cause of increasing resistance of bacteria to antimicrobial agents. (Pesavento *et al*., 2007).

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). 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.

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).

The resistance of pathogens causing mastitis to antimicrobial agents has two relevant implications. The first is a reduction in cure rates after treatment of clinical mastitis cases (Owen *et al*., 1997; Sol *et al*., 2000). The second issue is the potential impact of transmission of resistant bacteria to humans via the food chain (Ungemach, 1999). Antibiotic resistance patterns of *S.aureus* isolated from hospital environment, cattle and milk products in Nigeria have been reported (Kwaga and Adesiyun, 1984; Umoh *et al*., 1990; Nierman, 2001).

2.10 Staphylococcal Enterotoxins

2.10.1 Staphylococcal food poisoning

Staphylococcal food poisoning (SFP) is an intoxication that results from the consumption of foods containing sufficient amounts of one (or more) preformed enterotoxin (Dinges *et al*., 2000;

Le loir*et al*., 2003). Symptoms of SFP have a rapid onset (2–8 h), and include nausea, violent vomiting, abdominal cramping, with or without diarrhea (Balaban and Rasooly 2000; Murry, 2005; Tranter, 1990.) The disease is usually self-limiting and typically resolves within 24–48 h after onset. Occasionally it can be severe enough to warrant hospitalization, particularly when infants, elderly or debilitated people are concerned (Murray, 2005).

However, *S. aureus* is also present in food animals, and dairy cattle, sheep and goats, particularly if affected by subclinical mastitis, are likely contaminants of milk (Stewart, 2005). Air, dust, and food contact surfaces can also serve as vehicles in the transfer of *S. aureus* to foods.

2.10.2 *Staphylococcus aureus* enterotoxins

Staphylococcus aureus produces a wide variety of toxins including staphylococcal enterotoxins (SEs; SEA to SEE, SEG to SEI, SER to SET) with demonstrated emetic activity, and staphylococcal-like (SE*l*) proteins.

The *S. aureus* enterotoxins (SEs) are potent gastrointestinal exotoxins synthesized by *S. aureus* throughout the logarithmic phase of growth or during the transition from the exponential to the stationary phase (Betley *et al*., 1992; Bergdoll, 1979; Czop and Bergdoll, 1979; Derzelle *et al*., 2009; Otero *et al*., 1990). They are active in high nanogram to low microgram quantities (Larkin *et al*., 2009), and are resistant to conditions (heat treatment, low pH) that easily destroy the bacteria that produce them, and to proteolytic enzymes, hence retaining their activity in the digestive tract after ingestion (Bergdol, 1983; Evenson *et al*., 1988).

2.10.3 Staphylococcal enterotoxins and food poisoning outbreaks

Independent of their origin, enterotoxigenic *S. aureus* often differ in the number of mobile genetic elements and *se*/*sel* genes therein, as well as in the enterotoxins they produce. SEA, either alone or together with other SEs/SELs, is the enterotoxin most commonly reported in foods, and is also considered as the main cause of SFP, probably due to its extraordinarily high resistance to proteolytic enzymes (Balaban and Rasooly, 2000; Bergdol, 1988; Holmberg and Blake, 1984). The predominance of SEA is well documented in different countries. As relevant examples: (i) a comprehensive study of 359 outbreaks that occurred in the United Kingdom (UK) between 1969 and 1990 revealed that 79% of the *S. aureus* strains produced SEA (Wieneke *et al*., 1993). Meat, poultry and their products, particularly ham and chicken, were the vehicle in 75% of the incidents. SEA was detected alone in 56.9% of the outbreaks and, in conjunction with SED, SEB, SEC or SEB and SED in a lower number of outbreaks (15.4, 3.4, 2.5 or 1.1%, respectively); (ii) SEA was also the enterotoxin most frequently found among 31 SFP outbreaks in France (69.7%), which were associated with a great variety of foods including milk products, different types of meat, and salads, between 1981 and 2002 (Kerouanton *et al*., 2007). In agreement with this, *SEA*was the most common gene in the isolates tested, followed by *SED*, *SEG*,*SEI* and *SHE*; (iii) In Austria, an SFP outbreak that affected 40 children in 2007 was attributed to *S. aureus* isolates producing SEA and SED. Bovine milk products were identified as the source of the outbreak, and the cows, not the dairy owner, were the more likely reservoir of the SEs-producing *S. aureus* (Schmid *et al*., 2009); (iv) SEA was also the most common enterotoxin recovered from food poisoning outbreaks in USA (77.8% of all outbreaks) followed by SED and SEB (Casman, 1965); (v) A study of *S. aureus* obtained from dairy products, responsible for 16 outbreaks in Brazil revealed that the most frequently encountered enterotoxin

gene was *SEA* followed by *SEB* (Veras *et al*., 2008). Finally, (vi) several studies have investigated the distribution of SEs and *se*/*sel* genes in *S. aureus* from foods and SFP outbreaks in Asian countries. Among strains recovered from patients associated with SFP outbreaks during 2001-2003 in Taiwan, *SEA* was the most common gene, followed by *SEB* and *SEC* (Chiang *et al*., 2008). In Korea, about 90% of food poisoning isolates were reported to contain the *SEA* gene (Cha *et al*., 2006). SEA also was the most common SE associated to SFP in Japan (Shimizu *et al*., 2000). In this country, an extensive outbreak that occurred in 2000 was attributed to low-fat milk containing SEA (Asao *et al*., 2003), while a recent outbreak (2009) was due to crepes containing SEA and SEC (Kitamoto *et al*., 2009).

SEB, SEC or SED alone have been also implicated in SFP outbreaks through the world (Wieneke *et al*., 1993; Kerouanton *et al*., 2007; Veras *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Sampling Plan

3.1.1 Sample size

The sample size for this study was determined using the formula of ThrusField,(2005) at 95% confidence level and a reported 31%prevalence of mastitis from settled herds in Zaria (Umoh *et al*.,1990b).

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

Where, N= Sample size

Z= Statistics for a level of 95% confidence interval = 1.96

P= prevalence rate of mastitis from previous studies =31%

d = level of significance (allowable error) = 5% or 0.05

q= 1-p

Thus, $N = \frac{(1.96^2) \times 0.31 \times (1-0.31)}{(0.05)^2}$

$= \frac{3.8416 \times 0.31 \times 0.69}{0.0025}$

$= 328.6872$

The calculated sample size is 328.6872; but a total of 403 samples were collected from selected dairies farm

3.1.2 Study area

The study was conducted in five predetermined regions in Zaria, namely Bomo, Hanwa, Shika, Palladan and Wusasa from April to June 2015. Selection was based on the presence of farms that are producing and selling milk when the study commence.

3.1.3 Collection of samples.

Prior to quarter milk sampling, the teat ends were cleaned with absorbent cotton wool moistened in 70% alcohol. Two hundred ml of milk was collected in sterile container directly from the udder of individual cows. The sample were labelled and then transported immediately in ice-cooler boxes to Postgraduate Microbiology Laboratory Ahmadu Bello University, Zaria for bacteriological analysis.

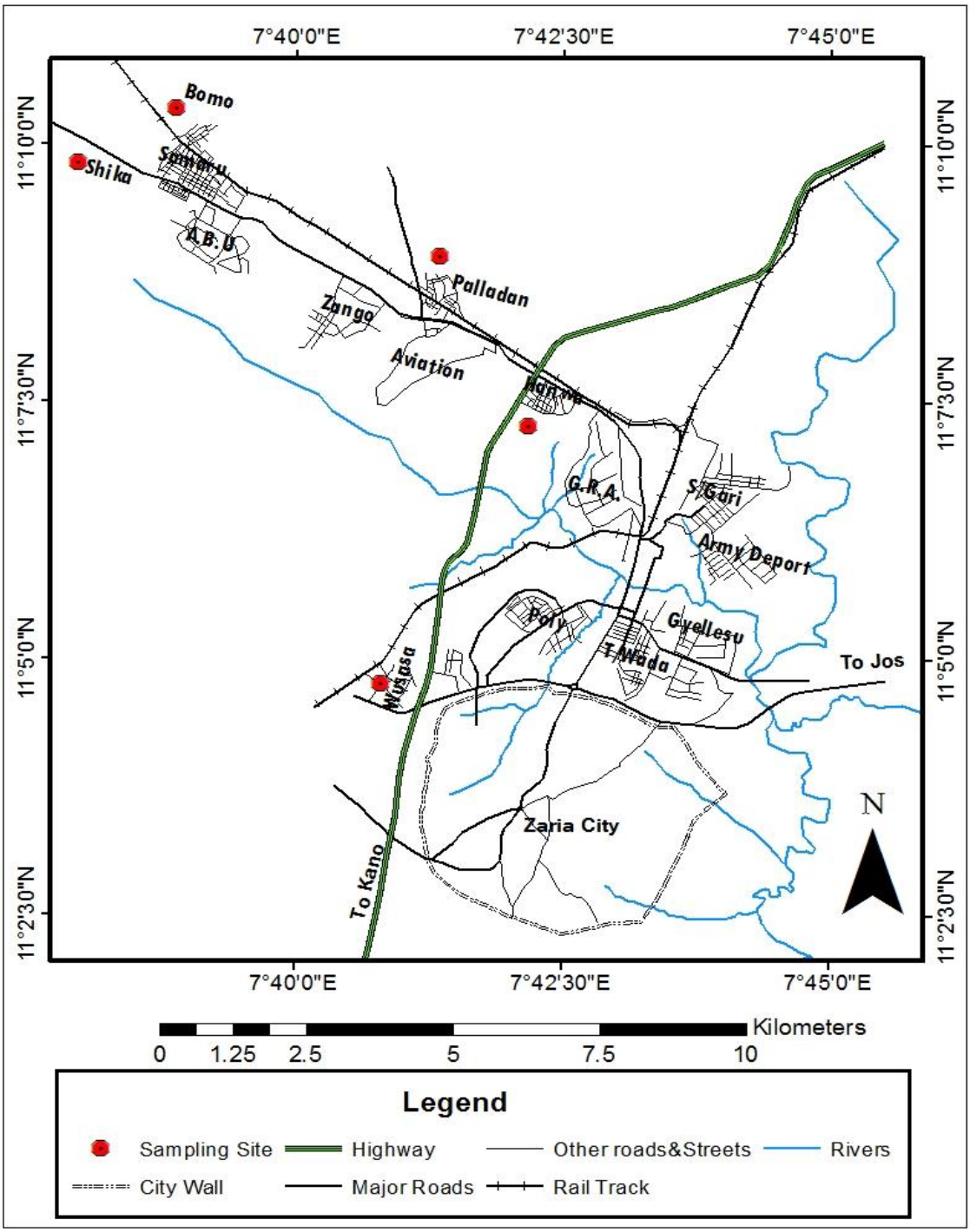


Fig: 3.1: Map of Zaria showing milk sampling sites.

Source: Zaria Satellite Image 2014.

3.2 Assessment of Physicochemical Quality of Milk

3.2.1 Physical inspection of the milk

The milk samples were checked for clots in which 5ml of the milk in test tube was placed in boiling water bath for 5min, and then examined for curd precipitate, it was also checked for flakes, discoloration and change in consistency. If the milk samples had clots, flakes discoloration and change in consistency, then it is indicative of a positive clinical mastitis.

3.2.2 Temperature

Temperature was determined using a clean thermometer. The thermometer was immersed in the milk samples and the readings were recorded.

3.2.3 pH

pH model was used to measure the pH of the milk. The pH model was immersed in the milk sample and the readings were recorded.

