

**ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCE
SCHOOL OF VETERINARY MEDICINE**

**STUDY ON CONTAMINATION OF BOVINE RAW MILK WITH STAPHYLOCOCCAL
SPECIES IN URBAN DAIRY FARMS OF ADDIS ABABA, ETHIOPIA**

BY

MEZGEBU MERID BAYABLE

JUNE, 2010

DEBRE ZEIT, ETHIOPIA

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A thesis submitted to the school of Graduate Studies of Addis Ababa University in partial fulfillment
of the requirements for the Degree of Master of Science in Tropical Veterinary Public Health

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my academic advisors Dr. Girma Zewde and Dr. Tesfaye Sisay for their overall guidance, helpful advice, close supervision and time devotion in correction of the manuscript.

I am highly indebted to Dr. Alehegne Wubete of the National Animal health Diagnostic and Investigation Center for his valuable advice during the work. I express my extreme appreciation to Teshome Belay and Genet Kinfu for their assistance in this study.

I wish to express my appreciation to Shola Regional Veterinary Laboratory for cooperativeness in transport in most time of sample collection from the urban dairy farms and all staff members especially Dr. Dereje Debebe for facilitating the work.

I am grateful to the Addis Ababa University, Faculty of veterinary medicine, Debre Zeit for supplying facilities during the work and also to all staff of Microbiology Laboratory especially Hirut Tesfaye and Seble Sinke for facilitating the laboratory work.

Amhara Regional State and Dejen woreda agriculture and rural development office are highly acknowledged for sponsoring me to attend this postgraduate programme and financial support throughout the study period.

I am grateful to Dr. Sisay Amare and Dr. Bedlu Ayalew for their unreserved help and encouragement during this work. All my families especially my sister Lemlem Merid is acknowledged for their appreciable heartily support during the work.

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LIST OF ABBREVIATIONS

API	Analytic Profile Index
Aw	Water activity
CMT	California mastitis test
CNS	Coagulase-negative staphylococci
CPS	Coagulase-positive staphylococci
CSA	Central Statistical Authority
FAO	Food and Agriculture Organization
FBD	Foodborne disease
H ₂ O ₂	Hydrogen peroxide
NIAID	National Institute of Allergy and Infectious Diseases
O-F	Oxidation-Fermentation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SCC	Somatic cell count
SFP	Staphylococcal food poisoning
SPSS	Statistical Program for Social Studies
TSS	Toxic shock syndrome
TSST	Toxic shock syndrome toxin
USA	United States of America
WHO	World Health Organization
χ^2	Chi-square

ABSTRACT

A cross-sectional study was undertaken from October 2009 to April 2010 to establish the prevalence of *staphylococcus* species in bovine udder milk, on teat and udder skin of lactating cows, milkers' hands and milking and milk collecting equipments and to assess the farm hygiene and pre-milking practices experienced in 25 randomly selected urban dairy farms of Addis Ababa, Ethiopia. A total of 604 samples comprising 270 bovine udder milk and 270 lactating cow skin swab samples (135 lactating cow teats and 135 udders) were collected by using cluster sampling technique. Swab samples from 19 milkers' hands, 22 milking buckets and 23 milk collecting containers were also collected from the selected farms. All the samples were examined for the presence of *staphylococcus* species by using standard bacteriological techniques. The prevalence of staphylococcus species were: coagulase-negative staphylococci (CNS) (53%), *S. aureus* (8.5%), *S. intermedius* (0.7%) and *S. hyicus* (0.7%) in the udder milk; CNS (38.5%), *S. aureus* (5.9%) and *S. hyicus* (0.7%) from udder skin and CNS (32.6%), *S. aureus* (4.4%), *S. intermedius* (1.5%) and *S. hyicus* (0.7%) from lactating cow teat skin swab samples. Only CNS species were isolated from milkers' hands, milking buckets and milk collecting containers with the respective prevalence of 42.1%, 13.6% and 8.7%. The hygienic status of the barns of most of the visited farms was poor and poor milking hygiene was practiced. The presence of staphylococci species on teat and udder skin of lactating cows, milkers' hands and milking and milk collecting equipments indicates the high chance of staphylococci species to contaminate milk leaving the udder of cow during milking. The high prevalence of staphylococci in the udder milk demonstrates its role in reducing milk quality and its potential risk for human consumption. This high contamination rate could be due to increased occurrence of intramammary infection and very poor hygienic conditions during milking. Educating or training of milking personnel and other non-milking dairy farm employees concerning the importance of good quality milk and the risk arising from contaminated milk; control of mastitis and intermammary infection; application of good farm hygiene and following standard milking procedures are important for minimizing contamination.

Key words: Addis Ababa, Bovine raw milk, Contamination, Staphylococci

1. INTRODUCTION

In developing countries various factors combine to compromise the hygienic quality of milk and milk products: the organization of milk supply chains, disfunctioning of the regulatory systems and quality control structures. The problem is compounded by local climatic conditions, where both heat and at times, humidity do not favor the preservation of the product in optimal conditions, when cold chain cannot be maintained. All studies done in Africa show that all the potential hazards linked to poor quality milk are present (Faye and Loiseau, 2000).

An overwhelming majority of dairy producers feel responsible for the safety and wholesomeness of the dairy products that leave their farms. Good animal health and hygienic conditions of the farm are important for the welfare of the animals and the profitability of the producers, as well as for the quality and wholesomeness of the dairy products leaving the farms for human consumption. Nevertheless, many dairy producers are unaware of the zoonotic potential of the most common bacterial contaminants in milk (Lejeune and Rajala-Schultz, 2009).

Worldwide, millions of people suffer from communicable and non-communicable diseases caused by contaminated food (Vander Vanter, 1999). The Center for Disease Control estimates that each year in USA alone, 76 million people get sick, more than 300,000 are hospitalized and 5,000 very young, elderly and immunocompromised Americans die as a result of foodborne illness. Recent change in food production and distribution system, microbial adoption, lack of support for public health resources and infrastructure have lead to the emergence of naive as well as traditional foodborne diseases (NIAID, 2005).

To date, around 250 different foodborne diseases have been described, and bacteria are the causative agents of two thirds of foodborne disease outbreaks (Loir, 2003). It has been reported that among the predominant bacteria involved in these diseases, staphylococci are the leading causes of gastroenteritis resulting from the consumption of contaminated food (Vargum and Vatansever, 2007).

Staphylococcal food poisoning (SFP) is due to the absorption of staphylococcal enterotoxins preformed in the food (Loir, 2003). Enterotoxin-producing *Staphylococcus aureus* plays an important role as causative organism of food intoxications. In many countries, *S. aureus* is considered to be the second or third most common pathogen causing outbreaks of food poisoning only outnumbered by *Salmonella* spp. and in competition with *Clostridium perfringens* (Atanassova *et al.*, 2001).

The quality of milk is determined by aspects of composition and hygiene. Due to its complex biochemical composition and high water activity milk serves as an excellent medium for the growth and multiplication of many kinds of microorganisms. The contamination of milk is largely due to human factor and unhygienic conditions. Usually milk gets contaminated with different kinds of microorganisms at milk collecting places (Parekh and Subhash, 2008).

Milk and milk products are common vehicles for staphylococcal food poisoning. They have frequently been implicated in staphylococcal food poisoning (SFP), and often contaminated raw milk is involved. These organisms can gain access to raw milk either by direct excretion from udders with clinical and sub-clinical staphylococcal mastitis or by contamination. The contaminants can also reach the products either during cooling or handling after cooking (Bendahou *et al.*, 2008).

The most important factors that are known to contribute to SFP outbreaks are inadequate refrigeration of food, preparing food far in advance of planned service, infected personnel, persons practicing poor personal hygiene during food preparation, inadequate cooking or heat processing and holding food in warming devices at bacterial growth temperatures (Jay, 2000). Thus, unhygienic treatment of food has to be considered as a major risk of contamination, and staphylococcal food poisoning is often associated with highly manually handled food (Atanassova *et al.*, 2001).

In developing countries, the surveillance system of foodborne diseases (FBD) hardly exists and it is, therefore, difficult to estimate the real magnitude of the problem. Even in countries where surveillance services are very efficient, the precise incidence of food poisoning is not known, as

outbreaks are often not reported to public health authorities (Jay, 2000; Acha and Szyfres, 2001). The incidence of FBD caused by *S. aureus* is thought to be much higher than reported since many cases remain undeclared (Hocking and Doyle, 1997).

In countries like Ethiopia, where surveillance system for foodborne disease does not exist and is difficult to estimate the amount of the cases, there is no specific concerned body to monitor and evaluate the incidence of the foodborne diseases (Cited by Senait, 2007).

As to our knowledge, in Addis Ababa, studies on the prevalence of staphylococcal species in bovine udder milk and other possible contamination sources of bovine raw milk in the urban dairy farms have not yet been conducted. Therefore, this study was undertaken with the following objectives:

- To establish the prevalence of *Staphylococcus* species in bovine udder milk, on teat and udder skin of lactating cows, milkers' hands and milking and milk collecting equipments
- To assess the farm hygiene and pre-milking practices experienced in the urban dairy farms

2. LITERATURE REVIEW

2.1. Microbiology of milk

2.1.1. Origin of bacterial contamination

Bacteria are present in raw milk as a result of milking mastitic cows, contamination from the environment during milking, contaminated milking and handling equipment and/or bacterial growth during storage. In order to achieve a high bacteriological quality at farm level, it is important for farmers or milk producers to be aware of the sources of contamination and to understand how they can be controlled (Suliman and Mohamed, 2009).

Commensal flora

Unless there is an intramammary infection or an animal has a systemic disease, milk in the mammary gland at the site of its production does not contain bacteria. However, as milk is excreted, it can become contaminated with bacteria that live as commensal microflora on the teat skin or on the epithelial lining of the teat canal, the duct in which the milk passes from the mammary gland to the teat orifice. In cattle, bacteria of the genus *Staphylococcus*, *Streptococcus*, *Bacillus*, *Micrococcus*, and *Corynebacterium* and occasionally coliforms colonize this location. Thus in healthy animal by the time the milk leaves the animal; it may contain numerous bacterial contaminants (Lejeune and Rajala-Schultz, 2009).

