Introduction

Mastitis in small ruminants is a momentous disease that affects not only meat and milk production, but the wellbeing of small ruminants and human health as well. Bacteria generally cause mastitis, it is the most common disease in livestock species, and it is one of the major diseases in the veterinary field (Abdullah, 2016; Tolone *et al.*, 2016).

Mammary infections can lead to various clinical or subclinical diseases in sheep. These are bacterial mastitis (‘mastitis’), mycoplasma mastitis (contagious agalactia) and lentiviral mammary infection (Gómez-Martín *et al.*, 2013; Minguijón *et al.*, 2015; Gelasakis *et al.*, 2015). In lactating dairy goats, the inflammation of the mammary gland is one of the most common infectious diseases. It is responsible for important economic losses and it can reduce yield and quality of the milk (Zaninelli *et al.*, 2015), Therefore, mastitis is one of the important pathologies in goats with serious financial consequences (Bourabah *et al.*, 2013). Furthermore mammary infections are the primary causes of ‘Milk-drop syndrome in ewes’ (>85% of all causes), the syndrome has been defined as a pathological entity at flock level, characterized by reduced milk yield of lactating ewes, with no clinical signs specific to a disease (Giadinis *et al.*, 2012).

In dairy-type flocks, mammary infections have an obvious financial significance, due to the reduction in milk yield, the downgrading of milk quality and the rejection of milk after antibiotic administration. Nevertheless, mammary infections are important also in meat production flocks, as reduced milk yield of ewes has been shown to lead to suboptimal growth of their lambs (Fthenakis and Jones, 1990). The conclusions of a recent meeting of a working group in ‘Welfare of sheep’ of the Animal Health and Welfare Panel of the European Food Safety Authority (EFSA), indicated that mastitis is one of the three most

important problems adversely affecting welfare of sheep across the range of sheep production and management systems (EFSA, 2015). Small ruminant production plays an important role as an income generating activity, particularly for the smallholders, whilst being a source of animal protein to support the national program (Windria *et al.*, 2016)..

It is estimated that over 80% of the world's goat population is located in Asia and Africa (Morand-Fehr *et al.*, 2004). Milk provides the major source of income in dairy farms. Therefore, every disturbance in producing optimal milk quantity and milk quality will reduce farm profitability. Milk quantity and quality are related to genetics (i.e., breed) and environment (i.e., nutrition and management) as well as to animal health (i.e., udder health) (Rovai *et al.*, 2014).

Objectives

a) Overall Objective

The aim of the current study was to report on microbial causes and antibiogram status of clinical mastitis in sheep and goats.

b) Specific Objectives:

- To isolate and identify the prevailing causal bacterial organisms from mastitis positive milk samples.
- To carry out the mode of response of isolated bacteria to the commonly antibiotics in use.
- To investigate and assess the effectiveness of classic bacteriological examination supported with usage of VITEK2 system in the diagnosis of clinical mastitis in sheep and goats.

Chapter One

Literature Review

1. Mastitis in Sheep and Goats

1.1. Definition

Mastitis is any inflammation of the mammary gland or udder regardless of its origin, severity or evolution (Bergonier and Berthelot, 2003). The term mastitis comes from the Greek words *mastos* (for breast) and *itis* (for inflammation). Mastitis is a disease that occurs in several different forms. In general, mastitis is divided into clinical, sub-clinical, and chronic (Doğruer *et al.*, 2010).

1.2 Types of mastitis

Clinical mastitis is the term used for bacterial infections of the mammary gland that are present with obvious symptoms. Visible indications of clinical mastitis include swelling, redness or necrosis of one or more half udders and abnormal discharge of milk (presence of clots or serum), as well as other symptoms such as anorexia, fever or agalactia. Usually the consequence of clinical mastitis is toxemia and gangrenous necrosis of the udder (Omaleki *et al*., 2011). Other definitions are available for the classification of clinical mastitis as the term mastitis indicates presence of an inflammatory process in the mammary gland; usually, a bacterial infection triggers an inflammatory reaction, which, in turn, leads to the development of variably detectable clinical signs (Bergonier and Berthelot, 2003). The clinical syndrome can follow a variable course, ranging from hyper-acute to chronic. The occurrence of chronic disease may produce clinical pictures, which become gradually less clinically evident (Bergonier *et al*., 2003). Depending on the clinical course, mastitis can be differentiated into:

1) Hyper-acute, presenting a serious udder inflammation accompanied by an evident systemic response.