3.2.4 Titratable acidity

Titratable acidity of the milk samples were determined according to the method of Association of Official Analytical Chemist (AOAC, 1990). Ten (10ml) of milk sample was pipetted into a beaker and 3-5 drops of 1% phenolpthalin indicator was added to it. The milk sample was then titrated with 0.1 N NaOH solution until a faint pink colour persisted. The tiaratible acidity, expressed as %lactic acid was finally calculated using the following formula.

$$\% \text{ Titratable acidity} = \frac{\text{Titre}}{\text{Volume of sample}} \times \text{Chemical factor (0.009)} \times 100$$

3.2.4 Specific gravity

A lactometer was used to measure the specific gravity of the milk. The lactometer was placed in a cylinder containing sufficient milk sample and allowed to float freely then the readings were recorded.

3.3 Proximate Composition of Milk

3.3.1 Moisture content

The moisture of the milk sample was determined according to Pearson (1976). Five grams of milk sample were placed into a dish and dried in the oven at 105^0c for 2 hours with the lid alongside. The lid was placed on the dish and transferred to the desiccators, the sample was weighed when the dish is cooled. The moisture percent of the milk sample was calculated from the following equation:

$$\text{Moisture (\%)} = \frac{(B - C) \times 100}{A}$$

Where:

A= sample weight in grams.

B= weight of dish + sample prior to drying.

C= weight of dish + sample after drying.

3.3.2 Ash content

The ash was determined according to Pearson (1976). A porcelain dish dried in an oven, cooled in desiccators and then weighed. Three gram of milk sample were placed into the dish and

burned in furnace at 550^0 C until free from carbon (residue appears grayish – white) about 2 hrs. It was transferred to the desiccators to cool down. The dish and sample was then re-weighted. The ash percent of the sample were calculated according to the following:

$$\text{Ash (\%)} = \frac{B - C}{A} \times 100$$

Where:

A= sample weight in grams before ashing.

B= weight of dish and sample after ashing.

C= weight of empty dish.

3.3.3 Fat content

The Gerber method was used to determine the milk fat content. Ten ml of sulphuric acid was dispensed into a butyrometer. Then, 11 ml of milk sample and 1 ml of amyl alcohol were added into the butyrometer having the sulfuric acid. The butyrometer was closed with rubber cork and the sample was shaken and inverted several times until all the milk was digested by the acid. Then the butyrometer was placed in a water bath at 65^0 C for 5mins. The sample was centrifuged for 5 mins at 1100 rpm. Finally, the sample was returned back to the water bath and kept for 5 mins at 65^0 C and fat percentage was read from the butyrometer scale (O' Connor, 1995).

3.3.4 Protein content

Total Protein content of the milk samples were determined by the Kjeldahl method (AOAC, 1990) according to the following procedures:

For digestion: Five gram of milk sample was warmed in a water bath at 38^0c and poured into a Kjeldahl flask. Fifteen gram potassium sulphate, 1.0 ml of copper sulphate solution and 25 ml of

concentrated sulphuric acid were added into the flask and mixed gently. The digestion was carried out in a digestion block until a clear solution appeared. Then it was allowed to cool to room temperature.

For distillation: The digestion flask was placed in the distillation equipment and then 30 ml of distilled water and 75 ml of 50% sodium hydroxide solution were added into it. Then, ammonia was distilled then 50 ml of 40% boric acid solution using bromocresol green indicator were added until blue colour appeared. Finally, the sample was titrated with 0.1 N hydrochloric acid solution from a burette until a faint pink colour solution was formed. Blank test was carried out using the above procedure except that water was used instead of test sample. The percentage of nitrogen in the milk samples were calculated as follows:

$$\%N = \frac{(Vs - Vb)\ HCl\ consumed \times NHCl \times 1.4007}{Sample\ weight} \times 100$$

$$\%CP = \%N \times 6.38$$

Where,

%N = percentage nitrogen by weight

Vs = volume of HCl used for titration of sample

Vb = volume of HCl used for titration of the blank

%CP = percentage of crude protein.

3.4 California Mastitis Test (CMT)

Two ml from each of the milk sample was placed in the CMT paddle. Three ml of CMT reagent was added to each cup. Then, gentle circular motion was applied to the mixtures in a horizontal plane for 15seconds. The reaction was interpreted based on the thickness of the gel formed by CMT reagent and milk mixture, and the test result were scored as negative (0), trace (T), + (weak

positive, ++ (distinct positive), +++ (strong positive) according to (Quinn *et al*., 2002). Quarters with CMT score of (+) or above were judged as positive.

3.5 Bacteriological Analysis of Milk

3.5.1 Total plate count

This was carried out according tostandard procedures recommended by APHA (1992) reported by (Tassew *et al*., 2010). For the total bacterial count, 10 ml of each milk sample was added into sterile test tube containing 90 ml of peptone water. Then thoroughly mixed, the sample was serially diluted up to 10^4 and 1 ml was transferred to 20 ml standard plate count agar solution and mixed thoroughly. The plated sample were allowed to solidify and then incubated at 37ºC for 48 h, after which the colonies were counted.

3.5.2 Total staphylococcal count

A dilution from 10^2 was surfaced plated on mannitol salt agar in duplicate plates for staphylococcal count. All the plates were incubated at 37º C for 48 h, after which colonies were counted.

3.5.3 Identification of *Staphylococcus aureus*

A sterile wire loop was used to streak the milk samples on MSA (Mannitol salt agar) plate and incubated at 37º C for 24 h. Cultures were examined for morphological, staining and cultural characteristics. Pure cultures that were Gram positive cocci in clusters were stored at 4°C for further biochemical characterization.

3.5.3.1 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 sixty seconds and thereafter decolourized with acetone and rinsed immediately with water. Safranine was added for thirty seconds and rinsed with water. The slide was blot-dried and oil immersion was added then viewed under the microscope.

3.5.3.2 Coagulase test

The tube coagulase test method was used. A sterile wire loop was use to collect the bacteria isolate and emulsified in a microtube containing 0.5ml of rabbit plasma. The mixture was incubated at 37°C and examined after1,3 and 6 h. Ability of the plasma to form a clot in the tube indicated a positive result.

3.5.3.3 Catalase test

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

3.5.3.4 Haemolysis

Pure culture was streaked on plates of blood agar to observe for the ability of the organism to produce haemolysin. Clear zone indicated positive haemolysis.

3.5.3.5 DNase

Pure culture was streaked on DNase agar and incubated at 37 ºC for 24 h. After incubation the plate was flooded with 10% hydrochloric acid. Clear zone indicated a positive result**.**

3.6 Confirmation of *S. aureus* using Microgen Staph-ID Identification

The microgen staph-ID system which comprises of a single microwell test strip having 12 standardised biochemical substrates which have been selected on the basis of extensive computer analysis for the identification of the genus staphylococcus was used. The dehydrated substrate in each well was reconstituted with a suspension of the organism to be identified. If the individual substrate are metabolized by the organism, a colour change occurs. The permutation of metabolized substrate was interpreted using the microgen identification system software (MID-60) to identify the test organism.

3.7 Antibacterial Susceptibility Test

Antimicrobialsusceptibility test was conducted on 54 isolates using 9 antimicrobial agents following the Kirby-Bauer disk diffusion method (Quinn *et al*., 1999). The antimicrobial agent and the concentration used were: Vancomycin (30ug), Cefuroxime (30ug), Methicillin (5ug), Norfloxacin (10ug), Ciprofloxacin (5ug), Penicillin (10ug), Chloramphenicol (30ug), Erytromycin (10ug), Gentamicin (10ug).

Each isolate was standardized using McFarland standard No. 0.5 and inoculated on Mueller Hinton Agar then the antibiotic disk was placed on the surface of the medium using a pair of sterile forcep. The plate was incubated at 37 ºC for 24 h, after which the diameter of each zone of inhibition was measured using a graduated ruler. Antibiotic susceptibility was reported as either sensitive, intermediate or resistant according to the standard diameter of zones of inhibition following the guidelines of the Clinical and Laboratory Standards Institute (CLSI).

3.8 Molecular Characterization using Polymerase Chain Reaction (PCR)

3.8.1 DNA extraction

DNA extraction was performed with DNA extraction kit, according to the manufacturer's instruction.

3.8.2 PCR amplification

The PCR was established using the primer (GSEAR-1 GGTTATCAATGTGCGGGTGG GSEAR-2 CGGCACTTTTTTCTCTTCGG and GSEBR-1 GTATGGTGGTGTAACTGC GSEBR-2 CCAAATAGTGACGAGTTAGG (Appendix 1) allowing the detection of genes encoding staphylococcal enterotoxin A and B. PCR was carried out in a Master cycler using the following conditions: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min), and elongation (72°C for 1 min). A final step (55°C for 2 min and 72°C for 5 min).

3.9 PCR Master Mix

A 30 microlitre reaction mixture was used for the DNA amplification, containing 1microlitre of primer 1, 1microlitre of primer 2, 0.6 microlitre of deoxynucleoside triphosphates, 3 microlitre of thermophillic buffer, 1.8 microlitre of $MgCl_2$, 0.1 microlitre of taq DNA Polymerase, 20 microlitre distilled water and 2.5 microlitre of template DNA in a 200 microlitre reaction tube.

3.10 Agarose Gel Electrophoresis of PCR Products

Twelve microlitre of PCR products mixed with 2ul of gel loading dye were loaded for electrophoresis in precast wells in 2% agarose gel with Tris acetate-electrophoresis (TAE). Gels were viewed and photographed using a gel documentation system.

3.11 Data Analysis

The results were analysed using SPSS version 20 and Microsoft excel.Chi square was used to test for association between qualitative variables.ANOVA was used for quantitative variable and Duncan multiple range test was used to analysed values at a different significance levels.$P<0.05$ was taken as significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Location of Dairy Herds with Number of Cows and Quarters

A total of 403 quarter milk sample was collected from 104 lactating cows from 17 dairy herds which include Palladan, Shika, Hanwa, Wusasa and Bomo (Table 4.1)

4.2 Milk Collected at Udder Quarters of Cow

The frequency distribution of milk collected at udder quarters of cow is summarised in Table 4.1. The result show that in Palladan 26.2% was collected from right rear and right fore while 23.8% was collected from left rear and left fore. In Shika, equal portion of milk (25%) was collected from each of the udder sites. In Hanwa, 26.1% was collected from left rear, left fore and right fore while 21.7% was collected from right rear. In Wusasa, 26.2, 25, 24.4 and 23.8% were collected from right fore, right rear, left fore and left rear respectively. In Bomo, 25.6% was collected from left and right fore while 24.4% was collected from left and right rear. On a total scale, 25.8% and 25.1% of milk were collected from right and left fore, while 24.6% was collected from left and right rear.

4.3 Results of Physicochemical Quality of Milk

4.3.1 Physical inspection of milk

All the 403 quarter milk samples had no clots, flakes, discolouration or change in consistency (100%).

4.3.2 Temperature of milk

The temperature range of milk samples measured at the point of collection was 23-37^{0}C. However in total, 92.1% of the milk had temperature in the range of 28-32^{0}C, while 4.5 and 3.5% had temperature in the range 33-37^{0}c and 23-27 ^{0}C respectively. 96.8% of the CMT positive milk samples and 90.6% of the CMT negative milk samples had their temperatures between 28-32^{0}C (table 4.3). There is no significant association between CMT reaction and temperature of milk (χ^2 =5.005, df=2, p=0.082).

4.3.3 pH of milk

The percentage distribution of pH of milk from the dairy herds in Zaria is presented in Table 4.4. On a total scale, 55.6% of the milk recorded pH values in the range of 6.5-6.7 while 26.3 and 18.1% respectively had pH values less than 6.5 and greater than 6.7. With respect to California mastitis test, 56.4% of the CMT positive milk samples had their pH greater than 6.7 while 63.8% of the CMT negative milk samples had their pH between 6.5-6.7. The differences observed was statistically significant (χ2=121.096, df=2, p=0.000).

