Detection of Staphylococcus aureus and other Coagulase Positive Staphylococci in Bovine Raw Milk in Khartoum State

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Department of Microbiology
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2010
Dedication

to my

father, mother, brothers and sisters

with love
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Abstract

The present study was undertaken to isolate and characterize coagulase positive staphylococci with emphasis on *Staphylococcus aureus*, an important food-borne pathogen, from bulk raw bovine milk in Khartoum State. Milk samples were collected from sale points in Khartoum, Omdurman and Khartoum North during January and February 2010. A total of 75 samples were collected; 25 samples from Khartoum (15 from Alshagara and 10 from Yathrib), 25 samples from Omdurman (all from Waddrow sale point) and 25 samples from Khartoum North (15 from Helat Kuku and 10 from a sale point near the Faculty of Veterinary Medicine, University of Khartoum). All samples were transported to the laboratory in an ice box and were cultured on mannitol salt agar on the same day.

A total of 163 coagulase positive *staphylococcus* isolates was obtained from all samples. Of these *S. aureus* constituted 32.6% of the isolates: 39.6% from Khartoum, 47.2% from Omdurman and 13.2% from Khartoum North. Other coagulase positive staphylococcus constituted 67.4% of the isolates. It appears that raw bovine milk contains a high percentage of pathogenic *S. aureus* which makes it inherently dangerous and should not be consumed before pasteurization or boiling.
اقة

١٠٠٠ ملتهب + فعال + حمض الرئيسي + واعز + بخصوص 

١٠٠٠ ملتهب + فعال + حمض الرئيسي + واعز (coagulase positive)

١٠٠٠ ملتهب + فعال + حمض الرئيسي + واعز ٧٥ كذبح. حالي + مخصومود + 

١٠٠٠ ملتهب + فعال + حمض الرئيسي + واعز ١٠ أ شتنف ليالي ١٥) كفى + مسما + + 

٩٠ ملتهب + فعال + حمض الرئيسي + واعز ٢٥ + ٢٥٢٥١ كفى + مسما + + 

١٠ أ شتنف ليالي ١٥) كفى + مسما

٠٠ ملتهب + يأخذت + ظنم + رؤية + سلم + ( Manitol - salt agar) ظنم + أذن + الأذن + ١٥١٠ سلم + " Guyerf
(coagulase positive) among the 163 units of blood tested, 32.6% were coagulase positive and 67.4% were coagulase negative. The coagulase-positive units were further analyzed to determine their prevalence in different populations. The study found that coagulase-positive units were more prevalent among women (47.2%) and among blood samples from patients with acute infections (39.6%). The prevalence was lower among blood samples from patients with chronic infections (13.2%).
INTRODUCTION

Milk is an excellent source of nutrients for human, and yet in a different context it provides a most suitable medium for microbial growth and metabolism (Richard, 2002). Bacteria in raw milk can affect the quality, safety and consumer acceptance of dairy products. Non pathogenic bacteria may affect milk and milk products quality (Sørhaug and Stepaniak, 1997; Barbano et al., 2006) and many countries have milk quality regulations, including limits on the total number of bacteria in raw milk, to ensure the quality and safety of the final product. The number and types of microorganisms in milk immediately after milking are affected by factors such as animal health, equipment cleanliness, season and feed. It is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk (Bramley and McKinnon, 1990).

Keeping fresh milk at an elevated temperature together with unhygienic practices in the milking process may result in microbiologically inferior quality milk. Several human microbial pathogens such as Staphylococcus aureus, Listeria monocytogenes, Salmonella spp, Campylobacter jejuni, and Mycobacterium tuberculosis have been found to be associated with milk and milk products (Flowers et al., 1993; Jayarao et al., 2006).
New Pathogenic bacteria in milk often emerge as a major public health problem, especially for these individuals who still drink raw milk. *Staphylococcus* spp are microorganisms that are naturally present in milk and dairy products and are often associated with food-borne disease outbreaks due to the ability of some strains to produce a thermostable enterotoxin. Diseases are usually associated with coagulase and thermonuclease positive *Staphylococcus* spp (Gabriela et al., 2009). Milk is a good substrate for *S. aureus* growth and enterotoxin production. Enterotoxins are very resistant to heat retaining some biological activity even after 28 min at 121\(^{\circ}\)C. The bacterium is also capable of producing several pathological conditions in human.

There are few studies on the prevalence of potentially pathogenic staphylococci in raw milk in the Sudan. The objective of this study was to determine the prevalence of *staphylococcus aureus* and other coagulase positive *staphylococcus* spp in bovine raw milk in Khartoum State.
Chapter I
Literature Review

Milk is an excellent source of nutrients for humans, and yet in a different context these same nutrients provide a most suitable medium for microbial growth and metabolism (Richard, 2002). The exact components of raw milk vary by species but it contains significant amounts of saturated fats, proteins and calcium as well as vitamin C (McGee et al., 2004). Raw milk obtained from cows is nutritious and can fulfill a person's needs. It contains helpful lactobacillus acidophilus bacteria that are useful for maintaining the healthy gastrointestinal tract. Normally, these bacteria can inhibit and even kill the pathogenic bacteria and yeast that are found in the gut (Shrinivas, 2010).

It was found that intake daily of lactobacilli-fermented milk is safe and effective in the prevention of antibiotic-associated diarrhea in hospitalized patients (Beausoleil et al., 2007).