2) Acute, when a serious udder inflammation is present but there is no systemic involvement.

3) Chronic, when there is no systemic involvement and only presence of fibrous lesions.

4) Sub-acute, when clinical signs are less evident compared to classical acute forms.

5) Subclinical, when clinical signs are not present and mastitis can only be detected by means of laboratory examination procedures (Olechnowicz and Jaśkowski, 2014).

Noteworthy, subclinical mastitis is characterized by inflammation of the udder detected by enumeration of inflammatory cells in milk (White and Hinckley, 1999). Ewes/does with mammary glands having no clinical abnormalities and giving apparently normal milk, which is bacteriologically positive and with a high SCC score, are considered to have subclinical mastitis (Kiossis *et al*., 2007).

1.3 Incidence and aetiology of Mastitis

Clinical mastitis incidence in does and ewes is usually below 5% per year. However, certain herds and flocks with management problems may experience rates above 30% (Hogan, 1999; Goodridge *et al*., 2004). Subclinical mastitis incidence and prevalence are defined by the bulk SCC of the herd. In French does, SCC values of 750,000, 1,000,000 and 1,500,000 cells/ml correspond to 30%, 39%, and 51% prevalence of subclinical mastitis (Hogan, 1999). Similarly, Spanish flocks with SCC values of 250 000 or 1,000,000 cells/Ml corresponded to subclinical mastitis prevalence of 16 and 35% respectively (Arroyo *et al*., 2011).

The incidence and aetiology of mammary abnormalities were examined in a study of Greek dairy ewes; abnormal milk and lumps, nodules, diffuse hardness, abscesses and cysts were detected in the udders, with a cumulative incidence of 5.1%. 47% of the cases developed during the first three weeks after the cessation of lactation (Saratsis *et al.*, 1998). In meat sheep, culling due to mastitis in Rambouillet sheep after one lactation in the USA was reported to be 46% (McFarland *et al.*, 2000; Kretschmer, 2007), and in the UK between 13% and 50% of ewes were reported to have udder lesions indicative of mastitis after inspection at the abattoir (Bocklisch and Wetzstein, 1994). In the USA, mastitis in dairy sheep was reported in up to 35% of ewes. As identified by a positive milk culture, and in 4 to 17% by positive SCC and milk culture (Menzies, 2001). In a study of 6500 ewes on a large German sheep breeding unit over two lambing, an average of 7% of the ewes suffered from clinical mastitis and 84% of the ewes had pathogenic bacteria in their udders (Bocklisch and Wetzstein, 1994). In a study of Irish ewes over three years, the incidence of acute clinical mastitis was reported to be 0.53% (Onnasch *et al.*, 2002). In the UK, the results from 2092 milk samples had been collected at three-weekly intervals from 358 ewes, the total incidence of subclinical mastitis was11.7% and its prevalence was between 5.5% and 7% during lactation, (Watkins *et al.*, 1991). The prevalence of subclinical mastitis increased with the age of the ewes, but was not influenced by the presence of teat lesions.

In previous study Smith, (1990) reported that 62% of occurrences of clinical mastitis were due to environmental pathogens as coli-form and several species of *Streptococci*. Globally, staphylococci are the most common mastitis-causing agents in cows, buffaloes, ewes, does and even woman (Rowan *et al.*, 1996; Moroni *et al.*, 2006); followed by

Streptococci and *Escherichia coli (E. coli)* which in some species may have a similar or higher prevalence than that of staphylococcal mastitis, less commonly, other Gram-positive bacteria (*Actinomyces* spp., *Corynebacterium* spp., *Bacillus* spp., *Mycobacterium* spp., *Enterococcus* spp., *Clostridium* spp.) and Gram-negative bacteria (*Klebsiella* spp., *Citrobacter* spp., *Serratia* spp., *Proteus* spp., and *Pasteurella* spp.). Mycoplasma may be involved in the aetiology of mastitis while cases due to moulds or yeasts are rare (Eberhart *et al*., 1987).