The types of organisms found in raw milk can be a reflection of the flora of the udder when proper procedures are not followed in milking and of the general environment of such animals. From both the udder and the hide, organisms can contaminate the general environment, milk containers, and the hands of handlers. The microflora on the hands and outer garments of handlers generally reflects the environment and habitats of individuals, and the organisms in question may be those from soils, waters, dust, and other environment sources. Additional important sources are those that are common in nasal cavities and the mouth and on the skin and

those from the gastrointestinal tract that may enter foods through poor personal hygienic practices (Jay, 2000).

Environmental contamination

One of the important factors in quality milk production is the protection of milk from external contamination at the time of milking and in the milking establishment. The dairy farm is an important reservoir for many foodborne pathogens (Lejeune and Rajala-Schultz, 2009).

Having limited the number of bacteria entering the milk during milking, it is essential that contamination from equipment situated between the cow and the refrigerated storage unit is kept to a minimum. Bacteria are also present in the air; dust and water, especially any water containing traces of milk residues provide a very good source of food for bacteria thereby enabling the bacterial counts to increase rapidly (Cawe, 2006).

The level of microbial contamination of cow's raw milk are influenced by dairy cow's health and hygiene, by the hygiene of the environment, where dairy cows are housed and milked, by methods of udder preparation and milking technique, methods of cleaning and sanitation of milking machines and milk cisterns, tenders' hygiene, speed of milk cooling to a required temperature and milk storage time (Hempen *et al.*, 2004; Cempirkova, 2006).

When cows are housed, bedding material and feed stuffs can be contamination sources, in both cases (housing and pasturing) feces, or dung is also an important contamination source. Contamination of bedding material can be very high, due to absorption of urine and feces. For mastitis causing bacteria, different bedding materials can be of influence as a vehicle of contamination. Air borne dust and droplets can contaminate milk with organisms such as staphylococci, rickettsia, and streptococci, which may produce either harmful toxins or direct infections among human consumers. Consequently, it is important to provide clean surroundings and to avoid dusty conditions during milking periods (Cawe, 2006).

Mastitis

Mastitis is defined as the inflammation of the mammary gland. The inflammation is the response of the tissue to injury. The purposes of the inflammatory response are to destroy or neutralize the injurious agent, most commonly invading bacteria, and allow healing and return to normal function. A key component of inflammation is the influx of white blood cells or leucocytes which resulted in an increase in the somatic (body) cell count (SCC) of milk, thus the SCC is a common measure of mammary gland health and milk quality (Cawe, 2006).

Bacteria multiply and produce toxins that cause injury to the milk secreting tissue (Khan and Khan, 2006). In spite of all kinds of scientific progress, mastitis remains the most common and ambiguity disease of dairy cattle throughout most of the world. It is estimated that one third of all dairy cows are infected with some form of mastitis in one or more quarters (Madut *et al.*, 2009).

Cawe (2006) reported that *Staphylococcus aureus*, the coliforms, streptococci and enterococci of environmental origin are the most common major pathogens that cause mastitis. He also indicated that these pathogens are found either in the udder as contagious pathogens or in the animal surrounding such as bedding area, manure and soil as environmental pathogens; and among these the most common are *Staphylococcus aureus* and *Streptococcus agalactiae* and these are spread from infected to clean udders during the milking process through contaminated milker's hands, cloth towels to wash or dry udder of more than one animal and possibly by flies (Haveri, 2008).

Kahan and Kahan (2006) indicated that sub-clinical mastitis is important than the clinical mastitis and it usually precedes the clinical form, is of longer duration, difficult to detect, adversely affects milk quality and production, constitutes a reservoir of microorganisms that lead to infection of other animals within the herd and poses a threat to human health.

2.2. *Staphylococci* species

The genus *Staphylococcus* is placed in the family *Micrococcaceae*. Some 33 species of staphylococci are known (Sandel and McKillip, 2002). They are Gram-positive cocci, 0.5 to 1.5 μm in diameter, which occur singly and in pairs or tetrads forming grape-like clusters. *Staphylococci* are aerobic and facultative anaerobic, catalase-positive, oxidase-negative, non-motile, non-spore forming and fermentative bacteria. Colonies appear smooth, raised, glistening, circular, and entire. Single colonies can attain a size of 4-6 mm in diameter on non-selective media. Colony color varies from grey or grey-white to orange depending on the culture media used (Hirsh and Zee 1999; Todar, 2005; Tsegmed, 2006).

Staphylococci are not highly fastidious in their nutritive requirements and grow readily on the usual meat extract peptone medium; growth is most profuse on sheep blood agar medium commonly used for isolation of the pathogenic forms (Jay, 2000). *S. aureus* gives good growth of creamy, often deeply pigmented colonies that are surrounded by the clear zones of β -hemolysis (Freeman, 1979).

Over the years, a range of selective and diagnostic media have been developed to assist in the detection and enumeration of *S. aureus* in routine food surveillance programmes and food poisoning investigations (Baird and Lee, 1995).

Selective bacteriological media containing one or more agents that are inhibitory to microorganisms other than the target pathogen (*S. aureus*) can be applied. The microorganism of interest is not inhibited by the presence of these components in the medium, and will therefore, form visible colonies during incubation. The two selective agents most commonly used for this pathogen are sodium chloride (NaCl) and potassium tellurite (K_2TeO_3). Hence, culture of the organism in mannitol salt agar, a selective medium with 7–9% NaCl that allows *S. aureus* to grow and produce yellow colored (golden coloured) colonies as a result of salt utilization and subsequent drop in the medium's pH (Soejima *et al.*, 2007).

Staphylococcus aureus is a hardy organism that withstands desiccation well, and can survive in dust and on dry metal, glass, or porcelain surfaces for long periods of time. This organism has a high heat resistance for a non-sporing bacteria, with D values at 60°C being reported to range from 2 to 50 minutes depending on the food. Most chemical sanitizers used routinely in the food industry, including chlorine, other halogens and quaternary ammonium compounds, will destroy *S. aureus* on surfaces when applied correctly. *Staphylococcus aureus* is considered to be a poor competitor in complex microbial populations and is frequently inhibited or overgrown by other faster growing micro-organisms (spoilage organisms) in foods. Therefore, foods that present the greatest risk of staphylococcal food poisoning are those where the normal microflora has previously been destroyed (e.g. cooked products) or inhibited (e.g. foods that contain a high concentration of salt) but that have subsequently been contaminated by *S. aureus* (Bremer *et al.*, 2004).

Staphylococci are widespread in nature and can be recovered from environment or as commensally inhabitants of the skin, mucous membranes and other body sites in humans and animals (Hempen *et al.*, 2004; Turkyilmaz and Kaya, 2005).

The principal reservoir of *S. aureus* is the human carrier. High proportions (from 30 to 35%) of healthy humans have staphylococci in the nasopharynx and on the skin. A carrier with a respiratory disease can contaminate foods by sneezing, coughing, or expectorating. Similarly, he may contaminate foods he handles if he has a staphylococcal skin lesion. However, even if not sick himself, the carrier may spread the agent by handling food ingredients, utensils, and equipment, or the finished food product (Arshad *et al.*, 2006; Cuomo, 2009).

The micro-organism can exist as a persistent or a transient *S. aureus* member of the normal flora without causing any symptoms in humans. The presence in foods is often related to improper manipulation by personnel, who are frequently contaminated with these micro-organisms (Acco *et al.*, 2003; Cawe, 2006; Haveri, 2008). Individuals, who either do milk animals or handle milk, can contribute additional organisms to the milk (Hempen *et al.*, 2004).

2.2.1. Requirements for growth

Staphylococci are typical of other gram-positive bacteria in having a requirement for certain organic compounds in their nutrition. Amino acids are required as nitrogen sources, and thiamine and nicotinic acid are required among the B vitamins. Although staphylococci are mesophiles some strains of *S. aureus* are known to grow at a temperature as low as 6.7 °C. Generally the optimum temperature required for growth is 37°C with a range of 7-47.8°C. Regarding pH, *S. aureus* can grow over a range of 4-9.8 but its optimum is in the range of 6-7 (Gay, 2000).

It can grow with high sodium chloride concentrations (up to 15% NaCl). These characteristics enable *S. aureus* to grow in a wide variety of foods. This, plus their ecological niche, can easily explain their incidence in foodstuffs that require manipulation during processing (Loir, 2003).

2.2.2. Requirements for toxin production

For toxic levels of enterotoxin to occur, extensive multiplication (growth) of staphylococci cells generally needs to have taken place in the food. Low temperatures and the presence of other bacterial species (spoilage organisms) prevent this from occurring (Bremer, 2004).

In general, enterotoxin production tends to be favoured by the optimum growth conditions of temperature and pH. It is well established that staphylococci can grow under conditions which do not favor enterotoxin production. With respect to a_w , enterotoxin production (except for SEA) occurs over a slightly narrower range than growth. The optimum temperature for toxin production is 35-40°C, the range being 10-45°C. The optimum pH for toxin production is 5.3 - 7.0, with minimum and maximum of 4.8 and 9.0, respectively. Greatest toxin production is in the presence oxygen but fewer toxins are also produced under anaerobic condition (Gay, 2000).

It is generally accepted that enterotoxin production is unlikely to occur at temperatures below 10°C. Reduced levels of water activity (a_w) may also inhibit toxin synthesis more than growth.

Optimum growth and toxin production occur at a_w levels > 0.99 . Toxin production has been reported at as low as $a_w = 0.86$ (Bremer *et al.*, 2004).

2.2.3. Virulence factors

The importance of staphylococci to both the clinical and food settings is due to the wide variety of specific virulence determinants common to this group of bacteria (Sandel and McKillip, 2004). Perhaps the most notable virulence factors associated with *S. aureus* are the heat stable protein enterotoxins and of the various metabolites produced by the pathogen; enterotoxins pose the greatest risk to consumer health. Enterotoxins cause diarrhoea and vomiting when ingested and they are responsible for SFP. Staphylococcal enterotoxins are superantigenic proteins secreted by certain strains of *Staphylococcus*, which, if allowed to grow in food may be responsible for SFP resulting in illness (Soejima *et al.*, 2007). In addition, they secrete toxic shock syndrome toxin (TSST) which is expressed systemically and is the cause of TSS (toxic shock syndrome) (Smith, 2007).

Staphylococcus aureus has a capacity to produce and expresses many cell surface associated and extracellular proteins that are potential virulence factors (Kaloreu *et al.*, 2007).