4.3.4 Titratable acidity of milk

The percentage distribution of titratable acidity of milk from the dairy herds in Zaria is presented in Table 4.5. On a total scale, 92.1% of the milk recorded titratable acidity of greater than 0.16% lactic acid, 5.7% had their titratable acidity in the range of 0.14-0.16% lactic acid while 2.2% had their titratable acidity of less than 0.14% lactic acid. 6.4% of the CMT positive milk sample and 5.5% of the CMT negative milk sample had their titratable acidity in the range of 0.14-0.16% lactic acid. There was no significant association between CMT reaction and titratable acidity of milk.

Table 4.1: Location of dairy herds with number of cows and quarters.

Location	No of herds	No of cows	No of quarters	No of cows with blocked teat canal
Palladan	4	22	84	4
Shika	3	22	88	0
Hanwa	3	18	69	3
Wusasa	3	22	84	4
Bomo	4	20	78	2
Total	**17**	**104**	**403**	**13**

Table 4.2: Frequency distribution of milk collected from udder quarters of cow

Dairy farm	Udder quarters								
	Right rear		Left rear		Right fore		Left fore		Total
	No of samples	%	No of samples	%	No of samples	%	No of samples	%	
Palladan	22	26.2	20	23.8	22	26.2	20	23.8	**84**
Shika	22	25.0	22	25.0	22	25.0	22	25.0	**88**
Hanwa	15	21.7	18	26.1	18	26.1	18	26.1	**69**
Wusasa	21	25.0	20	23.8	22	26.2	21	25.0	**84**
Bomo	19	24.4	19	24.4	20	25.6	20	25.6	**78**
Total	**99**	**24.6**	**99**	**24.6**	**104**	**25.8**	**101**	**25.1**	**403**

Table 4.3: Frequency Distribution of Temperature of milk from Dairy herds location in Zaria.

		Temperature		
CMT	**No examined**	**23-27^{0}C (%)**	**28-32^{0}C (%)**	**33-37^{0}C (%)**
Positive	94	0 (0)	91 (96.8)	3 (3.2)
Negative	309	14 (4.5)	280 (90.6)	15 (4.9)
Total	**403**	**14 (3.5)**	**371 (92.1)**	**18 (4.5)**

(χ^2=5.005, df=2, p=0.082)

Table 4.4: Frequency Distribution of pH of milk from Dairy herds locations in Zaria.

		pH		
CMT	**No examined**	**<6.5 (%)**	**6.5-6.7 (%)**	**>6.7 (%)**
Positive	94	14 (14.9)	27 (28.7)	53 (56.4)
Negative	309	92 (29.8)	197 (63.8)	20 (6.5)
Total	**403**	**106 (26.3)**	**224 (55.6)**	**73 (18.1)**

(χ^2=121.096, df=2, p=0.000)

Table 4.5: Frequency Distribution of Titratable acidity of milk from Dairy herds locations in Zaria.

		Titratable acidity (% lactic acid)		
CMT	**No examined**	**<0.14 (%)**	**0.14-0.16 (%)**	**>0.16 (%)**
Positive	94	0 (0)	41 (43.6)	53 (56.4)
Negative	309	0 (0)	83 (26.9)	226 (73.1)
Total	**403**	**0 (0)**	**124 (30.8)**	**279 (69.2)**

(χ^2=1.449, df=2, p=0.485)

4.3.5 Specific gravity of milk

The percentage distribution of specific gravity of milk from dairy farms in Zaria is summarised in Table 4.6. On a total scale, 70.5% of the samples had specific gravity between 1.028 and 1.032 while 29.5% had specific gravity less than 1.028. 77.7% of CMT positive milk samples had their specific gravity <1.028 while 85.1% of CMT negative milk samples had their specific gravity between 1.028-1.032. None of the milk sample had their specific gravity greater than 1.032. There is a significant association between CMT reaction and specific gravity ($\chi 2$=138.572, df=2, p=0.000).

4.3.6 Mean scores of physicochemical characteristics of milk according to califonia mastitis test.

Analysis of mean score of physicochemical characteristics of milk samples according to CMT is shown in Table 4.7. There was no significant association between mean value of temperature and titratable acidity. However, pH and specific gravity revealed a significant association. The mean score of pH for CMT positive samples (6.62 ± 0.47) was higher than that of CMT negative value.

4.4 Results of Proximate Composition of Milk.

The proximate composition (Moisture, Ash, Fat, and Protein content) of milk is summarized in Table 4.8. The moisture content ranged from 86.99 – 87.67%, ash content ranged from 0.66 – 0.69%, fat content ranged from 3.22 – 3.81% and protein content range from 3.27 – 3.45%. There is a statistical significance difference between means of protein and ash content. ($p<0.05$).

Table 4.6: Frequency Distribution of Specific gravity of milk from Dairy herds locations in Zaria.

		Specific gravity		
CMT	**No examined**	**<1.028 (%)**	**1.028-1.032 (%)**	**>1.032 (%)**
Positive	94	73 (77.7)	21 (22.3)	0 (0)
Negative	309	46 (14.9)	263 (85.1)	0 (0)
Total	**403**	**119 (29.5)**	**284 (70.5)**	**0 (0)**

(χ^2=136.480, df=2, p=0.000)

4.7:Mean scores of physicochemical characteristics of milk according to califonia mastitis test.

			physicochemical	Parameters	
CMT	**No examined**	**Temperature**	**pH**	**Specific gravity**	**Titratable acidity**
Positive	94	29.91 ± 1.42	6.62 ± 0.47	1.026 ± 0.002	0.18 ± 0.11
Negative	309	29.97 ± 1.83	6.44 ± 0.43	1.030 ± 0.002	0.17 ± 0.10
P-value		**0.784**	**0.001**	**0.000**	**0.461**

Table: 4.8:Proximate composition of milk from dairy herds locations in Zaria.

Dairy herds	Proximate Parameters			
	Moisture content	**Ash content**	**Fat content**	**Protein content**
Palladan	86.99±0.23	0.68±0.03	3.52±0.11	3.13±0.04[a]
Shika	87.67±0.30	0.68±0.02	3.48±0.14	3.45±0.11[b]
Hanwa	87.41±0.17	0.66±0.03	3.22±0.17[a]	3.19±0.06[a]
Wusasa	87.34±0.13	0.68±0.02	3.51±0.12	3.36±0.09[c]
Bomo	87.20±0.21	0.66±0.02	3.82±0.07[b]	3.25±0.04[a]
p-value	**0.258**	**0.031**	**0.038**	**0.963**

Values of proximate parameters are mean ± standard deviation. Means with different superscripts down the column are significantly ($P<0.05$) different.

4.5 Total Bacterial Count in Milk.

The total bacterialcount in milk from dairy farms in Zaria, expressed in log (CFU/ml) is summarised in Table 4.9. There is a statistical significant difference between the mean total bacterial count from the different location. The mean total bacterial count was 6.38 ± 0.11.

4.6 Total Staphylococcal Count in Milk.

The total staphylococcalcount in milk from dairy farms in Zaria, expressed in log (CFU/ml) is summarised in Table 4.10. There is a statistical significant difference between the mean total staphylococcus count from the different location. The mean total staphylococcuscount was 3.15 ± 0.14.

4.7 California Mastitis Test (CMT) on Milk.

Table 4.11 present the result of CMT conducted on 403 milk samples for the screening of subclinical mastitis. CMT scores of 0 and T are considered as negative while +, ++, and +++ as positive. The prevalence of subclinical mastitis ranged from 17.9% to 26.9% between the herd location. The result shows that 94(23.3%) quarter milk sample were CMT positive while 309 (76.7%) quarter milk sample were CMT negative.. The differences observed among the locations showed no significant association (χ^2=1.978, P=0.740).

4.8 CMT Reaction Based on Anatomic Site of Udder

Table 4.12 shows the results of the number of udder with respect to their anatomic sites and CMT reaction.The number of quarters are right rear 99 (24.6%), left rear 99 (24.6%), right fore 104 (25.8%) and left fore 101 (25.1%). The highest prevalence of 28.3% was recorded for milk samples collected at the right fore while the least prevalence of 18.8% was recorded for samples collected at the left rear udder. There was no significant association between CMT reaction and udder position (χ^2=4.348, P=0.226).

Table 4.9: Mean Total Bacterial Count from milk samples in relation to dairy herd locations.

Dairy farm location	**No of samples**	**Total bacterial count (Log_{10}cfu/ml)**	**F- value**	**P-value**
Palladan	84	6.43 ± 0.12^{a}	10.798	0.000*
Shika	88	6.41 ± 0.08^{a}		
Hanwa	69	6.38 ± 0.09^{a}		
Wusasa	84	6.22 ± 0.10^{b}		
Bomo	78	6.40 ± 0.08^{a}		
Mean total	**403**	**6.38 ± 0.11**		

Values of colony count are mean ± standard deviation. Means with different superscripts down the column are significantly ($P<0.05$) different.

Table 4.10: Mean Total Staphylococcal Count from milk samples in relation to dairy herds locations.

Dairy farm location	**No of samples**	**Total staphylococcal count (Log_{10}cfu/ml)**	**F- value**	**P-value**
Palladan	84	3.15 ± 0.11^{bc}	11.519	0.000*
Shika	88	3.11 ± 0.14^{c}		
Hanwa	69	3.21 ± 0.10^{ab}		
Wusasa	84	2.98 ± 0.13^{d}		
Bomo	78	3.24 ± 0.11^{a}		
Mean total	**403**	**3.15 ± 0.14**		

Values of colony count are mean ± standard deviation. Means with different superscripts down the column are significantly (P<0.05) different.

Table 4.11: Frequency distribution of California mastitis test (CMT) in relation to locations of dairy herds.

CMT scores									
Herd location	**No examined**	**0**	**T**	**+**	**++**	**+++**	**∑(CMT≥+) (%)**	**χ^2**	**P-value**
Palladan	84	59	5	8	11	1	20(23.8)	**1.978**	**0.740**
Shika	88	65	0	14	9	0	23(26.1)		
Hanwa	69	38	13	3	8	7	18(26.9)		
Wusasa	84	59	6	8	8	3	19(22.6)		
Bomo	78	61	3	10	4	0	14(17.9)		
Total	**403**	**282**	**27**	**43**	**40**	**11**	**94(23.3)**		

Table 4.12: The number of udder with respect to their anatomic sites and CMT reaction.

		Cmt reaction		
Udder	**Total sampled**	**Positive (%)**	χ^2	**p-value**
Right fore	99	28 (28.3)	**4.348**	**0.226**
Left fore	99	27 (27.3)		
Right rear	104	20 (19.2)		
Left rear	101	19 (18.8)		
Total	**403**	**94 (23.3)**		

4.9 Isolation and Identification of *S. aureus*

All the ninety four (94) mastitic milk sample were cultured for isolation of bacteria and isolated organisms subjected to microscopic and biochemical characterization. Eighty one were Gram positive cocci in clusters. Of the 81 Gram positive cocci in clusters, all were catalase and coagulase positive. Sixty four were positive for heamolysis while only 55 were positive for Dnase (Table 4.13). Sixty samples were selected for further confirmation using Microgen Staph-ID out of which 54 were confirmed as *S. aureus* ,5 were *S. hyicus* and 1 *S.intermidis.*

4.10 Result of CMT Scores in Comparison with Frequency of Isolation of *S. aureus* from Milk Sample.

All the milk sample with +++ CMT positive were positive for *S.aureus* while 9 (20.9%) and 34 (85%) of milk sample with + and ++ were positive (Table 4.14). 0 and T, CMT milk samples

4.11 Antibacterial Susceptibility Test.

The antibacterial susceptibility test result of 54 *S.aureus* isolates are summarised in table 4.15. The result indicates 100% of the isolates were sensitive to Vancomycin, Norfloxacin and Erythromycin, 7.4% were sensitive to Penicillin.

