Isolation and Identification of the Bacteria Associated with Bovine Mastitis and Detection of their Specific Antibodies in Milk and Sera

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نـَبِيَّ ﷺ
DEDICATION

To my mother
To my father
To my brothers and sisters
To my family, especially my uncles for their support.

With deep love
ACKNOWLEDGEMENTS

First of all my thanks and praise are due to Almighty Allah the Beneficent, the Merciful, for giving me the health and strength to accomplish this work.

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The aim of this study was isolation and identification of bacteria associated with bovine mastitis and detection of their specific antibodies in milk and sera. A total of 50 samples of milk and sera was collected from cows with clinical and subclinical mastitis in two different farms in Khartoum State (Alsalama, Khartoum and Shambat, Khartoum North). The samples were collected between December 2009 and June 2010. The bacteria were isolated from 38(76%) of the milk samples, and the total number of isolates was 45 with 3 yeasts. Sixteen (36%) bacterial isolates were obtained from cows with clinical mastitis, whereas 29(64%) bacterial isolates were obtained from cows with sub-clinical mastitis. Mixed infection was detected in 10 (20%) of the milk samples. Both Gram positive and Gram negative bacteria were isolated and identified to the species level, using cultural characteristics and biochemical tests. Most isolates (27) were Staphylococcus species all of them were coagulase positive (10 were Staph. aureus, 9 Staph. hyicus and 8 Staph. intermedius), other gram positive bacteria isolates were one isolates of Streptococcus agalactiae, two of Enterococcus species (one was avium, two mundtii and one was faecium), Listeria Ivanovii were two, Bacillus species three (two Bacillus licheniformis and one B. mycoides), and three strains of Corynebacterium psedotuberculosis. The gram negative bacterial isolates were three Enterobacteria species (two Citrobacter freundi and one Klebsiella pneumoniae). And Pseudomonas aeruginosa consisted of two strains. Agar double diffusion test was performed to detect antibodies specific to the
causative agents of mastitis in milk immunoglobulin fraction and serum collected from each cow against the whole-cell lysate (WCL) of the isolates. Only *Bacillus mycoides* gave clear precipitation lines with both milk immunoglobulin fraction and serum. Other bacteria gave precipitation lines with serum antibodies alone. The results revealed that the Gram positive bacteria, especially *Staphylococcus* species, were the common causative agent of bovine mastitis; the number of cows with subclinical mastitis was higher than cows with clinical mastitis. The bacteria associated with bovine mastitis can induce local and systemic specific antibodies response as measured by agar gel immuno-diffusion test.
The text in the image is a scientific study discussing the etiology of bovine mastitis. The study found that Staphylococcus, Escherichia coli, and Mycobacterium are the most common bacteria associated with mastitis.

- **Staphylococcus aureus** was the most prevalent, accounting for 60% of the samples.
- **Staphylococcus hyicus** was found in 20% of the samples.
- **Staphylococcus intermedius** was present in 18% of the samples.
- **Streptococcus agalactiae** was detected in 2% of the samples.
- **Enterococcus species** and **Listeria mycoides** were each found in 2% of the samples.
- **Escherichia coli** was present in 18% of the samples, followed by **Mycobacterium avium** in 12%.
- **Pseudomonas aeruginosa** was detected in 2% of the samples.

The study also highlighted the importance of testing the causative agents of mastitis to prevent the spread of these pathogens in dairy herds.
بالمعالجة، فإن الممكن من البقاء في الضرع المصاحب للمصابة بالبكتيريا. الالتماس الهلام يعترف بانتهاز الأورام العامة والمحصلة والتقنية. ينصح الامراض مع الحميات الحاكمة في البروكلي أو الدم لمضادة للالتهابات المتميزة.
INTRODUCTION

Mastitis is inflammation of the mammary gland due to the injury of any type. However, the udder disease of major concern is that associated with microbial infection. The microbes that are associated with mastitis are: Staphylococcus species, Streptococcus species and other Gram positive and Gram negative rods (Salih, 2008). Mastitis is characterized by physical, chemical and usually bacteriological changes in milk and by pathological changes in the glandular tissue (Salih, 2008). Loss of milk production lost, cows premature culling, milk discarded or down graded as well as veterinary expenses and large of some of money is lost to dairy farming each year through poor udder health (Blood et al., 1994). Together, these follows eroded milk income received by farmers and therefore, animal health affects both the well being of cows and profitability of dairy farms.

In the Sudan the disease has become one of the major problems in recent years, given the fact that many herd owners shifted to increasing milk production by selecting local breeds or importing foreign breeds that give higher milk production, but they are more susceptible to mastitis. Dairy farmers at present are moving from traditional farm toward a proper dairy farming that constitutes the nucleous for dairy industry in the Sudan. This requires the proper of health care in dairy farms specially the health of the udder.

Although there is considerable number of studies on bovine mastitis, no attention has been given to detect specific antibodies against the causative agents in milk and sera.
Objectives:

1. Isolation and identification of the causative agents of clinical and subclinical mastitis.

2. Detection of their specific antibodies in sera and milk of infected cows.
CHAPTER ONE
LITERATURE REVIEW

1.1 Definition

The term mastitis refers to inflammation of the mammary glands regardless of the cause. It is characterized by physical and chemical changes of the milk and usually bacteriological contamination of the milk and pathological change in the glandular tissue. The disease is responsible for decreased milk production, increased treatment costs, increased labour costs and increased culling of affected animals (Janzen, 1970; Asby et al., 1975; Dobbins, 1977; Blosser, 1979). The most important changes in milk are discolouration, the presence of clots and increased number of leukocytes (Blood et al., 1994).

The increased leukocyte count is a reaction of tissue to trauma. The disease may or may not be associated with systemic reaction with a decrease of at least 25% in dairy milk production (Shpigel et al., 1994).

Depending on the type of mastitis, it is manifested by swelling, heat, pain and indurations in the mammary gland in many cases but a large proportion of mastitic glands are not readily detectable by manual or visual examination of the milk using strip cup (Blood et al., 1994).

1.2. Classification of mastitis

Mastitis can be classified on different bases. According to time it is classified into acute and chronic although sub acute inflammation can exist for a short period of time. The severity of the inflammation
reaction depends upon the organism involved, its pathogenicity and
resistance of the host (Runnels, 1987). According to the character of
exudates it is classified into necrotic, gangrenous and hemorrhagic
mastitis. According to clinical signs it is classified into clinical and
sub clinical mastitis (Blood et al., 1994).

1.2.1. Clinical mastitis

This form of mastitis is characterized by apparent changes of
both milk and mammary glands (Abnormality of secretion and
abnormalities of size, consistency and temperature of the mammary
gland) and it is further classified according to the severity into
peracute, acute, sub acute and chronic mastitis.

1.2.1.1. Peracute mastitis

In this type there is a marked abnormality of milk and udder
with severe systemic reaction.

1.2.1.2. Acute mastitis

It is characterized by severe inflammation of the udder without
marked systemic reaction.

1.2.1.3. Subacute mastitis

It is characterized by a mild inflammation of the mammary
gland and abnormal milk secretion.

1.2.1.4. Chronic mastitis

In chronic mastitis there are no systemic sings and very few
external or apparent signs, but abnormal secretion of the gland occurs
intermittently.
1.2.2. Subclinical mastitis

This is an invisible abnormality of milk or udder which is characterized by an increase in somatic cell and/or leukocyte count (Rodostitis, Blood and Gat, 1994). It is a problem in the herd rather than the individual animal.

1.3. Etiology

The microbial causes of mastitis include a wide variety of bacteria, fungi, algae and viruses (Allen, 1985). The common bacterial causes of mastitis are broken down into:
- Contagious organisms that colonize the mammary gland and spread by milking machines and milkers.
- Environmental pathogens, that do not normally infect the mammary glands, but can do so when the cow's environment, the teats and udder, or the milking machine are contaminated with these organisms and they gain access to the teat cistern (William, 1995). The seasons play an important role in infection (Leslie, 2003). Ekesbo and Karjatalous (1972) observed the highest incidence of mastitis in summer, followed by spring and it was low in autumn and very low in winter.

In cattle the bacterial causes are *Streptococcus agalactiae*, *Staphylococcus aureus* and coliform. Other bacteria include: *Streptococcus uberis*, *Str. dysgalactiae Str. zooepidemicus*, *Str. Faecalis*, *Str. Pyogenes* and *Str. Pneumoniae*, Actenomyces pyogenes, Corynebacterium ulcerans, Mycobacterium bovis, Mycobacterium lacticola, Mycobacterium fortuitum, Bacillus cereus, Manhella multosida, Manhella Pasteurella hemolytica, Pesudomonas aeruginosa, Fusobacterium necrophorum, Bacteroides
funduliformis, Serratia marcescens, Mycoplasma bovis Mycoplasma Canadensis, Myco-plasma bovigenitalium, Mycoplasma alkalescens, Acholeplasma laidlawii, Nocardia asteroids, Nocardia braziliensis and Nocardia farcinicus (Blood et al., 1994). Teat injuries, poor hygiene, poor management, faulty milking machines and accumulation of milk and the presence of bacteria in or around the udder are all factors, which predispose cows to mastitis (Schalm et al., 1971; Addo et al., 1980; Ameh et al., 1993; Egwu et al., 1994). Arcanobacterium pyogenes is an opportunistic bacterium; it's normal inhabitant and it has been isolated from mucous membranes of cow (Pyorala, et al., 1994).