Potential virulence factors of *S. aureus* other than SEs according to (Foster, 1991) include:

- Surface proteins that promote colonization of host tissues;
- Invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase);
- Surface factors that inhibit phagocytic engulfment (capsule, immunoglobulin binding protein A);
- Biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production);
- Immunological disguises (protein A, coagulase, clotting factor);
- Membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin);
- Inherent and acquired resistance to antimicrobial agents.

2.3. Control strategies of milk contamination

Development of pre- and post-harvest control measures to effectively reduce contamination is critical to the control of pathogens in raw milk. One sure way to prevent raw milk-associated foodborne illness is for consumers to refrain from drinking raw milk and from consuming dairy products manufactured using raw milk (Oliver *et al.*, 2009).

2.3.1. Improved milking hygiene

Complete control of microbiological hazards (i.e. zoonotic pathogens) is challenging, if not impossible, in the dairy farm environment, because these organisms may have multiple reservoirs, they do not always produce identifiable disease; their transmission pathways are incompletely known; and cost-efficient sensitive diagnostic tests are not available. Dairy product food safety, however, can be enhanced by implanting excellent hygienic standards for housing and milking centers and cow cleanliness and through uniform adoption of milking practices that reduce contamination of milk (Lejeune and Rajala-Schultz, 2009).

2.3.2. Cooling

The components of milk and its physical and chemical properties provide very favorable conditions for the multiplication of microorganisms. When microorganisms are present in milk, their multiplication does not start immediately after milking thanks to milk's natural bacteriostatic properties. This protection is efficient for several hours following milking, so it is necessary to take advantage of this period to cool the milk to prevent microbial growth (Faye and Loiseau, 2000).

The multiplication of bacteria in milk is dependent on both the temperature and time of storage. After production, milk can be stored in cans and in bulk tanks before collection. The storage temperature influences the types of bacteria which grow and cause spoilage (Cawe, 2006).

Cooling reduces the bacterial growth, but cannot eliminate the microorganisms already present in the milk. It also favors the predominance of psychotropic bacteria, which are proteolytic agents, leading to degradation in the nutritional quality of the milk. In cooled milk, pathogenic bacteria can persist, as can deterioration bacteria, saprophytic bacteria, yeast, fungus and viruses. Some of these pathogens may be dangerous for humans and trigger non-specific digestive disorders (pathogenic strains of *Staphylococcus* species for example) or specific diseases (e.g. main zoonoses as tuberculosis or brucellosis) (Faye and Loiseau, 2000).

Staphylococci are known to resist freezing and are not eliminated by cooling. However, immediate cooling of milk is very important in the prevention of multiplication of staphylococci after milking and hence, prevents production of toxins which are highly resistant to heat (Loir, 2003; Cuomo, 2009).

2.3.3. Pasteurization

Pasteurization is the process of heating milk for a predetermined time at a predetermined temperature to destroy pathogens. The decreased frequency of bovine carriage of certain pathogens and improved milking hygiene can contribute considerably to decreased contamination of milk but have not and cannot fully eliminate the risk of milk born diseases (Lejeune and Rajala- Schultz, 2009).

Pasteurization is the most effective method of enhancing microbial safety of milk. The consumption of milk that is not pasteurized increases the risk of contrasting disease from a food staff that is otherwise very nutritious and healthy. Pasteurization improves the safety and lengthens the shelf life of a product by destroying pathogenic and spoilage organisms; however, it is not the same as sterilization. In spite of this public health benefit of pasteurization, an increasing number of people are consuming raw unpasteurized milk. Enhanced nutritional qualities, taste, and health benefits have all been advocated as reasons for increased interest in raw milk consumption (Lejeune and Rajal- Schultz, 2009; Oliver *et al.*, 2009).

Enterotoxins are relatively difficult to destroy by heating. The usual heat treatments used to cook food and destroy bacterial cells cannot be relied upon to inactivate the toxin. When foods are tested to assess their safety, both the presence of residual toxin as well as viable microorganisms are determined (Bermer *et al.*, 2004).

2.4. Staphylococcal food poisoning

Foodborne diseases are defined by the World Health Organization (WHO) as diseases of infectious or toxic nature caused by consumption of contaminated foods or water (Loir *et al.*, 2003). Foodborne infections are caused by the ingestion of viable (live) pathogenic microorganisms along with the food, while foodborne poisoning (food intoxication) is caused by the ingestion of toxins or other harmful substances that are produced by pathogenic microorganisms in the foods (Smith, 2007).

Staphylococcal food poisoning is one of the most common types of foodborne diseases worldwide. It has been identified in numerous outbreaks of food poisoning, but is believed to be under reported due to the self-limiting nature of the illness and the fact that most people recover within 1-2 days of becoming ill (Bermer *et al.*, 2004).

2.4.1. Staphylococci species of concern and incidence in foods

Staphylococcal enterotoxins are produced primarily by *S. aureus*. However, *S. intermedius* is the other well known enterotoxin producer and has been clearly involved in staphylococcal food poisoning (Loir, 2003). The coagulase-negative species *S. epidermidis* is also reported to have caused an outbreak (Vargun and Vatansever, 2007; Morandi *et al.*, 2007).

The other coagulase-positive and several coagulase-negative staphylococci are known to produce different types of enterotoxins but their public health importance is not well known (Gay, 2000). For example, among the coagulase negative species, *S. cohnii*, *S. epidermidis*, *S. xylosus* and *S. haemolyticus* have been isolated from ewe's milk and were found to produce one or several SEs (Loir, 2003).

According to different other authors, the proportion of enterotoxin-producing *S. aureus* strains of human origin varies between 18 and 75%. The proportion of toxigenic strains isolated from various sources (human, animal, and food) is very high. Strains of human origin predominate in epidemics, but animals are also reservoirs of the infection (Oliver *et al.*, 2009).

In general, staphylococci may be expected to exist, at least in low numbers, in any or all food products that are of animal origin or in those that are handled directly by humans, unless heat-processing steps are applied to effect their destruction. They have been found in a large number of commercial foods (Gay, 2000).

2.4.2. Staphylococcal enterotoxins (SEs)

The abundance of literature on SEs varies considerably among the types, according to the chronology of their identification and their importance in staphylococcal food poisoning. To date, 14 different SE types have been identified, which share structure and sequence similarities (Loir, 2003), of which the six antigenic types (named SE-A, B, C, D, E and G) are most commonly encountered in SFP (Kerouanton *et al.*, 2007).

In general, SE-A is recovered from food poisoning outbreaks more often than any of the others, with SE-D being second most frequent and the fewest number of outbreaks are associated with SE-E (Jay, 2000). Enterotoxins cause diarrhea and vomiting when ingested and they are responsible for SFP (Smith, 2007).

Staphylococcal enterotoxins are a group of single-chain, low-molecular weight proteins which upon hydrolysis, yield 18 amino acids, with aspartic, glutamic, lysine and tyrosine being the most abundant. The various enterotoxins differ in certain physiochemical properties, but each has about the same potency. Although, biological and serological activity are generally associated, it has been shown that serologically negative enterotoxin may be biologically active (Gay, 2000).

Heat resistance is one of the most important physical and chemical properties of SEs, which means that biological activity of toxins remains unchanged even after thermal processing of food. They are thought to be more heat resistant in foodstuffs than in a laboratory culture medium but can be inactivated by heat treatments used in the sterilization of canned foods when they are present at low concentrations (Holeckova *et al.*, 2002).

2.4.3. Incidence of SFP and vehicle foods

The foods that are most often involved in staphylococcal food poisoning differ widely from one country to another. In all cases of staphylococcal food poisoning, the food stuff or one of the ingredients was contaminated with an SE-producing *S. aureus* strain and was exposed, at least for a while, to temperatures that allow *S. aureus* growth. Most of the time the food stuff reaches this temperature because of a failure in the refrigeration process, or because a growth-permissive temperature is required during processing (e.g., cheese making). Many different foods can be a good growth medium for *S. aureus*, and have been implicated in staphylococcal food poisoning, including milk and cream, cream-filled pastries, butter, ham, cheeses, sausages, canned meat, salads, cooked meals, and sandwich fillings (Loir, 2003).

The main sources of contamination are humans (handlers contaminate food via manual contact or via the respiratory tract by coughing and sneezing) and contamination occurs after heat treatment of the food. Nevertheless, in foods such as raw meat, sausages, raw milk, and raw milk cheese, contamination from animal origins are more frequent and are due to animal carriage or infections (eg., mastitis) (Loir, 2003).

Milk is an excellent bacteriological medium. In fact, sterile skimmed milk is used routinely as a culture medium. A variety of diseases are potentially transmissible through milk (Pelczar *et al.*, 1986).

Milk and milk products have the potential to transmit pathogenic organisms to humans. All the nutritional components that make milk and milk products which are an important part of the human diet also support the growth of pathogenic organisms (Abdel Hameed *et al.*, 2007).

Milk is a good substrate for *S. aureus* growth, and dairy products are a known source of intoxication and are frequently contaminated with enterotoxigenic *S. aureus*, which is often involved in SFP (Hein *et al.*, 2005). *S. aureus* contamination can occur through the presence of *S. aureus* in the raw milk itself or during its processing (Morandi *et al.*, 2007).

Raw milk is a potential source of *S. aureus* in milk and milk products especially, in the case of defective pasteurization and, therefore, constitutes a major threat to public health (Thomas *et al.*, 2006; Kaloreu *et al.*, 2007).

Table 1: Principal factors that lead to the outbreaks of staphylococcal foodborne gastroenteritis in United States, 1973-1987

Causes	Number of outbreaks
Improper holding temperatures	98
Poor personal hygiene	71
Contaminated equipment	43
Inadequate cooking	22
Food from unsafe sources	12
Others	24

Source: Gay, 2000

2.4.4. Clinical signs

The incubation period is short, generally 3 hours after ingestion of the food involved. The interval between consumption of the enterotoxin and the first symptoms can vary from 30 minutes to 8 hours, depending on the quantity of toxin ingested and the susceptibility of the individual. The major symptoms are nausea, vomiting, abdominal pains, and diarrhea. Some patients may show low pyrexia (up to 38°C). More serious cases manifest prostration, cephalalgia, abnormal temperature, and lowered blood pressure, as well as blood and mucus in the stool and vomit. The course of the disease is usually benign and the patient recovers without medication in 24 to 72 hours. Recently, a toxic shock syndrome has been described. Symptoms

consist of vomiting, diarrhea, high fever, erythroderma, edema, renal insufficiency, and toxic shock (Cuomo, 2009).