4.12 Detection of Enterotoxin Genes (A and B) by PCR.

15 Isolate were selected for the identification of the gene Staphylococcal enterotoxin A and B (SEA and SEB), of which only two were positive for SEA and five were positive for SEB. None of the isolate harboured both gene for Staphylococcal enterotoxin A and B.

Plate 1 shows the amplicons of the Staphylococcal enterotoxin A (SEA) gene of *S.aureus* with a size of 102bp. Lane M 100bp DNA ladder.

Plate 2 shows the amplicons of the Staphylococcal enterotoxin B (SEB) gene of *S.aureus* with a size of 164bp. Lane M 100bp DNA ladder.

Table 4.13: Biochemical characterization of 81 *S. aureus* isolate from milk samples.

Biochemical test	No of positive isolate	%
Catalase	81	100
Coagulase	81	100
Haemolysis	68	84
Dnase	55	68

Table 4.14: Result of CMT scores in comparison with frequency of isolation of *S. aureus* from milk sample.

CMT scores	No examined	*S. aureus* positive (%)
+	43	9(20.9)
++	40	34(85)
+++	11	11(100)
Total	**94**	**54**

Table 4.15: Antibiotics sensitivity of 54 isolates of *S.aureus*

Antibiotics	**Sensitive (%)**
Vancomycin (30ug)	54 (100)
Cefuroxime (30ug)	45 (83.3)
Methicillin (5ug)	4 (7.4)
Norfloxacin (10ug)	54(100)
Ciprofloxacin (5ug)	48 (88.9)
Penicillin (10ug)	4 (7.4)
Chloramphenicol (30ug)	51 (94.4)
Erytromycin (10ug)	54 (100)
Gentamicin (10ug)	48 (88.9)

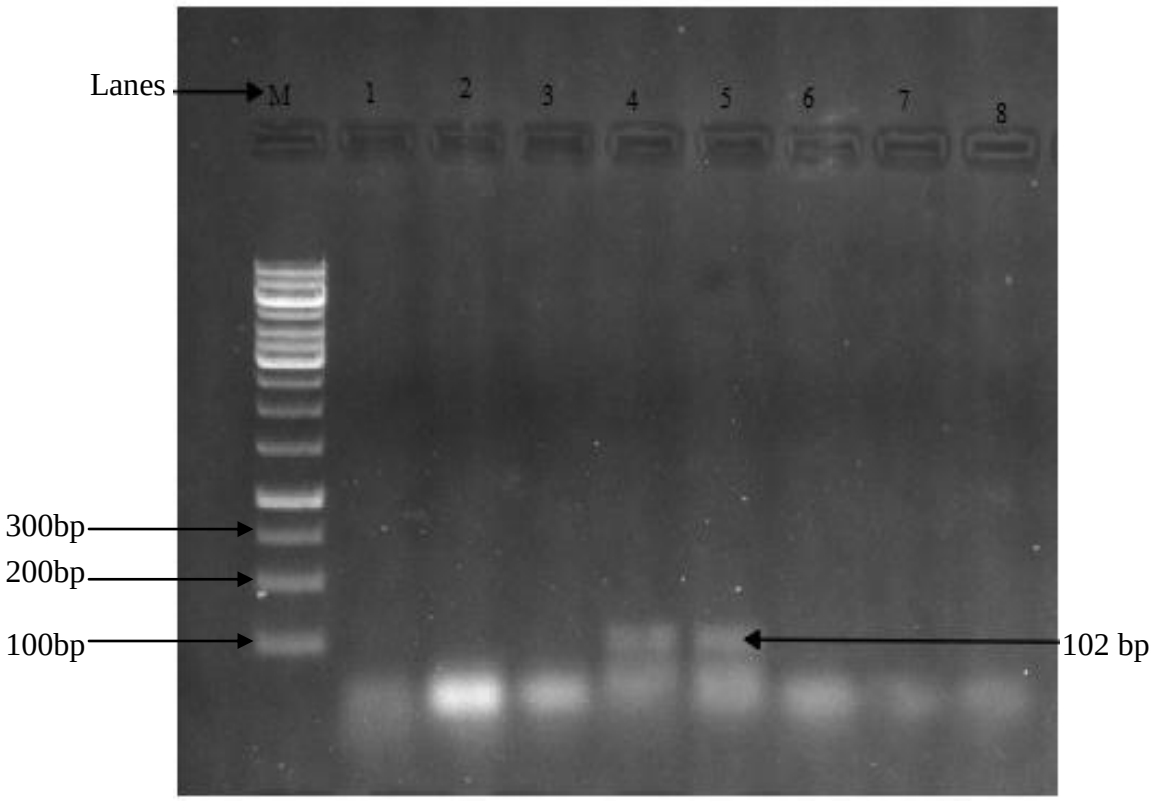


Plate 1: Amplicons of the Staphylococcal Enterotoxin A (SEA) gene of *S.aureus* with a size of 102bp. Lane M 100bp DNA ladder.

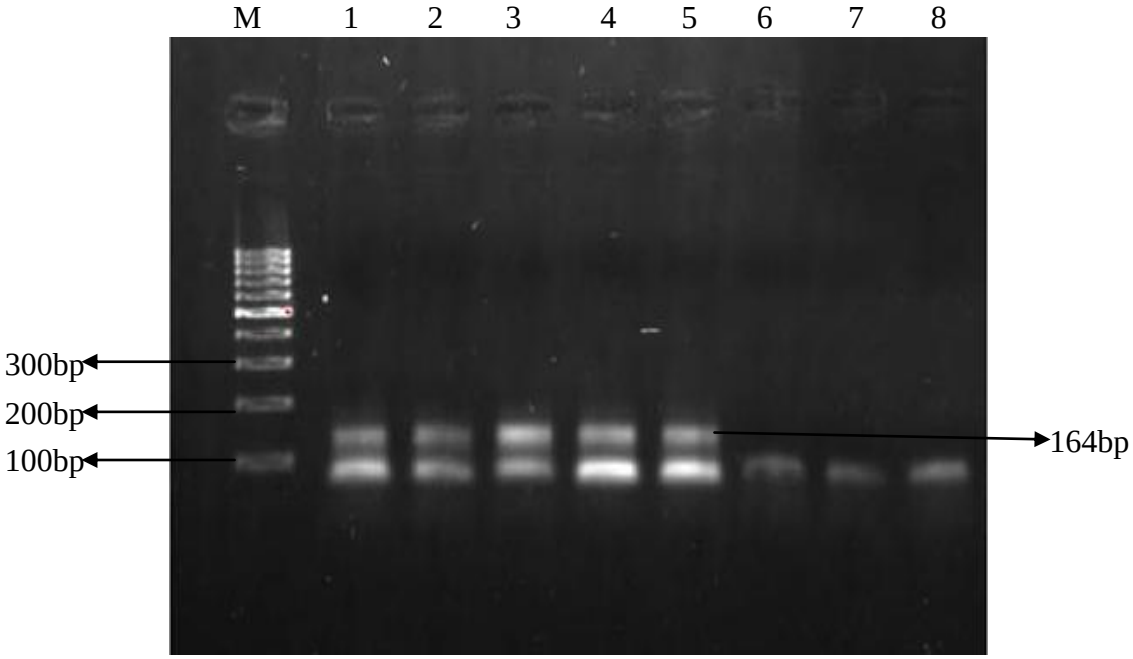


Plate 2: Amplicons of the Staphylococcal Enterotoxin B (SEB) gene of *S.aureus* with a size of 164bp. Lane M 100bp DNA ladder.

CHAPTER FIVE

5.0 DISCUSSION

Subclinical mastitis is hard to detect and is the main form of mastitis (Oliver *et al*., 2004; Pitkala *et al*., 2004). The findings in this research revealed that out of the 403 quarter sample assayed, 94 quarter samples were CMT positive therefore the prevalence of subclinical mastitis based on CMT was 23.3%. In a similar study, Suleiman *et al*., (2013) reported a 30.9% prevalence of subclinical mastitis based on CMT in Plateau state. The slight difference between this study and Suleiman *et al.,* (2013) could be due to traditional dairy husbandry practices whereby calvers are kept away from their dam over a long period of time and are only allowed to suckled for a short period as well as inadequate milk supply which leads to calves suckling vigorously, inducing teat injuries and subsequent infection of the mammary gland (FAO,1990). It could also be due to poor milk hygiene practices such as non- usage of disinfectant of udder, teat dipping and lack of instituting dry cow therapy.

Physicochemical characteristics of milk

All the samples had no clots, flakes, discoloration or change in consistency. The temperature of freshly drawn milk was about 38^0C. Bacteria multiply very rapidly in warm milk and milk sours rapidly if held at this temperature. If the milk is not cooled and stored at a temperature of 16^0C or below, the milk will spoil quickly (O'Connor, 1995). It is essential to cool the fresh milk in order to prevent or retard growth of bacteria in the milk.

The present study revealed that 73(77.7%) of mastitic milk had specific gravity below 1.028 which is below the standard range, while 21(22.3%) of mastitic milk had specific gravity within

the standard range (1.028-1.032). This agrees with the work of Ahmad *et al* (2005) that milk specific gravity decreases due to mastitis. In mastitic milk, specific gravity reduced due to increased chlorides and decreased lactose content (Badran *et al*., 1986; Haggag *et al.,* 1991).The result of statisticalanalysis shows a significant association (x2= 138.572) between CMT reaction and specific gravity of milk,

Specific gravity is the relation between the mass of a given volume of substance. However, lower specific gravity of milk indicate adulteration of milk and low quality of milk. A very high percentage of CMT negative milk sample 263(85.1%) had specific gravity within the standard range, and is indicative of the freshness of the milk.

Specific gravity and pH of milk are one of the best basis to assess the food value of milk for consumers and processors. The Ph may serve as the best indicator to assess the udder health status of the animal and food value of milk. As the severity of mastitis increased, pH values also increases, lowering the food value of the milk (Haggag *et al*., 1991).

Milk contains a large number of weak acid or weak base and its salt, so it behaves as a buffer solution. Fresh cow milk has pH of between 6.5 and 6.7 therefore, it is slightly acidic .Values higher than 6.7 connotes of mastitic milk while values lower than 6.5 connotes the presence of bacterial deterioration.pH value lower than 6.5 therefore indicate that considerable acid development has taken place. This is normally due to the bacteria activity (http://www.slideshare.net/mobile/farhana25/measuring-the-Ph-of-milk).

In this study, higher percentage of CMT positive milk 53(56.4%) had a pH range above 6.7, 27(28.7%) had a pH within the acceptable range and 14(14.9%) had pH below the acceptable range. This agrees with the work of Ahmad *et al*., (2005) who reported that pH increases due to

mastitis. There is a significant association between CMT reaction and pH. This also agrees with the work of Haggag *et al*., (1991) which reported that with severity of mastitis, pH of the sample increases.

Titratable acidity is a measure of freshness and bacterial activity in milk. Popescu and Angel (2009) reported that high quality milk has to have a <0.14% acidity. Normal fresh milk has an apparent acidity of 0.14 to 0.16% lactic acid (O'Connor 1995). This present study revealed that the mean titratible acidity of CMT positive milk is 0.18 ± 0.11 and for CMT negative milk is 0.17 ± 0.10 which are higher than the normal range, the slight increase in the titratible acidity might be due to bacterial activity in the milk. In a similar study, Asaminew and Eyassu (2011) reported a higher total acidity for milk samples collected from individual farmers (0.23±0.01) and dairy cooperatives (0.28±0.01) %lactic acid.