Milk is preserved due to its high nutritional value to prevent microbiological growth and chemical change and subsequent spoilage (Ibtisam et al., 2009). Methods of preservation include Pasteurization, thermalization and sterilization utilizing heat to kill microorganisms. These processes all involve the transfer of heat to in the product in order to raise the temperature to achieve closely controlled time - temperature process (e.g. 72 °c, 15s) for Pasteurization. Chilling and
freezing are commonly used to slow microbial growth and chemical change. Addition of 0.04 - 0.05% of hydrogen peroxide has been found to be effective and affordable means by which farms in tropical developing countries can extend the keeping quality of milk during transportation to the market (Odoi, 2003). Bacterial growth was reduced by 50% after addition of CO₂ and storage at 6.7°C for 48 h (Shipe et al., 1978; King and Mabbitt, 1982).

1.1. Health Hazards of Raw Milk

Milk contaminated with harmful bacteria has been linked to several serious diseases including typhoid fever, diphtheria, septic sore throat, scarlet fever, dysentery, Q-fever, and other kinds of food borne illness. Other diseases, including tuberculosis and undulant fever (brucellosis), can be transmitted to people via raw milk from diseased animals. Milk pasteurization was initially designed to kill the bacterium that causes tuberculosis as it was considered to be the most heat resistant pathogen found in raw milk. In 1960, the temperature at which milk is pasteurized was increased slightly to insure destruction of the bacterium Coxiella burnetii, which causes Q-fever.

In addition to the hazards historically associated with raw milk, scientists and some unfortunate consumers have recently become painfully aware of some new strains of harmful bacteria, called “emerging pathogens,” which also can get into milk and make people sick or even die (Donna, 1998).
1.2. Pathogenic bacteria in milk

During the milking process, presence of mastitis may introduce pathogenic bacteria in the milk. Bacteria present on the outside skin of the udders and unhygienic milking practices which allow the milk to come in contact with contaminants like feces may also introduce pathogenic bacteria in the milk. During the process of pasteurization, which is performed to cure and extend the milk's shelf life, probiotic lactobacillus bacteria are killed along with the pathogenic bacteria in the milk. However, the process fails to eradicate all of the pathogenic bacteria. Because of this one may find, through laboratory tests, different types of pathogenic bacteria in milk (Shrinivas, 2010). Pathogenic bacteria cause infectious diseases and can be found in milk and milk products, including ice cream and cheese. The characteristics of these bacteria vary between pathogens and so do the effects of the illnesses that they cause; some infections, if not treated correctly, can be deadly (Akeia, 2010). Pathogens that have been involved in communicable diseases associated with the consumption of milk include Salmonella, Listeria monocytogenes, Staphylococcus aureus, Campylobacter, Yersinia pathogenic Escherichia coli and Clostridium botulinum.

Detection of coliform bacteria and pathogens in milk indicates a possible contamination of bacteria either from the udder, milk utensils or water supply used.
Fresh milk dawn from a healthy cow normally contains a low microbial load (Ali et al., 2010).

1.3. Microbial quality of raw milk

The production of high-quality milk with low bacteriological counts begins at the farm and involves multiple factors related to cow, environment, and equipment hygiene (Elmoslemany et al., 2009).

Even for organisms that are not pathogenic, there can be effects on quality, flavor and shelf life of milk and dairy products. For example, high microbial counts in raw milk are responsible for quality defects in pasteurized milk, Ultra-high temperature (UHT) processed milk, dried skim milk, butter, and cheese (Sørhaug and Stepaniak, 1997; Barbano et al., 2006). Additionally, selecting raw milk of high quality has been associated with a decrease in consumer complaints caused by fluid milk quality (Keefe and Elmoslemany, 2007). Many countries have milk quality regulations, including limits on the total number of bacteria in raw milk, to ensure the quality and safety of the final products.

Microbiological quality of bulk tank milk is measured by means of several tests including, total aerobic bacterial count, preliminary incubation count, laboratory pasteurization count, and coliform count. These measures do not provide information about specific hygienic failure or the identity of specific microbial groups in the milk, but they indicate changes in the production, collection,
handling, and storage environment (Chambers, 2002). The number of coliforms in milk is an indicator of the cleanliness of cows and their environment (Jayarao and Wolfgang, 2003). Bulk tank somatic cell count (BTSCC) is an indicator of the level of subclinical mastitis present in a herd. Milk with high BTSCC has a higher level of proteolytic and lipolytic enzymes, which reduce cheese production and affect the flavor and shelf life of dairy products (Elmoslemany et al., 2009).

1.4. Staphylococci

Staphylococci are spherical cells about 1 mm in diameter. They grow in clusters because the cells divide successively in three perpendicular planes with the sister cells remaining attached to one another following each successive division (Kenneth, 2009).

Staphylococci are the most frequently isolated organisms from clinical specimens in the microbiology laboratory with the exception of the enterobacteriaceae. These bacteria are widespread in nature and can be recovered from environment or as commensally inhabitants of the skin, mucous membranes and other body sites in humans and animals (Heyla and Osman et al., 2006).

*Staphylococcus* spp are naturally present in milk and dairy products and are often associated with food-borne disease outbreaks due to the ability of some strains to produce thermostable enterotoxins. This ability is usually associated with coagulase and thermonuclease production, characteristics that are considered in the
microbiological analyses for the control of such microorganisms (Gabriela et al., 2009).

Animal staphylococci are broadly divided into 2 categories depending on whether or not they coagulate animal plasma.