2.4.5. Diagnosis

Symptoms of FBD associated with *S. aureus* are not suggestive and have little importance to warrant diagnosis (Johnson *et al.*, 2006). In the diagnosis of staphylococcal poisoning illness, proper interviews with the victims and gathering and analyzing epidemiologic data are essential. The presence of a common source, types of specific food and travel history should be investigated (Hagstad and Hubbert, 1986).

The most conclusive test is the linking of an illness with a specific food or in cases where multiple vehicles exist the detection of the toxin in the food sample (s). In cases where the food may have been treated to kill the staphylococci, as in pasteurization or heating, direct microscopic observation of the food may be an aid in the diagnosis (Nania *et al.*, 2006).

Incriminated foods should be collected and examined for staphylococci or the enterotoxins produced. The latter is especially important when foods that have been heated before consumption are implicated in the outbreak. For some outbreaks, food handlers are also tested to ensure whether they are carriers of the strain responsible (Hobbs and Gilbert, 1981).

Staphylococci can be identified on the basis of colony morphology, production of coagulase, detection of hemolysins, thermostable deoxyribonuclease, by various enzyme activities, and aerobic acid production from certain carbohydrates. Biochemical tests, which are commercially available, can be used to confirm the staphylococcal species (Quinn *et al.*, 2002). The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are catalase-positive. *Staphylococcus aureus* is catalase positive (meaning that it can produce the enzyme "catalase") and able to convert hydrogen peroxide (H_2O_2) to water and oxygen. Pathogenic staphylococci species can also be differentiated from most other staphylococci by the coagulase test: *S. aureus*, *S. intermedius* and *S. hyicus* are primarily coagulase-positive (causes

clot formation) while most other *Staphylococcus* species are coagulase-negative (Rowland *et al.*, 1994).

Commercial latex agglutination tests and Analytic Profile Index (API) Staph system (bioMérieux) are examples of assays available for identification of staphylococci. Recently, a real-time PCR assay was developed to identify common staphylococcal species. There are also automated systems, such as Vitek and Baxter-MicroScan, which incubate inoculated trays or cards, read and interpret results, and with the aid of their programmed computer, determine the identity of organisms (Tsegmed, 2006).

The presence of relatively large numbers of enterotoxigenic staphylococci is a good circumstantial evidence that the food contains toxins (Walderhaug, 2007).

Methods for detecting staphylococcal enterotoxins in foods must have sufficient sensitivity to recognize the protein at levels capable of causing SFP. The presence of SE in epidemiologically implicated foods, or the enterotoxigenicity of isolates, may be demonstrated immunologically based on monoclonal antibodies by application of a number of serological methods such as immunoassays (e.g. reverse passive latex agglutination or enzyme linked immunosorbent assay (ELISA)), nucleic acid based tests such as polymerase chain reaction, restriction fragment length polymorphism, or pulse field gel electrophoresis which, can detect approximately 1.0 ng of toxin/g of food (Walderhaug, 2007).

Phage typing is carried out for epidemiological purposes, particularly for *S. aureus* strains from human cases of food poisoning and occasionally, in reference laboratories, for *S. aureus* isolates from bovine mastitis (Quinn *et al.*, 1999).

2.4.6. Control and prevention of SFP

Staphylococcal food poisoning is not an infection, but an intoxication that results from ingestion of staphylococcal enterotoxins in food. The enterotoxins are produced when food contaminated

with *S. aureus* or other enterotoxigenic staphylococci is improperly stored under conditions that allow the bacteria to grow. Although contamination can originate from animals or the environment, food handlers with poor hygiene are the usual source. Effective methods for preventing staphylococcal food poisoning are aimed at eliminating contamination through common hygienic practices, such as wearing gloves, and proper food storage to minimize toxin production (Bermer *et al.*, 2004).

It is important to prevent the contamination of food with *Staphylococcus* before the toxin can be produced. There is no significant growth of *S. aureus* at temperature below 4.4 °C and the organisms are destroyed when kept at 77 °C for 20 minutes. Storing foods at temperature less than 4.4 °C or greater than 60 °C effectively prevents replication of *S. aureus* and significant toxin production (Salyers and Whitt, 2002).

Careful food preparation can prevent staphylococcal food poisoning. Anyone who has a skin infection should not prepare food for others until the infection heals. Food should be consumed immediately or refrigerated and not kept at room temperature (Bermer *et al.*, 2004).

3. MATERIALS AND METHODS

3.1. Study area

The study was carried out in urban dairy farms found in Addis Ababa. Addis Ababa is the capital city and administrative center of the Federal Democratic Republic of Ethiopia, comprising 10 administrative sub-cities. It is located in the central highlands of Ethiopia, covering an area of 530 km² with an altitude of 2500 meters above sea level. The average annual temperature is 21°C with relative humidity varying between 70 to 80% during the rainy season. The area receives an average of 1800 mm rain annually, with the major rains occurring between July and September (CSA, 2003).

The urban dairy production system is characterized by intensive production in which dairy animals are kept indoors at zero grazing. The greatest majority of dairy producers produce milk for the purpose of selling directly to the consumers and retailers (Fisseha, 2008).

3.2. Study population

The study population consists of samples of bovine raw milk from lactating cows, skin swab samples from lactating cows and dairy personnel and swabs from milking bucket and milk collecting containers from the selected 25 urban dairy farms. The farms selected were divided into three categories based on the number of lactating cows they have: small (less than 11), medium (11 – 20) and large (greater than 20). Most of the milking animals were exotic cross breeds.

3.3. Study design

The study was cross-sectional study involving milking cows, dairy personnel and milking equipments of 25 urban dairy farms from October 2009 to April 2010. On each sampling day, usually once a week, all the required samples were collected on one-trip.

Questionnaire was also designed and used to collect information from dairy cow owners and milking personnel of selected farms in one visit interview at the same day of sample collection. The information collected included the hygienic status of the barn and pre-milking practices experienced in dairy farms.

3.4. Determination of sample size

The sample size required for this study was determined according to Thrusfield (2005).

$$n = \frac{1.96^2 \times P_{exp} (1-P_{exp})}{d^2}$$

Where, n = the required sample size; P_{exp} = expected prevalence; d= desired absolute precision.

Therefore, by using estimated prevalence of 22.8% in bovine raw milk of dairy cows (Alehegne, 2009) and taking a confidence interval of 95% and 5% absolute precision, the minimum sample size required for this study was 270 lactating cows. A total of 540 samples comprising of 270 udder milk and 270 lactating cow skin swab samples (135 teat and 135 udder) were used for the study. Regarding teat swab sampling, one pooled swab sample involving all teats of each cow was collected. 19, 22 and 23 swab samples from selected farms were also collected from milking personnel, milking buckets and milk collecting containers, respectively (Table 2).

Table 2: Distribution of the type and number of samples collected

Type of sample	Number of samples
Milk	270
Skin swab	270
Lactating cow teat	135
Lactating cow udder	135
MHS	19
MBS	22
MCCS	23
Total	604

MHS=Milkers' hand swab; MBS =Milkig bucket swab; MCCS= Milk collecting container swab

3.5. Sampling techniques

Out of 10 sub-cities of Addis Ababa two (Yeka and Bole) were randomly selected by simple random sampling technique. Yeka and Bole have 75 and 35 dairy farms, respectively. After taking the list of farms of the two selected sub-cities, all large sized farms and 20% of randomly selected medium and small sized farms of each sub-city were selected also by simple random sampling technique. A total of 25 dairy farms were included in the study. A multistage cluster sampling technique was used to collect samples from lactating cows.

Almost all dairy farm owners milk their cows twice a day: in the morning from 4-6 am and in the afternoon from 3-4 pm. All samples were collected in the afternoon immediately before milking was started. First farmers were informed as milk samples would be collected, and this was to get their normal pre-milking preparations, but just after they completed their preparations all the required samples were collected.

The swab samples were collected before milk sampling by using sterile swabs. For every swab sample a sterile test tube filled with 4ml of phosphate buffered saline (PBS) was used. Prior to sampling, swab tips were moistened in the PBS and for swab sampling the swab was rotated and rubbed against the sampled surface several times. After completion of swabbing, the swab was

put inside into a test tube containing PBS and samples were transported in cool boxes to Shola Regional Veterinary Laboratory and they were stored in refrigerator at 4⁰C. In the next day, samples were transported in cool boxes on ice to the Microbiology Laboratory, Faculty Veterinary Medicine, Addis Ababa University, Debre Zeit and processed within 24 hours of collection. When they were not processed within 24 hours, they were stored in refrigerator at 4⁰C for a maximum of 2 days.

Milk samples were taken after collection of swab samples from teat apices and udder skin of lactating cows. Before collecting milk samples, teats were wiped with cotton wool soaked in 70% ethyl alcohol, paying particular attention to the teat orifices. Composite milk sample was collected by using sterile universal bottles from each lactating dairy cow after the first squirts of milk from each quarter was discarded according to the protocol described by Quinn *et al.* (2002). Each sample was labeled with necessary information, including date of sampling, name of the farm, type of sample and identification number of the animal from which the sample was obtained. Throughout the sampling process disposable gloves were used to avoid contamination.

3.6. Isolation and identification of Staphylococci

Staphylococci were isolated and identified according to standard techniques (Quinn *et al.*, 1999; ES ISO 6888-2, 2002; Quinn *et al.*, 2002). The isolation and identification of staphylococci needs different successive stages, which are described below and samples were processed and analyzed separately. The bacteriological media used were prepared according to the manufacturer's recommendations (Annex IV).

All the samples taken from milk and extramammary sites (teat apices, udder skin), milkers' hands and milking equipments) were directly streaked onto 7% sheep blood agar and incubated aerobically at 37⁰C for 24 – 48hrs (Quinn *et al.*, 2002).

After incubation, representative colonies were selected from each plate with bacterial growth based on morphology, color and hemolytic pattern. Different representative colonies were sub-cultured onto different blood agar plates and further incubated at 37⁰C for 24hrs. Typical colonies

were subjected to Gram stain to study the staining properties and cellular morphology. Gram positive pure cultures of a single colony type from the blood agar were transferred into nutrient agar plates and incubated at 37°C for 24 hours (Quinn *et al.*, 2002).