Proximate composition of milk

Milk composition and production are the interaction of many element within the cow and her external environment (O'Connor 1995). Milk composition and quality are important attribute that determine the nutritive value and consumer acceptability. Milk yield and composition are affected by breed, age, stage of lactation,season and plane of nutrition, interval between milking, milking method, health status and bacterial activities (Enavie 2001).

The significance of moisture content in milk is that, high moisture content implies high water activity which support microbial growth consequently reducing the shelf life of the milk sample (Ajai *etal*., 2012). Milk has high amount of water about 87%, so it is a good source of water in the diet (Krishna *etal*., 2015). Fresh cow milk is reported to contain about 88% water (Kataoka *et al*., 1991).

The ash content of milk samples obtained in this study is below the standard range which might be due to the feed of the animal. The ash content of cow milk remains relatively constant 0.7 to 0.8% and it is influenced by breed, stage of lactation and feed of the animal (O'Conner 1995). In a similar findings, Mahmood and Usman (2010) report a 0.60% of ash in cow milk. Oladapo and Ogunekun (2015) reports a higher ash content of 0.81 and 0.79% for Bunaji and Bokolooji breeds of cattles.

The fat content of the milk were within the standard range. There is a significant difference between means of fat content from the different locations ($p=0.038$). The fat content in milk has been found to vary considerably with the environment and stage of lactation (O'Mahony 1988). Eckles and Macy (2004) reported that milk fat is the most valuable constituent of milk, it is the food value of the milk. In a similar study, Tesfay *et al*., (2015) report a fat content of 3.86% and 3.83% of milk from dairy farms and milk vendors. A higher fat content of 4.0% for cow milk was reported by Mahmood and Usman (2010), also Oladapo and Ogunekun (2015) reports a higher fat content of 4.70 and 4.60 for Bunaji and Bokolooji breeds of cattles. The FDA requires not less than 3.25% milk fat for fluid whole milk (Raff, 2011).

There is a significant difference between means of protein content from the different location ($p=0.031$), this might be due to the plane of nutrition or health status of the cow. In a similar findings, Tesfay *et al*., (2015) reported a protein content of 3.42% and 3.27% for milk samples obtained from dairy farms and milk vendors. However, Debela *et al*., (2015) report a higher protein content of 4.08% and 3.81% for milk samples collected at open market and household milk producers.

Total bacterial and Staphylococcal count

The most widely used test as a general indication of good hygienic milk production is the standard plate count. Harding, (1999) reported that total bacterial count of raw milk from individual producers should not exceed 10^5cfu/ml and that for bulk milk should not exceed 3.0×10^5 cfu/ml while for pasteurized milk, the bacteria load should not exceed 2.0×10^4 cfu/ml (FDA, 2001). The total bacteria count of milk from all the locations ranged from 6.22 ± 0.10 - 6.43 ± 0.12 and for the staphylococcal count from 2.98 ± 0.13 - 3.24 ± 0.11 . The bacteria load of the milk were relatively high which reduces the quality of the milk and can cause quick spoilage of the milk. It can also lead to food poisoning after consumption of contaminated milk. This is in accordance with the findings of El deeb and Hassan (1987) who reported that total bacteria count increased when milk test positive for mastitis. Milk is highly nutritious food that serve as an excellent growth medium of wide range of bacteria (Ruegg and Reinermann, 2002).

At the onset of udder infection, mastitis is known to influence the chemical composition of milk constituents (Mohamed *et al*., 1997). In this study, subclinical mastitis based on CMT was higher in right fore and left fore. There was no association between CMT reaction and udder position. Ameh *et al*., (1999) reported that the number of cases found in fore and hind quarters shows no statistical difference.

Antibacterial susceptibility

The present study revealed that 54(13.4%) of *S. aureus* was isolated from mastitic milk. This does not agree with the work of Suleiman *et al.,* (2012) who isolated 30.2% *S. aureus* from mastitic milk in plateau state but agree with the report of Pitkala *et al*., (2004) who report an isolation rate of 10.2% for *S. aureus*. Recently, it was reported that 3% of all animals are infected

with *S. aureus* (Schullken *etal*., 2009). Moreover in a study conducted in Egypt, it was found that 16% of all mastitic cases are caused by *S. aureus* (Hameed *et al*., 2006). The isolation of *S. aureus* is of public health significance since is a commonly recovered pathogen in outbreaks of food poisoning due to milk and milk product (Culler, 1967).

Mastitis is one of the major causes of antibiotics usage in dairy cows (Mitchell *et al*., 1998; DANMAP, 2003). Antimicrobial therapy plays a role in mastitis control by reducing the levels of herd infection and preventing new infections. However, bacteriological cure rate against *S. aureus* for antimicrobial therapy is relatively low due to pathogen characteristics such as the ability to survive inside the host cell, ability to adapt to different environmental conditions, presence of virulence factors and pathological changes induced in chronic infection (Rabello *et al*., 2005; Waldvogel, 2000). Also extended use and inappropriate use of antibiotics in agricultural stock farming and in the treatment of human diseases were identified as the cause of increasing resistance of bacteria to antimicrobial agents (Pesavento *et al*., 2007).

In cases of mastitis, the wrong or incomplete use of antibiotic treatment of animals also contribute significantly to the development of bacterial resistance against them. A large number of the isolate were found to be resistant to an age long established antibiotic (Penicillin (92.6%) compared to the susceptibility of most of the isolates to the more recently developed antibiotic (Cefuroxime). In this study, the high resistance of *S. aureus* to penicillin and high sensitivity to vancomycin agrees with the work of Suleiman *et al*., (2012) who reported 100% sensitivity of *S. aureus* to vancomycin and completely resistant to penicillin.

The isolates were also highly sensitive to norfloxacin and erythromycin (100%), chloramphenicol (94.4%), gentamicin and ciprofloxacin (88.9%). Farooq *et al*., (2008) reported

that *S. aureus* was the major pathogen responsible for mastitis and norfloxacin was the most effective drug under invitro condition against the isolated pathogens. The proportion of *S. aureus* isolate sensitive to ciprofloxacin in this study agrees with the work of Nazneen *et al*., (2004) who reported 94.4% of *S. aureus* sensitive to ciprofloxacin. Also, isolate that are higly sensitive to gentamicin and chloramphenicol corroborate with the work of Redat *et al*., (2014) who reported that *S. aureus* was sensitive to gentamicin (92%) and chloramphenicol (65%).

The study shows that over 90% of the isolates were resistance to methicillin. For many years, methicillin resistance *S. aureus* (MRSA) was considered only a human pathogen, until a report of a MRSA infection in a dairy cow surfaced in 1972 (Devriese *et al*., 1972). There is now increasing evidence that MRSA can be transmitted in both directives, from human to animals and from animal to human. Once exposed to MRSA, animal can become colonized and may serve as reservoir to transmit the infection to other animals and also to their human handlers (Manian, 2003; Baptiste *etal*., 2005; Weese, 2005; Hanselman *et al*., 2006; Huijsdend *et al.,* 2006). Methicillin resistance *S. aureus* is an important human pathogen and may produce serious infections that may results in septicaemia (Pasavento *et al*., 2007). Infection due to this organism have been associated with increased morbidity, mortality and prolonged hospitalization (NNIS 2003).

Detection of staphylococcal enterotoxin genes using PCR.

In this study, 2 isolates harboured SEA and 5 isolates with SEB gene but none of the isolates harboured both gene for Staphylococcal enterotoxin A and B. The difference in this study and that of Suleiman *et al*., (2013) might be adduced differences in the nature of the study population

and in PCR screening methodologies (primers, amplification cycles and single versus multiplex PCR) which may contribute to such variations (Marwa and Mahmoud 2013).

The capacity to coagulate plasma, the principal characteristics of the *S. aureus* is highly correlated with the capacity to produce enterotoxins which is harmful to the tissue of the contaminated host. (Murray *et al*., 2006). The presence of *S. aureus* shows up unsanitary conditions in the cattle herd (Tortora *et al*., 2005). Normally, the production of enterotoxin is favored at temperature of 40 – 45°c, although Smith *et al*., (1982) detected production of toxins at temperature of 10 – 46°c. The predominant classical SE gene varied from country to country: Germany, SEA, SEC(Becker *et al*., 2003); New Zealand, SEB(Boerema *et al*., 2006); Poland, SEC(Bania *et al*., 2006) and in Egypt, SEA (Marwa and Mahmoud,2013).

In zaria, Umoh *et al.,* (1990) reported a relatively low prevalence of 19% enterotoxigenic *S. aureus* from bovine mastitic milk, also Suleiman *et al*., (2013) reported that out of 20 isolates tested for staphylococcal enterotoxin (ABCD), only 2 had SEA, none harboured SEB, C and D.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

From the 403 quarter milk samples collected, the result of physicochemical analysis shows that a high proportion of the milk had their pH and specific gravity within the standard range, but the total tiitratible acidity was slightly above the quality range. Also, the proximate composition shows that the milk sample are good source of fat and protein.

From the total bacterial counts and staphylococcal count of the milk samples, it indicate that the bacteria load in the milk sample were relatively high.

The prevalence of subclinical mastitis based on CMT was 23.3%. 54 *S. aureus* were isolated from the 94 CMT positive samples.

The Microgen Staph ID kit showed to be more efficacious for the detection and identification of *S. aureus* from mastitic cow milk.

The antibacterial susceptibility of the isolates shows that all the isolates were sensitive to vancomycin, norfloxacin and erythromycin.

The PCR technique detect the presence of Staphylococcal enterotoxin A and B in some strains but none of the isolate harboured both gene for SEA and B.

6.2 Recommendations

From the above findings, the following recommendations are given below:

1. There is need to educate dairy farmers on the need to control mastitis and make them aware of the risk of decreased milk production and reduced quality of the milk.
2. Dairy farmers should be encourage to practice proper milking hygiene at different steps of raw milk production in order to reduce the incidence of staphylococcal infection through consumption of contaminated milk product.
3. Consumption of raw cow milk should be discourage because of the risk of staphylococcal enterotoxin milk borne intoxication and would recommend the pasteurization of milk before consumption.

REFERENCES

Aarestrup, F. M., Wegener, H. C. and Rosdahl, V. T. (1995). Acomparative study of *Staphylococcus aureus* isolated from bovine subclinical mastitis during 1952-1956 and 1992. *Acta Veterinaria Standinavica*, 36:237-43.

Aarestrup, F. M., Larsen, H. D., Eriksen, H., Elsberg, C. S. and Jansen, N. E. (1999).Frequency of alpha-and beta-haemolysin in *Staphylococcus aureus* of bovine and human origin.A comparison between pHenotype and genotype variation in pHenotypic expression.*Acta pathogica Microbiologica Immunologica Scandinavia*, 107: 425-430.

Ahmad, T., Bilal, M.Q, Ullah, S. and Muhammad G. (2005) Effect of severity of mastitis on pH and specific gravity of Buffalo milk.*Pak. Journal of AgriculturalScience, Vol. 42(3-4).*

Ajai, A. I., Ochigbo, S.S., Ndamitso, M.M. and Ola, O.J. (2001). Proximate and Mineral Composition of different raw cow milk in Minna, Niger State, Nigeria. *European Journal of Applied Engineering and Scientific Research*, 1: 23-29.