1.3.1. Bacteria associated with bovine mastitis

1.3.1.1. Staphylococcus aureus

One of the most common types of chronic mastitis is caused by Staphylococcus aureus. It is often subclinical, where there is neither abnormal milk nor detectable change in the udder, but somatic cell count increases. Flare-up to clinical mastitis may occur in some cows especially after calving. The bacterium persists in mammary gland, teat canals, and teat lesions of infected cows and is considered contagious. The infection spreads at milking time, when Staphylococcus aureus contaminated milk comes into contact with teats of uninfected cows and the bacterium penetrates the teat canal. Once established, Staphylococcus aureus usually resist respond to antibiotics treatment and infected cows eventually must be segregated or culled from the herd. In some herds with Somatic cell counts (SCC) below 200.000 dairy managers have not been able to eradicate. Staphylococcus aureus, even when they practiced standard milking hygiene techniques (Roberson et al., 1994). In herds in which
Staphylococcus mastitis is a problem, more than 50% of cows may have chronic, subclinical infections. Staphylococcal mastitis leads to ductal obstruction with cells and cellular debris. If a new *Staphylococcus aureus* infection was not treated, the bacterium penetrates the mammary gland tissues and the cow attempts to wall off the area, forming an abscess and eventual scar tissue (Belschner *et al.*, 1996). The development of antibiotic resistance and formation of L-forms during treatment with some beta-lactam antibiotics (e.g., penicillin) are additional reasons for therapy failures. In Chronic mastitis caused by *Staphylococcus aureus*, cows usually has high Somatic cell counts, abnormal mammary tissue and recurrent cases causes of clinical mastitis (Merk, 1998).

### 1.3.1.2. *Streptococcus agalactiae*

*Streptococcus agalactiae* is an obligate pathogen that affects pre-milking heifers, as well as older cows in dairy herds. It is considered one of the major causes of economic losses in dairy farms without a control program.

Although *Streptococcus agalactiae* can live outside the udder for short periods of time in the right conditions, it is considered to be an obligate pathogen of the udder. A high percentage of cows may be affected in herds where control procedures are not implemented. Fomites such as strip cups, towels, milkers’ hands, cross suckling calves, milking machines and other milking equipment and unsanitary conditions are all potential sources of infection in cows. Even multi-use and hand-mixed antimicrobial mastitis preparations can be a potential source of infection for the udder.
Streptococcus agalactiae may be transmitted from udder to udder in many ways. *Streptococcus agalactiae* breaks the natural barriers of the udder, enters the teat canal, and ascends in the milk through the quarter. The bacteria penetrates the acinar epithelium, causing edema and extravasation of neutrophils into the lumen, resulting in subclinical or clinical mastitis as well as possible systemic infection. In later stages, the acini become filled with scar tissue which plugs the glandular- ductal system resulting in a chronic, smoldering infection which decreases milk production and increases the somatic cell count (SCC) of the quarter. Poor udder health due to *Streptococcus agalactiae* is slowly progressive over time, causing fibrosis and atrophy of the affected quarter. As a matter of fact, an individual cow with a high SCC typically has lower production that correlates with increased SCC of the herd (Carlton *et al.*, 1995).

**1.3.1.3. Coliform**

Smith (1990) reported that 62% of occurrences of clinical mastitis were due to environmental pathogens as coliform bacteria and most species of streptococci. Bovine mortality survey carried out in 1992, identified coliform mastitis as the single most important cause of death in dairy cows (Menzies *et al.*, 1992). The most common coliforms are *Escherchia coli*, *Enterobacter aerogenes* and *Klebsiella* spp. The secretion of the clinically affected quarter is usually brownish and watery (Merk, 1998).

**1.3.1.4. Pseudomonas aeruginosa**

*Pseudomonas aeruginosa* causes a herd wide infection have been repotted after expensive exposure to contaminated wash water,
teat cup and liners. Culling is recommended for cows infected with Pseudomonas (Merk, 1998).

1.3.1.5. Mycoplasma

It is a unique organism. Mycoplasma does not have cell walls, leaving them unaffected by most antibiotics that interfere with cell wall formation. Since no effective treatment is available, the best way to control this disease is to avoid purchasing cattle from known positive tested herds. In addition, if cattle are routinely purchased, the bulk tank and pot herd milk should be sampled monthly. Mycoplasma can be spread through the use of contaminated bottle mixes, syringes, and teat tubes in treating mastitic cows. Other infected cows are major sources of infection which can be transmitted by the milking machine components, hands of the operator, use of common rags and sponges, and directly from the environment. Teat dipping is essential for proper control. The spread of contagious organisms is controlled by teat dipping and are eliminated dry cow therapy. Herds with contagious mastitis problem usually have to get back to dip cups and cover the whole teat to the base of the udder to control the spread (Bray and Schearer, 1993).

1.3.2 Fungal infection

1.3.3 Viral infection

The most important viruses which can cause mastitis in cattle include, Ephemeral Fever virus, Foot and Mouth disease virus and Lumpy Skin Disease virus (Afsher and Bannister, 1979).

1.4 Normal milk flora

Micrococci, lactobacilli, mycoplasmas and diphtheroids, including *Corynebacterium bovis*, are frequently shed from the apparently normal mammary gland and some residues stay in the teat canal. Part of the organisms found in the mammary gland are not classified as normal flora, but are considered as commensals that are potential pathogens (Carter, 1986).

*Rowan et al.* (2003) observed that all strains of Bacillus spp. are implicated in mastitis in animals.

1.5. Epidemiology

The occurrence of mastitis involves the complex interaction among three major factor: the agent, the host and the environment (Radostitis *et al.*, 2000).

Host factors include stage of lactation, age, anatomy of udder, parity, intramammary defense mechanisms and pre parturient disease (Radostitis *et al.*, 2000).

At the pathogen level, factors such as the nature of the organism, its virulence and numbers, toxins and antimicrobial resistance are important. The management, climate, feeding, housing, milking technique and malfunction of milking machine are environmental risk factor (Anon, 1987). Milking machines damage the teat,
allowing pathogen access easily into the teat canal and transfer pathogens from one cow to another via contaminated clusters (DuPreez, 1991).

The rate of intramammary infections was significantly higher in dry period than during lactation (Eberhart, 1986). Bush and Oliver, (1987) observed the greatest increase susceptibility to mastitis occurred during the first three weeks of the dry period in which the new infection rate is higher than during the preceding lactation. The second period of heightened susceptibility occurs just prior to calving in the immediate postpartum. Invasion of the mammary gland by microorganisms are characterized by increased leukocyte count in the milk, the majority of cells being neutrophils.

1.6. Mastitis in dairy cows

In spite of the control efforts (including intensified treatment with antibiotics), mastitis is still the most important disease problem in the dairy industry (Fetrow and Mann, 1991). In Sudan mastitis was first reported in the 1953 (Annual Report of the Sudan Veterinary Service, 1953). Since then it was described as being fairly common (Annual Report of the Sudan Veterinary Service, 1953-1955 and Annual reports of the Department of Animal Production, 1956-1975). Later, prevalence of mastitis in dairy herds in Sudan was thoroughly investigated by Wakeem and Eltayeb (1962). The investigation was carried out to determine the incidence, prevalence rate of infection, the causative agent and the response to control efforts, which include treatment. Ninety six per cent of the milking cows were found to be infected. Mustafa et al. (1977) reported that clinical and sub clinical mastitis lead to 20% drop in milk production. The predominant and
causative organism were *Staphylococcus pyogenes* and *Streptococcus* spp. Oxytetracycline intramammary infusion was used to treat 12% of the cows which were infected with Staphylococci and 50% of those infected with Streptococci. They recommended some measures such as proper milking hygiene, dry cow therapy and culling for control of the disease in the herd.

Bagadi (1970) investigated both clinically and bacteriologically bovine mastitis in seven herds of cattle in three areas in Sudan. He found that *Staphylococcus aureus* was the most common causative agent representing 92.2% of the isolated bacteria from clinical cases and 44.2% from subclinical cases. Adlan *et al.* (1980) isolated *Streptococcus agalactiae*, *Bacillus cereus* and *Staphylococcus epidermidis* from bovine mastitis milk. *Staphylococcus aureus* was isolated from bovine clinical mastitis by Mamoun and Bakheit (1992). *Corynebacterium* spp. was isolated by Jha *et al.* (1994) from clinical mastitis. *Corynebacterium bovis* was isolated from clinical and subclinical cases of bovine mastitis Costa *et al.* (1998). *Actinomyces pyogenes* was isolated from 173 (9.8%) mastitic milk Ibrahim *et al.* (1997). They pointed out that seven hemorrhagic mastitis cases caused by *Actinomyces pyogenes* were observed among 108 dairy cows surveyed. *Staphylococcus aureus* was isolated from mastitic milk of some domestic animals Elsayed (2000). The out of 170 milk samples, 50 Actinomyces were isolated Abdel Rahman (2001). Fifty strains of Actinomyces spp. were isolated 170 milk samples (50 samples from normal milk and 120 samples from mastitic milk) Mohamed (2003). Nocardiae and related Actinomyces were isolated from caprine and bovine mastitis, respectively. Similarly, Kamal Eldeen (2003) and

1.7 Immune response in the bovine mammary gland

The immunity in the mammary gland can be, as in other systems, classified as innate or adaptive immunity.

1.7.1 The innate immunity

The innate immunity is the predominant defense during the initial periods of infection. It is the target of choice for selection against infectious diseases. The non-specifics responses are present or are quickly activated in the infection's site by numerous stimulations, and these responses are not enhanced by repeated exposure to the same agent (Carneiro et al., 2009).