After growth staphylococci were identified by using conventional bacteriological techniques on the basis of colony characteristics, pigment production, hemolysis and the following biochemical reactions: catalase test, oxidase test, O-F test with glucose and mannitol, coagulase test (by using rabbit plasma) and carbohydrate (mannitol and maltose) fermentation tests following the standard methods (Quinn *et al.*, 1999; ES ISO 6888-2, 2002).

Table 3: The main differentiating characteristics of the gram-positive cocci

Species	Coagulase	Catalase	Oxidase	O-F with glucose and mannitol
Pathogenic staphylococci	+	+	-	F
Non-pathogenic staphylococci	-	+	-	F
Enterococci	-	-	-	F
Streptococci	-	-	-	F
Micrococci	-	+	+/-	O

+ = positive, - = negative, F= fermentative, O = Oxidative, +/- =Positive or negative; Source: Quinn *et al.*, 1999

3.6.1. Primary identification of staphylococci species

Gram staining

Parts of all the suspected colonies of staphylococcal species were transferred and smeared using sterile inoculating loop on a glass slide. Then the bacterial smears were stained using the procedure of Gram's stain, and observed under oil immersion microscope to identify colonies based on their Gram's reaction, size, shape, arrangement etc.

Catalase test

A loopful of the typical suspected colony was taken from the top of the nutrient agar and placed on a clean microscopic slide. A drop of 3 percent hydrogen peroxide was added and an effervescence of oxygen gas, within a few seconds, was taken as a positive reaction.

Oxidase test

A piece of filter paper was moistened in a petridish with one percent aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride. The suspected colony of the bacteria was streaked firmly across the filter paper with a sterile platinum loop. The development of dark purple color along the streaked line within 10 seconds was taken as positive reaction.

Oxidation-Fermentation (O-F) test

The conventional O-F medium is most suitable for non-fastidious gram-negative bacteria. Modification of the O-F medium which is applied for the identification of staphylococci and micrococci was used according to Quinn *et al.* (1999). Test tubes filled with the O-F medium were heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen. The tubes were then cooled rapidly under cold running water. For each sample paired tubes were stab-inoculated with typical pure colony from the nutrient agar. Then sterile paraffin oil was layered on top of one of the paired tubes to a depth of about 1cm. The inoculated tubes were incubated at 37°C and examined in 24 hours and daily for up to 14 days. Inoculated paired tubes showing change of color from purple to yellow were considered as staphylococci.

3.6.2. Secondary identification of staphylococci species

Coagulase test

From the surface of each selected colony of staphylococci an inoculum was removed with sterile loop and transferred into a tube of brain-heart infusion broth and incubated at 37°C for 24hrs. After growth, 0.1ml of each culture was added aseptically to 0.3ml of the rabbit plasma and incubated at 37°C. Clotting was examined after 4 to 6hrs of incubation by tilting the tube and if the test was negative, re-examination was made after 24hrs of incubation. When the volume of the clot occupied more than half of the original volume of the liquid, it was considered as positive (ES ISO 6888-2, 2002).

Mannitol salt agar

Colonies of coagulase-positive isolates were streaked on to mannitol salt agar plate and incubated at 37°C and examined after 24-48hrs. The presence of growth and change of color (red to yellow) of the media was regarded as presumptive indication of coagulase-positive staphylococci.

Purple agar base

Purple agar base containing bromocresol purple as a pH indicator and 1% maltose was used to differentiate coagulase-positive staphylococci species. The interpretation of the result was done according to the method described by Quinn *et al.* (1999). Rapid fermenters of maltose that caused color change of the medium and colonies within 24hrs were taken as *S. aureus*; whereas weak and delayed fermenters were considered as *S. intermedius*. Isolates that did not cause fermentation and color change, rather that produced deep purple coloration around the colonies, were considered as *S. hyicus* (Table 3).

Table 4: Interpretation of results based on reactions of coagulase-positive staphylococci on purple agar base with 1% maltose.

Species	Maltose fermentation	Reactions on purple agar base with 1% maltose
<i>Staphylococcus aureus</i>	+++	Diffuse yellow color around colonies. Rapid reaction within 24 hours of incubation.
<i>S. intermedius</i>	±	Little change in the medium, slight yellow zone under colonies may have a yellowish tinge.
<i>S. hyicus</i>	-	Diffuse deep purple (alkaline) zone around the colonies.

+++ = rapid fermentation of maltose; ± = weak and slow fermentation; - = negative; Source: Quinn *et al.*, 1999

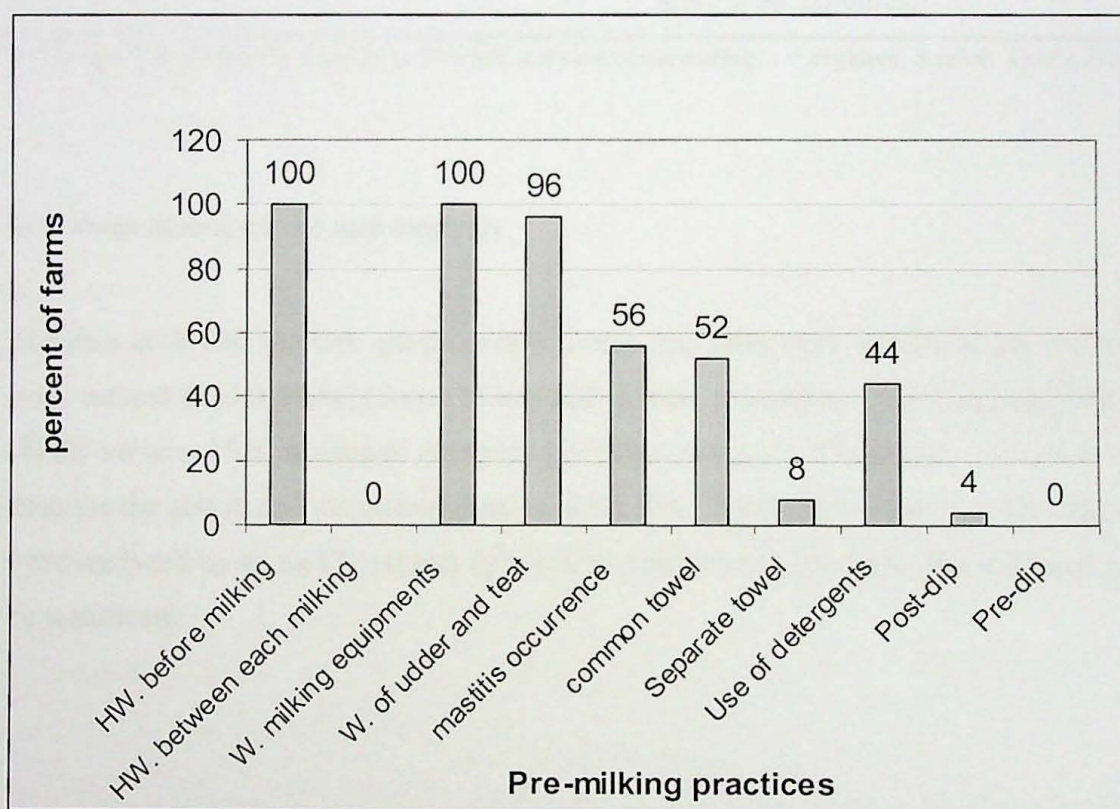
3. 7. Data management and analysis

The data collected through questionnaire survey and laboratory results of the collected samples were entered in to databases using Micro-Soft computer program Excel and analyzed using SPSS (SPSS version-15.0) statistical computer software programs. Descriptive statistics were used to describe the nature and the characteristics of the data. Comparisons between prevalence of groups were analyzed by using Chi-square (χ^2) test. For all tests p-value less than 0.05 was considered to be significant.

4. RESULTS

4.1. Questionnaire survey

Out of 25 dairy farms, the barns of 23 (92%) were poor hygienically. In 96% of all the farms the udder and teat of lactating cows was washed before milking, but 13 (52%) of them used common towel for washing more than one or all of the lactating cows. Additionally, washing was not complete and usually they used the towels to remove dirt and some moisture left on the lactating cow teat and udder skin surface. In all farms hand washing was practiced before milking, but none of them washed between each milking and only 11 (44%) of them used detergents (soap). Of all the farms dipping (i.e., post-dip) was practiced only in one farm. All the responses and observations to the questionnaire are summarized on figure 1.



W=washing; HW=Hand washing

Figure 1: Percentage of pre-milking practices

4.2. Prevalence of *staphylococci* species

4.2.1. Udder milk

A total of 270 udder milk samples were collected from 25 urban dairy farms and bacteriologically examined to identify *Staphylococcus* species. The overall prevalence of *Staphylococcus* species was: coagulase-negative *staphylococci* (CNS) (53%), *S. aureus* (8.5%), *S. intermedius* (0.7%) and *S. hyicus* (0.7%) (Figure 2). Similarly, species prevalence in the milk samples among different farm size was as follows: CNS (50.5%), *S. aureus* (6.9%), *S. intermedius* (1%) and *S. hyicus* (2%) in large scale; CNS (53.9%) and *S. aureus* (11.2%) in medium scale and CNS (55%), *S. aureus* (7.5%) and *S. intermedius* (1.3%) in small scale urban dairy farms (Figure 3).

