ADDIS ABABA UNIVERSITY

FACULTY OF VETERINARY MEDICINE

STUDY ON CONTAMINATION OF BOVINE RAW MILK WITH *STAPHYLOCOCCUS* SPECIES IN URBAN AND PERIURBAN DAIRY FARMS IN DIRE DAWA, ETHIOPIA

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements of Master Degree in Veterinary Public Health

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LIST OF ABBERVIATIONS

µ	Micro
^{0}c	Degree centigrade
AAU	Addis Ababa University
a_w	Water activity
BPW	Buffer Peptone Water
CNS	Coagulase Negative *Staphylococcus*
CPS	Coagulase Positive *Staphylococcus*
CSA	Central Statically Authority
DNA	Deoxyribose Nucleic Acid
DVM	Doctorate of Veterinary Medicine
ELISA	Enzyme Linked Immune Sorbent Assay
ES ISO	Ethiopian Standard International Organization for Standardization
EU	European Union
FAO	Food Agriculture organization
FBD	Food Borne Disease
FDA	Food Drug Administration
FVM	Faculty of Veterinary Medicine
HACCP	Hazard Analysis Critical Control Point
ICMSF	International Commission on Microbial Safety for Food
IgG	Immuno globulin
IMI	Intra Mammary Infection

ISO	International Organization for Standardization
MBS	Milk bucket swab
MCCS	Milk collecting container swab
MGE	Multiple mobile genetic elements
MHS	Milkers hand swab
MRSA	Multi resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
OIE	Office International Des Epizooties
PAB	Purple agar base
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Ppm	pathogen per milliliter
RNA	Ribonucleic acid
RPLA	Reverse passive latex agglutination
Scc	Somatic cell count
SCC	Staphylococci cassette chromosome
SE	Staphylococci enterotoxin
SFP	Staphylococci food poising
Spp	Species
TSST	Toxic Shock Syndrome Toxin
USA	United State of America
WHO	World Health Organization

ABSTRACT

A cross-sectional study was undertaken from October 2010 to April 2011 in urban and periurban dairy farms in Dire Dawa areas to isolate *Staphylococcus* spp. from raw milk at potential points of contamination with the organism. In the present study, survey using interview methods using structured questionnaire was made. In addition, bacteriological investigation of *Staphylococcus* species and determination of their prevalence and distribution was carried out. In this study, it was observed that 20(83.3%) of the farms has good hygienic status of bedding area. In 95.8% of all the farms the udder and teat of lactating cows was washed before milking; 33.3% of dairy establishment use separate towel, while 8.3 % use common towel for washing more than one or all of the lactating cows in the farm. In addition, 87.5% cleaned their hands before milking, however, only 58.3% conducted cleaning between milking; 79.1% of the dairy farms washed milking utensils. In 45.83% of the farm detergent or soap were routinely practiced; dipping was practiced in 20.8% dairy farms; 45.8% were not aware of milk contamination and food-borne disease. A total of 827 samples were cultured for isolation and identification of *Staphylococcus* species and know possible route of contamination. About 384 raw milk samples from bovine udder milk. Bovine raw milk revealed 184 (47.91%) isolates of *Staphylococcus* species. There were grouped into 109(28.4%) isolates of CNS, 42(10.93%) isolates of *S. aureus*, 18(4.7%) isolates of *S. intermedius* and 15(3.9%) isolates of *S. hyicus*. A total of 384 lactating cow skin swabs (192 udder swab samples and 192 teat swab samples) were collected from selected farms. Result showed that 85(44.3%) on pooled teat swab sample and 72(37.5%) from udder swab positive isolates of *Staphylococcus* species. They were grouped in *S. aureus* with 26(13.5%) isolates, *S. intermedius* with 6(3.1%) isolates, *S. hyicus* with 1 (0.52%) isolates and 52(27.1%) isolates yielded CNS. Udder swab samples yielded, *S. aureus* with 13(6.8%) isolates, *S. intermedius* with 7(3.6%); *S. hyicus* with 2(1.04 %) isolates and CNS with 50(26.04%) were identified. In addition, *Staphylococcus* species were detected at rates of 15% CNS and 10% *S. aureus* on the milking buckets, and 20% CNS species and 15% *S. aureus*, on bulk milk collecting containers. Five (26.3%) milkers' hands skin swabs were carriers of coagulase-negative Staphylococci. In conclusion, proper hygienic measures should be practiced to control staphylococcal contamination of raw milk and the subsequent public health hazard.

Key words: Contamination, Milk, *Staphylococcus*

1. INTRODUCTION

Prevention of food hazards, the first part of the food chain, is essential for the protection of human health and for improving the quality of life in all countries. Food safety has been indicated to play a significant role in the national economic and health development by safe guarding the health of the nation, enhancing tourism, national and international trade, production, distribution and consumption of safe food, preventing avoidable loss and conserving natural resources (Anonymous, 2007).

Worldwide, millions of people suffer from communicable and non communicable disease caused by contaminated foods. Food or water contaminated with infectious organisms or toxins secreted by such organisms act as a source of food poisoning. Food-borne disease (FBD) is the major public health concern, worldwide (Pal, 2001). Bacteria are the leading cause of food-borne disease and appear to be the causative agents of more than two thirds of the recorded food-borne disease outbreaks. The pathogenesis of the bacteria causing foodborne poisoning depends on their capacity to produce toxin after ingestion (in the digestive tract) or before (toxins preformed in foodstuff) (Haeghebaert *et al.*, 2003).

This disease takes a heavy toll in human life and do suffer more and many fatalities occur, particularly among infants, children, elderly, immunocompromised and other susceptible persons. They also create an enormous social, cultural and economic burden on communities and their health system (Le loir *et al.*, 2003).

They are many organisms, which cause food-borne disease in human beings, among which *Salmonella* species, *Listeria monocytogens*, *Clostridium perfringens* and *Staphylococcus* species are of important. *S. aureus* Gram positive bacteria are commonly carried on the skin or in the nasal passages of healthy people and animals (Pal, 2001). A person (or animal) that carries the bacteria on their body but does not exhibit signs of disease is considered to be "colonized". Approximately, 25 to 40% of human population is colonized by *S. aureus*, mainly in the nasal passages (Choi *et al.*, 2006).

Staphylococcal food poisoning (SFP) is one of the most prevalent causes of gastroenteritis, due to ingestion of contaminated food with thermostable staphylococcal enterotoxins (SE). The hazard to public health is particularly linked to the ability of 50% of *S. aureus* strains to produce thermostable enterotoxins associated with food poisoning (Yves *et al.*, 2003). Although their contamination can be readily avoided by heating of milk, nevertheless, it remains the most common foodborne disease worldwide with high occurrence second to Salmonellosis (Aycicek *et al.*, 2005).

S. aureus is a major pathogen in both nosocomial (hospital-acquired) and community-acquired infections, due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. Clinical signs range from minor skin conditions (e.g., pimples, boils and impetigo) to more severe disease such as cellulitis and postoperative wound infections (Pal, 2007). It can also cause pneumonia, endocarditis, osteomyelitis, septicemia, meningitis, sepsis, and toxic shock syndrome (Pal, 2007). On the other hand, some infections, such as staphylococcal food poisoning, rely on one single type of virulence factor, the staphylococcal enterotoxins (SEs) (Larkin *et al.*, 2009).

Milk, which is a major constituent of the diet, is considered essential to the health and well being of the community (Prejit-Nanu and Latha, 2007). The components of milk and its physical and chemical properties provide a very favorable media for the multiplication of microorganisms. The presence of many growth factors within this aliment will satisfy many of demanding microbial species, which are so difficult to grow in a less complete media (Guiraud, 1998).

Milk may contain some varieties of microorganisms at the exit of the udder; thus, milk can also be contaminated at various levels, either at cow itself, at milking time (whether it be manual or automated), milking equipment, food handler and dirt or by the middle of the impure water (Hayes *et al.*, 2001; Chye *et al.*, 2004). Detection of coliform bacteria and other pathogens in milk indicate about the possibility of contamination of the milk via the udder, by the milking equipment or via the water used (Bonfoh *et al.*, 2003).

Milk and milk products are common vehicles for staphylococcal food poisoning. The organism can get access to raw milk and milk product either by direct excretion from the udders having clinical or subclinical staphylococcal mastitis or by contamination from food

handlers' (Yilma *et al.*, 2007; Salandra *et al.*, 2008). Contamination of milk may also occur due to fraudulent practice during milking, storage, transport and processing (Smith, 2007).

SFP is a common disease whose real incidence is probably underestimated for a number of reasons, which include misdiagnosis, lack of surveillance or unreported minor outbreaks, improper sample collection and improper laboratory examination (Le loir *et al.*, 2003). The control of this disease is of social and economic importance. In fact, it represents a considerable burden in terms of loss of working days and productivity, hospital expenses and economical losses in food industries and catering companies (Rosec and Gigaud 2002; Chiang *et al.*, 2008).

The safety of milk with respect to SFP is great concern around the world. This is especially true in developing countries like Ethiopia, where production of milk often takes place under poor unsanitary condition, inadequate refrigeration and heat processing, poor personal hygiene during food preparation and; consumption of raw milk which is typically produced in small dairy farm under unsatisfactory hygienic conditions is common practice (Wubete, 2004). In spite of the aforementioned prevailing situation and the presence of number of public health hazard due to consumption of raw milk and milk product in Ethiopia, however there is paucity of information and difficulty to estimate the amount of SFP cases, which is due to absence of surveillance system and specific concerned body to monitor and evaluate the incidence (Senait, 2007).

As to our comprehension, studies on the isolation and identification of *Staphylococcus* species in bovine raw milk and assessment of the possible contamination source in Dire Dawa urban dairy farms have not yet been conducted. Therefore, this study was designed to fulfill the following main objective:

- To establish the prevalence of *Staphylococcus* species in bovine udder milk and other possible contamination sources of bovine raw milk in urban and periurban dairy farms of Dire Dawa town.

2. LITERATURE REVIEW

2.1. Milk and milk products contamination

Milk is a complex biological fluid containing a wide variety of constituents and possessing unique physical and chemical properties (Huth *et al.*, 2006). It is considered as a complete food which contains proteins, fat, carbohydrates, minerals, vitamins and water (Prejit-Nanu and Latha, 2007). In addition, inclusion of these products in the diet aids in the prevention of certain diseases, such as obesity, hypertension and diabetes. Also it is rich source of calcium, which is important for growing bones and the prevention of osteoporosis (McCarron and Heaney 2004; Jayarao *et al.*, 2006; Le Jeune and Rajala-Schultz, 2009).

Health risk to consumers can be associated with milk, due to the presence of zoonotic pathogens and antimicrobial drug residues (Bradely, 2002; Pal, 2007). Spoilage of milk occurs when microorganisms or their enzymes degrade the carbohydrates, proteins, fats and produce noxious end products (Hayes *et al.*, 2001). Moreover, the rich nutrient composition and neutral pH make milk a good medium for the survival and growth of bacteria (Morgan *et al.*, 2001).