Akineden, O, Annemuller C, Hassan, A.A, Lammler C, Wolter, W and Zschock M. (2001). Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. *Clinical and Diagnostic Laboratory Immunology*, 8: 659- 964.

Al-Majali, A. M., Al-Qudah, K. M., Al-Tarazi, Y. H., and Al-Rawashdeh, O.F. (2008). Risk factors associated with camel brucellosis in Jordan. Trop. Anim. Health Prod. 40: 193-200.

Ameh, J. A., Edgbe-nwiyi, T. and Zaria, L. T (1999).Prevalence of Bovine mastitis in Maiduguri, Borno state, Nigeria.*Veterinary Archive*, 69:67-95.

American Public Health Association (APHA) (1992). Standard method for the examination of dairy product, 16thed, APHA, Washington. Pp 213-220.

Argudin, M. A, Mendoza, M. C, and Rodicio M. R .(2010). Food poisoning and *Staphylococcus aureus* enterotoxins. Toxin, 2: 1751-1773.

Asaminew,T. and Eyassu, S. (2011). Microbial quality of raw cow's milk collected from farmers and dairy cooperatives in Bahir Dar Zuria and Mecha district, Ethiopia. Agric. Biol. J. N. Am. 2(1):29-33.

Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa, H. and Kozaki, S. (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in

Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. Epidemiol. Infect. 130:33–40. [PMC free article] [PubMed]

Atasever, S.(2012). Estimation of correlation between somatic cell count and coagulation score of bovine milk.*Int J Agric Biol*, 14: 315-317.

Badran, A.E., M.A. Sharaby, and G.A. Hassan.(1986). Susceptibility of cows and buffaloes to mastitis infection. II. Milk quality and mastitis infection. Indian veterinary J. 63: (12): 107-1022.

Balaban, N. and Rasooly, A. (2000).Staphylococcal enterotoxins.Int. J. Food Microbiol. 61:1-10 [PubMed]

Bania, J., Dabrowska, A., Korzekwa, K., Zarczynska, A., Bystron, J., Chrzanowska, J. and Molenda, J. (2006): The profiles of enterotoxin genes in *S. aureus* from nasal carriers. Lett. Appl. Microbiol., 42(4): 315–320.

Baptiste, K.. E., Williams, K. and Williams,M. J. (2005).*Emerg Infect Dis*. 1:1942-1944

Barabas, J. (1994). Milk hygiene with particular reference on milk handling and preservative. Proc. Regional work shop on Raw milk handling and preservation. University of Alexandria, Eqypt.

Becker, N.G. (1989). Analysis of infectious disease data. Chapman and Hall, London.

Becker, K. Friedrich, A.W., Lubritz, G., Weilert, M., Peters, G. and Von Eiff, C. (2003): Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *S. aureus* isolated from blood and nasal specimens. J. Clin. Microbiol., 4(4): 1434–1439.

Belschner, A. P., J. W. Hallberg, S. C. Nickerson, and W. E. Owens.(1996). *Staphylococcus aureus* mastitis therapy revisited. Proceedings, National Mastitis Council annual meeting, Madison, Wis., 116-22

Bergdoll, M.S. (1979) Staphylococcal intoxications. In: Reimann H., Bryan F.L., editors. Foodborne Infections and Intoxications. Academic Press Inc; New York, NY, USA: 1979. pp. 443–494.

Bergdoll, M.S. (1983). Enterotoxins. In: Easman C.S.F., Adlam C., editors. Staphylococci and Staphylococcal Infections.Vol. 2.Academic Press Inc; London, UK. pp. 559–598.

Bergdoll, M.S. (1988). Monkey feeding test for staphylococcal enterotoxin.Meth.Enzymol. 165:324–333. [PubMed]

Betley, M.J., Borst, D.W. and Regassa, L.B. (1992). Staphylococcal enterotoxins, toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. Chem. Immunol. 55:1–35. [PubMed]

Blasko, B. (2011). World importance and present tendencies of dairy sector.University of Debrecen.

Bradley, A. J**.** (2002). Bovine mastitis: an evolving disease. *Veterinary Journal* **164**: 116-128.

Bramley, A.J. and Dodd, F.H., (1984). Reviews of the progress of dairy science: mastitis

control—progress and prospects. J. Dairy Res. 51, 481-512.

Blood, D. C. and Radostits O.M., (1989) *Veterinary Medicine*. A textbook of the disease of cattle, sheep, pigs, goats and horses.university printing house, Oxford. 7th edition. Pp 501-550.

Boerema, J.A., Clemens, R. and Brightwell, G. (2006): Evaluation of molecular methods to determine enterotoxigenic status and molecular genotype of bovine, ovine, human and food isolates of *S. aureus*. Int. J. Food Microbiol., 107(2): 192–201.

Burton, J.L., Madsen, S.A., Yao, J., Sipkovsky, S.S. and Coussens, P.M.(2001).An immunogenomics approach to understandingperiparturient immunosuppression and mastitis susceptibility in dairycows.*Acta Veterinaria Scandinavica* 42: 407-424.

Byron, H. W. Arnold, H. J. and Johan, A.A. (1994).Fundamentals of dairy chemistry.2nd ed., Westport, Conneticut.The Avi publishing company, Inc pp 2-8.

Cabral, KG, Lammler, C, Zschock M, Langoni H, De Sa M EP, Victoria C, Da Silva AV (2004). PHeno and genotyping of *Staphylococcusaureus*, isolated from bovine milk samples from Sao Paulo State, Brazil. Can. J. Microbiol., 50: 901-909.

Calvinho, L. F., Toselli, F.G., Weimann, W.R., Canavesio, V.R., Neder, V.E and Iguzquiza, A.I. (2002). Antimicrobial sensitivity of coagulase-positive staphylococcal strains isolated from bovine mastitis in the central dairy catchment area of Argentina.*Review of Argentina Microbiology*, 34:171–175.

Carlos, A. D. M. (1990): Characteristics of *Staphylococcus aureus* from subclinical bovine mastitis in Brazil. *Brazil Veterinary Journal*, 146: 443-448.

Casman, E.P. (1965). StapHylococal enterotoxin. Ann. N.Y. Acad. Sci. 1965;128:124–131. [PubMed]

Cha, J.O., Lee, J.K., Jung, Y.H., Yoo, J.I., Park, Y.K., Kim, B.S. and Lee, Y.S. (2006).Molecular analysis of *Staphylococcus aureus* isolates associated with staphylococcal Food poisoning in South Korea. J. Appl. Microbiol. 101:864–871. [PubMed]

Chiang, Y.C., Liao, W.W., Fan, C.M., Pai, W.Y., Chiou, C.S., Tsen, H.Y. (2008).PCR detection of staphylococcal enterotoxins (SEs) N, O, P, Q, R, U, and survey of SE types in *Staphylococcus aureus* isolates from food-poisoning cases in Taiwan. Int. J. Food Microbiol. 121:66–73. [PubMed]

Culler, S. (1967).Mastitis and its influence upon reproductive performance in dairy cattle. In: Proc. Inter,Symp.Bovine mastitis Indianapolis. 176-180.

Czop, J.K. and Bergdoll, M.S. (1974).Staphylococcal enterotoxin synthesis during the exponential, transitional, and stationary growth pHases. Infect. Inmun. 9:229–235. [PMC free article] [PubMed].

DANMAP (Danish Integrated Antimicrobial Resistance Monitory and Research Programme). (2003). Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and himans in Denmark 2003.DANMAP, Soborg, Denmark.

Debela, GT. Eshetu, M. and Regassa, A. (2015). Physico-chemical qualities of raw cow milk in ethiopia. The case of borana zone, Yabello district.Global journal of dairy farming and milking production. ISSN:2449-1861 Vol 3(2), pp.86-91.

De Oliveira, A.P., Watts, J.L., Salmon, S.A. and Aarestrup, F.M. (2000). Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. *Journal of Dairy Science*, 83:855–862.

Derzelle, S., Dilasser, F., Duquenne, M. and Deperrois, V. (2009). Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. Food Microbiol. 26:896– 904.[PubMed].

Devriese, L. A., Van Damme, L. R. and Fameree, L. (1972). Zbl Vet Med B 19:598-605.

Ebliny, L. T., Fox, L.K., Bayles, K.W., Bohach, G.A., Byrne, K.M., Davis, W.C., Ferens, W.A. and Hillers, J.K. (2001). Bovine mammary response to an experimental intramammary infection with *Staphylococcus aureus* strain containing a gene for *Staphylococcal* enterotoxin C1. *Journal of Dairy Science*, 84: 2044- 2050.

Eckles, C.H. and Comb, W.B. (2004). Milk and milk product 4thed. New delhi.

Egwaikhide, P.A., Malu, P.S.,Lawal, U.,Adelagun, R.O. and Andrew, C. (2014).. Physicochemical and Microbiological analysis of Fermented cow milk (nono) consumed within Kaduna town, North western, Nigeria. *s*, 29: 2225-2557

El Deeb, SA and Hassan, HN. (1987). Changes in the cow's milk composition as affected with mastitis infection.*Alexandria. Journal of Agricultural Research*, 32: 163- 174.

Enavie, K. (2001). Effects of supplementation on the milk yield. Proceeding of the 26th Annual conference of Nigeria society for animal production, march 2001, ABU Zaria Nigeria. Pp 353-355.

Eriskine, R. J. (2001). Intramuscular administration of ceftiofursodiu versus intrammamary Infusion of penicillin/novobiocin for treatment of *streptococcus agalactiae* mastitis in dairy cows. J. AM. Vet. Med. Assoc. 208:258-260.

Erskine, R. J., Walker, R.D., Bolin, C. A., Bartlett, P.C. and White, D.G. (2002).Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period.*Journal of Dairy Science*. 85:1111–1118.

Erskine, R. J., Cullor, J., Schaellibaum, M., Yancey, B. and Zecconi, A. (2004). Bovine mastitis pathogens and trends in resistance to antibacterial drugs. *InProceedings of the 43rd National Mastitis Council Management.*Charlotte, North Carolina. National Mastitis Council, Madison,WI. Pp. 400–414.

Evenson, M.L., Hinds, M.W., Bernstein, R.S. and Bergdoll, M.S. (1988). Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. Int. J. Food. Microbiol. 7:311–316. [PubMed].

Farooq, A.A., S. Inayat., M.S., Akhtar and M. Mushtaq. (2008). Prevalence of mastitis and antibiotic sensitivity of bacterial isolate recovered from Nili-Ravi buffaloes. J. Anim. Pi. Sci 18(2-3). University of vet and animal sciences, Lahore.

Food and Agriculture organization (FAO) (1990), Animal production and health papers. 85:9-24

Food Outlook- Global Market Analysis (2012): food and Agriculture Organization of the United Nations Rome. pp8, 51-54.

FDA (2001).Grad A pasteurized milk ordinance. Center for food safety and applied nutrition. Section 1-7 cited in http://eee.cfsan.fda.gov/ear/pmo01-2.html.

Gentilini, E., G. Denamiel, P. Lorente, S. Godaly, M. Rebuelto, and O. De Gregorio.(2000). Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina.*Journal of Dairy Science*, 83:1224–1227.

Grohn, Y.T., Wilson, D.J., Gonzalez, R.N., Hertl, J.A., Schulthe, H., Bennett, G. and Schukken, Y.H. (2004).Effect of pathogen-specific clinical mastitis on milk yield in dairy cows.*Journal ofDairy Science,* 87:3358-3374.

Haggag, H.F., L.F. Hamzawi, G.A. Mahran and M.M. Ali.(1991). PHysico-chemical properties of colostrums and clinical and sub-clinical mastitic buffalo milk.*Egyptian Journal of Dairy Sci*ence, 19(1): 55-63.