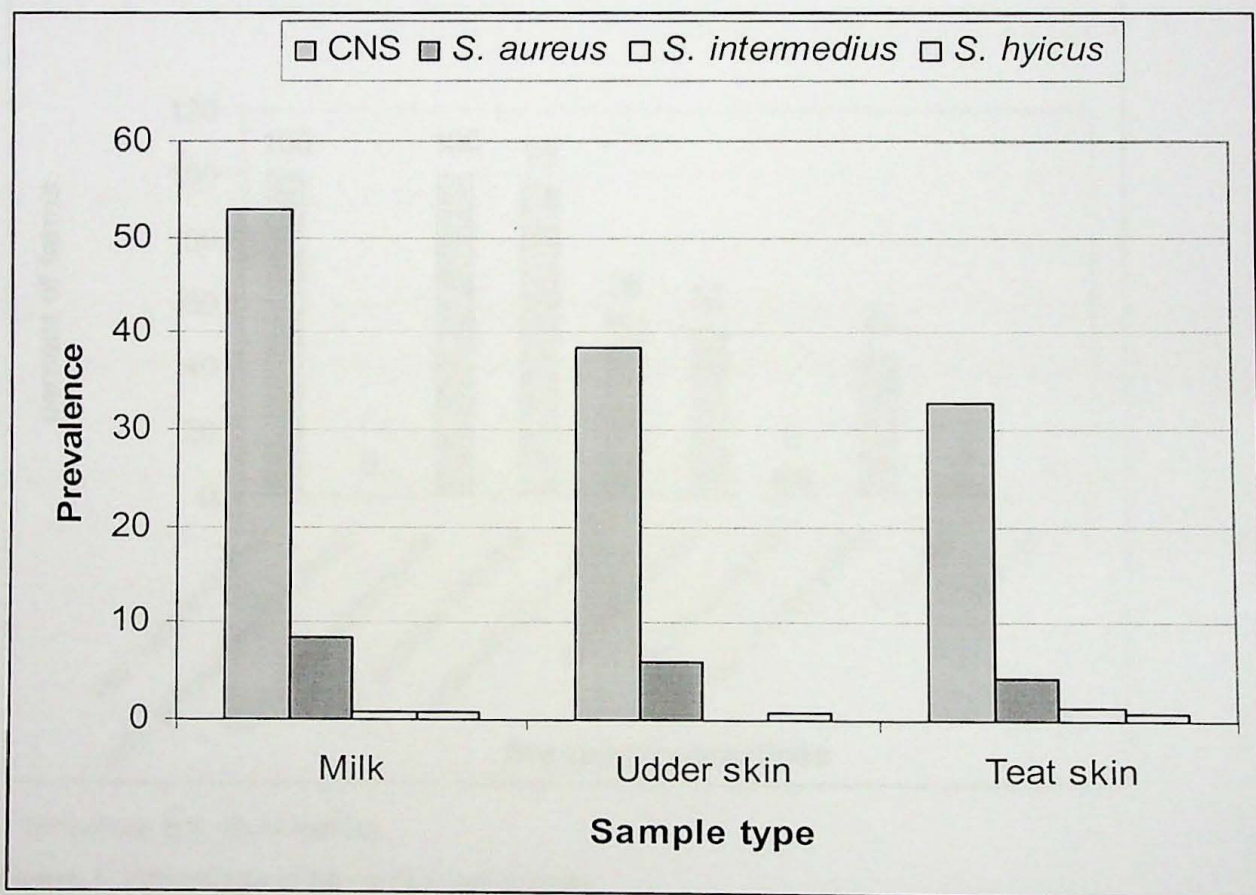


Figure 2: Prevalence of staphylococci species in udder milk, udder skin and teat skin

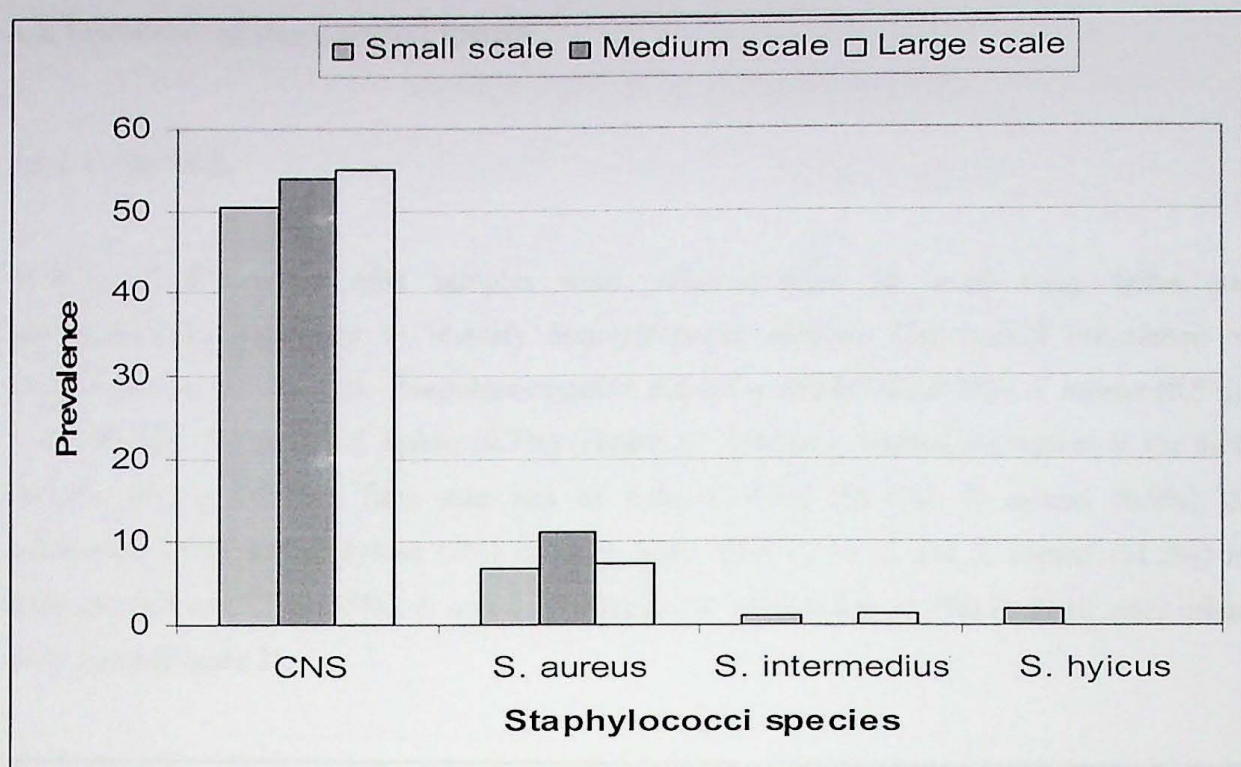


Figure 3: Prevalence of staphylococci species in bovine udder milk samples in large, medium and small scale urban dairy farms

Comparison was made between different farm sizes with respect to prevalence of each *Staphylococcus* species in udder milk samples. There was no statistically significant difference in prevalence between the farms for all *Staphylococcus* species ($p > 0.05$) (Table 5).

Table 5: Comparative prevalence of *Staphylococcus* species in bovine udder milk between farm sizes

staphylococci species	Farm sizes			χ^2	df	P-value
	Small scale	Medium scale	Large scale			
CNS	50.5	53.9	55.0	0.41	2	0.813
<i>S. aureus</i>	6.9	11.2	7.5	1.28	2	0.528
<i>S. intermedius</i>	1.0	0.0	1.3	1.03	2	0.597
<i>S. hyicus</i>	2.0	0.0	0.0	3.37	2	0.185

4.2.2. Udder and teat skin

Out of 270 lactating cow skin swab samples (135 lactating cow teats and 135 udders) examined, the prevalence of *Staphylococcus* species identified were: CNS (38.5%), *S. aureus* (5.9%) and *S. hyicus* (0.7%) in case of udder and CNS (32.6%), *S. aureus* (4.4%), *S. intermedius* (7%) and *S. hyicus* (7%) in teat (Figure 2).

The comparison of prevalence of *Staphylococcus* species in udder and teat skin between farm sizes showed statistically significant variation between farms for CNS species for teat ($\chi^2 = 6.90$, $p = 0.033$) and for udder ($\chi^2 = 7.76$, $p = 0.021$), but no statistically significant difference was seen between farms for the rest species in both teat and udder ($p > 0.05$) (Table 6).

Table 6: Comparative prevalence of *Staphylococcus* species in the udder and teat skin swab samples between farm sizes.

Sample type	<i>Staphylococcus</i> species	Farm sizes			χ^2	df	P-value
		Small scale (n=48)	Medium scale (n=44)	Large scale (n=43)			
Teat	CNS	18.8	43.2	37.2	6.90	2	0.033
	<i>S. aureus</i>	2.1	4.5	7.0	1.30	2	0.527
	<i>S. intermedius</i>	0.0	0.0	4.7	4.34	2	0.114
	<i>S. hyicus</i>	0.0	2.3	0.0	2.10	2	0.353
Udder	CNS	22.9	45.5	48.8	7.76	2	0.021
	<i>S. aureus</i>	4.2	9.1	4.8	1.18	2	0.554
	<i>S. intermedius</i>	0.0	0.0	0.0	0.00	2	0.000
	<i>S. hyicus</i>	2.1	0.0	0.0	1.83	2	0.401

Analysis of comparison of prevalence of each *Staphylococcus* species between udder and teat skin showed a statistically significant difference only for CNS species ($\chi^2 = 10.93$, $p = 0.001$) (Table 7).

Table 7: Comparison of prevalence of *Staphylococcus* species between udder skin and teat skin

<i>Staphylococcus</i> species	Prevalence		χ^2	df	P-value
	Udder	Teat			
CNS	38.5	32.6	10.93	1	0.001
<i>S. aureus</i>	5.9	4.4	0.40	1	0.529
<i>S. intermedius</i>	0.0	1.5	0.00	0	0.000
<i>S. hyicus</i>	0.7	0.7	0.01	1	0.931

The comparison of prevalence of *Staphylococcus* species between teat skin and udder milk showed statistically significant variation for CNS species in between teat skin and udder milk ($\chi^2=16.90$, $p = 0.000$) (Table 8), but no significant difference was observed in species prevalence between udder skin and udder milk (Table 9).

Table 8: Comparison of prevalence of *Staphylococcus* species between udder milk and teat skin

<i>Staphylococcus</i> species	Prevalence		χ^2	df	P-value
	milk	Teat			
CNS	51.9	32.6	16.90	1	0.000
<i>S. aureus</i>	6.9	4.4	1.01	1	0.315
<i>S. intermedius</i>	0.7	1.5	0.015	1	0.902
<i>S. hyicus</i>	1.5	0.7	0.02	1	0.902

Table 9: Comparison of prevalence of *Staphylococcus* species between udder milk and udder skin

<i>Staphylococcus</i> species	Prevalence		χ^2	df	P-value
	milk	Udder			
CNS	51.9	38.5	1.16	1	0.282
<i>S. aureus</i>	6.9	5.9	0.61	1	0.436
<i>S. hyicus</i>	1.5	0.7	0.02	1	0.902

4.2.3. Milkers' hands and milking and milk collecting equipments

Out of 19 milkers' hands skin, 22 milking bucket and 23 milk collecting container swab samples examined, only CNS species were identified with the respective prevalence of 42.1%, 13.6% and 8.7% (Figure 4).