Environmental organisms include: *E. coli*, *Enterobacter aerogenes* and other Gram-negative bacteria and (CNS), environmental Streptococci (*Str.*), yeast or fungi are isolated and from the skin, teat ends, and teat canals where they can gain access to the gland causing IMI (Watts, 1988).

1.4 Epidemiology

In dairy animals, mastitis causing pathogens are classified depending on their epidemiological behaviour (Eberhart *et al*., 1987). However, such description is not as clear for small ruminants as it is for dairy cattle. During the milking of ewes, the likely source of coagulase-negative staphylococci (CNS) is the skin on the teats along with skin on the inner side of legs; thus, many CNS infections become long-term chronic infections (Piessens *et al*., 2011). CNS may leak out to the milk from the infected udder and then spread via the milking equipment to other ewes. Thus, the source of mastitis pathogens in small ruminants should not be assumed based simply on the behaviour of these pathogens in dairy cows (Olechnowicz and Jaśkowski, 2014). Nonetheless, in clinical mastitis in small ruminants the highest incidence of clinical mastitis has been reported is 22% (Fthenakis, 1994; Las Heras *et al*., 2002; Bergonier *et al*., 2003; Barber *et al*., 2006; Arsenault *et al*., 2008; Koop *et al*., 2010), particularly in outbreaks associated with particular

environmental conditions or in association with other infectious diseases, such as orf disease (Burriel, 1997). According to Moulk, (1954) poor mothering of lambs, either because of a reduced mothering instinct or because of udder problems, including those caused by intra-mammary infection, blind teats or poor nutrition, has been implicated as the most significant contributor to the high mortality rate in lambs.

Some less frequently occurring problems like udder injuries, sanguineous mammary secretion, mammary hematomas, mammary cysts, mammary neoplasias, mammary oedema and rupture of the suspensor ligament of the udder have to be considered in the differential diagnosis (Fragkou *et al*., 2014).

The most important two causative agent of the mastitis are *Staphylococcus spp*. and *Mannheimia haemolytica*, together accounting for over 80% of isolates from field cases of the disease (Bergonier *et al*., 2003). Although it has been repeatedly confirmed that the teat is the portal of entry of the causal agents, the epidemiology of these two organisms has not been fully described, although various theories regarding their source(s) have been proposed. For example, in the study of Scott and Jones (1998), has been suggested that *M. haemolytica*, which is part of the bacterial flora of the tonsils and naso-pharynx of lambs may invade the mammary gland of ewes during suckling; where staphylococci have been considered to originate from milkers' hands or from the teat skin (Bergonier and Berthelot, 2003). The teats duct may also harbour bacteria, which under certain circumstances could invade the mammary parenchyma and cause mastitis. Nevertheless, in some studies the epidemiology of the predominant pathogens has been described clear. In flocks of meat sheep, the transmission of *Staphylococcus aureus* (*S. aureus*) between ewes could be a result of the herdsman during

manual udder control, or the udder being exposed to bedding material contaminated from infected ewes (Watson and Buswell, 1984). The main reservoir of the contagious pathogens is the mammary gland. *S. aureus* and *Str. ssp* are considered among the contagious pathogens. Consequently, these bacteria are spread from animal to other or between udders of the same animal during the milking process. However, its prevalence in caprine herds is low and transmission between does is infrequent (Contreras *et al.*, 2003). Apparently this refer to that *S. aureus* is germ capable of contaminating different surfaces and easily isolated from the skin and mucosa of healthy animals. The role of environmental sources of *S. aureus* is less important than its presence in the mammary gland habitat, but may help in the spreading of infection from milking of infected animals, milking machines, dairy workers or even flies can transmit it, but are not the origin of these infections (Roberson, 1999). The bacteria causing mastitis are often classified as "contagious" if it is believed that the source of infection of milk is an infected mammary gland with subclinical mastitis pathogens, or "environmental", if the bacteria are considered to be opportunistic pathogens that normally reside in the environment around the animals (Ruegg, 2011).