1.4.1. Coagulase positive staphylococci (CPS)

This group so far comprises 4 staphylococcus species; S. aureus, S. intermedius, S. delphini and S. hyicus and are regarded as potentially serious pathogens.

i) S. aureus: the major pathogen of a number of important human and animal diseases (Elnazeir, 2000). Since its early discovery as an opportunistic pathogen, it continues to be a major cause of a variety of infections. In the late 1950 and early 1960, S. aureus caused considerable morbidity and mortality as a nosocomial pathogen (Martin and Stanley, 2006).

ii) S. delphini: was first isolated from purulent skin lesions of dolphins. Then it was isolated from a variety of different animals and may be more clinically important than was previously thought (Jeanette et al., 2009). The new species is established and differentiated from the other coagulase-positive Staphylococcus species primarily on the basis of its deoxyribonucleic acid-deoxyribonucleic acid hybridization relationships, its cell wall composition, its bacteriolytic activity pattern, its penicillin-
binding protein profile, its biochemical reactions, and the relatively high guanine-plus-cytosine content of its deoxyribonucleic acid (Pietroe et al., 1988). *S. dilphini* was isolated from human face and abcess (Hind, 1997) From medical laboratories environment (Huda, 1997) and from human nose (Reem, 2000).

iii) *Staphylococcus hyicus*: has been isolated from the skin of pigs with and without exudative epidermitis, intact skin and mange lesions of cattle and bovine udder lesions (Walter, 1981). *Staph hyicus* strains can be separated into virulent and a virulent strains, Strains isolated from the skin of diseased piglets have been shown to produce substances which causes exfoliation of the skin of piglets. Furthermore isolates of *S. hyicus* from other porcine sources such as, arthritis, necrosis of the ear, and milk as well as from the skin of bovines have been shown not to produce such substances. So far no in vitro assay has been developed that can distinguish between virulent and avirulent strains (Henrik, 1993). It was isolated by Kamal (1995) from eye, nose and ear, Nura (1997) from abcess.

iv) *S. intermedius*: is a serious opportunistic pathogen found principally in dogs, fowl and pigs. Occasionally has been isolated from other animal species
including bovine milk. *S. intermedius*, a veterinary flora and pathogen, has been isolated from humans with infected dog bite wounds. It appears that *S. intermedius* is a true zoonotic opportunistic pathogen (Talan *et al*., 1989) it has been long regarded as the common cause of pyoderma in dogs (Ross, 2009). It was isolated from; human eyes (Hind, 1997), human nose (Raad, 1997), skin (Kamal, 1995) and from abcess (Nura, 1997).

### 1.4.2. Coagulase negative staphylococci (CNS)

Originally thought as non-pathogenic commensals, but during the last 25 years their role in a number of important diseases has been and is still illucidated (Elnazeir, 2000).

### 1.5. *Staphylococcus aureus*

*S. aureus* is a facultative anaerobic Gram-positive coccus that is catalase positive and oxidase negative. Under the microscope, it usually appears as grape-like clusters. It can be found in the air, dust, water and human faeces, and can be present on clothing and utensils handled by man.

The carrier rate varies with different populations and studies have found a carriage rate of 10-40% in adults outside the hospital environment. Carriage may be
intermittent or continuous over a long period of time. Approximately 15-20% of humans carry enterotoxin producing staphylococci.

Various types of skin eruptions and inflammations and wounds can harbor large numbers of these micro-organisms. Animals and poultry can also carry *S. aureus* on various parts of their bodies. Udders and teat canals in cows are a source of *S. aureus*. It can be isolated from the milk of healthy cows and high levels are found in milk from cows suffering from mastitis. Strains from animal sources are less likely to produce endotoxins than strains from human sources (Heyla and Osman, 2006).

*Staphylococcus aureus* causes pneumonia, phlebitis, meningitis, urinary tract infections, osteomyelitis, endocarditis and superficial skin lesions such as furunculosis. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* also causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome (Heyla and Osman, 2006). It is a major causative agent of intramammary infections in dairy cows (Sutra and Poutrel, 1994).

Despite being a hardy organism *S. aureus* grows poorly in complex microbial ecosystems and it can be inhibited or overgrown by other organisms present in food. This poor competitive ability probably limits the growth of the organism in
many foods. To date 11 different toxins have been identified and implicated in food borne illness (Hocking, 2003).

1.5.1. Virulence characteristics of *S. aureus*

*S. aureus* is one of the causal agents of mastitis in dairy cows. Its large capsule protects the organism from attack by the cow's immunological defenses. Enterotoxins are produced by *S. aureus* isolated from mastitic cows. The organism is fairly heat sensitive; however, heat cannot be relied on to make food safe as the toxin is heat stable (Hocking, 2003).

*S. aureus* has the ability to produce several exoenzymes that contribute to virulence (Dinges *et al.*, 2000). For example, Catalase production by *S. aureus* functions to inactivate toxic hydrogen peroxide and free radicals formed by the myeloperoxidase system within host phagocytic cells after ingestion of the microorganisms (Kloos and Banner, 1999). Moreover, free and bound coagulase, sometimes called the clumping factor, may act to coat the bacterial cells with fibrin shielding them from opsonization and phagocytosis, and protein A, normally a component of the cell peptidoglycan, may also be secreted into the medium during growth and counter elimination of the pathogen by host polymorphonuclear cells. Each of these features may be considered key virulence determinants of *S. aureus*. Deoxyribonuclease, Thermonuclease, hyaluronidase, lipases, and hemolysins
produced by *S. aureus* also contribute to virulence. However, the most notable virulence factors are the enterotoxins. Staphylococcal food poisoning results from consumption of one or more preformed enterotoxins resulting in symptoms of intoxication (Mary *et al.*, 2004).