Raw milk of a good sanitary quality if allowed to stand under condition that permits bacterial growth will rapidly undergo a series of chemical changes. The principal change is lactose fermentation to lactic acid by acidic lactic organisms (Huth *et al.*, 2006).

Milking performed under hygienic conditions with strict attention to sanitary practices will reduce the entry of microorganisms into the milk (Chye *et al.*, 2004; Oliver *et al.*, 2009).

2.1.1. Sources of microbial contamination in Milk

Milk can be contaminated at any stage in production-to-consumption continuum. The milk secreted into an uninfected cow's udder is sterile (Oliver et al., 2009). Contamination of raw milk can be derived from the environment or the human hand, during and after milking and from the cow itself, due to the presence of udder or systemic infections (Bradely, 2002; Esron et al., 2005).

Contamination via the exterior of teats

The dairy farm environment is an important reservoir for many food-borne pathogens (Oliver *et al.*, 2005). The most common sources of microorganism in the farm environment are feed, feces, bedding material, soil, and producing animal body itself. Bacteria from these sources are transferred to milk in a number of steps (Vissers and Driehuis, 2007).

Producing animal body, its flank and tail hair of all animals harbors organisms and dust often fall from these areas into the milking pails can cause marked deterioration of raw milk (Esron *et al.*, 2005). Bacteria that live as commensal microflora on the teat skin or on the epithelial lining of the teat canal, the duct that conveys the milk from the mammary gland to the teat orifice can be a source of considerable contamination (Baazize, 2005). The first few streams of milk from each teat should be collected separately and discarded. This flushes out the organisms that have entered the teat through the teat opening (Wilson *et al.*, 1997).

Basically two overlapping routes or contamination pathways for bacteria can be distinguished. The first contamination pathway starts with feed. The presence of pathogen in milk depends on ingestion of contaminated feed such as silages followed by amplification in bovine hosts and fecal dissemination in the farm environment (Baazize, 2005). Contamination of bedding material is high due to absorption of feces and urine. During milking, feces, bedding material and microorganism on the surface of teats are transmitted to farm tank milk (Te Giffel, 2003). Teat cleaning prior to milking only partly reduces attached dirt and spores (Vissers and Driehuis, 2007).

The second contamination pathway starts with soil. Especially during grazing, soil can contaminate the exterior of teats and, as in the first pathway; bacteria originating from soil are transmitted to milk during milking (Wilson *et al.*, 1997). The mixture of feces, bedding material and/or soil attached to the teats is referred to as dirt. Dried dirt and filth is picked up by all movements and carried about as dust with organisms such as *Streptococcus*, *Staphylococcus* and *Rickettsia* in the atmosphere. For this reason, dust may be the source of almost every kind of contamination (Bonfoh *et al.*, 2003).

Contamination via the interior of teats:

Contamination via the interior of teats is associated with mastitis. Mastitis is one of the most complex diseases of dairy cows, that are generally involves interplay between management practices and infectious agents, having different causes, degrees of intensity, variations in duration, residual effects and responses between animals (Wallenberg *et al.*, 2002; Dohoo *et al.*, 2003).

Mastitis is defined as inflammation of the mammary gland, clinical (grossly evident changes to milk, the gland or the whole animal) or as subclinical (diagnosed using ancillary tests such as the somatic cell count), in response to tissue injury for the purpose of destroying or neutralizing intramammary bacterial infection (IMI) and to prepare the way for healing and return to normal function (Compton *et al.*, 2007; McDougall *et al.*, 2007).

Among the several causes of mastitis, microbial infection is important; usually bacteria, that invade the udder, multiply in the milk-producing tissues, and produce toxins that are cause immediate injury. Milk from a cow with an infected udder is likely to contain a large number of bacteria of the genera *Staphylococcus*, *Streptococcus*, *Bacillus*, *Micrococcus*, and *Corynebacterium* and, occasionally, coliforms colonize this location (Le Jeune and Rajala-Schultz, 2009). These pathogens are mostly found either in the udder (contagious pathogens) or the cow's surroundings (environmental pathogens), such as bedding, manure, and soil (McDougall *et al.*, 2007).

Contagious mastitis pathogens (*S. aureus* and *Str. agalactiae*) were the most commonly isolated bacteria (Tenhagen *et al.*, 2009). The primary sites of these bacteria are the milk of infected quarters and therefore, they are spread from infected udders to clean udders during the milking process through, milkers' hands, cloth towels used to wash or dry more than one cow, contaminated teat cup liners and possibly by flies (Roberson *et al.*, 1994).

Recently published work has shown that 3 percent of all animals are infected with *S. aureus* (Schukken *et al.*, 2009). *S. aureus* causes one of the most common types of chronic mastitis (Carter and Kerr, 2003). Infection is usually subclinical, causing elevated somatic cell counts (SCC) but no detectable changes in milk or the udder (Piepers *et al.*, 2007). The bacteria persist in mammary glands, teat canals, and teat lesions of infected cows and are contagious.

Once established, *S. aureus* infections do not respond well to antibiotic therapy and infected cows must be segregated or culled from the herd (Tenhagen *et al.*, 2009).

This type of mastitis is an important threat affecting the world's dairy industry, it impairs alveolar function and reduces milk yield, and has a deleterious effect on milk composition due to increased milk somatic cell counts (Scc) and antibiotic residue in milk, also it have direct economic cost due to higher culling rate and occasional fatalities and veterinary and antibiotic costs (Ruegg, 2003). Further public health concern includes the potential for spread of antibiotic resistant *S. aureus* from cattle to human populations via milk (Juhasz-Kaszanyitzky *et al.*, 2007; Tenhagen *et al.*, 2009).

Contamination via surfaces of the milking equipment

High rate of milk contamination by exogenous sources (milking utensils and equipment), occurs when microorganisms and milk residues adhered to surfaces in the milking equipment (such as pails, strainers, milking machines, cans, pipes, bottles, and other equipment used for handling) are not removed completely during cleaning (McKinnon *et al.*, 1990).

In general, *S. aureus* are more resistant to cleaning procedures and hardly the organism resists desiccation (Saran, 1995). During milking adhered microorganisms are released into passing milk and increase the microbial contamination of tank milk. Especially cracked and decayed rubber parts are sensitive to accumulation of microorganisms (Te Giffel, 2003).

Low total bacteria counts of raw milk may reflect a good sanitation practices applied during milking, good hygienic environment in milking premises, dairy cow's health and hygiene and method of cleaning and sanitation of milking machine and storage technique applied. The essential requirements are, to use milking equipment with smooth milk contact surfaces with minimal joints and crevices, an uncontaminated water supply, detergents to remove deposits and milk residues and method of disinfection to kill bacteria (Vissers and Driehuis, 2007).

2.2. Staphylococci species

2.2.1. Microbiology, pathogenesis and epidemiology

Taxonomically, the genus *Staphylococcus* is in the Bacterial family *Staphylococcaceae*, which includes three lesser known genera, *Gamella, Macrococcus* and *Salinicoccus*. Over 30 species of *Staphylococcus* are known, the most pathogenic species for humans is *S. aureus* which can be differentiated from other *Staphylococcus* species colonising humans (e.g. *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *S. capitis*) in the diagnostic laboratory by a positive coagulase reaction (Kenneth, 2004).

Bacteria of the genus *Staphylococcus* are Gram-positive cocci that are microscopically observed as individual organisms, in pairs, and in irregular grapelike clusters, which is about 1 µm in diameter. The term *Staphylococcus* is derived from the Greek term *staphyle*, meaning "a bunch of grapes" (Orwin *et al.*, 2001; Ryan and Ray, 2004). Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation of glucose, mannose, galactose, maltose, inositol, xylose, dulcitol and mannitol sugars that yields principally lactic acid, but do not utilized creatinin (Khan and Rind, 2001). *S. aureus* is non-motile, non-spore forming, non-capsulated, oxidase-negative, catalase and coagulase positive; they usually reduce nitrate to nitrite (Pal, 2007; Leonard and Markey, 2008). The biochemical characteristics of *Staphylococcus* species are summarized in (Table 1).

Colonies characteristic of most strains of *Staphylococcus* are pigmented, ranging from cream-yellow to orange color, smooth, translucent, slightly raised, glistening, circular and entire margin. *S. aureus* forms a fairly large colony usually attain 4-6 µm in diameter. It produced β haemolysis on 5% sheep blood agar. However, many strains may appear dirty white and nonhemolytic (Stewart, 2005). It also gives a positive mannitol fermentation and deoxyribonuclease test. It produced uniform turbidity in nutrient broth, whereas it did not grow on MacConkey's agar medium (Kluytmans, and Wertheim, 2005).

The major criterion for identification is the organism's ability to clot plasma. There are three coagulase-positive *Staphylococcus* species: *S. aureus* in humans and animals, and *S. intermedius*, and *S. hyicus* in animals. The presence of the enzyme coagulase separates the virulent pathogen, *S. aureus*, from the less virulent coagulase-negative staphylococci species (Matthews *et al.*, 1997).

S. aureus strains display a selective affinity for particular animal species strains can be classified into biotypes according to their human or animal origin. Biotype schema, includes six different biotypes (human, non-β-hemolytic human, avian, bovine, ovine, and nonspecific), based on biochemical characteristics (Quinn *et al.*, 2002).

Table 1: The main differentiating characteristics of the gram-positive cocci

Species	Coagulase	Catalase	Oxidase	O-F test
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.*, (2002)

S. aureus consists of cytosol, a single cytoplasmic membrane, and the surrounding cell wall. The cell wall of *S. aureus* is resistant to lysozyme and sensitive to lysostaphin, which is mainly peptidoglycan, composed of repeating disaccharide N-acetylglucosamine-N-acetylmuramic acid (GlcNAc-MurNAc) units with attached teichoic acids (Navarre and Schneewind, 1999). The main function of peptidoglycan is to provide a rigid envelope for the cell content and increased resistance to physical and chemical stress (Larkin *et al.*, 2009). In addition it has endotoxic properties (Matthews *et al.*, 1997; Holtfreter and Broker, 2005).

Staphylococcus is ubiquitous in nature living alongside humans and animals as an opportunistic pathogen as well as on most food surfaces, it can be found as commensals on the skin, mucous membranes of the upper respiratory tract (Pal, 2007) and lower urogenital tract and as transients in the digestive tract of all warm-blooded animals (Salandra *et al.*, 2008). Bacteriological culture of anterior nares and skin of normal individuals invariably yields Staphylococci (Choi *et al.*, 2006). Up to 20% of healthy humans persistently carry *S. aureus* in their nose, with no symptoms, and are considered to be colonised, and another 60% are intermittent carriers with no symptoms (Peacock, *et al.*, 2001; Choi *et al.*, 2006).