Enterobacteria, mainly *E. coli*, were obtained from 7.3% of the clinically affected udder halves. However, the number of clinical cases caused by Gram negative bacteria and *A. pyogenes* may be underestimated because the samples were frozen before bacteriological analysis (Schukken *et al.*, 1989; Sánchez *et al.*, 2003).

The prevalence of infection and the causative agents may vary with the husbandry and environment of the ewes (Quinlivan, 1968; Kirk *et al.*, 1980; Shoop and Myers, 1984; Hueston *et al.*, 1986; McCarthy *et al.*, 1988; Bor *et al.*, 1989; Peris *et al.*, 1991; Larsgard and Vaabenoe, 1993;

Ahmad *et al.*, 1992a,b; Fthenakis, 1994). The bacterial agents most commonly isolated vary with clinical or subclinical mastitis, and include CNS, other *Staphylococcus* species, *Pasteurella* species, *Actinomyces (Corynebacterium) pyogenes, Streptococcus ssp* and *E.coli* (Watson and Buswell, 1984; Arranz and de Heredia, 1989; Bor *et al.*, 1989).

In order to clarify the epidemiological picture of the predominant bacterial causes of clinical mastitis a study by Lafi and Hailat, (1998) in lrbid Governorate, Jordan, showed that *S. aureus* is the predominant cause of mastitis in cows and ewes, this may be due to the mechanism of virulence in Staphylococcal infections and the ability of the organism to invade tissues rather than to excrete substances. The contamination of milker's hands, wash cloths, milking machine cups and bedding grounds may increase the incidence of *S. aureus*.

The predominant position of *Streptococcus agalactiae* as a cause of clinical mastitis has been usurped by *S. aureus*e specially, in areas such as Jordan where the treatment of mastitis with penicillin has been practiced intensively and machine milking has replaced hand milking (Farah, 1992). The change in balance away from Gram-positive cocci to Gram-negative bacteria has been significant because they are resistant to hygienic control measures (Radostits *et al.*, 2000).

1.5 The important Causative agents of Clinical Mastitis in Sheep and Goats

1.5.1 Bacterial causative Agents

1.5.1.1 Gram positive agents

Staphylococcus species

Staphylococcus species are Gram-positive cocci in clusters, non-motile, non-sporing, aerobic and facultative anaerobic. It is catalase-positive; usually oxidase-negative. Hydrolyse arginine; produce acetone.

Attack sugars by fermentation (Barrow and Feltham, 2004). Considering the fact that *S. aureus* and CNS are a part of important causative agents of clinical and subclinical mastitis, respectively (Windria *et al*., 2016), *S. aureus* isolated from mastitis (11 ovine strains) exhibited an ability to adhere to epithelial primary cultures from ovine mammary gland and epithelial cell line (Iturrlde *et al*., 1993). On the basis of elevations of activities of CMT *S. aureus* was the most pathogenic in clinical and subclinical mastitis. *S. hyicus* showed only marginal pathogenicity (Maisi and Riipinen, 1991).

Streptococcus species

Str. species are Gram-positive cocci in pairs or chains, non-motile, non-spore forming, aerobic, facultative anaerobic, catalase and oxidase negative. Ferment carbohydrates (Barrow and Feltham, 2004). Despite the pathological importance of streptococci in mastitis, they do not represent a group of especially prevalent pathogens on goat farms and are usually reported in somewhat less than 5–10% of cases of caprine mastitis. They mostly cause clinical mastitis, and a number of epidemiological studies on subclinical mastitis even report the absence of these pathogens. This situation seems clearly different in certain areas of dairy sheep production, in which major problems on farms are diagnosed due to *Str. agalactiae* (Contreras *et al*., 2003). Because of this absence of *Str. agalactiae* in aetiology of goat's mastitis, most of the diagnosis is related to environmental streptococci. Therefore, these occasional diagnoses of streptococcal mastitis in dairy goats must be associated with problems of environmental contamination, particularly from poor bedding conditions, as for streptococci (Contreras *et al*., 2003).