Pascal and Celine (2005) reported that innate immunity includes the keratin plug, the humoral factor and the phagocytic cell:

1.7.1.1 The keratin plug

The first obstacle to be faced by the agent is the barrier represented by the teat sphincter and the keratin plug. This canal is sealed between milking, and during the dry period, by the keratin plug derived from the stratified epithelial lining of the canal. Probably the major role of this waxy plug is to achieve a physical barrier preventing the penetration of bacteria.
When the pathogenic agent crosses the teat canal and reaches the teat cistern, the humoral factors and the phagocytic cells starts do act.

1.7.1.2 The humoral factors

1.7.1.2.1 Complement

The contribution of the complement system to the defense of the bovine mammary gland has recently been reviewed. Complement is present in milk of healthy uninflamed glands at low significant concentrations. The classical pathway is not functional due to the lack of C1q, but the alternative pathway can operate, with two consequences: deposition of opsonic C3b and C3bi on bacteria, and generation of the pro-inflammatory fragment C5a. The chemotactic fragment C5a has been shown to induce the migration of neutrophils through the mammary epithelium in vitro and vivo, but the role of C5a in the initiation of the inflammatory response of the mammary gland remains to be specific.

1.7.1.2.2 Lactoperoxidase

This enzyme in the presence of thiocyanate and hydrogen peroxide, inhibit or kills bacteria of many species, including the most common mastitis causing pathogens.

Immunoglobulins in mammary secretion are derived from blood serum or are made locally by cells of the lymphocyte-plasma cell series situated close to the glandular epithelium. The major immunoglobulin in colostrum and milk of ruminants, IgG1, is derived from the blood and is transferred into secretion selectively relative to IgG2, probably by a mechanism requiring specific receptor sites on the basal or intercellular membrane of the glandular epithelium. Acute
inflammation causes suppression of selective transfer of IgG1, but there is a marked increase in the transfer of proteins, such as IgG2 and serum albumin, which enter secretion nonselectively. Infusion of antigen into the mammary gland of ruminants some weeks before parturition induces a persisting local production of antibody, most of which is associated with IgA and IgM. IgA cells in the mammary gland probably originate in the intestine, and prior antigenic stimulation of the gut may be required for maximal IgA antibody responses in the gland.

1.7.1.2.3 Lysozyme (N-acetylmuramyl hydrolase)

Is a bactericidal protein cleaving the peptidoglycans of cell wall of Gram positive and Gram negative bacteria. Only a few bacterial species are killed and lysed by lysozyme, but this enzyme can synergize with antibodies, complement or lactoferrin.

1.7.1.2.4 Lactoferrin (Lf)

Is the a protein which exerts several functions related to innate immunity. Lf was first known for its iron chelating properties, the basis of two of its activities, bacteriostasis and protection against oxygen radicals catalyzed by free iron.

1.7.1.2.5 Trasferrin

Is another iron-binding protein which is found in milk. The milk of ruminants contains only low concentrations of transferring, from 1mg/ml in milk compared to 4-5mg/ml in serum.

1.7.1.2.6 Xanthine

An enzyme of the membrane of milk fat globules, catalyses the formation of nitric oxide from inorganic nitrite, which under aerobic
conditions leads to generation of peroxynitrite, a powerful bactericidal agent.

1.7.1.2.7 Cytokine

Glycoproteins acting on local and/or systemic regulatory factors. They are produced by many cell types in the skin, such as keratinocytes, melanocytes, Langerhans cells and dermal endothelial cells. They possess a broad range of overlapping biological activities and are potent at very low concentrations \((10^{-10} \text{ M})\). They act via specific cell surface receptors and result in a cascade of cellular events with final synergistic or antagonistic effects. A large number of cytokines are produced in the skin under normal and pathological conditions and play an important role in the pathogenesis of chronic inflammatory disorders. They include IL-1, IL-6, IL-7, IL-8, IL-12, G-CSF, M-CSF, GM-CSF TNFα, TGF α and β, VEGF, FGF, PDGF, IGF, immunosuppressive factors (IL-IRA, IL-10) (Claudy et al., 1996).

1.7.1.3 The phagocytic cell

The non-specific cellular defenses are represented by neutrophils, macrophages, natural killer cells (NK) and dendritic cells. To these leukocytes, another cell type should be added, the membraneous epithelial cell, which is at the interface between the body and its environment (Pascal and Celine, 2005).

1.7.1.3.1. Neutrophils

Present in normal milk and their role in mastitis is not clear. One the one hand, their concentration is too low for an efficient phagocytosis in suspension.
1.7.1.3.2 Macrophages

Are a major cell type in milk, secretion of the involuted udder, and mammary tissue. Milk macrophages are phagocytic cells which can ingest the common mastitis pathogens. They are less active than milk neutrophils at phagocytosis, and both milk cell types are less efficient than their blood counterparts.

1.7.1.3.3 Natural killer cells (NK)

Are large granular lymphocytes that have cytotoxic activity independent of major histocompatibility complex through antibody dependent cell-mediated cytotoxicity. Although neutrophils and macrophages are well equipped to seek out and eliminate extracellular pathogens, NK cells are critical to the removal of intracellular pathogens. NK cells are also capable of killing bacteria by releasing bactericidal proteins belonging to the saposin-like protein family upon stimulation.

If these mechanisms have been functioning adequately, the majority of pathogens will be eliminated in a short time, before the specific immune system be activated. The fast elimination of the microorganisms will not allow these alterations in the amount or quality of produced milk. The best understanding of the defense mechanisms of the mammary gland and its alterations during the critical periods of infection, is an useful tool in devising and developing methods to control mastitis, the major illness of dairy ruminants (Pascal and Celine, 2005).
1.7.2 Adaptive immunity

1.7.2.1 Antibodies

Immunoglobulins in mammary secretion are derived from blood serum or are made locally by cells of the lymphocyte-plasma cell series situated close to the glandular epithelium. The major immunoglobulin in colostrum and milk of ruminants, IgG1, is derived from the blood and is transferred into secretion selectively relative to IgG2, probably by a mechanism requiring specific receptor sites on the basal or intercellular membrane of the glandular epithelium. Acute inflammation causes suppression of selective transfer of IgG1, but there is a marked increase in the transfer of proteins, such as IgG2 and serum albumin, which enter secretion nonselectively. Infusion of antigen into the mammary gland of ruminants some weeks before parturition induces a persisting local production of antibody, most of which is associated with IgA and IgM. IgA antibodies in the mammary gland probably originate in the intestine, and prior antigenic stimulation of the gut may be required for maximal IgA antibody responses in the gland (Lascelles, 1979).

The immune response in mammary glands of cattle was measured after intestinal, local, and systemic immunization with T4 bacteriophage. Nonlactating pregnant cows were immunized by infusions into the intestine or mammary gland and by subcutaneous injection in the region of the prescapular or external inguinal lymph nodes. Titers of antibodies of different isotypes were measured in serum and in lacteal secretions by enzyme-linked immunosorbent assay and numbers of cells producing antibodies of each isotype were determined in lacteal secretion by the Jerne plaque assay. Substantial
increases in immunoglobulin G subclass 1 (IgG1) and IgG2 antibody titers were detected in serum and lacteal secretions of animals immunized through an intestinal fistula. IgM and IgA antibody responses were low or undetectable. Low numbers of IgA and IgG1 plaque-forming cells were occasionally detected. It is proposed on the basis of these data that migration of antigen – stimulated IgG lymphoblasts and perhaps of antigen, to spleen and peripheral lymph nodes may be dominant events after intestinal immunization of ruminants. This is consistent with the pre-dominance of serum-derive IgG antibodies in colostrums and milk. Intramammary infusion of antigen gave rise to increases in antibody titers in all classes which were greater not only in lacteal secretions but also in blood serum than with their systemic route used. Comparison of IgA titers in secretions from the immunized glands with those in serum also suggest that locally synthesized IgA antibodies might have contributed in some measure to serum titers. Local synthesis in both immunized and non-immunized glands was also reflected by the presence of increased numbers of IgA and IgG1 plaque-forming cells. It was hypothesized that antibody-forming cells responsible for local synthesis originated in lymphoid tissue within the mammary gland or from peripheral lymph nodes, depending upon the route of immunization (Chang et al., 1981).

1.8 Diagnosis of mastitis

Tentative diagnosis of bacterial mastitis depends on symptoms and changes in milk. However; confirmatory diagnosis depends on isolation and identification of the causative agent.
1.8.1 Physical examinations

All physical examinations according to Kelly (1984) include in

1.8.1.1 Visual examination

Clinical mastitis may be detected by examining the udder for swelling, a symmetry of the infected quarters and redness which are indicative of acute mastitis.

In chronic mastitis the glands reveal reduction in size and symmetry of the infected quarter. In gangrenous mastitis the glands reveal the presence of swelling and blue colour of the udder.

1.8.1.2 Palpation of the udder

In acute mastitis palpation reveals an increase in local temperature, pain, abnormal texture and increase in size and local temperature of supramammary lymph node. In chronic mastitis palpation reveals abnormal texture, no pain, normal local temperature and increase in size of supramammary lymph nodes. In gangrenous mastitis palpation reveals a decrease of local temperature, abnormal texture and increase in size of supramammary lymph nodes. In late stage of gangrenous mastitis desquamation of the udder from the body and smelling offensive odour are observed (Anon, 1987).