### 1.5.2. Toxins

Depending on the strain, *S. aureus* is capable of secreting several toxins, which can be categorized into three groups. Many of these toxins are associated with specific diseases.

Pyrogenic toxin superantigens these have super antigen activities that induce toxic shock syndrome (TSS). The staphylococcal enterotoxins, which cause a form of food poisoning, are included in this group.

#### 1.5.2.1. Exfoliative toxins (EF)

Implicated in the disease staphylococcal scalded-skin syndrome, which occurs most commonly in infants and young children. It also may occur as epidemics in hospital nurseries. The protease activity of the exfoliative toxins causes peeling of the skin observed with scalded-skin syndrome.
### 1.5.2.2. Other toxins

Staphylococcal toxins that act on cell membranes include alpha-toxin, beta-toxin, delta-toxin, and several bicomponent toxins. The bicomponent toxin Panton-valentine leukocidin (PVL) is associated with severe necrotizing pneumonia in children.

### 1.5.3. Protein A

Protein A is a protein that is anchored to staphylococcal peptidoglycan pentaglycine bridges by the transpeptidase Sortase A. Protein A is an IgG-binding protein which binds to the Fc region of an antibody, which is involved in the anchoring of several staphylococcal surface proteins (Scheewind *et al.*, 1995).

### 1.6. Mastitis

Mastitis is an inflammation of the mammary glands of dairy cows that can be caused by physical or chemical agents, with the majority of cases caused by bacterial infection. Mastitis is the most common and expensive disease affecting the dairy industry worldwide (Harmon, 1994; Quinn *et al.*, 1994; Moussaoui *et al.*, 2004; Park *et al.*, 2007).

More than 130 microorganisms are related to bovine mastitis, with mastitis-causing bacteria broadly classified as contagious or environmental pathogens (Watts, 1988; Quinn *et al.*, 1994; Park *et al.*, 2007). *S. aureus* is a major cause of bovine...
mastitis. Since gene expression of many bacteria is known to be regulated by the environment, milk may play an important role in the regulation of the early steps in the pathogenesis of bovine mastitis by *S. aureus*. To get insight into the response of *S. aureus* to the milk environment, a Tn917-lacZ mutant library was generated and screened for genes specifically expressed during growth in milk (Aart, 2000).

### 1.7. Microbial Contamination from the Exterior of the Udder

The exterior of the cows’ udder and teats can contribute to microorganisms that are naturally associated with the skin of the animal as well as microorganisms that are derived from the environment in which the cow is housed and milked (Bramley, 1982; Bramley and McKinnon, 1990; Hogan *et al*., 1989; Zehner *et al*., 1986). Organisms associated with bedding materials that contaminate the surface of teats and udders include streptococci, staphylococci, Gram positive spore-formers, coliforms and other Gram-negative bacteria. Both thermoduric and psychrotrophic strains of bacteria are commonly found on teat surfaces (Bramley and McKinnon, 1990). Several studies have investigated pre-milking udder hygiene techniques in relation to the bacteria count of milk (Bramley and McKinnon, 1990; Galton *et al*., 1984; Pankey, 1989).
1.8. Staphylococci and food poisoning

Staphylococcal food poisoning is one of the most common types of food borne disease worldwide. It has been identified as the causative agent in numerous outbreaks of food poisoning, but is believed to be under reported due to the self-limiting nature of the illness and the fact that most people recover within 1-2 days of becoming ill. The onset of symptoms in staphylococcal food poisoning can be very rapid, generally around 3 hours after ingestion of the food but may be as early as 1 h or as late as 6 hours, depending on individual susceptibility to the toxin, the amount of contaminated food eaten, the amount of toxin in the food, and the general health of the individual. The most common symptoms are nausea, vomiting, retching, abdominal cramping, and prostration. Some individuals may not demonstrate all of the symptoms associated with the illness. In more severe cases, headache, muscle cramping, and transient changes in blood pressure and pulse rate may occur. Blood may be observed in stools and vomits. Recovery generally takes two days. However; it is not unusual for complete recovery to take three days or longer in severe cases (Bremer et al., 2004).

A number of factors contribute to the virulence of \textit{S. aureus}, including deoxyribonuclease (DNase), Catalase, lipases and hemolysins. However, the most notable virulence factors are the enterotoxins. Staphylococcal food poisoning results from consumption of one or more preformed enterotoxins resulting in
symptoms of intoxication (Mary et al., 2004). Milk products, as well as other products with a high protein content, are a good substrate for growth of coagulase positive staphylococcus. Milk products were involved in 26 % of the outbreaks due to a staphylococcal food-borne intoxication. S. aureus can gain access to milk either by direct excretion from udders with clinical and subclinical staphylococcal mastitis or by environmental contamination during the handling and processing of raw milk (Eurobian Commition, 2003). Other coagulase positive Staphylococci as S. hyicus was isolated from minced meat, S. intermedius was isolated from sausage and S. delphini was also isolates from sausage by (Samia, 1997).
Chapter II

Materials and Methods

2.1. Study area

The study was conducted in Khartoum State, Sudan including Khartoum, Khartoum North and Omdurman, during Januarys and February, 2010.

2.2. Raw Milk Samples

Seventy five samples of raw bovine milk were collected randomly in the morning from commercial tanks at sale points in Khartoum, Omdurman and Khartoum North during January and February of 2010. A total of 75 samples were collected; 25 samples from Khartoum (15 from Alshagara and 10 from Yathrib), 25 samples from Omdurman (all from Wad drow sale point) and 25 sample from Khartoum North (15 from Helat kuku and 10 from a sale point near the Faculty of Veterinary Medicine, University of Khartoum). Samples were collected in clean sterile containers and transported immediately in an ice box to the bacteriological laboratory, Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, where examination of the milk samples was done.
2.3. Sterilization Procedures

2.3.1. Hot air oven

Glassware and metal instruments were sterilized in hot air oven at 180° C for half an hour.