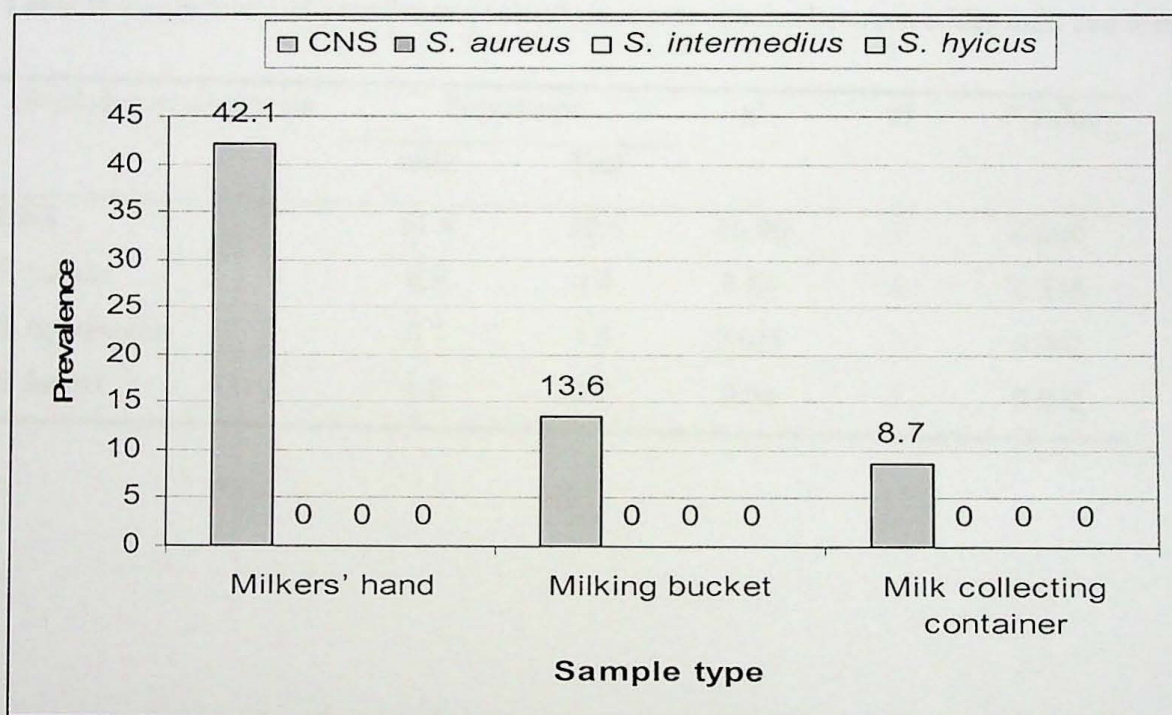


Figure 4: prevalence of *Staphylococcus* species in milkers' hands, milking bucket and milk collecting containers

5. DISCUSSION

Staphylococcal food poisoning is a major concern worldwide (He *et al.*, 2010; Compton *et al.*, 2008). It results from the consumption of food in which enterotoxigenic staphylococci have grown and produced toxins (Loir, 2003). Staphylococci are common contaminants of raw milk produced in dairy farms. These organisms in milk may originate from the infected (mastitic) cow, udder and teat skin, milkers' hands, milking and milk storage equipments. The extent of contamination varies widely from one farm to another, but is dependant primarily on the milking management used in dairy farms (Green and Bradley, 2004). Raw milk is the potential source of staphylococci in milk and milk products (Thomas *et al.*, 2006).

The staphylococci species isolated in the udder milk of lactating cows in this study could be associated with sub-clinical or clinical mastitis. In the absence of intramammary infection or systemic disease, at its site of production milk does not contain bacteria. Milk from cow udders infected with *staphylococci* can contaminate numerous milk products (Oliver *et al.*, 2009). The presence of even one pathogen in milk may have impact on human health; under optimal conditions one pathogen per milliliter of milk could reproduce to a level of more than 1,000,000 per milliliter in less than seven hours. If one farm doesn't prevent bacterial growth, which contaminated milk it can also spread bacteria to other high quality milk when they are mixed (Tybor, 1992).

5.1. Prevalence of *Staphylococcus* species

5.1.1. Udder milk

The majority of the *Staphylococcus* species contaminating bovine udder milk in this study are coagulase-negative staphylococci with the prevalence of 53%. This is higher than that reported by Alehegne (2009), who noted a prevalence of 37.6% in bovine udder milk of lactating cows in smallholder dairy farms in Debre Zeit. Bishi (1998) and Nesru (1999) reported a prevalence of

54% and 41.9%, respectively. Gizat (2004) recorded 49.6% in Bahir Dar. All reported higher proportional prevalence of CNS compared to other bacterial causes of mastitis in the udder milk samples collected from lactating cows with clinical or sub-clinical mastitis. Atyabi *et al.* (2006) in his four-year investigation showed that CNS were the most frequent organisms causing contamination of milk in industrial dairy cattle around Tehran and he also indicated in most cases, they caused sub-acute mastitis.

These variable results by different workers concerning the occurrence of staphylococci in bovine udder milk may be due to the difference in the number of samples, the isolation and identification techniques used and the place of origin of the samples collected.

The second most prevalent species in the study was *S. aureus* with the prevalence of 8.5% and this is lower than Alehegne (2009) (15.8%), but is in agreement with what Haveri (2008) and Abdel Hameed *et al.* (2007) found who indicated a prevalences of 10.2% and 7.34%, respectively. From udder milk of lactating cows with sub-clinical and clinical mastitis, Getahun (2006) and Gizat (2004) reported a proportional prevalence of 20% and 17.9% for *S. aureus*, respectively. Atyabi *et al.* (2006) indicated a prevalence of 2.89% for *S. aureus* in udder milk samples collected from CMT positive cows. The prevalence of *S. intermedius* and *S. hyicus* was 0.7% for each in this study. The findings of Alehegne (2009) for *S. intermedius* and *S. hyicus* were 13% and 1.3%, respectively.

The primary entry points of a pathogen into the milk supply are cows; cows with mastitis contaminate the milk. According to Lejeune and Rajala-Schultz (2009) mastitis is the single disease that has the most significant impact on milk quality. Cows suffering from mastitis discharge large numbers of pathogens like staphylococci, *E. coli* and *Clostridium perfringens* into the milk (Tabor, 1992).

Staphylococci are the most common causes of bovine intramammary infections and mastitis in modern dairy farms worldwide (Haveri, 2008). The high prevalence of coagulase-negative staphylococci (CNS) in this study cannot be ignored, only by taking their minor pathogenic effects compared to the coagulase-positive *Staphylococcus* species. In the past, coagulase-

negative staphylococci were often regarded as non-pathogenic, but newly generated data now indicates that these species have become the predominant emerging mastitis pathogens in several countries (Pyorala and Taponen, 2009). CNS can cause persistent udder infections, which result in increased milk somatic cell count (SCC) and decreased milk quality. CNS infection can damage udder tissue and leads to decreased milk production. Additionally, CNS can contaminate milk and many of them are known to be enterotoxigenic and they have also been reported to cause staphylococcal food poisoning. Furthermore, the strains of several CNS species (i.e., *Staphylococcus epidermidis*, *S. haemolyticus*, *S. warneri*, *S. hominis* and *S. saprophyticus*) were found to have high levels of resistance to various antibiotics (Vargun and Vatansever, 2007).

S. aureus is mainly involved in intramammary infection and mastitis in lactating cows. Intramammary infection due to *S. aureus* are generally sub-clinical, nevertheless, intramammary infection and mastitis of sub-clinical form cause considerable economic losses, particularly milk losses ranging from 10-25% of the total yield according to the intensity of infection (Green and Bradley, 2004; Arshad *et al.*, 2006). Jerry (2003) indicated that the infected mammary gland is the primary and most important reservoir of *S. aureus*). According to Abdel Hameed *et al.* (2007) milk from cows with sub-clinical mastitis has a high chance to contaminate raw milk than clinical mastitis and is a public health hazard.

In several investigations, it was possible to isolate from skin lesions and cow's milk the *S. aureus* phage type 80/81, which is related to epidemic infections in man. One of the studies proved that phage type 80/81 produced interstitial mastitis in cows. The same phage type was found among animal caretakers, which indicates that the bacterium is intertransmissible between man and animals and that the latter may reinfect man (Oliver *et al.*, 2009). According to recent investigations, a high proportion of strains isolated from staphylococcal mastitis produce enterotoxin A, which causes many outbreaks in humans (Oliver *et al.*, 2009).

5.1.2. Udder and teat skin

In this study, a total of 135 lactating cow teat skin and 135 udder skin swab samples were collected and bacteriologically analyzed. The samples were collected immediately after the dairy men completed their usual pre-milking preparations. All farms (except one) washed the udder and teat of cows before they start milking and this is their usual pre-milking practice.

Out of the total of the teat and udder skin swab samples examined, the prevalence of CNS was 38.5% and 32.6% and that of *S. aureus* was 5.9% and 4.4% for udder and teat, respectively. White *et al.* (1989) identified CNS species on teat skin, and the species identified were *S. chromogenes* and *S. warneri* in 62% and 61% of the sampled heifers, respectively. The other identified species in this study were *S. intermedius* (1.5%) and *S. hyicus* (0.7%) on teat skin and *S. hyicus* (0.7%) on udder skin.

Such a high prevalence figure after washing of udder and teat before milking in this study indicates the high chance of staphylococci species to contaminate milk leaving the udder of cow during milking. Cows are normal carriers of many pathogens either on the hair or skin due exposure to soil, manure, water and other environmental surfaces. Cross-contamination of the milk can occur due to poor milking hygiene practice, if udders and teats are not properly cleaned, dried and sanitized. Dirty teats and udders can contaminate the milk during milking. In addition, organisms can find their way into the udder and teat where they can multiply and cause infections; the residual water “magic water” on teats and udders is also a bacterial source and risk to milk safety. Both the exterior and interior of the teats and udder can become infected with staphylococci species and contaminate the milk during milking.