1.8.1.3 pH indicator paper

Test strips detect the more alkaline pH in quarters with mastitis. Normal milk has pH of approximately 6.5 to 6.7, whereas mastitic milk often approaches the plasma pH of 7.4 (William, 1995).
1.8.2 Chemical examinations

1.8.2.1 California Mastitis Test (CMT)

The California Mastitis Test (CMT) which is called Rapid Mastitis Test (RMT) is used for the detection of mastitis, this test was more sensitive than the strip cup test and enables subclinical mastitis to be detected (Bramley, 1975). The CMT was commonly used for detection of mastitis and has proved to be highly efficient (Blood et al., 1994). The CMT can be useful in indicating and controlling mastitis since it focuses attention on the individual quarters that are secreting milk with high number of leukocytes (Poso and Mantysarri, 1996). This is an indirect test that grossly measures the amount of DNA, primarily a function of the number of nucleated white blood cells in the milk (Quinn, et al., 1994). CMT contains Alkayl aryl sulphonate that breaks down DNA of the cells and precipitate them and bromocresol purple that impart shade to the tested milk and reveals the alkalinity or acidity of milk (Sukla and Supekar, 1982). The CMT is most helpful in detecting subclinical mastitis and serves little purpose in acute clinical mastitis. The test tends to have a high score in recently fresh cows and in cows at the end of lactation just prior to drying off. CMT score is also elevated in secretion from cows whose milk production has dropped precipitously due to illness (William, 1995). For reliable results, the CMT therefore, should be conducted just before milking, after stimulating the cow and having discarded the fore milk. Based on amount of gelling that occurs as equal amount of milk and reagent interact, the test (reaction) is subjectively read (scored) as 0 (negative), 1 (slight), 2 (moderate),
3 (heavy). These scores equate well with somatic cell levels (Philpot and Nickerson, 1991).

1.8.2.2 Modified Whiteside test

The test is performed by adding 1-2 drops of N. sodium hydroxide solution 0.4% to 5 drops of cold milk on glass on black background and then stirring the mixture vigorously for 20 seconds. In positive reaction the milk will separate to water and shreds or flakes but in negative reaction the mixture remains uniformly opaque (Kelly, 1984).

1.8.2.3 Somatic cell counts

Somatic cell count which is measured by direct count or indirect assays, remains the cornerstone of mastitis diagnosis.

Particia et al. (1953) reported 75% of the variation in average leucocyte counts of herd milk might be explained in terms of percentage mastitic animals within the herd. They also reported that Streptococcus agalactiae infection was associated with higher leucocyte count in herd milk than were the corresponding percentages of infection caused by other organisms. Black-burn (1968) found that there were variations in the cell count of cow's milk through out lactation and from one to another and he noted the average total cell count of samples from which Staphylococci, Streptococci or Coliform organisms were isolated, increased from the first to seventh lactation due to increase in number of polymorphs within infected samples. Mononuclear cells contribute to the somatic cell count, but neutrophils comprise the majority of cells. Somatic cells also include epithelial cells that make up the internal lining of the mammary gland tissue and
are normally replaced during the events of lactation (Harmon and Lang Lois, 1986). The somatic cell counts have become the most widely used index of the level of infection within individual cows and herds (Bartlett et al., 1992). The direct microscopic somatic cell counts is the produce of evenly spreading measured volume of milk over calibrated area of a microscope slide, staining the film and counting somatic cells in a specific area of the film (Packard et al., 1992). The count is then converted to cells per milliliter by a factor, which is determined by magnification and area counted. Somatic cells consist primarily of leukocytes that are present in the udder in response to infection and to repair damaged tissue. When the udder or teat is severely injured there are large increases in somatic cell counts (De Graaf and Dwinger, 1996). Clinical mastitis caused by *Staphylococcus aureus* has been observed more frequently in herds with a bulk milk cell count lower than 150,000 cells /ml Elbers et al. (1998). Low somatic cell count herds are considered to have higher levels of environmental mastitis (Peeler et al., 2000).

1.8.2.4 Isolation of microorganism in culture

Bacteriological cultures and biochemical tests are required for isolation and identification of bacteria from mastitic milk samples from individual quarters of cows to determine the etiological agents involved (Anon, 1987). In herds with a large number of subclinical cases of mastitis, a reliable diagnosis can be made by culturing samples of milk from cows selected on basis of increased CMT score or SCC. Most of the bacteria that cause mastitis grow on ox or sheep blood agar. A MacConkys agar plate is streaked in parallel to detect *Enterococcus faecalis* and any Gram-negative bacteria that are able to
grow on the medium. Edwards medium is highly selective for streptococci and also act as an indicator medium for haemolysis and for the hydrolysis of aesculin. A Sabouroud dextrose agar plate can be inoculated if a fungal pathogen is suspected (Quinn et al., 1994; Carter, 1996).

1.8.2.5 Serological tests for bovine mastitis
1.8.2.5.1 ELISA

The production of *Staphylococcal enterotoxins* (SE) and toxic shock syndrome toxin-1 (TSST-1) was studied in 81 strains of *Staphylococcus aureus* isolated from cases of mastitis in cattle, goats and sheep. SE and TSST-1 were detected by ELISA double antibody sandwich technique. More *Staph. aureus* strains isolated from sheep produced enterotoxins than those from goats and cattle. Enterotoxin producing Staph were the predominant types in all isolates from these animal species. The highest proportion of strains producing TSST-1 were obtained from sheep, twice as many as those from goats or cows (Orden, 1992).

1.8.2.5.2 Agglutination test

A total of 59 mastitis staphylococcal strains were tested for gross agglutination upon supplementation of growth media with ovine and bovine milk whey and mammary secretions from dry cows. Differences were observed when comparing bacterial species or origins (ovine vs. bovine) of bacteria and whey. All of the ovine and bovine *S. aureus* strains tested, but only 4 among 22 other ovine mastitis staphylococcal strains, showed growth agglutination in Todd Hewitt broth (THB) supplemented with ≥30% (v/v) ovine milk whey. None of the strains agglutinated during growth in regular THB.
medium. Ovine whey had an agglutination induction capacity higher than bovine whey, concerning the number of responsive ovine and bovine *S. aureus* strains. There were no differences between whey samples from different ewes with regard to their capacity to induce agglutination. Ovine *S. aureus* strains were more responsive than bovine strains of this bacterial species, concerning the number of responsive strains to bovine whey (≥30% in THB), the proportion of responsive strains at low (10%) ovine whey concentrations, and the strength of reaction (precipitation timing and clump sizes). Secretions from dry cows systematically induced agglutination in all of the bovine and ovine *S. aureus* strains tested (Baselga and Amorena, 1990).

### 1.8.2.5.3 Precipitation

Cultural characteristics and serological relationships of mycoplasmas from, mastitic milk, and other sources were studied. All strains were haemolytic for red blood cells of the guinea pig, rabbit, sheep, cow and horse. Satellite growth enhancement was seen only with the strains associated with mastitis.

Rabbit antisera to each mycoplasma were tested against homologous and heterologous strains for precipitins and for growth inhibitory antibodies. Four serotypes were distinguished among the bovine isolates by agar-gel double-diffusion technique. Growth inhibitory antibodies were detected in homologous antisera against only four of the strains studied (Jain *et al*., 1967).

### 1.8.2.6 Molecular diagnosis of the etiology of bovine mastitis

Molecular probes reacting in PCR with bacterial DNA from bovine milk, providing direct and rapid detection of *Escherichia coli*,
Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus parauberis, and Streptococcus uberis, have been developed. Two sets of specific primers were designed for each of these microorganisms and appeared to discriminate close phylogenetic bacterial species (e.g., S. agalactiae and S. dysgalactiae). In addition, two sets of universal primers were designed to react as positive controls with all major pathogens of bovine mastitis. The sensitivities of the test using S. aureus DNA extracted from milk with and without a pre-PCR enzymatic lysis step of bacterial cells were compared. The detection limit of the assay was 3.125 × 10² CFU/ml of milk when S. aureus DNA was extracted with the pre-PCR enzymatic step compared to 5 × 10³ CFU/ml of milk in the absence of the pre-PCR enzymatic step. This latter threshold of sensitivity is still compatible with its use as an efficient tool of diagnosis in bovine mastitis, allowing the elimination of expensive reagents. The two PCR tests avoid cumbersome and lengthy cultivation steps, can be performed within hours, and are sensitive, specific, and reliable for the direct detection in milk of the six most prevalent bacteria causing bovine mastitis (Renée Riffon et al., 2001).

1.9 Control of infection

The objective of any dairy farm is to produce as much milk as possible. Mastitis not only reduces the productive capacity of cows, it is also expensive to treat. Therefore, its prevention should be a priority. Prevention of mastitis depends primarily on good hygiene (before, during and after milking) practices and effective animal management which include treatment of clinical cases as they occur, use of udder disinfection and pre milking strip cup, post milking teat dipping and dry cow therapy (Radostitis et al., 2000). Vaccination
against mastitis has long been an active field of research, but for the time being, the panoply of mastitis vaccines is neither well stocked nor very efficient. Another approach to the control of mastitis is the selection of more resistant animals.