2.3.2. Autoclaving

Culture media were sterilized by autoclaving at 121°C for 15 minutes or 115° C for 20 minutes. The discarded cultures were sterilized at 121°C for 30 minutes.

2.3.3. Steaming

Sugars solutions were sterilized by steamer at 100°C for 30 minutes.

2.3.4. U.V. light

It was used to sterilize the media pouring room.

2.3.5. Red heat

Nichrome wire loops and straight wires placed in benzene burner flame and the entire loop/wire up to the loop holder were heated to red hot.

2.4. Disinfectants

Seventy percent alcohol was used to disinfect the surfaces of benches before and after use.

2.5. Solutions, chemicals and reagents
2.5.1. Normal saline solution

This solution was prepared by dissolving 8.5 g of NaCl in 1 liter of DW, then 3 ml volumes were distributed into cotton capped test tubes and Sterilized by autoclaving at 121°C for 15 minutes, then stored at 4-8°C in sealed basket.

2.5.2. Oxidase test reagent

This reagent was prepared by dissolving 0.1 g of tetra methyl-\(p\)-phenylenediamine dihydrochloride in 10 ml sterile DW.

2.5.3. Potassium hydroxide solution, 40% w/v

This solution was prepared by transferring 40 g of Potassium hydroxide (KOH) pellets to chemical screw-cap bottle, then 100 ml DW was added and mixed well until the chemical was completely dissolved.

2.5.4. Andrade’s indicator

This indicator was prepared by dissolving 5 g of acid fuchsin in 1 liter of DW, then 150 ml of sodium hydroxide N-NaOH was added to solution, mixed well and allowed to stand at room temperature, and frequently shaken for 24 hours. Straw-yellow color was attained after 24 hours.

2.5.5. Hydrochloric acid, 1 mol/l (1N)

This reagent was prepared by adding 91.4 ml of DW to 250 ml flask, and then 8.6 ml of concentrated hydrochloric acid was added to the water.
2.5.6. Alpha naphthol solution, 5% w/v

Five grams of $\alpha$-naphthol was dissolved in 100 ml absolute ethanol. The final solutions had a straw color.

2.5.7. Hydrogen peroxide 3% aqueous solution

This solution was prepared by adding 50 ml of 6% Hydrogen peroxide to 50 ml DW.

2.5.8. EDTA

Was used as anticoagulant for human plasma.

2.6. Culture media for bacteriological examinations

Several solid, Semi Solid, liquid, selective and differential media were used for this purpose. All media were prepared according to (Oxoid, 1973; cowan and steel’s and Barrow and Felltham, 1993). And the ingredients are given below.

2.6.1. Solid media

2.6.1.1. Nutrient agar (Oxoid Code: CM0003 (Powder)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g/liter)</th>
</tr>
</thead>
</table>
Lab-Lemco’ powder  1.0
Yeast extract  2.0
Peptone  5.0
Sodium chloride  5.0
Agar  15.0

pH 7.4 ± 0.2

Twenty-eight grams of powder were added to one liter of distilled water, brought to boil to dissolve completely, then sterilized by autoclaving at 121 °C for 15 minutes and poured aseptically in 20-25 ml volumes into sterile petri dishes. Nutrient agar slopes were also prepared in crew capped bottles, and then stored at 4-8°C in sealed plastic bags.

2.6.1.2. Sheep blood agar base: Code: (Oxoid CM0854)

A Blood Agar Base that has been specifically formulated to give improved hemolytic reactions with sheep blood.

ingredients  Quantity ( g/ liter )
Forty grams of dehydrated blood agar base were added in to one liter of distilled water and brought to the boil to dissolve completely, and then sterilized by autoclaving at 121°C for 15 minutes. Fifty milliliters of defibrinated sheep blood were added to 50°C cooled base, then mixed with gentled rotation and poured aseptically in 20-25 ml volumes into sterile petri dishes, and stored at 4-8°C in sealed plastic bags.

2.6.1.3. Deoxyribonuclease (DNase) medium

Code (OxoidCM0321):

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>14.0</td>
</tr>
<tr>
<td>Peptone Neutralised</td>
<td>4.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4.5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Final pH 7.3 ± 0.2
Tryptose 20.0
Deoxyribonucleic acid 2.0
Sodium chloride 5.0
Agar 12.0
pH 7.3 ± 0.2

Thirty nine grams of dehydrated medium were suspended in one liter of DW and brought to boil to dissolve completely, then sterilized by autoclaving at 121 °C for 15 minutes, and poured aseptically in 20-25 ml volumes into sterile petri dishes. The plates were stored at 4-8°C in sealed plastic bags.

2.6.2. Semi-Solid media

2.6.2.1. Hugh and Leifson's (O/F) medium (Barrow and Feltham, 1993)
<table>
<thead>
<tr>
<th><strong>Ingredients</strong></th>
<th><strong>Quantity (g/liter)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>2</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.3</td>
</tr>
<tr>
<td>Agar</td>
<td>3</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.2 % aq.sol 15 ml</td>
</tr>
</tbody>
</table>

The ingredients were dissolved in one liter of distilled water in a water bath, and the medium was filtered. Then indicator was added and thin mix up, the media was sterilized by autoclaving at 115°C for 20 minutes. Thirty minutes steam sterilized glucose solution was added to cooled medium give final concentration of 1%. The media was then mixed well and distributed aseptically in 10 ml volumes into sterile cotton plugged test tubes of not more than 16 mm diameter, and stored at 4-8°C.