Staphylococcus species is intrinsically, physically and chemically robust; it can be survive in a variety of environments and desiccation (drying). It is able to grow in a wide range of temperatures (7° to 48.5°C with an optimum of 30 to 37°C, pH (4.2 to 9.3, with an optimum of 7 to 7.5) and sodium chloride concentrations (up to 15% NaCl) (Bergdoll, 1989; Schmitt *et al.*, 1990). Resistance to heat and desiccation is dependent upon surface and surrounding matrices. *S. aureus* suspended in 0.9% NaCl is rapidly inactivated at 46°C, however, when protected by proteins (such as in milk or in pus) it can survive for more than 50 min at 60°C (Clements, and Foster, 1999). These characteristics plus their ecological niche can easily explain their incidence in foodstuffs that require manipulation during processing, including fermented food products (Kenneth, 2004).

S. aureus can acquire genes conferring resistance to specific classes of disinfectants such as cationic substances (e.g. quaternary amines, triclosan) by an efflux mechanism. However, the recommended application concentrations of the corresponding disinfectants, overcomes this resistance under ideal conditions (Russell, 2002).

S. aureus growth and toxin production would be powerful tools for microbial risk assessment in food industries. Up to now, in most countries, microbial risk assessment for *S. aureus* relies on the identification and the quantification of coagulase positive isolates in end products. As *S. aureus* is widely spread in raw ingredients and since low contamination levels do not induce FBD, the microbial norms in most countries tolerate *S. aureus* contamination (Le loir *et al.*, 2003).

S. aureus is one of the commonest and most important Gram-positive hospital-acquired organisms, which are transmitted through aerosol or direct contact with fomites, infected animals, or infected people (Pal, 2007). Moreover, it is an extremely versatile pathogen that causes superficial lesions (boils, furonculosis), deep-seated and systemic infections (endocarditis, meningitis, osteomyelitis, pneumonia) (Pal, 2007), and toxemic syndromes (Kluytmans, and Wertheim, 2005).

2.2.2. Requirement for growth and toxin production

S. aureus is considered an exigent bacterium in terms of nutritional requirements. Valine is necessary for growth and arginine and cystine are necessary for both growth and toxin production in five strains of *S. aureus* that produce SEA, SEB or SEC. The necessities for

other amino acids vary with the strains (Onoue and Mori, 1997). Glucose has been shown to have an inhibitory effect on SE production. This inhibitory effect has been attributed to a drop in pH, as a consequence of glucose metabolism (Bergdoll, 1989; Schneewind *et al.*, 1995).

SE production is optimal in neutral pH and decreases in acidic pH. Usually, SE production is inhibited in pH below 5. At a given pH, substances used to acidify the medium may have more or less effects. High concentrations of sodium chloride increase the inhibitory effect of acidic pH, with no SE production at salt concentrations above 12%, independent of the pH (Notermans and Heuvelman, 1983). On the other hand, alkaline pH also decreases the production of entrotoxin via decreased expression of *agr* (Regassa and Betley, 1992).

S. aureus is quite sensitive to microbial competition. This feature has been particularly well studied in fermented food products. Genigeorgis (1989) demonstrated that the higher the concentration of competing microorganisms in milk, the lower the rate of *S. aureus* growth and SE production. Competition with lactic acid bacteria has been reported in other research on cheese (Vernozy-Rozand *et al.*, 1998) and fermented sausage production (Sameshima *et al.*, 1998). The effect of lactic acid starter is mainly due to lactic acid production, lowered pH, oxygen peroxide production, competition for nutrients and due to the synthesis of antimicrobial substances, such as bacteriocins (Maria-Angeles *et al.*, 2010).

2.2.3. Virulence factors

S. aureus is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. This bacterium is a significant cause of nosocomial infections, as well as community-acquired diseases (Larkin *et al.*, 2009). The spectrum of staphylococcal infections ranges from pimples and furuncles to toxic shock syndrome and sepsis, most of which depend on numerous virulence factors (Stewart, 2005).

On the other hand, the most common types of some infections, such as Staphylococcal food poisoning, rely on one single type of virulence factor, the SEs. SEs belongs to a family of the so-called pyrogenic toxins which include SEs, TSST, Exfoliatins A and B and *Streptococcus* pyrogenic toxins originating from *Staphylococcus* and *Streptococcus* species (Balaban and Rasooly, 2000).

The *S. aureus* genome can also carry multiple mobile genetic elements (MGE) that encode a wide range of virulence and resistance determinants which are capable of horizontal transmission between strains (Lindsay, and Holden, 2004). *S. aureus* strains have been shown to express several virulence factors, such as lipases, proteases, coagulase, haemolysins and DNase, which could contribute to virulence, even if the role of some enzymes on the pathogenesis still remains unclear (Larkin *et al.*, 2009).

Prospective virulence factors including toxins of *S. aureus* and their pathogenic effects according to (Jay, 2000; Kenneth, 2004) includes:

- Surface proteins which binds Fc portion of IgG and inhibits opsonization that promote colonization of host tissues (Protein A).
- Invasins and cytolytic destruction of phagocytes that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase);
- Biochemical properties that enhance their survival in phagocytes (carotenoids, catalase)
- Coagulase production that promote conversion of fibrinogen to fibrin. Fibrin deposition may shield Staphylococci from phagocytic cells
- Immunological disguises (Protein A, coagulase);
- Membrane-damaging toxins that lyse eucaryotic cell membranes (hemolysins, leukotoxin)
- Inherent and acquired resistance to antimicrobial agents.

2.3. Control strategies of milk contamination

Food producers are responsible for the safety and quality of their products (European Commission, 2000). The quality, safety and economic performance of milk and dairy products depend on the quality of raw milk, processing, distribution and storage conditions (Codex Alimentarius, 2004). A chain management approach is required to ensure safe and high-quality dairy products. In the dairy chain, dairy farms have an important role. Amongst other factors, the safety and shelf life of dairy products is determined by the contamination of farm milk with microorganisms (De Buyser *et al.*, 2001).

To produce safe foods, the hazard analysis critical control points (HACCP) approach is implemented throughout the dairy chain (Jadhav and Pal, 2001). HACCP is a science-based quality-management system developed to ensure the safety of foods (Codex Alimentarius, 2004). As an alternative to HACCP, codes for good farming practices have been developed (Morgan, 2004). Good animal health and hygienic conditions on 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 raw food products leaving the farms for consumption (Ruegg, 2003).

In order to define an effective strategy for controlling the contamination of milk by microorganisms it is essential to know of the microbial sources and routes of contamination. Development of pre-harvest (pre-milking), harvest, and post-harvest control measures to effectively reduce contamination is critical control of human pathogens in raw milk (Bradley *et al.*, 2007).

2.3.1 Pre-harvest control

Exclusion of milk for supply from diseased cows

Milk for processing is only to be harvested from healthy animals (De Buyser *et al.*, 2001). These require the identification, isolation and withholding of milk from supply of animals with clinical diseases communicable to humans, animals suffering severe weight loss, severe injury or fever, or withholding of milk for supply from mammary glands of cows that are inflamed or injured until healed or the clinical signs have resolved. It also requires adequate record-keeping by the farm dairy operator and monitoring of the farm and animal health by a veterinarian (Anonymous, 2006).

Enhanced animal health and Vaccination programmes

Management programmes including vaccination against infections disease supervised by a registered veterinarian, should be a condition of supply of milk for processing into raw-milk products. These programmes should cover all replacement stock, milking cows and service bulls. Correct use of these vaccines combined with other risk management procedures should eliminate the risk of milk contamination by many organisms (Rasmussen *et al.*, 1991).

Farm hygiene control

Animals should be kept clean by maintaining hygiene of their environment. This includes ensuring that where animals are housed for lying down, there is sufficient area for each cow and the bedding material is kept clean and dry. Concrete races, holding and feeding areas should be free of accumulations of dung and slurry, and paddocks, tracks and gateways should be well maintained and free of accumulations of mud, dung and slurry (Dairy Hygiene Inspectorate, 2006). Irrigation of farm dairy effluent onto pastures presents a risk for infection of grazing cattle or contamination of udders and teats (Wang *et al.*, 2004). Sufficient farm area needs to be available for effluent irrigation, to allow a minimum paddock "withholding period", thus allowing pathogen numbers on grazed areas to naturally reduce.

Raw milk supplier and farmers can be aware and aquire extra training for maintaining high farm hygiene standards. Monitoring tools, including an assessment of farm and animal hygiene, need to be expanded for harvesters of milk intended for processing into raw milk products (Anonymous, 2006).

Dietary management

This requires that producers must not offer cows any feed that will contaminate milk with residues, contaminants or taints. Processors may also require producers to cease feeding any material to cows that may contaminate milk with toxins, residues or any other harmful substance, including feed grown on land where human waste has been applied. Fermented feeds (including silages) are offered to stock supplying milk for production of raw milk products should be of an acceptable quality (Pahlow *et al.*, 2003; Vissers and Driehuis 2007). A feed testing regimen must be implemented to test pH (as an indicator of safety) prior to opening, and during the use, of stacks or bales of these feeds. Feed with pH >5.0 should not be fed to animals supplying milk for raw milk products (Cooper and Walker 1998).

Mastitis management

Dry cow therapy is an important part in control of all, but particularly infectious pathogens associated with mastitis, such as *S. aureus* and *Strep. agalactiae* (Bradley *et al.*, 2007). The dairy farm operators supplying milk for raw-milk products must have in place a programme or procedures for the management of mastitis (Jorgensen *et al.*, 2005).

2.3.2. Harvest control

Examination of foremilk prior to milking

This practice, commonly known as "foremilk stripping", is used to quickly identify cows with clinical mastitis, or other abnormalities e.g. blood in the milk, to prevent such milk entering the bulk tank and human food supply. Cows with clinical mastitis shed vast numbers of bacteria in the milk from affected glands, and hence pose a serious risk for contamination of tank milk if not identified prior to milking (Hassan *et al.*, 2001). Removal of milk from the streak canal before cup attachment may also serve the purpose of flushing out many potential human pathogens present there (Hassan *et al.*, 2001).

Pre and Post-milking teat disinfection

This is a key component for control of environmental, fecal- and urine-associated human pathogens on teats. Faeces, bedding and soil matter from teats, udders and tails are important sources of milk contamination (Anonymous, 2006). Effective premilking teat disinfection both reduces new intramammary infections (Oliver *et al.*, 1994) and improves the microbiological quality of raw milk (Rasmussen *et al.*, 1991; Hassan *et al.*, 2001). Summarizing a number of studies, was concluded that pre-milking antiseptic teat dipping with manual drying was a key component to control contagious mastitis pathogens (*S. aureus and Strep. agalactiae*) (Magnusson *et al.*, 2006; McDougall *et al.*, 2008). Further, only teats, rather than the udder, should be routinely cleaned either by water or an effective disinfectant, followed by drying with single-use paper towels, which additionally removes disinfectant residue (McKinnon *et al.*, 1990; McDougall *et al.*, 2008).