Halleron, R. (1997). Liner slips cause 10 to 15 percent of new infections. *Hoard's Dairyman* 142 (August): 624. Fort Atkinson, Wis.: W. D. Hoard and Sons Company. *www.hoards.com*.

Hanselman, B. A., Kruth, S. A., Rousseau, J. (2006). *Emerg Infect Dis* 12:1933-1938.

Harding, F. (1999).Milk quality.A chapman and hall food science book.Aspen publishers Inc. Gaithersburg, Maryland. First edition pp. 44-59.

Hameed, KGA, Sender G and Korwin-Kossakowska A, (2006).Public health hazard due to mastitis in dairy cows.*Animal Science Papers and Reports*.25: 73- 85.

Hemme, T. and Otte, J. (2010). Status and prospects for smallholder milk production. A global perspective. Food and agriculture organization of the united nations

Hoblet, K.H., Schnitkey,G.D.,Arbaugh, D., Hogan, J.S., Smith, K.L., Schoenberger,P.S., Pritchard, D.E., Bowman. G.L., Heider, L.E., Brockett, B.L. and Conrad, H.R. (1991). Cost associated with selected preventive practices and with episodes of clinical mastitis in nine herds with low somatic cell Counts. *Journal of American Veterinary Medical Association*, 199:190-196.

Houben, E.H.P., Dijkhuizen,A.A., Van Arendonk, J.A.M. and Huirne, R.(1993). Short and long term production losses and repeatability of clinical mastitis in dairy cattle. *Journal of Dairy Science*, 76:2561-2578.

Holmberg S.D. and Blake P.A.(1984). Staphylococcal food poisoning in the United States.New facts and old misconceptions.JAMA. 251:487–489. [PubMed].

Huijsdend, X. W., Van Dijke, B. J., Spalburg, E, (2006). *Ann Clin Microbiol. Antimicrob*. 1476-07.

Hussain, R., Javed, M. T and Khan, A. (2012.) Changes in some biochemical parameters and somatic cell counts in the milk of buffalo and cattle suffering from mastitis. Pak Vet J, 32: 418-421.

International Dairy Federation.(1999). Suggested interpretation of mastitis terminology. Bull. Int. Dairy Fed. 338, 3-26.

Jones, G.M., Bailey, T.L. and Roberson, J.R. (1998) *Staphylococcus aureus* mastitis: Cause, Detection, and Control. Virginia Cooperative Extension.Publication number 404-229.

Jones, G. M. and Bailey, T. L. "Understanding the Basics of Mastitis".Virginia Cooperative Extension.Retrieved 4 February 2010.

Kandasamy, S, Green, BB., Benjamin, AL, Kerr, DE. (2011). Between-cow variation in dermal fibroblast response to lipopolysaccharide reflected in resolution of inflammation during *Escherichia coli* mastitis. J Dairy Sci. Dec;94(12):5963-75. doi: 10.3168/jds.2011-4288. PubMed PMID 22118085.

Kataoka, K. Nakal, T. and Imamura, T. (1991). Japan journal od dairy science. 20,222-232.

Kérouanton, A., Hennekinne, J.A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A.and De Buyser M.L. (2007). Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. Int. J. Food Microbiol. 115:369–375. [PubMed]

Kirk, J. H., S. L. Berry, I. A. Gardner, J. Maas, and A. Ahmadi.(1997). Dry cow antibiotic treatment in a herd with low contagious mastitis prevalence.Proceedings, National Mastitis Council annual meeting, Madison, Wis., 164.

Kitamoto, M., Kito, K., Niimi, Y., Shoda, S., Takamura, A., Hiramatsu, T., Akashi, T., Yokoi Y., Hirano, H., Hosokawa, M., Yamamoto, A., Agata, N. and Hamajima, N. (2009)Food poisoning by *Staphylococcus aureus* at a university festival. Jpn. J. Infect. Dis. 62:242 243. [PubMed]

Krishna, DG. Al-aamri, MS. Al-kinde, HS.Al-amri, KE. And Kethani devi, CH. (2015). Proximate and mineral composition (major and minor elements) of commercial, cattle raw milk and dairy products.International journal of inorganic and bioinorganic chemistry. ISSN: 2249-8540. 5(1):19-22

Kwaga, J.K.P. and Adesuyin.A.A (1984). Antibiogram of Staphylococcus aureus isolates from Ready to eat products. Journal of Food Protection, 47:805-867.

Lafi, S.O., Al-rawashdeh, O.F., Erafaj, K.I. and Hallat, N.O.(1994). Incidence of clinical and prevalence of sub clinical mastitis in Joadanian dairy cattle.*Preventive Veterinary medicine,*18 98.

Larkin, E.A., Carman, R.J., Krakauer, T. and Stiles, B.G. (2009). *Staphylococcus aureus*: the toxic presence of a pathogen extraordinaire. Curr. Med. Chem.16:4003–4019. [PubMed]

Le Loir, Y., Baron, F. and Gautier, M. (2003).*Staphylococcus aureus* and food poisoning. Genet. Mol. Res. 2:63–76. [PubMed]

Li, F., Park, S.Y., Ayers, T.L.., Miller, F.D., MacFadden, R., Nakata, M., Lee, M.C. and Effler,

P.V. (2005).Methicillin resistant *S. aureus.*Hawaii 2000-2002.*Journal of Emerging Infectious Disease,*11(8):1265-1210.

Mahmood, A. and Usman, S. (2010). A comparative study on the physicochemical parameters of milk samples collected from buffalo, cow, goat and sheep of Gujrati, Pakistan. Pakistan journal of nutrition 9(2):1192-1197.

Manian, F. A.(2003). *Clin Inf Dis* 36:E26-E28.

Marwa I.and Mahmoud, M. (2013). Association between *agr* alleles and toxin gene profiles of *S. aureus* isolates fromhuman and animal sources in Egypt. International journal of advanced research , volume1, issue 8, 133-144. ISSN NO 2320-5407.

Mattews, KR, Roberson, J, Gillespie, BE, Luther, DA, Oliver, SP. (1997)."Identification and Differentiation of Coagulase-Negative *Staphylococcus aureus* by Polymerase Chain Reaction.*Journal of Food Protection***60** (6): 686–8.

Miller, G.Y., Barlett, P.C., Anderson, S. and Heider, L. E. (1993).Costs of clinical mastitis and mastitis prevention in dairy herds.*Journal of American VeterinaryMedical Assocition*, 202:1230-1236.

Mitchell, JM, Griffiths, MW, Mc Ewen, SA, Mc Nab, WB and Yee, AJ. (1998). Antimicrobial drug residues in milk and meat: causes concerns, prevalence, regulations, tests, and test performance. J. food prot. 61: 742-756

Montville, T. J, Mattews, K. R (2008).*Food Microbiology*: An introduction. 2nded, ASM press Washington DC.

Mohamed, IE, El Owni OAO and Mohamed GE, (1997).Effect of bacteria causing mastitis on milk constituents.*Sudan Journal of Veterinary Science and Animal Husbandry*, 36: 125-136.

Murray, R.J. (2005). Recognition and management of *Staphylococcus aureus* toxin-mediated disease.Intern. Med. J. 2:S106–S119. [PubMed].

Murray, P.R., Rosenthal K.S. and Pfaller, M.A. (2006).Microbiologia Medica. Rio de Janeiro, Elseveir.979.

National Nosocomial infections surveillance system (NNIS) (2003). NNIS system, data summary report from January 1992 through June 2003, issued August 2003. *American Journal of Infection andControl*, 31:481-498.

Nazneen, N.I., Zinat Farzana, A.M. Masudul azad chowdhury, Adnan Mannan, K.M. Kamaruddin, A.M. Zonaed siddiki and Inkeyas Uddin (2014).Characterization of bovine subclinical mastitis caused by s.aureus in southern Bangladesh by bacteriological and molecular approaches.*Asian Journal of Biological Sciences*, 7:1-12.

Nickerson, S. C., Owens, W.E and Boddie, R.L. (1995). Mastitis in dairy heifers: Initial studies on prevalence and control. *Journal of Dairy Science* 78(7): 1607-18.

Nickerson, S. C., E. P. Hovingh, C. Peterson, S. Brannock, E. Schaffer, and P. W. Widel. (2008). Administration of a Staphylococcus aureus bacterin to dairy heifers reduces new infection rate and somatic cell counts at time of calving. Paper presented at the American Dairy Science Association annual meeting, Indianapolis, Ind. *Journal of Dairy Science* 91:300, E-supplement 1, abstract No. 247.

Nierman, P. (2001). Methods to detect abnormal milk in standard methods for the examination of diary product.17th edn. American Public Health Association, Washington DC, USA 2004,281-292.

Nooruddin M, Ali ML and Debnath NC (1997).Retrospective epidemiologic study of periparturient diseases in dairy cows. 1. Clinical mastitis. *The Bangladesh Veterinarian* 14: 43-47.

O'Connor C B. (1995). Rural Dairy Technology ILRI Training Manual I, International Livestock Research Institute, Addis Ababa, Ethiopia.

O'Mahony, F. (1988). 'Rural dairy technology- Experiences in Ethiopia',Dairy technology unit, ILRI Manual No. 4., international livestock centre for Africa, Addis Ababa, Ethiopia. 64.

Ogston A. (1984). "On abscess".Classics in infectious diseases.*Review of Infectious Diseases,* 6(1):122-128.

Oladapo, AF. And Ogunekun, TO. (2015). Quality assessment of fresh milk from traditionally managed Nigerian Bunaji and Bokolooji breeds of cattles. *The Pacific Journal of Science and Technology*, 16(1).pp.280-285.

Oliver, S. P., Lewis, M. J., Gillespie, B.E and Dowlen, H.H. (1992). Influence of prepartum antibiotic therapy on intramammary infections in primigravid heifers during early lactation.*Journal of Dairy Science* 75(2): 406-14

Oliver, S.P., Gillespie, B.E., Headrick, S.J., Moorehead, H., Lunn, P., Dowlen, H.H., Johnson, D.L., Lamar, K.C., Chester, S.T., Moseley, W.M. (2004). Efficacy of extended ceftiofur intramammary therapy for treatment of subclinical mastitis in lactating dairy cows. J. Dairy Sci. 87, 2393-2400.

Otero A., García M.L., García M.C., Moreno B. and Bergdoll M.S.(1990). Production of staphylococcal enterotoxins C1 and C2 and thermonuclease throughout the growth cycle. Appl. Environ. Microbiol. 56:555–559. [PMC free article] [PubMed]

Owen, W. E., Ray, C.H., Watts, J.L. and Yancey, R.R. (1997). Comparison of success of Antibiotic therapy during lactation and results of antimicrobial susceptibility tests for bovine mastits. Journal of Dairy Science, 87:2433-2441.

Pasavento, G., Ducci, B., Comodo, N. and Lo Nostro, A. (2007). Antimicrobial resistance profile Of *Staphylococcus aureus* isolated from raw meat: A research for methicillin resistant *Staphylococcus aureus* (MRSA). *Food control*, 18:196-200.

Pearson, A.J.W. (1976). The chemical analysis of foods. Seventh ed, J. and Achurchill.

Pinchuk, I. V, Beswick E. J. and Reyes V. E (2010) Staphylococcus enterotoxins.Toxins 2:2177- 2197.

Pitkala, A.,Haveri, M., Pyorala, S.,Myllys, V. and Honkanen-Buzalski T.(2004). Bovine Mastitis in Finland - Prevalence, Distribution of Bacteria, and Antimicrobial Resistance.*Journal of Dairy Science*, 87:2433-2441.