### 2.6.3. Liquid media

#### 2.6.3.1. Nutrient broth (Oxoid Code: CM0001)
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lab-Lemco’ powder</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>pH 7.4 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Thirteen grams of the medium were suspended in one liter of distilled water, Mixed well and distributed 5 ml volumes into cotton plugged test tubes and Sterilized by autoclaving at 121°C for 15 minutes.

2.6.3.2. Methyl Red - Voges Proskauer medium (MR – VP)

Seventeen grams of dehydrated medium were suspended in one litre of distilled water. Mixed well, distributed in 5 ml volumes into cotton plugged test tubes, and sterilized by autoclaving at 121°C for 15 minutes.

2.6.3.3. Carbohydrate fermentation media (Barrow and Feltham, 1993)
This medium was prepared by dissolving 13.5 g of dehydrated peptone water medium in 900 ml of distilled water, Mixed well and the pH value was adjusted to 7.1 - 7.3. Ten milliliter of Andrade’s indicator was added, and then the media was sterilized by autoclaving at 121°C for 15 minutes. Hundred milliliter 100 ml of 10% sugar solution (glucose and maltose) were added to the cooled mixture, mixed well and distributed in 5 ml volumes into sterile cotton plugged test tubes containing inverted Durham’s tubes, and sterilized by steaming for 30 minutes and stored in refrigerator until used.

2.6.4. Selective and differential media
2.6.4.1. Mannitol Salt Agar (Code: Oxoid CM0085)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity ( g/ liter )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab-Lemco’ powder</td>
<td>1.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Mannitol</td>
<td>10.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>75.0</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.025</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

pH 7.5 ± 0.2

One hundred and eleven grams of powder were suspended in one liter of distilled water and boiled to dissolve completely. The medium was sterilized by autoclaving
at 121°C for 15 minutes and poured aseptically in 20-25 ml volumes into sterile petri dishes, then stored at 4-8°C in sealed.

2.7. Cultural methods
2.7.1. Media used for primary isolation
Seventy five milk samples were directly streaked aseptically with a sterile wire loop on Mannitol Salt Agar and incubated aerobically for 24-48 hours at 37°C.

2.7.2. Incubation of culture
All inoculates of solid and liquid media were incubated aerobically at 37°C for 24-48 hours, except for carbohydrate media which were incubated up to 7 days. Oxidation fermentation media were incubated 10-14 days.

2.7.3. Examination of cultures
Visual examination of all cultures on solid media was performed for the detection of growth, colonial morphology, diagnostic or enzymatic reaction and any changes in the media. The liquid media were similarly examined for turbidity and colour change.

2.7.4. Purification and storage of maintenance medium
Inoculated Mannitol Salt Agar plates were incubated aerobically for 24-48 hours at 37°C. Colonies on manitol salt agar with bright yellow zones that indicated toleration of 7.5% NaCl and mannitol fermentation or were colony with cream
white or pink color were purified by sub culturing on nutrient agar. The inoculated nutrient plates were incubated aerobically at 37°C for 24-48 hours.

Pure isolates were then transferred to nutrient agar slopes and the growth was kept in the refrigerator at 4°C.

**2.8. Identification of isolates**

Identification of purified isolates was performed according to Barrow and Feltham (1993) and Scheme of El Sanousi (1996).

**2.8.1. Microscopic examination**

This was done microscopically to identify the shape, arrangement and Gram reaction using the Gram staining technique.

**2.8.1.1. Gram’s stain**

This was done on direct smears from milk samples and from milk cultures.

**2.8.1.1.2. Staining procedure**

Smears were prepared directly from milk samples by spreading a loop full of milk on a slide before culture the smears were dried and fixed by heating. From young nutrient agar cultures a colony of each isolate was emulsified in a drop of normal saline on glass slide, dried and fixed by heating. The smears were flooded by crystal violet for 1 minute and then washed with tap water. Lugol’s iodine solution was applied for 30 seconds, and then washed with tap water. The smears were decolorized with few drops of acetone for 2-3 seconds and washed thoroughly in
water. Then smears were flooded with safranin for 30 seconds, and then washed with tap water. The slides were blotted dry and examined under light microscope using oil immersion lens (X100).

2.8.1.1.3. Motility test

Colonies from young nutrient agar cultures of isolates were stabbed in the center of tubes of semisolid nutrient agar and incubated at 37°C up to 6 days. Nonmotile organisms gave growth that was confined to the stab line, while motile bacteria gave diffuse, hazy growth throughout the medium.

2.8.2. Biochemical tests

All biochemical tests were done according to Barrow and Feltham (1993).

2.8.2.1. Oxidase test

The organism was grown on nutrient agar. Oxidase reagents were added to a piece of filter paper. A colony of the test organism was picked with a sterile bent glass rod and rubbed on the filter paper. Recorded result: A dark purple colour that developed in 5 to 10 seconds was considered as a positive result.

2.8.2.2. Catalase test

A drop of 3% aqueous solution of hydrogen peroxide was placed on a clean slide and a small amount of the bacterium colony was placed on the drop using a glass rod. Recorded result: Production of gas bubbles indicated a positive result.
2.8.2.3. Sugar fermentation test

The sugar media were inoculated with bacteria and incubated at 37°C aerobically and then examined daily for up to 7 days. Acid production was indicated by the development of a pink colour in the medium.