1.9.1 Vaccine of bovine mastitis

A number of problems are uniquely associated with vaccination of dairy cows for mastitis. One of these is that the number of mastitis pathogens is numerous and heterogeneous. Vaccine efforts have concentrated mainly on the major mastitis pathogens. While at least one *S. aureus* bacterin has been commercially available for a number of years, no large-scale, independent field trials have been published in refereed journals which support the efficacy of this vaccine. Experimental vaccines for *S. aureus* composed of pseudocapsule-enriched bacterins supplemented with α- and/or β-toxoids appear promising, but none of these has been commercialized. With *S. uberis*, some protection against homologous strain challenges was reported recently with a live strain and a bacterin, but other data from the same laboratory showed this vaccine would not protect against heterologous challenge strains. At this time there is only one highly effective vaccine for mastitis, the core-antigen vaccine for coliform mastitis. All of the commercially available vaccines for this indication are bacterins of rough mutants of *E. coli* strain J5 or Salmonella spp. Preliminary success with an experimental vaccine based on the plasminogen activator of *S. uberis* is a very different approach for a mastitis vaccine. Little success has been reported with vaccination against other mastitis pathogens (Yancey, 1999).
1.10 Treatment of mastits

A program for mastitis treatment starts with clinical cases and treats in earliest stage. In subclinical mastitis quarters are identified using survey or representative sampling during a routine check. Another treatment during dry period the objective from it cures the infections and to protect from a new infection which may occur during dry period. Choice of intramammary infusion is very important. A broad spectrum antibiotic is essential for the dry period intramammary infusion (Blood et al., 1994). The preferable antibiotic, is the due to binding to mammary tissue and be in a long acting base.

1.11 Economic losses

Mastitis is the widest spread infectious disease in cattle and it is considered from an economic aspect the most damaging diseases (Dodd, 1985). The loss of milk yield due to clinical cases may reach up to 40% where in subclinical mastitis it may reach 60% (Dijkuizen and Stelwagen, 1981). The effects on animal depend on severity of mastitis with systemic signs. Their signs vary according to the type of mastitis and the causative agents (Aiello, 1998). Early diagnosis of mastitis is vital because changes in the udder tissue take place much earlier than they become apparent.
CHAPTER TWO
MATERIALS AND METHODS

2.1. Collection of samples

A total of 50 milk samples and 50 serum sample were collected in Khartoum State from dairy farms at Alsalama (Khartoum), and Shambat (Khartoum North) during the period from December 2009 to June 2010. Only milk samples from cows that reacted positively to California Mastitis Test (CMT) were collected and subjected to bacteriological and immunological investigation in the laboratory.

The study was made during the period from December 2009 to August 2010.

2.2. Collection methods, transport and storage of milk samples

Udder were first washed with water and then the teats and teat orifices were disinfected with pieces of cotton wool soaked in 70% ethyl alcohol and then dried with fresh pieces of cotton wool. Approximately 3-5ml of milk from infected quarter were taken (after discarding the fore milk) aseptically in sterile Bijou bottles for bacteriological investigation and labeled, and from infected cows 100 ml milk were taken in clean bottles and 10 ml blood were taken for serum in syringe for immunological investigation and labeled. Samples were placed in ice and transport immediately to laboratory where they were kept in refrigerator at 4°C until used.

2.3 Media

For the identification of isolated pathogens, different type of media were used (solid, semi solid and liquid media) all media were prepared according to methods described by the manufacturers.
2.3.1 Solid Media

2.3.1.1. Nutrient agar (OXOID, CM 3)

Twenty – eight grams of powder were added to 1 liter distilled water. Dissolved and pH was adjusted to 7.4. The medium was sterilized by autoclaving at 121°C for 15 min- be for being poured into sterile Petri - dishes in 20ml volumes.

2.3.1.2. Blood agar (OXOID, CM55)

To every 90 ml of sterile nutrient agar 10 ml of sheep defibrinated blood were added aseptically at 45-50°C, mixed and poured into sterile Petri –dishes in 15 – 20 ml volumes.

2.3.1.3. Chocolate agar (OXOID, CM367)

Blood agar was placed in an incubator and sat at 65°C for 30-60 min until the medium assumes a uniform chocolate colour and poured into sterile Petri –dishes in 15 – 20 ml volumes.

2.3.1.4. MacConkey’s agar (OXOID, CM7)

Fifty tow grams of medium were suspended in a liter of distilled water. Dissolved and pH was adjusted to 7.4. The medium was sterilized by autoclaving at 121°C for 15 min, and then poured aseptically in sterile Petri –dishes in 15 – 20 ml volumes.

2.3.1.5. Starch agar

Ten gram of potato starch was triturated with 50 ml distilled water to a smooth cream and added to 1000 ml molten Nutrient Agar. Mixed, and sterilized at 115°C for 10 min. and poured into sterile Petri –dishes in 15 – 20 ml volumes.

2.3.1.6. Urea agar (OXOID, CM53)

2.4 grams of medium were suspended in 95ml of distilled water, which was brought to boil to dissolve the powder completely. The medium was then sterilized by autoclaving at 121°C for 15 min,
cooled to 50°C and aseptically we add introduced 5 ml of sterile 40% urea solution, mixed and then distributed in 10 ml volumes into sterile bijou bottles and allowed to solidify in a slant position.

2.3.1.7. Simmon’s citrate Aagar (OXOID, CM155)

Twenty – three grams were suspended in one liter of distilled water, boiled to dissolve completely and the pH was adjusted to 7.0. The medium was sterilized by autoclaving at 121°C for 15min, and distributed into bijou bottles in portion of 5ml each and left to solidify in a slope position.

2.3.1.8. Ammonium salt sugars (ASS)

Twenty gram of agar, 0.2 gram yeast extract, 0.2 potassium chloride, 1 gram ammonium phosphate and 0.2 watered magnesium sulphate were dissolved in 1000 ml distilled water by steaming then 4 ml Bromcresol purple, 0, 2% aq.soln were added as indicator and sterilized at 115°C for 20 min. The basal medium was allowed to cool to about 60°C and appropriate sugar was added as sterile solution to give the final concentration of 0.5- 1%. They mixed and distributed aseptically into sterile tubes which are inclined so that the medium set as slopes.

2.3.1.9. Aesculin-bile agar

Forty gram of ox bile, dehydrated, 0.5 gram ferric citrate, 15 gram Agar and 1000 ml Nutrient broth were dissolved by heating and allowed to cool then 1 gram of aesculin was added and dispense in screw-capped bottles, sterilized at 115°C for 20 min. and allowed to set as slopes.

2.3.1.10. Nutrient gelatin media (OXIDCM135a)

Four gram of gelatin was soaked in 50 ml distilled water and when thoroughly softened 1000 ml of Nutrient agar was added and
mixed and sterilized at 115°C for 10 min. and poured into sterile Petri dishes in 15 – 20 ml volumes.

2.3.2. Semi-Solid Media

2.3.2.1. Hugh and leifson’s (O.F) medium (OXOID)

The medium was prepared by dissolving 10.3 grams of solid in 1 liter of distilled water by heating, and pH was adjusted to 7.1 filtered bromothymol blue 0.2% aqueous solution was added and then the medium was sterilized at 115°C for 20 minutes. Sterile solution of glucose was aseptically added to give a final concentration of 1%. The medium was mixed and distributed aseptically as 7 ml volume in sterile test tubes. For Staphylococcus, Streptococcus and Micrococi a modification was done to this media Collef (1996).

2.3.2.2. Motility medium (OXOID)

Thirteen grams of nutrient broth was add to 4 grams of agar and dissolved in 1 liter of distilled water and the pH was adjusted to 7.2. The medium was distributed as 5 ml volumes in test tubes containing carigie- tubes and sterilized by autoclaving at 115°C for 15 minutes.

2.3.3. Liquid Media

2.3.3.1. Nutrient broth (OXOID CM1)

Thirteen grams of the medium were suspended in 1 liter of distilled water, dissolved and the pH was adjusted 7.4. The medium was distributed as 5 ml volumes in test tubes and sterilized by autoclaving at 121°C for 15 min.

2.3.3.2. Peptone water (OXOID CM9)

Fifteen grams of the medium were dissolved in 1 liter of distilled water and the pH was adjusted to 7.2. The medium was distributed into 5 ml volumes and sterilized by autoclaving at 121°C for 15 min.
2.3.3.3. **Glucose phosphate broth (M.R-VP medium) (OXOID CM43)**

Five grams of peptone water and 5g of potassium phosphate were dissolved in 1 liter of distilled water, the pH was adjusted to 7.5. Five grams of glucose were then added, mixed and the medium was distributed into 5ml volumes in test tubes and sterilized by autoclaving at 121°C for 15 minutes.

2.3.3.4. **Peptone water sugars**

Nine hundred ml of peptone water was prepared and the pH was adjusted to 7.1-7.3 before 1 ml of Andrade's indicator were added. Ten grams of appropriate sugar were added to the mixture, which was distributed into tubes, 5 ml of each one. They were sterilized by autoclaving at 115°C for 10 minutes.

2.3.3.5. **Serum water sugars**

Four gram of peptone and 0.8 gram of sodium phosphate were dissolved in 800 ml distilled water, the mixed was stem at 100°C for 15 min. and filter. Then 200 ml of sterile serum was added and stem for a further 15 min. and the pH was adjusted to 7.5-7.8 before 10 ml of Brom cresol purple, 0, 2% aq.soln were added as indicator. They were sterilized by autoclaving at 115°C for 10 minutes. 0.5-1% appropriate sugar was added aseptically as sterile solution and distributed into sterile tubes.

2.3.3.6. **Nitrate broth**

One gram of nitrate was dissolved in 1 liter of nutrient broth, then distributed into tubes and sterilized by autoclaving at 115°C for 20 minutes.
2.3.3.7. Aesculin broth

One gram aesculin, 0.5 gram ferric citrate were dissolved in 1000 ml distilled water, and sterilized at 115°C for 10 minutes and distributed into sterile tubes.

2.3.3.8. Arginine media

Five gram Peptone (tryptone), 5 gram yeast extract, 2 gram potassium phosphate, 0.5 gram glucose and 3 gram arginine monohydrochloride were dissolved in 1000 ml distilled water by heating and the PH was adjusted to 7.0, boiled, filtered and sterilized at 115°C for 20 minutes and distributed into sterile tubes.