Popescu, A. AND Angel, E. (2009).Analysis of milk quality and its importance for milk processors. Lucrari Stiintifice Zootehnie Si Biotehnologii, vol. 42(1), Timisoara. P.501-506

Pyorala, S. and E. Pyorala.(1997). Accuracy of methods using somatic cell count and N-acetyl-beta-D-glucosaminidase activity in milk to assess the bacteriological cure of bovine clinical mastitis.*Journal of Dairy Science* 80(11): 2820-25.

Pyörälä, S.(2002).New strategies to prevent mastitis.*Reproduction in Domestic Animals* 37: 211-216.

Quinn, P.J., Carter, M.E., Markey, B. & Carter G.R., (1994). In: Clinical Veterinary Microbiology. 1st ed., Wolfe Publishing, London.

Quinn, P. J., M. E. Carter, B. Markey and G. R. Carter.(1999). *Clinical VeterinaryMicrobiology*. Mosby Publishing, London. 327-344 p.

Quinn, P. J., M. E. Carter, B. Markey and G. R. Carter.(2002). Veterinary Microbiology Microbial Diseases, Bacterial causes of bovine mastitis, 8thEd. Mobsy International Limited, London. Pp.465-467.

Rabello, RF, Souza CRVM, Duarte RS, Lopes RMM, Teixeira LM.And Castro ACD (2005). Chareaterization of staphylococcus aures isolates recovered from bovine mastitis in Riode Janeiro, Brazil. J. Dairy sci. 88: 3211-3219

Radostits, O.M. and Blood, D. C., (2000). *Veterinary Medicine*.A textbook of the disease of cattle, sheep, pigs, goats and horses.9th edition. (Eds) Blood, D.C., Radostits, O.M., Gay, C.C., Hinchcliff,K.W., W.B. Saunders Company Limited, NewYork Pp 626-630.

Radostits, O. M., Gay, C. C., Hinchcliff, and Constable, P. D. (2007). Mastitis In: *Veterinary Medicine*: A Text book of disease of cattle, sheep, pigs, goats, and horses 10th edition, Ballier, Tindall, London. 674-762 p

Raff, H. (2011). Market implications on changing fat content of milk and dairy product, fat content and composition of animal products. J. Food Science and Technology 5 (2): 6-17.

Rasmussen, M.D., E.S. Frimer, D.M. Galton, and L.G. Petersson. (1992). The influence of premilking teat preparation and attachment delay on milk yield and milking performance. *Journal of Dairy Science,* 75:2131.

Redeat Belayneh, Kelay belihu and asamenew Testaye. (2014). Microbiological study of bacterial causes of bovine mastitis and its antibiotic susceptibility patterns in east showa zone, Akaki district, Ethiopia.Journal of vet medicine and animal health.Vol 6(4) pp116-122.

Robert, T.M. Edmondson J.E. and Barry S. (2006).Using the California Mastitis Test. University of Missouri Extention.1993 to 2006 Curator of University of Missouri, all right reserved.

Roy, J. P., D. Du Tremblay, L. DesCoteaux, S. Messier, D. Scholl, and E. Bouchard. (2007). Effect of precalving intramammary treatment with pirlimycin in nulliparous Holstein heifers.*Canadian Journal of Veterinary Research* 71(4): 283-91.

Ruegg, P.L. and D.J. Reinemann (2002). Milk quality and mastitis test. Bovine practitioner, 36: 41-45.

Ruuw and Berts, D. (2004).Youghurt production.Issues of dairy production. http:/www.gironet-nt/home/berding/dtpvol.72html

Ryan, KJ. and Ray, CG. (2004). *Sherris Medical Microbiology* (4th ed.). McGraw Hill.ISBN 0-8385-8529-9.

Sabour, P. M., J. J. Gill, D. Lepp, J. C. Pacan, R. Ahmed, R. Dingwell, and K. Leslie. (2004).Molecular typing and distribution of *Staphylococcus aureus* isolates in Eastern Canadian dairy herds. *Journal of Clinical Microbiology,* 42:3449–3455.

Samad MA (2008). Animal Husbandry and Veterinary Science, volume II, LEP pub no.11, Bangladesh Agricultural University campus, Mymensingh.

Sandholm, M., Honkanen-Buzalski, L., Kaartinen S. & Pyörälä, S. (1995a). Detection of inflammatory changes in milk.*The bovine udder and mastitis*.University of Helsinki, Faculty of Veterinary Medicine. Helsinki.

Schantz E.J., Roessler W.G., Wagman J., Spero L., Dunnery D.A. and Bergdoll M.S. (1965). Purification of staphylococcal enterotoxin B. Biochemistry. 1965;4:1011–1016. [PubMed]

Seegers H, Fourichon C and Beaudeau F (2003). Production effects related to mastitis and mastitis economics in dairy cattle herds> Veterinary Research 34: 475-491.

Seo K. S, Bohach G. A (2007) *Staphylococcus aureus*. Ch 22 in: Doyle M. P, Beuchat L. R (eds) Food Microbiology: Fundamentals and frontiers. 3rd ed, ASM press Washington D C.

Shimizu A., Fujita M., Igarashi H., Takagi M., Nagase N., Sasaki A., Kawano J. (2000).
Characterization of *Staphylococcus aureus* coagulase type VII isolates from staphylococcal food poisoning outbreaks (1980–1995) in Tokyo, Japan, by pulsed field gel electropHoresis. J. Clin. Microbiol. 38:3746–3749. [PMC free article] [PubMed]

Shitandi A., Anakalo G., Galgalo T. and Mwangi M. (2004).Prevalence of bovine mastitis amongst smallholder dairy in Kenya.Egerton University Njoro 20107 Kenya.*Journal of Dairy Science,* 87:4145-4149.

Shopsin, B., Mathema, B., Martinez, J., HA, E., Campo, M.L., Fierman, A.,Krasinki, K., Kornblum, J., Alcabes, P., Waddington, M., Riehman, M. and Krieswirth (2000). Prevalence of methicillin –resistant and methicillin-susceptible *Staphylococcus aureus* in the community.*Journal of infectious diseases* 182:359-362.

Smith, J. L., Buchanan R. L. and Palumbo, S. A. (1982). Effect of food environment on staphylococcal enterotoxins synthesis: review. J Food protection 46: 545-555.

Smith, J. L., Todhunter, D.A. and Schoenberger, B.V. (1985). Environmental mastitis: cause, prevalence and prevention. *Journal of Dairy Science,* 68:1531- 1553.

Sol, J., O. C. Sampimon, J. J. Snoep, and Y. H. Schukken. (1997). Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus*. *Journal of Dairy Science* 80(11): 2803-08.

Sol, J., Sampimon, O. C., Barkema, H. W. and Schukken, Y. H. (2000). Factors associated with Cure after therapy of clinical mastitis caused by *Staphylococcus aureus. Journal of dairy Sciences*, 83:278-284.

Sordillo, L. M., & Streicher, K.L. (2002).Mammary gland immunity and mastitis susceptibility. *Journal of Mammary Gland Biology and Neoplasia* 7(2):135–146.

Stewart G.C. (2005). *Staphylococcus aureus*. In: Fratamico P.M., Bhunia A.K., Smith J.L., editors. Foodborne pathogens: Microbiology and Molecular Biology. Caister Academic Press; Norfolk, UK: 2005. pp. 273–284.

Suleiman, A. B., Umoh, V. J., J. K. P. Kwaga, S. J. Shuaibu (2012). Prevalence and antibiotic resistance profiles of methicillin resistance s.aureus (MRSA) isolated from bovine mastitic milk in plateau state, Nigeria. *International Research Journal of Microbiology* (IRJM) (ISSN:2141-5463). Vol 2(8). Pp 264-270.

Suleiman, A. B., Umoh, V. J., J. K. P. Kwaga, S. J. Shuaibu (2013). Enterotoxigenicity and antibiotic resistance of s. aureus isolated from sub-clinical bovine mastitic milk in Plateau state Nigeria. *ResearchJournalofMicrobiology,* 8:101-107.

Tesfay,T. Kebede, A. and Eyassu, S. (2015). Physicochemical properties of cow milk produced and marketed in dire dawa town, Eastern Ethiopia. Food science and quality management. ISSN:2224-6088. Vol 42, pp56.

Tranter H.S. (1990). Foodborne staphylococcal illness.Lancet. 336:1044–1046. [PubMed].

Thrusfield, M. (2005). Veterinary Epidemiology . 3rd Ed. Blackwell Science ltd, a Blackwell Publishing Company, UK. 188-265.

Ungemach, F.R. (1999). Einaz von Antibiotika in der veterinary medizin: Konsequenzen Und Rationale Umgang. Tierarztl Prax, 27: 335-340.

Umoh V.J., Adesiyun, A.A. and Gomwalk, N.E. (1990). Enterotoxigenicity of staphylococci

isolated from raw milk obtained from settled herds around Zaria, Nigeria. *Revue Élev.Méd.Vét.Pays, Trop.,* 43, 43-47.

Veras J.F., do Carmo L.S., Tong L.C., Shupp J.W., Cummings C., Dos Santos D.A., Cerqueira M.M., Cantini A., Nicoli J.R. and Jett M. (2008). A study of the enterotoxigenicity of coagulase-negative and coagulase-positive staphylococcal isolates from food poisoning outbreaks in Minas Gerais, Brazil.*International Journal of Infectious Diseases*, 12:410–415. [PubMed]

Vintov, J., Aarestrup, F. M. Zinn, C. E. and Olsen J. E. (2003). Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. *Veterinary Microbiology,* 95:133–147.

Waldvogel, F.A. (2000).*Staphylococcus aureus* (including staphylococcal toxic shock).In principles and practice of infectious diseases G.I. Mandell, J.E. Bennnette, and R. Dolin, (editors). Churchill livingstone. Philadelphia, Pennsylvania, USA. 2069 – 2092.

Watts, J. L. and Salmon, S.A. (1997). Activity of selected antimicrobial agents against strains of *Staphylococcus aureus* isolated from bovine intramammary infections that produce *β*-lactamase. *Journal of Dairy Science,* 80:788–791.

Wellenberg, G. L., van der Poel, W. H. M. and Van Orschot, J. T. (2002). Viral infections and bovine mastitis: a review. *VeterinaryMicrobiology* 88: 27-45

Weese J. S. (2005). *J Am Anim Hosp Assoc* 41:150-157.

Wieneke, A.A., Roberts, D. and Gilbert, R.J. (1993) Staphylococcal food poisoning in the United Kingdom, 1969–90.Epidemiol. Infect. 110:519–531. [PMC free article] [PubMed]

Wilson, D. J. Gonzalez, R.N. and Sears, P.M. (1995). Segregation or use of separate milking units for cows infected with Staphylococcus aureus: Effects on prevalence of infection and bulk-tank somatic cell count. *Journal of Dairy Science* 78(9): 2083-85.

Wilson, DJ, Gonzalez, R.N. and Hertl, J. (2004) Effect of clinical mastitis on the lactation curve: a mixed model estimation using daily milk weights. *Journal of Dairy Science*, 87, 2073-2084. World Dairy Cow Numbers [FAO] jan 14, 2014 retrieved march 23, 2014.

APPENDIX I: NUCLEOTIDE SEQUENCES AND PREDICTED SIZES OF PCR PRODUCTS

Gene	Primers	oliginucleotide sequence	Size of amplified product	References
Sea	GSEAR-1	GGTTATCAATGTGCGGGTGG	102	Mehrotra *et al*., 2000
	GSEAR-2	CGGCACTTTTTTCTCTTCGG		
Seb	GSEBR-1	GTATGGTGGTGTAACTGC	164	Mehrotra *et al*., 2000
	GSEBR-2	CCAAATAGTGACGAGTTAGG		