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 (Ruegg, 2003). Dairy product food safety, however, can be enhanced by implementing excellent milking management, covers all aspects of milking cows quickly and effectively, while assuring the health and hygiene of the cows, quality of the milk, hygienic standards for milking centers, through uniform adoption of milking practices (Vilar *et al.*, 2007).

Hygiene of staff and equipment at milking is an important control point for reducing contamination of raw milk from faecal-and urine-associated pathogens (Anonymous 2006), and from human-associated pathogens. All persons involved in the milking process must be in good health and must be careful in their personal cleanliness. Each person must always carry a clean handkerchief and use it to prevent the spraying of nasal and oral discharges into the atmosphere, equipment or products (Brightling *et al.*, 2000).

The hands and forearms of milking staff should be cleaned before milking starts and kept clean during milking and milk handling. Exposed skin wounds should be hygienically covered. Best milking practice includes use of gloves by milking staff, especially during the search for and treatment of cow's with clinical mastitis (Brightling *et al.*, 2000; Te Giffel, 2003; Jorgensen *et al.*, 2005).

Farm dairy water quality

Maintaining continuous potable water supplies in dairy processing operations is important for ensuring good milk plant hygiene, for use in pre-milking teat cleaning and for preparation of teat disinfectants. The microbiological quality of water is tested before it is used in a dairy plant and use must be either an approved, piped supply or chlorinated (50 ppm) water. Water from surface supplies is contaminated by dust, animals, plants, people, and other agents could be additional source of contamination (Bonfoh *et al.*, 2003). Presumptive coliform test is the most common test, to reveals fecal or sewage contamination or the absence of *E. coli* in a 100 ml sample.

2.3.3. Post-harvest control

Milking plant hygiene

This is an important step for control of multiplication of all pathogens in milk transport and storage areas of the plant. Milk must be protected from contamination during transfer and storage. Bulk milk tanks should be cleaned and disinfected after each milk collection and kept in good condition. A potential source of bacterial contamination of raw milk is failure to adequately clean and disinfect milking equipment and bulk milk tanks (Anonymous, 2006).

Milk filtering and cooling

Milk must be adequately filtered to meet requirements for removal of sediment and foreign matter. Adequate chilling of raw milk is also required as a key component for quality of milk for processing, as well as control of multiplication of human pathogens in tank milk. Time since milk harvesting and temperature standards are also set out and processors may reject milk from collection if these standards have not been met (Anonymous, 2006). These standards include pre-cooling of milk to 18°C and cooling of the milk in the vat to 7°C within 3 hours of collection.

Pasteurization

Pasteurization is the process of heat treatment for a predetermined time at a predetermined temperature to destroy pathogenic organisms that might be in milk, without adversely affecting the food's flavor and colour. It improves the safety and lengthens the shelf life of a product by destroying pathogenic organisms; however, it is not the same as sterilization. It become the cornerstone of milk safety, because of challenges related to preharvest eradication of pathogens and the ineffectiveness of environmental hygiene screening to adequately control microbial risks in milk (Holsinger *et al.*, 1997).

It was originally developed for the control of infection due to brucellosis and tuberculosis from infected cattle, but also controls a wide range of other human pathogens. However, consumption of raw milk or its products is common or traditional in many countries despite public health risks (Le Jeune and Rajala-Schultz, 2009).

2.4. Staphylococcal food poisoning

Food-borne diseases (FBD) are defined as "diseases of infectious or toxic nature caused by, or thought to be caused by the consumption of food or water" and are major concern worldwide (Haeghebaert *et al.*, 2003). To date, around 250 different food-borne diseases have been described, and bacteria are the causative agents of two thirds of food-borne disease outbreaks (María-Ángeles *et al.*, 2010).

Among the predominant bacteria involved in these diseases, *S. aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food (Yves *et al.*, 2003; Jorgensen *et al.*, 2005). Human intoxication is caused by ingesting enterotoxins produced in food by some strains of *S. aureus*, usually because the food has not been kept hot enough (60°C, 140°F, or above) or cold enough (7.2°C, 45°F, or below) (Jablonski and Bohach 1999; Dinges, *et al.*, 2000).

2.4.1. Enterotoxigenic potential of other *Staphylococcus*

Several Staphylococcal species other than *S. aureus* reportedly produce SEs. Among coagulase negative species *S. cohnii*, *S. epidermis*, *S. xylosus* and *S. haemolyticus* have been isolated from ewe's milk and were found to produce one or several SEs (Le loir *et al.*, 2003). The coagulase positive *S. intermedius* is the predominant non-*S. aureus* species that has been clearly involved in staphylococcal food poisoning outbreaks; some strains have been shown to produce SEs (Pal, 2001).

2.4.2. *Staphylococcus* aureus enterotoxins

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 (Pal, 2001; Derzelle, *et al.*, 2009). SEs is short proteins secreted in the medium, which is soluble in water and saline solutions. 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 such as pepsin or trypsin, hence retaining their activity in the digestive tract after ingestion (Thomas *et al.*, 2006). They are rich in lysine, aspartic acid, glutamic acid, and tyrosine residues. Although several staphylococci can produce SEs, the majority of SFP cases are attributed to *S. aureus* (Balaban and Rasooly, 2000).

The abundance of literature on SEs varies considerably among the types, according to the chronology of their identification and their importance in SFP (Rosec and Gigaud 2002). To date, *S. aureus* produces a wide variety of toxins including 14 different *staphylococcal* enterotoxins (SEs; SEA to SEE, SEG to SEI, SER to SET) with demonstrated emetic activity, and staphylococcal-like (SEI) proteins, which are not emetic in a primate model (SEIL and SEIQ) or have yet to be tested (SEIJ, SEIK, SEIM to SEIP, SEIU, SEIU2 and SEIV). SEs and

SEIs have been traditionally subdivided into classical (SEA to SEE) and new (SEG to SEIU2) types. All possess superantigenic activity and are encoded by accessory genetic elements, including plasmids, prophages, pathogenicity islands, vSa genomic islands, or by genes located next to the staphylococcal cassette chromosome (SCC) (Larkin *et al.*, 2009).

In contrast to *S. aureus*, SEs is remarkably heat resistant (D-values of 3-8 min at 121°C). They may be present in foods than in a laboratory culture medium (Bergdoll, 1989), but can be inactivated by heat treatments used in the sterilization of canned foods when they are present at low concentrations (Dinges *et al.* 2000).

In recent decades, the spread of antibiotic resistance in bacteria, including staphylococci, is increasing and may represent a hazard for human health. Among antibiotic-resistant staphylococci, multidrug-resistant *S. aureus* strains (MRSA) are of great public concern as resistances make more difficult the treatment of infections (Huys *et al.* 2005; Maria-Angeles *et al.*, 2010).

SEA 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. Also, SEC and SED are usually identified in SFP associated with the consumption of dairy products in many countries (Maria-Angeles *et al.*, 2010).

2.4.3. Food incriminated in SEP

The origins of Staphylococcal food poisoning differ widely among countries; this may be due to differences in the consumption and food habits in each of the countries (Le loir *et al.*, 2003). High-protein products that require considerable handling during preparation and are kept at slightly elevated temperatures after preparation are frequently involved in SFP. Many different foods can be a good growth medium for *S. aureus*, and have been frequently incriminated in staphylococcal intoxication include meat and meat products, poultry and egg products, salads, bakery products, particularly cream-filled pastries and cakes and sandwich fillings (Wienke, *et al.*, 1993; Tamarapu, *et al.*, 2001). Salted food products, such as ham, have also been implicated, according to the capacity of *S. aureus* to grow at relatively low water activity a_w (Miller, 2000).

Food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination, via manual contact or through respiratory

severe, particularly in infants, elderly or debilitated people, characterized by headache, muscle cramping, and transient changes in blood pressure and pulse rate (Yves *et al.*, 2003).

2.4.5. Public health significance of Staphylococcal food poisoning

Food-borne disease are extremely costly, Health experts estimate that the annual cost of food-borne disease in united state is about 5 to 6 million dollars in direct expense and loss of productivity. People infected with food borne microbes may have no symptoms or developed symptoms ranging from intestinal discomfort to bloody diarrhea. Food-borne illnesses not only affect the health of individual who become ill, but it can also have dramatic economic impact to eating establishment (Maria-Angeles *et al.*, 2010).

In developing countries like Ethiopia where surveillance system for food borne disease is not existence and it is difficult to estimate the problem. Even in countries where surveillance systems are very efficient, the precise incidence of food poisoning is not known, as outbreaks are often not reported to public health authorities (Jay, 2000).

2.4.6. Diagnosis of Staphylococcal food poisoning

The most conclusive test in diagnosis of SFP is 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), so it is based on proper interviews of the victims and gathering and analyzing epidemiological data is essential (ICMSF, 1996).

The presence of enterotoxin together with relatively large numbers of enterotoxigenic staphylococci in the clinical specimen is good circumstantial evidence that the food contains toxin (Pal, 2001), although this clinical sample is only very rarely available for analysis. In addition to the presence of enterotoxin, there are usually $>10^6$/g of an enterotoxin producing *S. aureus* present in implicated food (María-Ángeles *et al.*, 2010).

Currently available methods for detection and identification bacteria in food samples are conventional bacteriological methods, which is compared and validate. For food and environmental samples, the use of selective media and suitable growth condition are provide and other enrichment methods are employed to limit the growth of contaminating microorganisms to reasonable number and multiplication of foodborne bacteria to the level that are enough for their detection (Mackay 2007).

The *Staphylococcus* organism usually identified on the basis of colonial appearance, haemolytic pattern, biochemical profiles (catalase test, coagulase (fibrin clot formation, positive for *S. aureus*), DNAse (zone of clearance on nutrient agar), lipase (a yellow color and rancid odor smell), and phosphatase (a pink color) tests) and ribosomal RNA gene restriction patterns (Quinn *et al.*, 2002).

Methods of detecting Staphylococci enterotoxin is must be sufficient sensitive to recognize the protein at the level of capable of causing SFP. Detection of trace amounts of staphylococcal enterotoxin in foods incriminated in food poisoning, before identification by specific precipitation with antiserum (antienterotoxin), first the toxin must be separated from food constituents and concentrated (Chiang *et al.*, 2008).

Two principles are used for the purpose of detecting small amounts of enterotoxin in food according to (Chiang *et al.*, 2008), include: The selective adsorption of the enterotoxin from an extract of the food onto ion exchange·resins and the use of physical and chemical procedures for the selective removal of food constituents from the extract, leaving the enterotoxin(s) in solution.