2.3.3.9. Gluconate broth

One point five gram peptone 1 gram yeast extract, 1 gram potassium phosphate and 40 gram potassium gluconate were dissolved in 1000 ml distilled water by heating. The PH was adjusted to 7.0, filtered, and sterilized at 115°C for 20 minutes and distributed into sterile tubes.

2.3.3.10. Malonate-phenylalanine medium

Two gram ammonium sulphate, 0.6 gram potassium phosphate, 0.4 gram potassium dihydroge phosphate. Two gram sodium chloride, 3 gram sodium malonate, 2 gram yeast extract were dissolved in 1000 ml distilled water by heating, filtered. 12.5 ml bromthymol blue, 0.2% aq.soln was added as indicator solution and sterilized at 115°C for 20 minutes and distributed into sterile tubes.

2.4. Biological materials

2.4.1. Sheep blood

Sheep blood was used for the preparation of blood agar.
2.4.2. Human plasma

This was used for the detection of coagulase production by staphylococci.

2.5. Reagents

2.5.1. California mastitis test reagent (EURO FARM)

2.5.2. Hydrogen peroxide

It was prepared as 3% aqueous solution, protected from light and stored in cool place. It was used for catalase test.

2.5.3. Oxidase test reagent

Tetramethyl p-phenylenediaminedihydrochloride was prepared as 1% aqueous solution and used for oxidase test.

2.5.4. Kovac’s regent

This regent is composed of (5g) of Para- dimethyl- amino Benz aldehyde; 75 ml amyl alcohol and 25ml concentrated hydrochloric acid. The cooled and the acid were added carefully. The regent was stored at 4°C for later use in indole test.

2.5.5. Methyl red solution

This solution was prepared by dissolving 0.04 g of methyl red powder in 40 ml ethanol and the volume was made up to 100 ml with distilled water.

2.5.6. Alpha- naphtol solution

It was prepared as 1% aqueous solution and also used for V.P test.

2.5.7. Andrade’s indicator

Dissolve (5g) of acid fuchsin in 1 liter distilled water, and then added 150 ml alkali solution (NaOH). It was used in peptone sugar medium.
2.5.8. Bromothymol blue solution

This indicator was prepared by dissolving 0.2 gram of bromthymol blue powder in 100 ml distilled water. It was used for oxidation fermentation test.

2.5.9. Normal saline

Physiological or isotonic saline was prepared by dissolving 8.5 grams of sodium chloride in 1 liter of distilled water to obtain 0.85% concentration.

2.5.10. Nitrate test reagent

This reagent was composed of two types of solution:
Solution A: Sulphanilic acid 0.33% in 5 N-acetic acid dissolved by gentle heat.
Solution B: Dimethyl- α-naphthyl amine 0.6% in 5N- acetic acid.

The complete reagent was used to detect nitrate reduction.

2.5.11. Nessler's reagent

Five gram of potassium iodine was dissolved in 5 ml of freshly distilled water, cold saturated mercuric chloride solution was added until a slight precipitate remains permanently after thorough shaking 0.40 ml of 9N-NaOH was added and the mixed was diluted to 100 ml with distilled water and was allowed to stand for 24 h. was used to detect Arginine hydrolysis.

2.5.12. Benedict's qualitative solution

17.3 gram of sodium citrate, 10 gram of anhydrous sodium carbonate were dissolved in 60 ml distilled water and 1.73 gram of copper sulphate was dissolved in 20 ml distilled water and it was added to the first solution with constant stirring. And the volume was completed to 100ml by addition of 20 ml distilled water was used to detect Gluconate oxidation.
2.6. California Mastitis Test (CMT)

This test was done at the farm. About 3 ml of milk from each quarter and an equal amount of CMT reagent was mixed by circular rotation of paddle held horizontally. The result of the test was interpreted as follows:

1. No slime or slight precipitate that dissolved with mixing was scored as negative.
2. Slime gel, thick gel or viscus and tacky gel formation, was scored as positive.

Based on the degree of gelling, the test was subjectively read as:
- 0 (negative), -1 (slight), 2 (moderate), and 3 (heavy).

2.7. Sterilization

2.7.1. Flaming

Flaming was used to sterilize slides, cover slips and glass rods.

2.7.2. Hot air oven

For one hour for glass ware such as pipettes, Petri dishes, tube, Flasks, glass rods were sterilized in the hot air oven at 160ºC.

2.7.3. Moist heat (autoclaving)

For sterilization of media, solutions, screw-capped bottles, eppendorf tubes, rubber stop pared flasks, plastic ware were sterilized by autoclaving at 121ºC for 15 min and 110ºC for 10 min for sugar media.

2.7.4. Disinfection

Alcohol (70%) and phenolic solution were used for disinfecting working place in the media preparation room and in the floors and bench in laboratory.
2.8. Cultural methods

2.8.1. Primary isolation

The swabs were cultured on blood agar, chocolate agar and MacConkey's agar. The plates were incubated aerobically at 37ºC for 1-2 days.

2.8.2. Examination of culture

Visual examination for detection of growth, pigmentation and colonial morphology.

2.8.3. Purification and storage isolates

Isolated bacteria were purified by repeated subculture on blood agar and nutrient agar plates and incubated at 37ºC for 24 hours until pure colonies were obtained. The purified bacteria were stored at 4ºC.

2.8.3.1. Identification of isolates

Identification of isolates was carried out according to Barrow and Feltham (1993).

2.8.3.1.1. Primary identification

2.8.3.1.1.1 Preparation of smears

Smears were prepared by emulsifying small inoculums of the bacterial culture in a drop of sterile normal saline and spreading them on clean slide. The smears were allowed to dry and fixed by gentle heating.

2.8.3.1.1.2 Gram’s stain

This was done as described by Barrow and Feltham (1993). It was used to study morphology, shape and gram staining reaction of each isolates.

Gram-positive bacteria appeared purple, while Gram-negative bacteria appeared red.
2.8.3.1.3. Biochemical tests

The entire following biochemical test were conducted and preformed according to Barrow and Feltham (1993).

2.8.3.1.3.1. Primary test

2.8.3.1.3.1.1. Catalase test

To differentiate those bacteria that produce the enzyme catalase, a drop of 3% hydrogen peroxide was placed on a clean slide and a colony of test organism cultured on nutrient agar was picked by glass rod and added to the drop of solution. A positive result was indicated by immediate production of air bubbles.

2.8.3.1.3.1.2. Oxidase test

Commercial oxidase paper was used. The test organism was picked using sterile bent glass rod and rubbed on a filter paper, saturated with oxidase reagent. The development of dark purple color within 10 seconds indicated appositive result.

2.8.3.1.3.1.3. Oxidation fermentation test (O.F)

This test was used to determine the way by which the bacterium attacks a carbohydrate (by fermentation or by oxidation).

The test performed by growing the bacterium in tow tubes of Hugh and Leifson’s medium. One of them was covered with a layer of sterile paraffin oil.

All tubes were incubated at 37°C and examined daily for 14 days. Fermentative organisms produced a yellow color on both tubes while oxidative organisms produced a yellow color only on open tube. When the two tubes were green, the result was negative.

2.8.3.1.3.1.4. Motility test

By a sterile wire, a small piece of colony was picked and stabbed in the center of the semi-solid agar in the Craigie tube. This
preparation was incubated at 37°C overnight. The growth outside Craigie tube and turbidity in the medium indicated the organism was motile.

2.8.3.1.3.1.5. Gas from glucose

Tubes of glucose sugar medium were inoculated with the test culture and incubated for up to 7 days at 37°C. Production of acid was indicated by the development of pink color in the medium and gas production was indicated by the presence of empty space in the inverted Durham’s tube.

2.8.3.1.3.2. Secondary test

2.8.3.1.3.2.1. Vogues- proskauer (VP) test

Pure culture inoculated in test tubes or screw – capped bottle containing glucose phosphate broth and incubated at 37°C over night. 0.2ml of 40% KOH and 0.6 ml of 5% alpha-naphthol solution were added to one ml of culture, shacked and the tubes were placed in slope position and examined, positive test was indicated by strong red color within half an hour.

2.8.3.1.3.2.2. Indole test

Peptone water was inoculated with the isolate under test and incubated at 37°C for 48 hours. Kovac’s reagent was added to culture and shacked well. The development of red ring on the surface of the culture indicated the positive result.

2.8.3.1.3.2.3. Methyl red test (MR)

Culture in peptone water was incubated over night and then 3-5 drops of methyl red added to the culture, production of red color in the medium indicate positive test.

2.8.3.1.3.2.4. Urease test
The test bacteria were inoculated on a slope of urea agar medium incubated at 37°C and examined for up to 5 days. The change of color of the medium to pink indicated a positive result.

2.8.3.1.1.3.2.5. Citrate utilizing test

Simmon’s citrate medium was inoculated with the test organism, incubated at 37°C and examined daily for 7 days. The color changed to blue was positive or yellow was negative.

2.8.3.1.1.3.2.6. Acid production from carbohydrate

Peptone water sugar (glucose, lactose, sucrose, maltose, xylose, mannitol, rhamanose, salisin, lactose, raffinose, glycerol, starch, arabinose, fructose, adonitol and sorbitol) medium was used. It was incubated with the organism under test, incubated at 37°C, and examined daily up to 7 days for acid production. Development of pink or red color indicated positive result. For Bacillus and Pseudomonas (ASS) and for Streptococci (Serum water sugar) development of yellow color indicated positive result.