2.8.2.4. Oxidation fermentation test (O.F)

Duplicate tubes of Hugh and leifson’s (O/F) medium were inoculated by test organism with straight wire. Sterile paraffin oil was added to one tube to a depth about 3cm above the medium to seal it from air, and then both tubes were incubated at 35°C for up to 14 days. The oxidizer organisms showed acid production which was indicated by a yellow color in the sealed tube only; fermenters showed acid production in both tubes; organisms that didn’t utilize carbohydrate showed no change in medium or a blue color in the sealed tube.

2.8.3. Secondary tests

2.8.3.1. Tube coagulase test

2.8.3.1.1. Collection of Plasma

Blood for plasma was collected from humans in glass tubes containing Etheline Diamine Tetra Acetic acid (EDTA). After mixing, the tubes were centrifuged for 5-10 minutes and the supernatant was collected and the deposit was discarded.

2.8.3.1.1.2. Test procedure
Colonies were picked up aseptically with a sterile wire loop and inoculated into sterile nutrient broth and incubated under aerobic condition at 37°C for 24 hours. Then 0.5 ml of the broth cultures was added to 0.5 ml of undiluted human plasma and incubated at 37°C for 4 hours the tubes were examined after 1 and 4 hours for clotting of the plasma. Negative tubes were incubated at room temperature overnight and reexamined.

2.8.3.2. Deoxyribonuclease (DNase) test

The plates were inoculated by spotting the organisms onto the surfaces of the agar media so that a thick plaque of growth is evident after 18 hours incubation. The plates were flooded with 1N HCl and allowed to stand on the bench (lids uppermost) for a few minutes. Zones of clearing around the colonies indicated a positive test, and no clear zone indicated a negative test.

2.8.3.3. Acetylmethylecarbinol (acetoin) production (Voges-proskauer reaction)

The test organisms were inoculated in MR VP media incubated at 37°C for 2 days before 0.6 ml of 5% w/v alcoholic α-naphthol solution and 0.2 ml of 40% w/v KOH solution were added respectively. The tubes were shaken well Then sloped (to increase the area of the air liquid interface), and examined after 15 minutes and 1 hour. A positive reaction was indicated by a strong red color.
2.8.3.4. Maltose fermentation test

Maltose media were inoculated, incubated at 37 °C and examined daily for up to 7 days. A pink color in the medium indicated acid production. Gas formation was detected in the inverted Durham tubes.

2.8.3.5. Haemolysis on blood agar

Isolates which didn’t ferment maltose were cultured on blood agar to detect the type of coagulase positive *staphylococcus* spp.
Chapter III

Results

3.1. Isolation of bacteria

From Seventy five raw milk samples cultured on manitol salt agar, 163 coagulase positive staphylococcus spp were obtained. Of these 53 (32.6 %) isolates were identified as staphylococcus aureus, 55 (33.7 %) as staphylococcus delphini, 32 (19.6 %) as staphylococcus hyicus and 23 (14.1 %) as staphylococcus intermedius (Table 1). Gram- positive cocci which were negative for the coagulase test were not investigated further.

All the isolates of coagulase positive staphylococcus spp. were found to be non motile, catalase positive, oxidase negative and phosphatase positive. All isolates identified as S.aureus were Deoxyribonuclease positive. They attacked carbohydrates by fermentation and produced acid without gas from sugars. Growth in liquid media was characterized by the formation of turbidity after 24 hours of incubation at 37°C. (Table 2).

On mannitol salt agar S.aureus colonies showed bright yellow zones around the colonies due to mannitol fermentation (Fig. 1) as were other coagulase positive Staphylococci gave white, cream, or pink colonies with no fermentation or coloration of the medium (Fig. 2).
On nutrient agar coagulase positive *staphylococcus spp* gave creamy buff or golden yellow colonies, about 1-3 mm in diameter, smooth, low convex opaque, glistening, and of a butyrous consistency. On blood agar the colonies were creamy, buff, or golden yellow in color, 1-3 mm in diameter, smooth, low convex opaque, glistening and of a butyrous consistency and Surrounded by narrow zones of clean haemolysis (Fig. 3).
Table 1: Frequency of coagulase positive *staphylococcus* spp in raw milk in Khartoum State

<table>
<thead>
<tr>
<th>Locality</th>
<th><em>S. aureus</em></th>
<th><em>S. delphini</em></th>
<th><em>S. hyicus</em></th>
<th><em>S. intermedius</em></th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khartoum</td>
<td><em>21 (39.6%)</em></td>
<td>18 (32.7%)</td>
<td>8 (25.0%)</td>
<td>9 (39.1%)</td>
<td>56 (34.4)</td>
</tr>
<tr>
<td>Omdurman</td>
<td>25 (47.2%)</td>
<td>18 (32.7%)</td>
<td>13 (40.6%)</td>
<td>6 (26.0%)</td>
<td>62 (38.0)</td>
</tr>
<tr>
<td>Khartoum North</td>
<td>7 (13.2%)</td>
<td>19 (34.5%)</td>
<td>11 (34.4%)</td>
<td>8 (34.7%)</td>
<td>45 (27.6)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>53 (32.6%)</td>
<td>55 (33.7%)</td>
<td>32 (19.6%)</td>
<td>23 (14.1%)</td>
<td>163 (100)</td>
</tr>
</tbody>
</table>

*No. of isolate
Discussion

*Staphylococcus* spp. are microorganisms that are naturally present in milk and dairy products and are often associated with food-borne disease outbreaks due to the ability of some strains to produce thermostable enterotoxins. This ability is usually associated with coagulase and thermo nuclease production, characteristics that are considered in the microbiological analyses for the control of such microorganisms.