Recent genetic advances have enabled reliable and rapid techniques for detection of SE in epidemiological implicated food, or the enterotoxigenicity of isolates, may be demonstrated immunological based on monoclonal antibodies by application of a number of serological methods such as immunoassays (e.g. reverse passive latex agglutination), nucleic acid based test such as polymerase chain reaction, restriction fragment length polymorphism (Chiang *et al.*, 2008; Francois and Schrenzel 2008).

Recently real-time PCR is increasingly employed in clinical laboratories as a rapid technique for the identification and characterization of clinical isolates of *S. aureus* (Mackay 2007; Francois and Schrenzel, 2008).

Several enzyme-linked immunosorbent assay (ELISA) methods have been proposed for the identification of SE and are currently the most commonly used methods. Among them noncompetitive, double antibody sandwich ELISA, appears to be the most popular for routine toxin identification (Carmo *et al.*, 2003).

Phage typing may also be useful when viable *staphylococci* can be isolated from the incriminated food, from victims, and from suspected carrier such as food handlers (Bennet and Monday, 2003).

2.4.7. Prevention of *Staphylococcus* food poisoning

It is important to prevent contamination of food with *Staphylococcus* before the toxin is produced. The primary control measure for *Staphylococcus* food poisoning formulation of product to prevent growth of the organism, further toxin production, hence maintaining time and temperature, pH and water activity and anaerobic conditions (lack of oxygen) restricts the growth and toxin production by *S. aureus* (Pal, 2001).

S. aureus can indeed be easily eliminated from foodstuffs by heat treatment (in pasteurized foods) or by competition with other flora (in fermented foods), whereas SEs can remain biologically active after most of the treatments used during food processing. Because of the stability of SE cooking and other processes, the toxins can be present in food in the absence of viable organisms (Le loir *et al.*, 2003). Pasteurization killed the staphylococci but had no effect on the SEs. These, and many other examples, illustrate the importance of the elimination of any contamination sources during the processing and refrigeration of food and food ingredients whenever possible (Le Jeune and Rajala-Schultz, 2009).

Successful method of prevention SFP aimed at elimination of contamination through good hygiene practice, especially by food handlers, person who have a nose or eye infection or purulent infections on your hands or wrists do not prepare food until proper medical eradication therapy. Also the use of disposable aprons and gloves by staff reduces skin-to-skin contact and, therefore, further reduces the risk of transmission (Pal, 2007).

Optimal storage condition and food preparation can prevent SFP. Store cooked food in a wide, shallow container and refrigerate as soon as possible. Keep kitchens and food-serving areas clean and sanitized. Quaternary ammonium can be used in conjunction with ethanol to increase the duration of the sanitizing action (Pal, 2007).

Comprehensive education program for the consumer and food handler, both in commercial establishment, about the origin and personal and environmental hygiene is of paramount importance

3. MATERIALS AND METHODS

3.1. Study area

The study was carried out in urban dairy farms found in and around Dire Dawa. Dire Dawa is one of the two chartered urban settlement of Federal Democratic Republic of Ethiopia. It is located in eastern part of Ethiopia, which is about 515 Km far from Addis Ababa, which lies 950 – 1250 meter above sea level. Dire Dawa is second largest administrative city covering with an estimated area of 1,213.20 square kilometers (CSA, 2005).

The climatic condition of Dire Dawa seems to be greatly influenced by its topography, which is characterized by warm and dry climate with a relatively low level of precipitation. The mean annual temperature of Dire Dawa is about 25.4^{0}C. The average maximum and minimum temperature is 31.4^{0}C and 18.2^{0}C respectively. The region has two rain seasons; that is, a small rain season from March to April, and a big rain season that extends from August to September. The aggregate average annual rainfall that the region gets from these two seasons is about 604 mm. In the area floods are fairly common during the major rainy season occurring between June and September (CSA, 2005).

The livestock population of the area in 2005 estimated to be 40,400 head of cattle, 46,280 sheep, 118,770 goats, 5,070 camels (CSA, 2005). 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 (CSA, 2005).

3.2. Study population

The study population represented lactating exotic cross breed cows, dairy personnel and milking utensil (milking bucket and milk collecting containers). The samples consists of bovine raw milk and lactating teat and udder skin swab, milker's hand skin swab, milking bucket and milk collecting containers swabs from the selected 24 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).

3.3. Study design

A cross-sectional study was conducted from October 2010 to April 2011. Sampling was carried out usually twice a week in selected 24 urban dairy farms involving milking cows, dairy personnel and milking equipments. On each sampling day, all the required samples were collected.

3.4. Questionnaire survey

Questionnaire was also designed and used to collect information from dairy cow owners and milking personnel of selected farms in one visit interview after collection of the required sample. The personal observation and information collected included the hygienic status of the barn and pre-milking procedures practiced in dairy farms.

3.5. 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 50 % in bovine raw milk of dairy cows and taking a confidence interval of 95% and 5% absolute precision, the minimum sample size required for this study was 384 lactating cows. A total of 768 samples comprising of 384 udder milk and 384 lactating cow skin swab samples (192 teats and 192 udders) were used for the study. Concerning teat swab sampling, one pooled swab sample involving all teats of each cow was collected. 19, 20 and 20 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	384
Skin swab	384
-Lactating cow teat	192
-Lactating cow udder	192
MHS	19
MBS	20
MCCS	20
Total	827

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

3.6. Sampling techniques

The town comprises two woredas; from the two woredas, the town woreda which consists of 25 kebeles, out of 25 kebeles 3 kebele namely; sabeyan, number one and addis ketema were purposively selected for this study based on the abundance of dairy farm and milk production level. Sabian, number one and addis ketema have 19, 14 and 8 dairy farms, respectively. After taking the list of farms from selected kebeles, all large sized farms and 20% of randomly selected medium and small sized farms of each kebele were selected also by simple random sampling technique. A total of 24 dairy farms were included in the study. A multistage cluster sampling technique was used to collect samples from lactating cows.

Milking practice was done twice a day in all dairy farms: 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 dairy farm owners and their employee were informed as milk samples would be collected, and so as 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.

Milk samples were taken after collection of swab samples from teat apices and udder skin of lactating cows. Before collecting milk samples, the udder and the teats were washed thoroughly and teats were rubbed with cotton wool soaked in 70% ethyl alcohol, paying particular attention to the teat orifices. After the first couple of squirt milk were discarded, about 10 ml of milk sample was collected by using sterile universal bottles from each lactating dairy cow according to the protocol described by Quinn *et al.* (2002).

All samples were collected aseptically using disposable gloves to avoid contamination; the sample were 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.

Samples were kept in icebox containing icepack and taken immediately to Dire Dawa Diagnostic and Investigation Veterinary Microbiology Laboratory, which is usually ranging between ½ an hour to one hour. Upon arrived the sample were stored overnight in a refrigerator at 4^0C.

The samples were processed on the next day of collection for primary isolation and identification of staphylococci species. After primary isolation and identification of staphylococci species, presumptive colonies of staphylococci were transported using nutrient broth to School of Veterinary Medicine, Addis Ababa University, Debre Zeit for secondary identification of staphylococci species.

3.7. Isolation and identification of *Staphylococcus*

Staphylococci were isolated and identified according to standard techniques (ES ISO 6888-2, 2002; Quinn *et al.*, 2002). Samples were processed and analyzed separately using the following procedure.

3.7.1. Primary identification of *Staphylococcus* species

Colony appearance

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 37^{0}C for 24 – 48 hrs. The bacteriological media used were prepared according to the manufacturer's recommendations (Annex IV). The representative colonies of *Staphylococcus* species usually appeared opaque, white or cream color and with 1-2mm haemolysis were selected from each plate.

Representative colonies were sub-cultured onto different blood agar plates and further incubated at 37^{0}C for 24 hrs. After growth presumptive colonies were identified by using conventional bacteriological techniques on the basis of colony characteristics, pigment production and hemolysis. Final identification of the organisms and species assignment were done based on gram staining, catalase test, oxidase test, carbohydrate (mannitol and maltose) fermentation and coagulase test(by using rabbit plasma) (ES ISO 6888-2, 2002).

Gram staining

All suspected colonies of *Staphylococcus* species were transferred to glass slide and smeared using sterile inoculating loop on a glass slide. Then the smears were stained using the procedure of Gram's staining and observed under a light microscope using oil immersion objective. The gram's stained smears from typical colonies that showed gram-positive cocci occurring singly, in pair and in irregular clusters resembling bunched grape were taken as presumptive *Staphylococcus* species. Gram positive pure cultures of a single colony type from the blood agar were transferred into nutrient agar plates and incubated at 37^{0}C for 24 hours (Quinn *et al.*, 2002).

Catalase test

Pure colonies of the isolates were picked from the top of the nutrient agar and mixed with a drop of 3% hydrogen peroxide (H_2O_2) on a clean microscopic slide. If the organism was positive, bubbles of oxygen gas were liberated within a few seconds, while catalase negative did not produce an effervescence of oxygen. The catalase positive cocci were considered either as staphylococci or micrococci.

Oxidase test

The suspected colony of the bacteria was streaked firmly across a piece of moistened filter paper with 1% aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride with a sterile platinum loop in a Petri-dish. 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.* (2002).

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 37^0C 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.

3.7.2. Secondary identification of *Staphylococcus* species

Coagulase test

There are two different tests that can be performed to detect the presence of coagulase: a tube test to detect free coagulase and a slide test to detect bound coagulase. From the surface of each selected colony of staphylococci an inoculums was removed with sterile loop and transferred into a tube of brain-heart infusion broth and incubated at 37^0C for 24hrs. After growth, 0.1ml of each culture was added aseptically to 0.3ml of the rabbit plasma and incubated at 37^0C. Clotting was examined after 4 to 6 hrs of incubation tilting the tube and if the test was negative, re-examination was made after 24 hrs 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

The colonies that were identified by primary identification test and coagulase-positive isolates were streaked on to mannitol salt agar plate and incubated at 37^0C and examined after 24-48hrs. The presence of growth and change of pH in the media (red to yellow) were regarded as confirmative indicative of the salt tolerant staphylococci. Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* cause yellow discoloration of the medium. Colonies that develop weak or delayed yellow color after 24h of incubation were taken as *S. intermedius* and colonies that failed to develop any change in the medium were considered as *S. hyicus* and coagulase negative *Staphylococcus* (CNS).

Purple agar base

The suspected colony from nutrient broth was inoculated on purple agar base (PAB) media plate with 1% maltose and incubated at 37 ^{0}c for 24-48 h to differentiate coagulase-positive isolates(Quinn *et al.* 2002). The identification was based on the fact that *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* gives a weak or delayed reaction and *S. hyicus* did not ferment maltose but attracks the peptone in the medium producing an alkaline reaction (deep purple coloration) around the colonies (Table 3).