2.8.3.1.1.3.2.7. Nitrate reduction test

The test organism grown into 5ml nutrient broth and incubated for 48 hours after that one ml of nitrate reagent A was added followed by one ml of reagent B. Development of the red color indicated the reduction of nitrate to nitrite.

2.8.3.1.1.3.2.8. Gluconate oxidation

The test organism grown into gluconate broth and incubated for 48 hours after that to 5 ml of this culture 1 ml of Benedict's qualitative solution was added, mixed and was boiled for 10 min. The formation of brown, orange, or tan precipitate constitutes a positive reaction.
2.8.3.1.1.3.2.9. Gelatin hydrolysis

Nutrient gelatin was inoculated with a straight wire by the test organisms and was incubated at 37°C up to 14 days; every 2-3 days was placed in a refrigerator for 2 h and then was examined for liquefaction as appositive result.

2.8.3.1.1.3.2.10. Arginine hydrolysis

The test organism grown into 5 ml arginine broth and incubated for 24 hours after that 0.25 ml of Nessler's reagent was added. Arginine hydrolysis was indicated by development of brown colour. For Strepptococci, 0.5 ml of culture was added to 4.5 ml distilled water, the mixer was shaken and added to 0.25 ml Nessler's reagent. Arginine hydrolysis was indicated by development of brown colour.

2.8.3.1.1.3.2.11. Malonate utilization

The test organisms were inoculated in Malonate–phenylalanine medium lightly and incubated for 18-24 h. Examined for colour change and the culture was kept for the phenylalanine deamination test. A positive malonate reaction was indicated by a deep blue colour.

2.8.3.1.1.3.2.12. The camp test

The test was performed by streaking a known Staphylococcus culture across a 10% sheep blood agar plate and then the test organism was inoculated at right angle to it with out touching between them, and after overnight incubation at 35-37°C the test organism was presumed to be Streptococcus agalatiaeif there is interaction with the Staphylococcal haemolysin as shown by an arrow-head shaped area of haemolysis (Monica 1984). The test was also done to Listeria.
2.8.3.1.1.3.2.13. Aesculin test

Aesculin broth was inoculated by test organism and was examined daily up to 5 days for blackening; this was indicated hydrolysis of aesculin.

2.8.3.1.1.3.2.14. Aesculin bile test

This test was done for Streptococcus. The test organisms were inoculated or streaked in aesculin bile agar and were incubated for 24 h for growth and blackening of the medium as appositive result.

2.8.3.1.1.3.2.15. Growth at 45ºC test

All isolates of Streptococci were Subjected to incubation temperature at 45ºC. The inoculated plates (chocolate agar) were examined daily for growth. Ability of test isolate to grow at 45ºC was taken as positive.

2.8.3.1.1.3.2.16. Starch hydrolysis Test

The test was done to streptococci. The test isolated were cultured on starch agar medium, the plates were incubated at 37ºC in air with added carbon dioxide for 2 days, the plates were flooded with lugal's iodine solution. A positive starch reaction gave clear zones around the growth of each isolates.

2.8.3.1.1.3.2.17. Coagulase test

Plasma of rabbits or human was used. Plasma contains fibrinogen that is converted to fibrin by the staphylococcal coagulase enzyme.

2.8.3.1.1.3.2.17.1. Slide coagulase test

A drop of normal saline was placed on clean slide and small amount of test organism was emulsified in each of the drop to make two thick suspensions.
A drop of undiluted human plasma was added to one of the suspensions and mixed gently. The development of clumping within 10 seconds was reported as positive result.

2.8.3.1.3.2.17.2. Tube coagulase Test

This to detect free Coagulase, fresh plasma was diluted 1:10 in physiological saline. Half ml of diluted plasma was placed in sterile test tube and 0.5 ml of overnight broth culture of tested organism was added. The tube then was incubated at 37°C and examined after 1, 3, and 24 hours. Positive test was indicated by Coagulation of the tube content.

2.9. Collection of blood, serum and plasma

Blood for enrichment media was collected by venous puncture of the jugular vein of a healthy sheep. Blood was de fibrinated by using heparin as anticoagulant. Serum for precipitation test was collected by venous puncture of the jugular vein of a miasmatic cow, and then centrifuges this sample to separate the serum from other components after that aspirate the serum and placed in sterile eppendorftubes.

Blood was Plasma for coagulase test was aspirated from any human. Using tube containing an anticoagulant (Heparin), then centrifuge this sample to separate the plasma from other components after that aspirate the plasma and placed in sterile tubes.

2.10. Immunological methods

2.10.1. Isolation of milk immunoglobulin

Thirty ml of milk were centrifuged at 4000 rpm for 30 minutes at 28°C in two 15 ml falcon tubes, the whey and cell depress were removed and the protein was taken and put in clean peter and mixed with 20% sodium sulphate and was stirred with magnetic stirrer for 30
minutes and centrifuged at 4000 rpm for 30 minutes at 28°. The deposit was dissolved in PBS and then concentrated by poly ethylene glycol and was kept at -20°C until used (Butler, 1983).

2.10.2. Preparation of whole cell lysate of bacterial isolates

The isolates were grown on blood agar and the colonies were put in 2 ml normal saline a,1 ml of excess fluid was transferred to other blood agar plate using pipette. The plate was incubated at 37°C for 24-48 h. Then colonies were covered with 0.5 % formalin saline and were left overnight at 4°C to kill the bacteria,then the pellet was washed 2 times with normal saline at 4000 rpm for 30 minutes at 28°C,The deposit was suspended in PBS and sonicated in sonicator (MSE-England) for 30 seconds stroke and in short intervals, the amplitude was kept 18 rpm per cycles (Srivastava, 1998) and then concentrated by poly ethylene glycol and were kept at -20°C until used.

2.10.3. Detection of antibody in milk immunoglobulin fraction and sera

Agar gel immunodiffusion (AGID) test was done to detect the presence of antibodies in milk and sera using Whole cell lysate of the isolates.

2.10.3.1. Agar gel immunodiffusion test

The agar was contain 3 wells, in one well was placed 30µl of whole cell lysate (WCL) of bacterial antigen and on the other 2 wells was placed 30µl of the antibodies (one was serum and the other was milk antibodies). The gel was incubated in humidity chamber for 24-48 hours. The test was read against the illuminator chamber.
CHAPTER THREE
RESULTS

3.1. Bacteria isolated from mastitic milk

In this study 50 milk samples were collected and bacteria was isolated from 38 (76%) of the milk samples. The bacteria was identified to the species level as shown in tables (1-8).

3.2. Bacteria isolated from cows with clinical mastitis

Bacteria were isolated from all the thirteen (26%) milk samples collected from cows with clinical mastitis. Mixed infection was encountered in 3 (6%) samples only. The main bacteria isolated were Staphylococcus species.

3.3. Bacteria isolated from cows with subclinical mastitis

Bacteria was isolated from 29 (64%) of milk samples collected from 37 cows with subclinical mastitis as a previously detected by California mastitis test. Mixed infection was found in 7(14%) of sample. The main bacteria isolated were Staphylococcus species.

3.4. Mixed bacteria infection

Mixed infection was found only 10 milk samples (20%) of both clinical and subclinical mastitis.

Most isolates were Staphylococcus 27 (60%) all of them were coagulase positive (10 (22%) Staphylococcus aureus which show double zone of hemolysis on the blood agar (Fig. 1), 9 (20%) Staphylococcus hyicus and 8 (18%) Staphylococcus intermedius) (Table 1), Streptococcus agalactiae was 1 (2%) of the isolate (Table 2, and Fig. 2), Enterococcus species were 4 (9%) (1, (2%) Enterococcus avium, 2 (4%) Enterococcus mundtii and 1 (2%) Enterococcus faecium (Table 3), Listeria Ivanovii were 2 (4%) (Table
4), Bacillus Species were 3 (7%) (2 (4%) *Bacillus licheniformis* and 1 (2%) *Bacillus mycoides* that show heamolysis on blood agar (Table 5 and Fig. 3), three strain of *Corynebactrium psedotuberculosis* (7%) were found with three strains strain 1 (2%) was differed from the main genus by acid from xylose and hydrolysis of aesculin the two were found positive in this strain but were negative in the main strain, strain 2 (2%) was differed from the main genus by acid from xylose, Salicin and hydrolysis of aesculin the three were found positive this strain opposite to the main strain and the last strain 3 (2%) was differed from the main strain by xylose, salicin, hydrolysis of Aesculin and VP they all were found positive in this strain but were negative in the main strain (Table 6), Enterobacteria species were 3 (7%) 2 (4%) *Citrobacter freundii* and 1 (2%) *Klebsiella pneumoniae* (Table 7), the two strain of *Pseudomonas aeruginosa* (alkali producers) (4%) but they were differed from the main genus by acid from glucose it was negative in the primary biochemical test in this strain but positive in the main strain however the all (the 2 strain and the main genus) were read on the secondary table of the biochemical test after suppose that the acid from glucose is negative (Table 8).

3.5. Detection of specific antibody to the causative agent of mastitis in sera and milk

The serums' antibodies of all isolates gave positive result in a form clear precipitation lines on AGID test (27%) exception that of the Staphylococcus, Streptococcus and Enterococcus species on the other hand the milk's antibody of all isolates gave negative result on AGID test exception that of the *Bacillus mycoides* species (0.02%) (Table 9 and Fig. 4).
Table (1): Biochemical reaction of *Staphylococcus* species isolates

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th><em>Staphylococcus aureus</em></th>
<th><em>Staphylococcus intermedius</em></th>
<th><em>Staphylococcus hyicus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Sphere (cocc)</td>
<td>Sphere (cocc)</td>
<td>Sphere (cocc)</td>
</tr>
<tr>
<td>Spore</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate (F\O\ -)</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>VP</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coagulase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Maltose</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from Mannitol</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid from Xylose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

O= Oxidative, F= Fermentative, VP= The Voges –Proskauer.