Common towel was used to wash udders and teats of lactating cows in most of the visited farms. The towel was soaked and washed in the same bucket water and washing was not complete beyond removing dirt and some moisture left on the teat and udder skin surface. All these conditions may increase the chance of staphylococci to contaminate the milk leaving the udder of cows. Milking was performed in the same shade for there were no separate milking areas.

The other reason for defected pre-milking preparation was the low number of employees in the farms serving both cleaning and milking. In most of the farms only one individual was seen to do all farm practices including milking.

In this study, there was no variation in the prevalence of staphylococci species in bovine udder milk in the three dairy farm categories ($p>0.05$) and this was probably due to the similar management systems practiced in the farms.

5.1.3. Milkers' hands and milking and milk collecting equipments

The results of this study indicated out of 19 milkers' hands skin swab samples examined, 8 (42.1%) were found to be carriers of coagulase-negative staphylococci. Milkers can carry the pathogenic bacteria, since many of the organisms have their origin as commensal flora in the skin and mucous membranes, in human feces or infections. They can directly contaminate the milk, the cow or the equipment through poor personal hygiene. Milkers can also become contaminated from soil and manure on the cow and in turn cross-contaminate the milk or equipment.

The employees who milk the cows, "milkers," have the important yet routine job of harvesting the milk from the udder of the cows in a manner that maintains milk quality and protects the udder from infections. In addition, the milkers have the responsibility of washing and sanitizing milk equipments, cooling and properly storing the milk. They handle a complex set of equipment and chemicals that affect milk quality.

Although the most important foodborne pathogen, *S. aureus*, has not been recovered from the hands of milking personnel in the study, the presence of CNS (42.1%) contaminants in this findings suggest the possibility of *S. aureus* in the hands of milkers after hand washing cannot be ruled out. Similar to udder and teat, hand washing was not complete in the visited farms and only 44% of them used soap to wash their hands before milking.

It is recognized that food handlers are the major sources of contamination with staphylococci. High frequency of carrier status among food handlers has been identified by several investigators.

Many studies conducted on staphylococci carrier status in humans in many countries showed that 30 to 50% of them were carriers at any given time. Pereira *et al.* (1994) examined 55 healthy food handlers in large industries in Brazil and found that 32 (58.2%) were carriers of *S. aureus* and 17(30.9%) carried enterotoxigenic strains in their nose, throat, and under fingernails (Carmo *et al.*, 2003). Carmo *et al.* (2003) in their findings also indicated that out of 5 food handlers examined, 4 were found to be colonized by enterotoxigenic bacteria.

The result of this study also showed that out of 22 milking buckets and 23 milk collecting containers examined, 13.6% and 8.7% were found to harbor CNS species, respectively; but no other species was recovered from both. Milking and milk storage equipments can be source for milk contamination and the situation is aggravated, if the equipment is not properly cleaned and sanitized after use. Milk residues left on equipment and utensil surfaces provide nutrients to support the growth of many microorganisms including staphylococci (Hempen *et al.*, 2004).

6. CONCLUSIONS AND RECOMMENDATIONS

Consumers, processors and regulatory agencies are increasingly interested in the safety and wholesomeness of milk resulting in increased emphasis on the farm management to ensure the production of high quality milk. Despite technical advances in milk processing, the quality of milk is still determined at the dairy farm. The control of staphylococci contamination of raw milk to avoid problems related to public health hazards is very important and constitutes a challenge to food hygienists.

The result of this study suggests that staphylococci species present in bovine udder milk, udder and teat skin of lactating cows, hands of milking personnel and milking and milk storage equipments potentially contaminate milk. This may result in low milk quality and might potentially cause food poisoning. The numerous farm sources of *staphylococci* are potential sources of contamination of milk in which the bacteria can unintentionally enter the milk.

Infections are spread from infected to non-infected cows during milking via contaminated milkers' hands and teat washing and cleaning towels used on several cows. Cross-contamination of the milk can also occur from poor cow hygiene before milking, if udders and teats are not properly cleaned, dried and sanitized. It seems important to consider the possible presence of toxigenic strains of *S. aureus* and handle milk carefully during milking, collection, transportation, processing, and consumption. Based on these conclusive remarks the following recommendations are forwarded:

- ✓ The high prevalence of staphylococci in the udder milk, on teats and udders of lactating cows, milkers' hands and milking and milk collecting equipments suggests that improvement of sanitation on dairy farms should be made to minimize the contamination of raw milk.
- ✓ In order to reduce the contamination of raw milk with staphylococci species at the farm level, it is necessary to introduce appropriate hygienic milking practices. Udders and teats should be washed thoroughly with soap and disinfected with chlorine or

iodine solution and wiped dry before milking and milkers should also wash their hands properly with soap prior to milking.

- ✓ More attention should also be given to clean clothes and a clean milking place. All equipment that gets in contact with milk should be properly washed with sanitizer and be disinfected afterwards.
- ✓ Effective programmes for mastitis control that promote dairy food safety are important based on identifying the pathogens present and developing effective tools to control *mastitis* pathogens. Controlling mastitis is important because the condition has significant adverse effects on public health.
- ✓ All these changes of common practices can only be achieved through practical training of all those who are involved in the production and collection of milk. Milk producers and processors must be trained in order to improve the quality of the dairy products, they produce.
- ✓ Public awareness has to be made concerning the importance of good quality milk and of the risk of human infection arising from contaminated milk and milk products.
- ✓ The distribution of enterotoxigenic staphylococcus species and also staphylococcal enterotoxins in milk production chains should be studied in order to prevent consumers from staphylococcal food poisoning.

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8. ANNEXES

Annex I: Questionnaire used for interviewing dairy farm owners

Date _____

Name of the owner _____

Number of cows

Name of the farm _____

Total _____

Address _____

Lactating _____

1. Hygienic status of bedding area

Poor _____

Good _____

2. Clearing of hands before milking

No _____

Yes _____

3. Clearing of hands between each milking

No _____

Yes _____

4. Washing of milking utensils before milking

No _____

Yes _____

5. Supply of towel

No _____

Common _____

Separate _____

6. Use of detergents

No _____

Yes _____

7. Previous occurrence of mastitis

No _____

Yes _____

8. Dipping practice before and after milking

No _____

Before _____

After _____

Both _____

9. Washing of udder and teat before milking

Yes _____

No _____

Annex II: Primary identification tests of *Staphylococcus* species

No	Sample code	Cellular morphology			Primary identification tests			Genera
		Staining	Shape	Arrangement	Catalase	Oxidase	O-F test	

Annex III: Secondary identification tests of *Staphylococcus* species

Secondary identification tests			Species
Coagulase test	Mannitol salt agar	Purple agar base	

Annex IV: Media and Reagents used for the isolation and identification staphylococci

1. **Agar powder, Bacteriological 500 g** (Himedia laboratories pvt Ltd., Mumbai-400 086, India). Recommended for routine bacteriological work where compatibility with other ingredients is not a critical criteria. Gelling temperature not more than 40°C.

2. Tryptone peptone (pancreatic digest of casein) (Becton Dickinson, France). A microbiological peptone for use in culture media.

3. Nutrient agar 500 g (Oxoid, Basingstoke, Hampshire, England).

Composition (g/l): 'Lab-Lemco' powder 1.0; Yeast extract 2.0; Peptone 5.0; Sodium chloride 5.0; agar 15.0. PH 7.4 ± 2 at 25°C

Preparation: suspend 28 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

3. Blood agar base (infusion agar) 500 g (HiMedia laboratories pvt. Ltd. 23, Vadhani ind. Est, LBS Marg, Mumbai – 400.086, India. MO73). For isolation and cultivation of many fastidious pathogenic microorganisms after addition of blood.

Composition (g/l): Lab-lemco powder 10.0; peptone 10.00; Sodium Chloride 5.00; agar 15.00. Final PH (at 25°C) 7.3 ± 0.2 .

Preparation: suspend 40.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (121°C) for 15 minutes and cool to 50°C .

4. Purple agar base (Difco TM)

Composition: Proteose peptone No. 3=10.0 gm; Beef extract= 1.0; Sodium chloride=5.0 gm; Agar=15.0 gm; Bromocresol purple=0.02 gm. Final pH for laboratory use= 6.8 ± 0.2 .

Preparation: Suspend 31gm of the powder in 1litre of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes. When preparing 0.5-1% carbohydrate fermentation sugars, dissolve 5-10 gm of the desired carbohydrate in the basal medium prior to autoclaving or dissolve 31gm Purple Agar Base in 900 ml of purified water and aseptically add 100 ml of a sterile 5-10% carbohydrate solution (w/v) after autoclaving.

5. Mannitol salt agar

Recommended for the selective isolation cultivation and enumeration of staphylococci from clinical and non-clinical specimens.

Composition: gm/lit Sodium chloride 75.00; Agar, 15.00; D-mannitol 10.00; Pancreatic digest of casein 5.00; Pancreatic digest of animal tissue 5.00 Beef extract 1.00; Phenol red 0.075.

Preparation: Add 111.02 gm powder to distilled /deionized water. Bring volume to 1.0 liter and mix thoroughly. Gently heat while stirring and bring to boiling. Autoclave at 15 psi pressure at 121⁰C for 15 minutes. Final pH at 25⁰C 7.4±0.2.

6. **Brain heart infusion broth 500 g** (HiMedia Laboratories Pvt. Ltd. 23, Vadhani Ind. Est., LBS Marg, Mumbai – 400 086, India, M210- 500 G):

Composition g/l: Calf brain infusion solids 12.5; beaf heart infusion solids 5.0; peptose peptone 10.0; glucose 2.0; sodium chloride 5.0; Di-Sodium phosphate 2.5.

7. **Yeast extract (Oxoid LTD,BASINGSTOKE, HAMPSHIRE, ENGLAND)**

Typical analysis (%W/W); pH (.5% solution) 7.0±0.2 at 25⁰C; Total nitrogen= 10.0-12.5; Aminonitrogen 5.1; Sodium chloride 0.3.

A water soluble autolysate in powder form. For use in culture medium as a source of nitrogen and water soluble B vitamins. Final pH at 25⁰C 7.4±0.2.

CURRICULUM VITAE

Personal profile

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8. DECLARATION

I, the undersigned, declare that “This thesis is my original work and has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknowledged”.

Name: Mezgebu Merid Bayable

Signature:



Date of submission: _____

This thesis has been submitted for examination with our approval as academic advisors.

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