Table 3: Interpretation of results based on reactions of coagulase-positive staphylococci on purple agar base with 1% maltose

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

+++ = rapid fermentation of maltose; ±= weak and slow fermentation; - = negative;

Source: Quinn *et al.*, (2002)

3.8. 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. Prevalence and distribution of *Staphylococcus* species

4.1.1. Udder milk

In the present study, a total of 384 udder milk samples were collected from 24 urban and periurban dairy farms to determine the prevalence and distribution of *Staphylococcus* species. About one or more *Staphylococcus* species were detected by routine bacteriological investigation. The investigation revealed 184 (47.91%) isolates of *Staphylococcus* species. All the isolates identified as *Staphylococcus* were tested for species assignment using biochemical characteristics. They were grouped into 109(28.4%) isolates of CNS, 42(10.93%) isolates of *S. aureus*, 18(4.7%) isolates of *S. intermedius* and 15(3.9%) isolates of *S. hyicus* (Table 4).

Similarly, species prevalence in the milk samples among different farm size was as follows: CNS (28.3%), *S. aureus* (10.0%), *S. intermedius* (3.9%) and *S. hyicus* (4.8%) in large scale; CNS (28.1%), *S. aureus* (12.4%) *S. intermedius* (6.7%) and *S. hyicus* (2.2%) in medium scale; CNS (29.2%), *S. aureus* (12.3%), *S. intermedius* (4.6%) and *S. hyicus* (3.1%) in small scale dairy farms (Figure1).

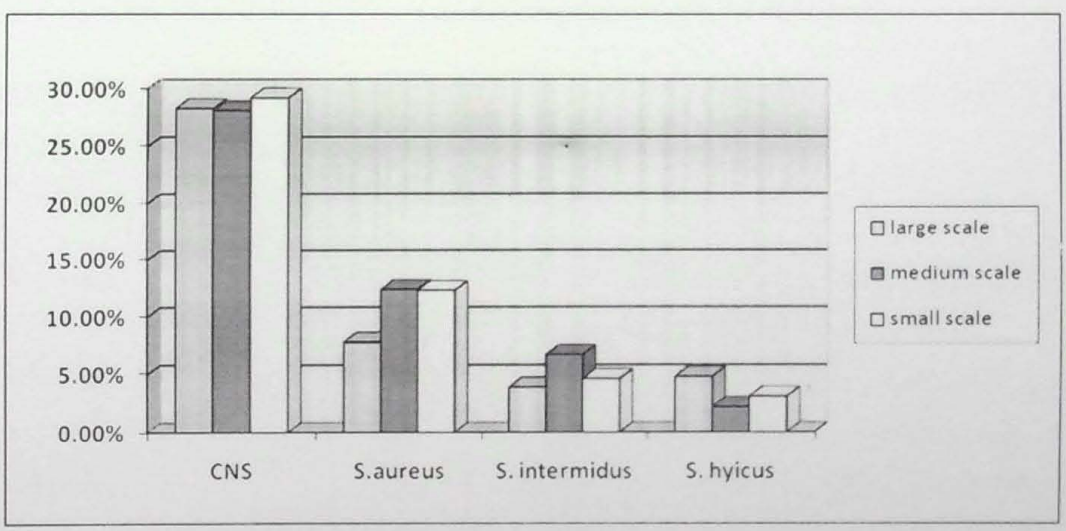

Figure 1: Prevalence of *Staphylococcus* species in bovine udder milk samples in large, medium and small scale urban dairy farms

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 Staphylococci species ($p>0.05$) (Table 4).

Table 4: Comparative prevalence of staphylococci species in bovine udder milk between farm sizes

Staphylococcus species	Farm sizes			χ^2	df	P-value
	Large scale	Medium scale	Small scale			
CNS	28.3%	28.1%	29.2%	0.28	2	0.986
S. aureus	7.8%	12.4%	12.3%	0.512	2	0.772
S. intermedius	3.9%	6.7%	4.6%	1.150	2	0.563
S. hyicus	4.8%	2.2%	3.1%	1.242	2	0.537

4.1.2. Prevalence of *Staphylococcus* species in lactating cow skin swabs

Out of 384 lactating cows, skin swab samples (192 udder swab samples and 192 teat swab samples) were tested for identification of *Staphylococcus;* the prevalence of staphylococci species identified were: 85(44.3%) from pooled teat swab sample and 72(37.5%) from udder swab. The overall *Staphylococcus* was tested for species determination using biochemical characteristics (Table 5).

The prevalence rates of *Staphylococci* species identified from teat swab were: *S. aureus,* 26(13.5%) isolates, *S. intermedius*, 6(3.1%) isolates, *S. hyicus*, 1 (0.52%) isolate and CNS, 52(27.1%) isolates. Regarding to udder swab samples the prevalence rates were *S. aureus,* 13(6.8%) isolates, *S. intermedius,* 7(3.6%) isolates; *S. hyicus*, 2(1.04 %) isolates and CNS, 50(26.04%) isolates.

The comparison of prevalence of staphylococci species in udder and teat skin between farm sizes showed no statistically significant difference ($p>0.05$) (Table 5).

Table 5: Comparative prevalence of Staphylococci species in the udder and teat skin swab samples between farm sizes

Sample type	staphylococci species	Farm sizes			χ^2	df	P-value
		Large scale (n=115)	Medium scale (n=44)	Small scale (n=33)			
Teat	CNS	26.1%	31.8%	24.2	0.692	2	0.707
	S. aureus	10.4%	15.9%	21.2%	2.817	2	0.244
	S. intermedius	2.6%	6.8%	0.0%	3.148	2	0.207
	S. hyicus	0.9%	0.0%	0.0%	0.673	2	0.714
Udder	CNS	25.2%	26.7%	28.1%	0.122	2	0.941
	S. aureus	5.2%	8.9%	9.4%	1.103	2	0.576
	S. intermedius	3.5%	2.2%	0.3%	0.887	2	0.642
	S. hyicus	1.7%	0.0	0.0	1.313	2	0.508

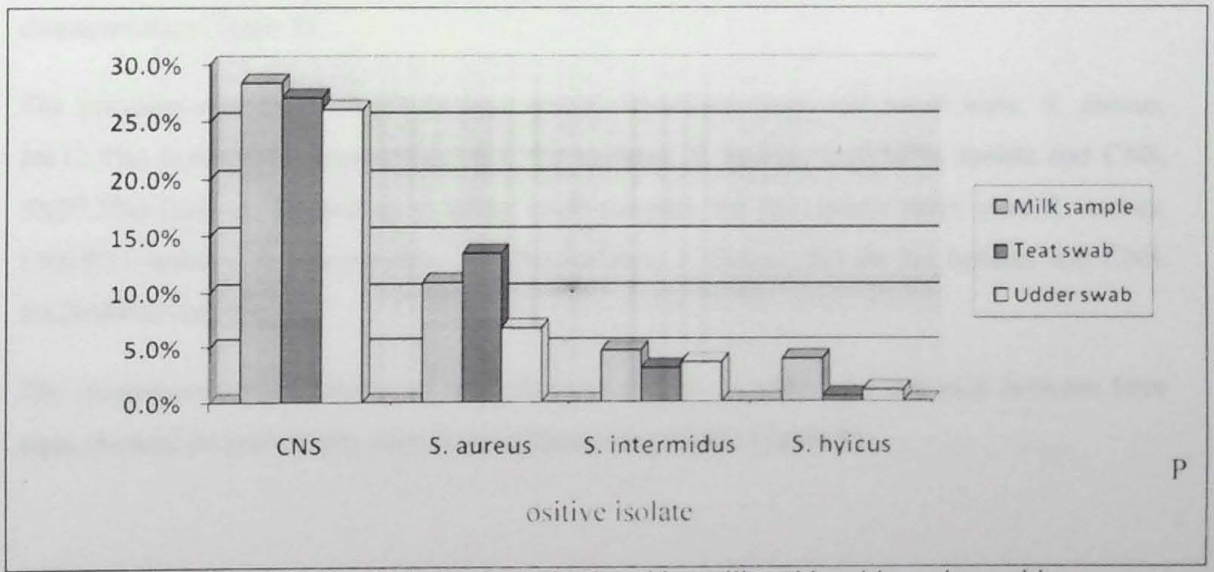

Figure 2: Prevalence of Staphylococci species in udder milk, udder skin and teat skin

4.1.3. Milkers' hand and milking and milk collecting equipments

Out of 59 swab samples (20 milking buckets, 20 milk collecting containers and 19 milkers' hands skin swabs) were examined. Bacteriological culture showed that 5 (25%) from milking buckets, 7(35%) from milk collecting containers and 5(26.3%) from milkers' hands skin were positive for staphylococcus. The overall proportional distribution of *Staphylococcus* species from 58 swab samples are summarized in (Figure 3).

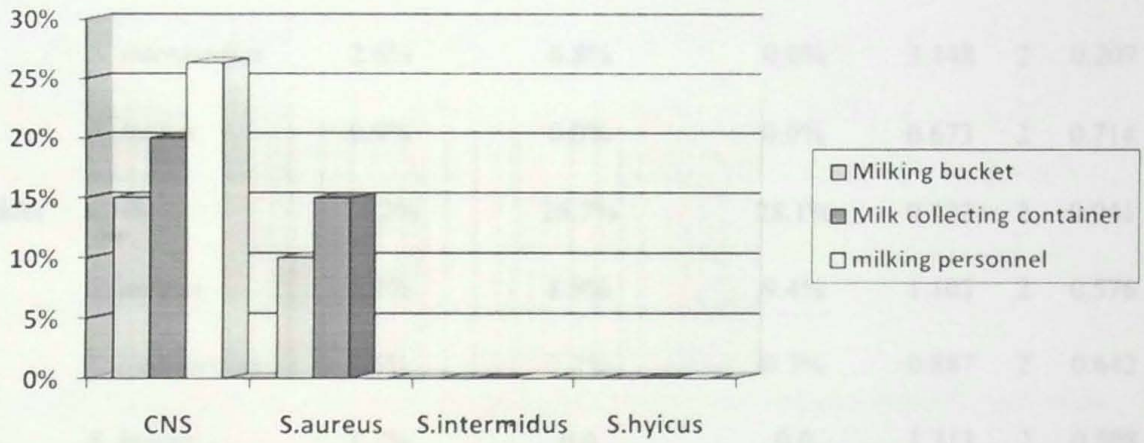

Figure 3: Proportional distribution of *Staphylococcus* species in milking and milk collecting equipments

4.2. Questioner survey

In questioner survey, 24 urban and priurban dairy farm managers and employee were participated in the interviews. These establishments were observed for hygienic status of the barn and milking premises, pre-milking procedures practiced in dairy farms, health status of workers, their protective clothing, source of milk contamination, and knowledge of food poisoning.