Table (2): Biochemical reaction of *Streptococcus agalactiae*

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th><em>Streptococcus agalactiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Sphere (cocc)</td>
</tr>
<tr>
<td>Spore</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate (F\O\ -)</td>
<td>F</td>
</tr>
<tr>
<td>CAMP test</td>
<td>+</td>
</tr>
</tbody>
</table>

CAMP=Christie, Atkins and Munch - Petersen
### Table (3): Biochemical reaction of Enterococcus Species

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Enterococcus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) Avium</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Sphere (cocci)</td>
</tr>
<tr>
<td>Spore</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate (F\O\ -)</td>
<td>F</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 45</td>
<td>+</td>
</tr>
<tr>
<td>Yellow pigment</td>
<td>+</td>
</tr>
<tr>
<td>Bile-Aesculin</td>
<td>+</td>
</tr>
<tr>
<td>VP</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of Aesculin</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of Arginine</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of Starch</td>
<td>-</td>
</tr>
<tr>
<td>Fermentation of Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation of Adonitol</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation of Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation of Lactose</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table (4): Biochemical reaction of *Listeria ivanovii*

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th><em>Listeria ivanovii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Spore</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>+ (gas)</td>
</tr>
<tr>
<td>Carbohydrate (F\O\ -)</td>
<td>F</td>
</tr>
<tr>
<td>B-haemolysin</td>
<td>+</td>
</tr>
<tr>
<td>CAMP test</td>
<td>-</td>
</tr>
</tbody>
</table>
Table (5): Biochemical reaction of Bacillus spp.

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Bacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) Mycoides</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Spore</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate (F\O\ -)</td>
<td>-</td>
</tr>
<tr>
<td>In ammonium salt media acid from Glucose</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Glactose</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Salicin</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Xylose</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>VP</td>
<td>+</td>
</tr>
<tr>
<td>Indol</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate</td>
<td>+</td>
</tr>
</tbody>
</table>
## Table (6): Biochemical reaction of *Corynebacterium psedotuberculosis*

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th><em>Corynebacterium psedotuberculosis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Spore</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>+ (gas)</td>
</tr>
<tr>
<td>Carbohydrate (F\O\ -)</td>
<td>F</td>
</tr>
<tr>
<td>Acid from Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Salicin</td>
<td>-</td>
</tr>
<tr>
<td>Acid from Starch</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Xylose</td>
<td>+</td>
</tr>
<tr>
<td>VP</td>
<td>-</td>
</tr>
<tr>
<td>Ausculin hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Arginine hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduced</td>
<td>+</td>
</tr>
</tbody>
</table>
Table (7): Biochemical reaction of Enterobacteria

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th>Klebsiella Pneumoniae</th>
<th>Citrobacter freundii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Spore</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>+ (gas)</td>
<td>+ (gas)</td>
</tr>
<tr>
<td>Carbohydrate (F:O: -)</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Maconkey growth</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gluconate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Negative</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Glycerol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Maltose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Mannitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Raffinose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Rhamanose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Salicin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid from Sorbitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Sucrose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Xylose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Starch</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table (8): Biochemical reaction of *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Biochemical test</th>
<th><em>Pseudomonas aeruginosa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>-</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Spore</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Growth in air</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Glucose(acid)</td>
<td>Negative</td>
</tr>
<tr>
<td>Carbohydrate (F\O\ -)</td>
<td>O</td>
</tr>
<tr>
<td>Maconkey growth</td>
<td>+</td>
</tr>
<tr>
<td>Green Pigment</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate</td>
<td>+</td>
</tr>
<tr>
<td>Simmon's Citrate</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>Gluconate</td>
<td>+</td>
</tr>
<tr>
<td>Malonate</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Glucose</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Maltose</td>
<td>-</td>
</tr>
<tr>
<td>In ammonium salt media acid from Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Fructose</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>In ammonium salt media acid from Xylose</td>
<td>+</td>
</tr>
</tbody>
</table>
Table (9): Detection of specific antibody to causative agent in sera and milk of infected cows in agar double diffusion test

<table>
<thead>
<tr>
<th>Bacteria species</th>
<th>Serum Antibodies</th>
<th>Milk Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus intermedius</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus hycus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus mundtii</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus avium</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Listeria Ivanovii</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus mycoides</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus licheniformis</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Corynebactrium</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella Pneumoniae</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. (1): Growth of *Staphylococcus aureus* on blood agar causing double zone of hemolysis.
Fig. (2): Growth of *Streptococcus agalactiae* on the blood agar.
Fig. (3): Growth of *Bacillus mycoides* notice haemolysis of blood agar
Fig. (4): Sera from cows with mastitis gave specific precipitation lines with respective whole cell lysate of corresponding isolate.
CHAPTER FOUR
DISCUSSION

Fifty milk and serum samples were collected and bacteria were isolated from only 38 (76%) milk samples.

The most common organism isolated in this study was Staphylococcus spp. Staphylococci were (60%) of the total of isolates and all of them were coagulase positive. Staphylococcal mastitis is world wide distribution (Elgadasi, 2003). Staphylococcus spp is the cause of contagious mastitis that is mainly sub-clinical and chronic but may be per acute and lead to gangrene of udder involved mammary quarters.

Coagulase positive staphylococci (60%) were the most frequently isolated bacteria in this study and all of them were isolated from clinical cases. These findings were in agreement with the findings other authors (Dever Siege, 1979; Bramley, 1975; Rahman and Box, 1983).

Radostits et al. (2000) mention that Staphylococcus aureus is first microorganism incriminated in bovine mastitis. A predominance of Staphylococcus aureus mastitis in cows has been reported by Watts (1988); Falade et al. (1989) and Carlos (1990). Elsayed, (2000) was isolated Staphylococcus aureus (8.85%) and Staphylococcus hyicus (8.85%) from 499 milk samples from different domestic animals: cows, sheep, goat and camels and these agreed with AlAyies (2004) who isolated Staphylococcus aureus (73.7%) and Staphylococcus hyicus (6%) from 100 bovine mastitic milk samples. Staphylococcus
intermedius was isolated and these agreed with Chaffer (1999). Corynebacterium pseudotuberculosis was isolated in this study and this in agreement with Jozef (1999). Bacillus licheniformis was isolated and this is in agreement with Logan (1988). Listeria ivanovii was recovered from milk of mastitic cattle and these agreed with Rawool et al. (2007). Streptococcus agalactiae was isolated and this is in agreement with David (1997).

In this study Bacillus mycoides was isolated from clinical case and Bacillus licheniformis were isolated from sub-clinical case These findings were in agreement with the findings of other authors (Elgadasi,2003) who reported that Bacillus spp. were isolated in both clinical and subclinical case.

Douglas (2005) mention that almost half of all clinical cases of mastitis are caused by Gram-negative bacteria. Among these bacteria, intramammary infection by Pseudomonas aeruginosa and this deferent from this study in which Pseudomonas aeruginos were found in subclinical case.

Isolation of Klebsiella spp. this is in agreement with Cullor (1992), who found that 20% of bovine mastitic case, in Nordic countries caused by coliform of witch about 85% were E. colli, in the rest Klebsiella spp., and other Enterobacteria isolated. Jackson and Bramle (1983), mentioned that the Coliform such as E. coli, Klebsiella Pneumoniae, Klebsiella oxytoca, Enterobacter cloatae, Enterobacter arogenes and Citrobacter freundii are all associated with bovine mastitis and this is in agreement with this study in witch Klebsiella pneumoniae and Citrobacter freundii were isolated.
Jayarao et al. (1991) found Enterococcus spp. such as Enterococcus faecium and Enterococcus faecalis related to this study which found Enterococcus faecium, Enterococcus mundtii and Enterococcus avium.

Mammary gland immunity is a rapidly evolving field of research in veterinary medicine (Carneiro et al., 2009). In this study whole cell lysate of bacterial isolates was tested against sera and milk, in order to detect specific antibodies against the isolates. Streptococci, Enterococci and Staphylococci gave no precipitation line with milk or sera. This failure of interaction with their specific antibodies might be explained by the presence of protein A and Streptoprotein G that interfere with the serology of these organisms. However other Bacterial isolates gave frank precipitation lines with their respective serum antibodies but not with milk. In contrast, Bacillus mycoides gave clear precipitation lines with sera and milk although it is not common within the bacteria that cause bovine mastitis (Blood et al., 1994). These results are in agreement with the finding of (Carneiro et al., 2009). The significance of these antibodies in protection against mastitis and the immunology of the udder were reviewed by Carneiro et al., 2009).
CONCLUSION AND RECOMMENDATIONS

Conclusions

From this study it can be concluded that:

1. This study has shown that Gram positive bacteria, especially Staphylococcus species were the common causative agent of bovine mastitis.

2. The number of cows with subclinical mastitis was higher than cows than cows with clinical mastitis.

3. The bacteria associated with mastitis can induce local and systemic specific antibodies response as measured by agar gel immune-diffusion test.

Recommendations

1. To study local udder immune responses to the bacteria associated with mastitis.

2. To investigate the role of local antibodies and systemic antibodies in protection of the udder with a view to formulate protective vaccines.
REFERENCES