The present study investigated the prevalence of *S. aureus* and other coagulase positive *Staphylococcus* spp in raw bulk milk samples randomly collected in Khartoum State. The results of 75 pooled raw milk samples showed a high recovery rate of coagulase positive *S. aureus* (32.6%) and other coagulase positive *Staphylococcus* spp (67.44%) in all samples. Elnazeir (2000) reported the isolation of *S. aureus* from 84% and other coagulase positive *Staphylococcus* spp from 60% of 26 milk samples obtained from cows suffering from clinical and subclinical mastitis. His figures were high for *S. aureus* and this may be attributed to the small number of samples he examined, his use of several media for primary isolation and because these samples were from infected cows. Mohammad *et al.* (1997) reported
a high count for \textit{S. aureus} in milk from cows with clinical mastitis followed by that from cows from sub clinical mastitis compared with milk from healthy cows. The clinical history of cows from which bulk milk in this study was examined was unknown. The figures of Elnazeir (2000) for other coagulase positive were lower than these obtained in this study and this may be attributed to differences in hygienic procedures followed in farms during milking.

\textit{S. aureus} has been reported as an important cause of mastitis, especially where machine milking and treatment with Penicillin are practiced. It is considered a contagious organism easy to spread among cows during milking (Farah, 1992; Paape \textit{et al.}, 2000) and an important human pathogen isolated from raw milk (Harvey and Gilmour, 1985).

In the present study, \textit{S. aureus} was found in 32.6\% of raw bulk milk samples examined in Khartoum State. According to locality, \textit{S. aureus} was most frequently isolated from milk in Omdurman area as it was recovered from all examined 25 samples (47.2\%). In Khartoum area \textit{S.aureus} was recovered from 21 (39.6\%), where as it was in 7 (13.2\%) of the samples from Khartoum North. Variation in frequency of isolation might be due to either clinical or subclinical infections of dairy cows in different localities or frequent contamination of milk by milkers or distributers.
Other coagulase positive *Staphylococcus* spp were isolated from 67% of milk samples and included *S. delphini* (33.7%), *S. hyicus* (19.6%) and the least frequently isolated was *S. intermedius* (14.1%). According to locality, the other coagulase positive Staphylococci were found in 62.5% of Khartoum samples, 59.7% of Omdurman samples and 84.4% in Khartoum North. These variations in prevalence might be due to variation in clinical or subclinical infection of dairy cows or to the hygienic measures applied in milk production cycle.

*S. delphini* was first isolated from purulent skin lesions of dolphins (Pietro *et al.*, 1988). It has been isolated from skin infections of horses, cows, mink, pigeons and dolphins highlighting its broad host range (Jody, 2008). This organism maybe more clinically important in man than previously thought. Watts and Owens (1988) isolated *S. hyicus* from bovine mammary glands in four dairy herds. It was the predominant organism isolated from cows in a herd with a bulk milk somatic cell count (SCC) greater than 900 X 10. Furthermore, *S. hyicus* has been isolated from pigs, cattle, and goats, and has been shown to produce enterotoxins other than A to E, producing an emetic response in the monkey feeding tests (European Commission, 2003).

*Staphylococcus intermedius*, produces beta and delta toxins and has been associated with bovine mastitis infection. Donald *et al.* (1985) reported five
isolates of *S. intermedius* among 93 staphylococcal isolates from intramammary infections from dairy cows from 26 California dairy herds. Due to contaminated milk *S. intermedius* has been involved in one outbreak caused by butter blend and margarine involving over 265 cases in 1991 in United States (European Commission, 2003). It was also considered to be the etiologic agent in an outbreak of food poisoning (Isabelle *et al.*, 1997).
4.2. Conclusions

- From seventy five raw milk samples cultured on mannitol salt agar, 163 coagulase positive *Staphylococcus* spp were obtained of these 53 (32.6%) were identified as *S. aureus*, 55 (33.7%) as *S. delphin*, 32 (19.6%) as *S.hyicus* and 23 (14.1%) as *S.intermedius*.

- The isolation of *S. aureus* and other coagulase positive in the investigated raw bulk milk was found as one of the commonest and serious problems.

- Identification of coagulase positive *S. aureus* and other coagulase positive in raw milk are important to knowledge. The milk maybe harbor pathogenic organism.

- Sub clinical and clinical infections on dairy cows have significant effect on the level of milk constituents. This affects the nutritive value, processing and keeping qualities because it may contain pathogenic bacteria as reported previously.

4.3. Recommendations
• A good management practice should be directed; such as cleaning, applying personal and equipment hygiene during milking process. Also milk must be kept in clean and cold steal or plastic tank to avoid contamination while transferring from farm is also be recommended to avoid contact of \textit{S. aureus} and other coagulase positive \textit{Staphylococcus} spp in milk.

• Pasteurization and boiling milk before drinking or manufacturing other milk products are recommended to kill all the present pathogenic organisms before multiplication and toxin production.
References


Fig. 1: colonies of *staphylococcus aureus* on manitol salt agar showing fermentation and yellow discoloration.
Fig. 2: *Staph. aureus* on Manitol salt agar showing Positive manitol fermentation (left), coagulase positive *Staph. hyicus* showing negative manitol fermentation (right).
Fig. 3: *Staphylococcus intermedius* on blood agar showing clear hemolysis.