Out of 24 dairy farms, 19 (79.2%) of the farms have poor barn and premises sanitation. In 87.5% of the entire farm cleaned their hands. Regarding to cleaning of hand between each

milking, 58.3% conducted this procedure. Concerning to washing of milking utensils before milking, 79.1% of the dairy farm conducted the procedure.

Washing of the udder is important steps in prevention of contamination and removal of dust and filthy. Among the respondents 95.8% of the dairy farms practiced this pre-harvest control procedure, but 8.3 % used common towel for washing more than one or all of the lactating cows in the farm and 33.3% of them used separate towel to individual cows. The manager or/and employee interviewed responded that out of 24 dairy farms 45.83% of the dairy farms routinely use detergent, nevertheless 54.2% were not practicing.

Among the 24 respondents, 91.6% of them recognized previous mastitis in their dairy establishments, while 8.3% did not observe the case. Dipping practice was experienced in 20.8% dairy farms. When enquired about the general awareness on milk contamination and food borne disease, 45.8% informed that they were not aware, the rest 54.2% were familiarized with that.

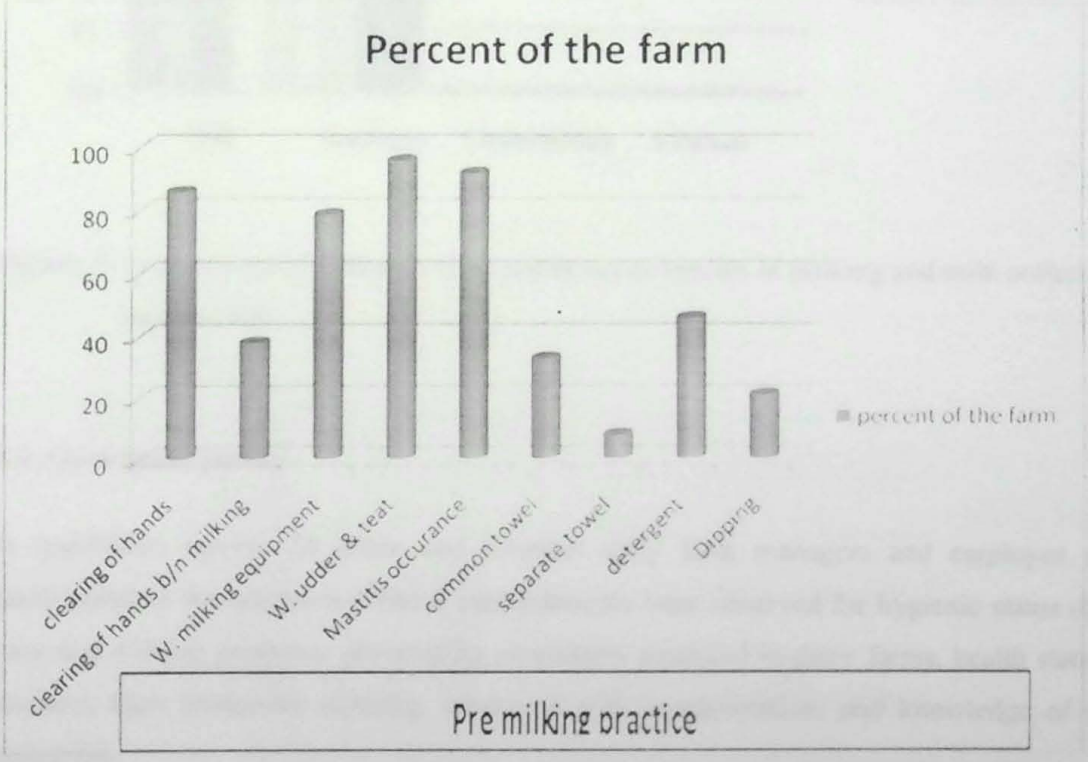

Figure 4. Percentage of pre-milking practice

W=washing

5. DISCUSSION

Staphylococcal food poisoning is a major apprehension worldwide. This form of food poisoning is known as "intoxication" or "poisoning" and common cause of gastroenteritis, due to absorption of preformed thermostable *Staphylococcus* enterotoxin. *S. aureus* is now recognized as the main agent of Staphylococcal food poisoning, due to production of enterotoxin. Indeed, Staphylococcal toxin is unique because it is not destroyed by heating, even by canning. This is illustrated by a large outbreak caused by consumption of canned food product in many countries (Maria-Angeles *et al.*, 2010).

Staphylococci are common contaminants of raw milk produced in dairy farms. These organisms can gain access to raw milk by direct excretion from udders having clinical and subclinical Staphylococcal mastitis or by contamination from udder and teat skin, air-borne dust or droplets at the site of production and processing, unclean milking and milk storage equipments and the milk handlers, which pose public health risk to consumers (Yilma *et al.*, 2007). The extent of contamination varies widely from one farm to another and by geographical differences, but is dependent primarily on the milking management used in dairy farms (Green and Bradley, 2004; Thomas *et al.*, 2006).

5.1. Prevalence of Staphylococci species

5.1.1. Udder milk

The majority of the staphylococci species contaminating bovine udder milk in this study are coagulase-negative Staphylococci with the prevalence of 28.4%. This result is lower than that reported by Alehegne *et al.* (2009) and Mezegebu (2010), who noted a prevalence of 53% and 37.6% in bovine udder milk of lactating cows in dairy farms in Addis Ababa and Debre Zeit, respectively. Bishi (1998) and Nesru (1999) reported a prevalence of 54% and 41.9%, respectively. 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 dairy cattle around Tehran and he also indicated in most cases, they caused sub-acute mastitis.

Because mastitis is a complex disease involving interactions of several factors, mainly of management, environment, and factors relating to animal and causative organisms, its prevalence is expected to vary from place to place (Dohoo *et al.*, 2003). Also literature review shows very variable results concerning the occurrence of staphylococci in bovine udder milk (McDougall *et al.*, 2007). This is probably due to the difference in the number of samples, the isolation and identification techniques used and the place of origin of the samples collected.

Staphylococci are the most common causes of bovine intramammary infections and mastitis in 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 staphylococci 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).

Coagulase-negative *Staphylococcus* species (CNS) are isolated frequently from cows and primiparous heifers with clinical and subclinical mastitis worldwide (Lejeune and Rajala-Schultz, 2009; Oliver *et al.*, 2009). Severe local and systemic signs have been reported in animals with CNS intramammary infections. CNS intramammary infection is generally associated with a slight to moderate increase in the number of somatic cells in milk, and has a deleterious effect on milk quality and composition (Schukken *et al.*,2009), and also impairs alveolar function that reduces milk production (Piepers *et al.*, 2007).

CNS is also known to cause persistent intramammary infection that can last for several months throughout lactation in the absence of intervention (Schukken *et al.*, 2009). 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.

The role of CNS in the bovine mastitis syndrome has come under increased scrutiny since more CNS associated with clinical and subclinical intramammry infection are being reported. Except for a few reports, researchers have generally regarded CNS associated with bovine mastitis as one homogenous group; however, CNS consists of more than 30species and

subspecies. Recently studies have investigated species-specific differences in CNS isolated from bovine mastitis.

A study by Taponen *et al.* (2008) reported that *S. simulans* and S. *chromogenes* were the most commonly isolated CNS from both clinical and subclinical bovine mastitis in Finland. Taponen *et al.* (2008) also reported that *S. chromogenes* was seen most often in the first lactation and *S. simulans* was the predominant species in subsequent lactations. Furthermore, the strains of several CNS species (i.e., *S. 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).

The second most prevalent species in the study was *S. aureus* with the prevalence of 10.93%. This result was in agreement with Mezegebu (2010), Haveri (2008) and Abdel Hameed *et al.* (2007) who indicated prevalence rates of 10.7%, 10.2% and 7.34%, respectively. Nevertheless this result is lower than Alehegne *et al.* (2009) who reported 15.8%. From udder milk of lactating cows with sub-clinical and clinical mastitis, Getahun (2006) reported a proportional prevalence of 20% for *S. aureus*. Atyabi *et al.* (2006) indicated a prevalence of 2.89% for *S. aureus* in udder milk samples collected from CMT positive cows.

The present study also proved that prevalence of 4.7% *S. intermedius* and 3.9% *S. hyicus*. The result is in conformity to Mezegebu's (2010) finding, who reported 4.9% of *S. intermedius* and 3.9% of *S. hyicus*. The findings of Alehegne *et al.* (2009) for *S. intermedius* and *S. hyicus* were 13% and 1.3%, respectively.

The major reservoirs of *S. aureus* are infected udders, teat canals, and teat lesions. The bacteria are spread to uninfected quarters by teat cup liners, milkers' hands, washcloths, and flies. Staphylococci do not persist on healthy teat skin but readily colonize damaged skin and teat lesions. The organisms multiply in infected lesions and result in increased chance of teat canal colonization and subsequent udder infection (Schukken *et al.*, 2009).

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. Lejeune and Rajala-Schultz (2009) reported that mastitis is the single disease that has the most significant impact on milk quality. Cows suffering from mastitis discharge large numbers of pathogens like *Staphylococcus*, *E. coli* and *Clostridium perfringens* into the milk (Tenhagen *et al.*, 2009).

S. aureus is mainly involved in intrammary infection and mastitis in lactating cows. Intramammary infection due to *S. aureus* are generally sub-clinical nevertheless, it is the most costly disease, which cause considerable economic losses to the dairy industry worldwide, particularly milk losses ranging from 10-25% of the total yield according to the intensity of infection: Arshad *et al*., 2006 reported that losses estimated 2 billion dollars per year in the United States alone. These relevant economic losses are also attributable to rejected milk, reduced milk quality, early culling, drug costs, veterinary expenses, and increased labor costs (Green and Bradley, 2004; Arshad *et al*., 2006).

Inadequate hygienic condition of dairy environment, poor animal health service, and lack of proper attention to health of the mammary gland were important predisposing factors of mastitis in the area. Milk and milk products can become contaminated unless good hygiene (including mastitis control) is practiced on farms and precautions are taken to prevent contamination and subsequent growth of staphylococci during collection and storage. Strains of *S. aureus* from cows are implicated in outbreaks of food poisoning, following consumption of raw milk.

Further public health concern, which deserves special attention, is the potential for spread of antibiotic resistant *S. aureus* strains from cattle to human populations via milk. Concern exists over the possible transmission of these strains to man. On several occasions, resistant strains have been found both in animals (cows, swine, and fowl) and in their caretakers, with the same antibiotic resistance. Moreover, "human" strains (phage typed) have on occasion been isolated from the nostrils and lesions of other species of domestic animals (Compton *et al*, 2008; Oliver *et al*., 2009).

5.1.2. Udder and teat skin

A total of 192 lactating cow teat skin and 192 udder skin swab samples were collected and bacteriologically analyzed. The samples were collected immediately after the dairy men completed their usual pre-milling preparations.

Out of the total of the teat and udder skin swab samples examined, the prevalence of CNS was 27.1% and 26.0% and that of *S. aureus* was 13.5% and 6.8% for teat and udder, respectively. The present result is less than from the finding of Mezegebu (2010) who reported that prevalence of CNS was 38.5% and 32.6%. However, the result for *S. aureus* is higher than that reported by Mezegebu (2010), who noted a prevalence of 5.9% and 4.4% for udder and

teat, respectively. White *et al.* (2002) 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* (3.1%) from the teat skin and (3.6%) udder; and *S. hyicus* (0.5%) on teat skin and (1.07%) on udder skin. Mezegebu (2010) also identified *S. intermedius* (1.5%) and *S. hyicus* (0.7%) on teat skin and *S. hyicus* (0.7%) on udder skin.

Cows harbor many pathogenic organisms either on their flank, tail hair, teat and udder 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. Both the exterior and interior of the teats and udder can become infected with Staphylococci species and contaminate the milk during milking.

In semi-intensive and extensive dairy farms of the study area, cows were maintained in dirty and muddy common barns with bedding materials that favor the proliferation and transmission of mastitis pathogens from the udder and teat skin. The bacteria are spread to uninfected quarters by common washcloths, used to wash udders and teats of lactating cow's more than one cow. In most of the visited farms the use of common cloth towel was dominated; towels soaked and washed in the same bucket water and washing was limited to 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. In addition in all dairy farms there was no isolated milking premise; cows were milked in their barns.

The other reason for the development of pre-harvest, harvest, and post-harvest control measures to effectively reduce contamination to a critical control in raw milk was the low number of employers and the resulted load of duty. Also lack of awareness, technical information and motivation about route of milk contamination and the resultant public health concern is the major obstacle to implement good farm hygiene. 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' hand and milking and milk collecting equipments

The results of this study indicated out of 19 milkers' hands skin swab samples examined, 5(26.3%) were found to be carriers of coagulase-negative Staphylococci. Milk can be contaminated by microorganisms directly from the milk handlers who have direct or indirect contact with the milk, especially if these persons are in the process of shedding pathogenic organisms. Pathogens and other organisms can gain access to milk as a result of the milk handler's activities such as coughing, sneezing, scratching and from body surfaces in contact with milk, particularly the fingers.

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 storaging the milk. They handle a complex set of equipment and chemicals that affect milk quality. Personal cleanliness of the milk handler is necessary particularly during milking process and distribution. Milkers can also become contaminated from soil and manure on the cow and in turn cross-contaminate the milk or equipment. In the present study, the majority of cows are kept in unclean barns. Milk handlers as well as the cows lack regular medical checkups.

Although the most important food borne pathogen, *S. aureus*, has not been recovered from the hands of milking personnel in the study, the presence of CNS (26.3%) contaminants in this findings suggest the possibility of *S. aureus* to occur in the hands of milkers after hand washing. Similar to udder and teat, hand washing was not complete in the visited farms.

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 (Kluytmans and Wertheim, 2005).

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 20 milking buckets and 20 bulk milk collecting containers examined, the result proven that 15% CNS and 10% *S. aureus* on the milking buckets, and 20% CNS species and 15% *S. aureus*, on bulk milk collecting containers but no other species was recovered from both.

Milk containers, receptacles and equipments which have direct physical contact with milk are most likely to be a source of microbial growth and multiplication, if they are not properly cleaned and sanitized after use. Milk utensils and equipment are specially designed and made in order to mitigate conditions that are favorable for microbial growth and multiplication and to facilitate easy cleaning and sanitizing process. 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

Good animal health and hygienic conditions on 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 raw food products leaving the farms for human consumption. Despite technical advances in milk processing, the general hygiene at milking affects the numbers of bacteria in the milk, so, 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 results of the present study suggest that the 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 and result in low milk quality and might potentially cause food poisoning. Although CNS was the dominant isolates from all types of samples, *S. aureus* was also recovered from raw milk, but not from the farm workers. Further studies on the potential effects of staphylococcal isolates of all species on human health are needed.

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.

- Contagious mastitis control measures.should continue even in herds with low to non-existent levels of *S. aureus* intramammary infection. 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.

- To produce safe foods, the hazard analysis critical control points (HACCP) approach should be implemented throughout the dairy chain.

- Consumers of milk are also advised to pasteurize or heat the milk prior to consumption. Sale and distribution of raw milk should be banned

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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 p.v.t. L.t.d., Mumbai-400 086, India). Recommended for routine bacteriological work where compatibility with other ingredients is not a critical criteria. Gelling temperature was not more than 40°C.

2. Tryptone peptone (pancreatic digest of casein) (Becton Dickinson, France).

   A microbiological peptone used for 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 liter of distilled water.
   - Bring to the boil to dissolve completely.
   - Sterilize by autoclaving at 121°C for 15 minutes.

4. Blood agar base (infusion agar) 500g (HiMedia Laboratories Pvt. Ltd. 23, Vadhani ind. Est, LBS Marg, Mumbai – 400.086, India. MO73). Used 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.

5. 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=0.02gm. 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 5-10gm of the desired carbohydrate in the basal medium prior to autoclaving or dissolve 31gm Purple Agar Base in 900ml of purified water and aseptically add 100mlof a sterile 5-10% carbohydrate solution (w/v) after autoclaving.

6. Mannitol salt agar

It is recommended for the selective isolation, cultivation and enumeration of *Staphylococcus* from clinical and non-clinical specimens.

Composition:

- Sodium chloride 75.00 gm/lit
- 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 111.02gm powder to distilled /deionized water.
- Bring volume to 1.0 liter and mix thoroughly.
- Gently heat while stirring and bring to boiling.
- Autoclave at 15psi pressure at 121^{0}C for 15 minutes. Final pH at 25^{0}C 7.4±0.2.

7. Brain heart infusion broth 500g (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
- Beef heart infusion solids 5.0
- Peptose peptone 10.0; glucose 2.0
- Sodium chloride 5.0
- Di-Sodium phosphate 2.5.

8. Yeast extracts (Oxoid LTD, Basingstoke, Hampshire, England)

A water soluble autolysate in powder form, it is used for in culture medium as a source of nitrogen and water soluble B vitamins.

Composition

- Typical analysis (%W/W)
- pH (.5% solution) 7.0±0.2 at 25^{0}C:
- Total nitrogen= 10.0-12.5:
- Aminonitrogen 5.1
- Sodium chloride 0.3

Annex V

Catalase test

This test detects the presence of the enzyme catalase that converts hydrogen peroxide to water and gaseous oxygen. The reagent 3 percent hydrogen peroxide should be stored at 4°C in a dark bottle. Extra care must be taken if the bacterium has been grown on blood agar, because the presence of red blood cells can lead to a false-positive reaction. A loopful of the bacterial growth is taken from the top of the colonies on nutrient agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3 percent hydrogen peroxide is added. An effervescence of oxygen gas, within a few second, indicates a positive reaction.

Oxidase test

This test depends on the presence of the cyrochrome oxidase in a bacterial cell. Anaerobes are oxidase negative. The regents used in the test should be colorless and stored in a dark bottle at 4^0C. The solutions must not be used if they became dark blue. Auto-oxidation of the reagents may be retarded by the addition of the reagents may be retarded by the addition of 1 percent ascorbic acid. Other precaution include testing colonies on non- selective media that do not contain glucose or nitrate and the use of a glass rod or platinum loop to pick up the bacterial growth. A conventional nichrome loop contains iron and if used, can result in a false-positive test. A piece of filter paper is moistened in a petridish with 1 percent aqueous solution of tetramethyl-p- phenylenediamine dihydrochlotde. The test bacterium is streaked firmly across the filter paper with a glass rod. A dark purple colour along the streak line within 10 seconds indicates a positive reaction.

Oxidation-fermentation (O-F) test

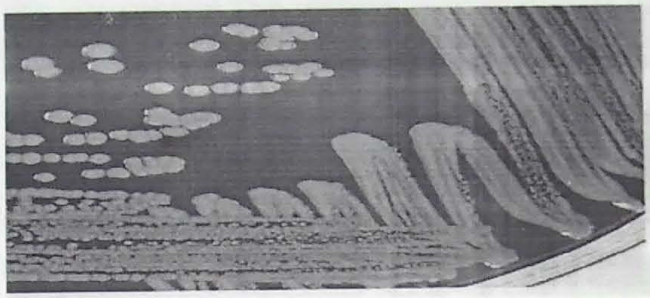
This test is used to determine the oxidative or fermentative metabolism of a carbohydrate by the bacterium. The medium is semi-solid and usually contains glucose as the test sugar and bromothymol blue as the PH indicator. The uninoculated medium (pH 7.1) is green and if acid is produced by the bacterium, as a result of glucose utilization, the medium becomes yellow (pH 6.0). Bacteria that can metabolise glucose under either aerobic or anaerobic conditions are facultative anaerobes and in this test are said to be fermentation. The aerobes that require atmospheric oxygen for growth and metabolism are called oxidative. Some

bacteria are uncreative in the conventional O-F medium, either because they are unable to grow in the basal medium or because they cannot attack glucose.

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.* (2002). 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 *Staphylococcus*.

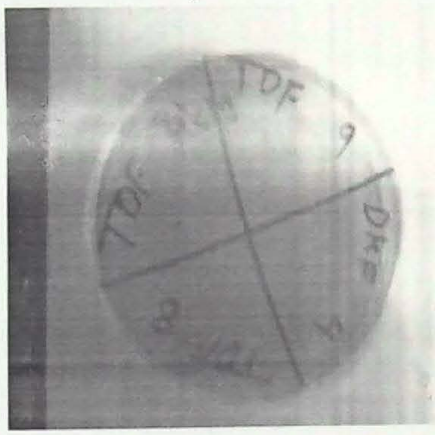
Annex VI

Typical colonies of *Staphylococcus* species

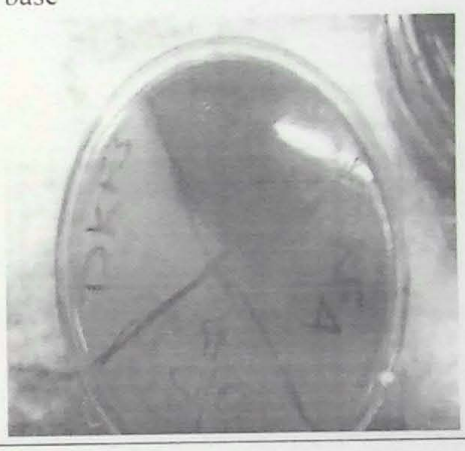


S. aureus on blood agar

Fermentation of *Staphylococcus* on mannitol salt agar



Fermentation of *Staphylococcus* on purple agar base



9. SIGNED DECLARATION

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

Dr. Daniel Shiferaw

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Advisors:

Dr. Tesfaye Sisay

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