Enterococcus species

Enterococcus are Gram-positive cocci in pairs or short chains. Non-motile (except two species), non-sporing, aerobic, facultative anaerobic, catalase and oxidase-negative. Attack carbohydrates fermentative (Barrow and Feltham, 2004). Those Gram positive bacteria are associated with mastitis in sheep, goats and cattle; include *Enterococcus faecium* and *Enterococcus faecalis* (Contreras and Rodríguez, 2011).

1.5.1.2 Gram-Negative agents

E. coli

E. coli is Gram-negative rods, motile, aerobic and facultative anaerobic. Catalase-positive, Oxidase-negative, ferment sugars and produce gas (Barrow and Feltham, 2004**)**. *E. coli* was the most commonly isolated bacteria from clinically mastitic goats in an investigation made in seven government established goat farms in Nigeria (Ameh *et al.*, 1993). *E. coli* are organisms commonly found in the environment and considered opportunists. *E. coli* can be associated with a very hot watery mastitis with toxic signs (Ahmad *et al.*, 1992a).

***Pasteurella* species**

Pasteurella (*P*) are Gram-negative rods, Non-motile, aerobic and facultative anaerobic. Usually catalase-positive, oxidase-positive. Sugars are attacked by fermentation (Barrow and Feltham, 2004). The main source of infection from *P. haemolytica* for the teat of the ewe is the mouth of the lamb. In dairy sheep, where ewes are milked by hand or machine after lambs are removed, the incidence of *P. haemolytica* mastitis is dramatically reduced after lambs are weaned because *P. haemolytica* is no longer present on the teat of the ewe .The results clearly demonstrated that *P. haemolytica*is is a major mastitis pathogen, a

fact not previously appreciated because the traditional view had been that most ovine mastitis was caused by *S. aureus* (Jones and Watkins, 1998).

Enterobacter Species

Enterobacter are gram-negative rods; motile, aerobic and facultative anaerobic. Catalase-positive; oxidase-negative, ferment the sugar and produce gas. VP-positive, gluconate-positive (Barrow and Feltham, 2004). In a previous investigation of mastitis in goats in Tiaret region (Western Algeria), showed a prevalence of 33.9% among the tested goats. Enterobacteriaceae were the predominant bacteria isolated (54.02%) as all, while *Enterobacter spp*. isolated were (17%) (Bourabah *et al*., 2013).

1.5.1.3 Mycoplasma species as causative agents

Mycoplasma is a genus of bacteria that lack a cell wall around their cell membrane. They do not possess rigid cell walls, they are resistant to antibiotics such as penicillin, which interfere with the synthesis of bacterial cell walls. Mycoplasma are the smallest bacterial cells yet discovered typically, about 0.1 µm in diameter. It can be present in the milk of sheep and goats and can cause mastitis in the animal (Quinn and Markey, 2003). Contagious agalactia (CA) is a serious disease of small dairy ruminants that has a substantial economic impact on the goat and sheep milk industries, the main aetiological agent of the disease is *Mycoplasma agalactiae*, although other species, such as *Mycoplasma mycoides* ssp *capri*, *Mycoplasma capricolum ssp capricolum* and *Mycoplasma putrefaciens*, are pathogenic in goats. There are two clinical–epidemiological states of CA in sheep and goats; herds and flocks may exhibit outbreaks of CA or may be chronically infected, the latter with a high incidence of subclinical mastitis and only occasional clinical cases (Gómez-Martín *et al*., 2013). All of the mycoplasmas

isolated were identified as *Mycoplasma agalactiae*, known to be responsible for most cases of caprine CA detected in Spain (Gil *et al*., 1999). Furthermore, in another study 19 herds of Murciano-Granadina goats were examined. A clinical outbreak of classic CA was declared in herd 11 in March. The disease practically affected all lactating goats, leading to a notable drop in milk production and considerably increased cell counts. In herds 6, 9, 13, and 17, mycoplasma-infected animals were culled, with no episodes of CA or observed repercussions on milk yield (Corrales *et al*., 2004).

1.5.2 Viral agents

Deng *et al*., (1986), indicated that the mammary gland is more susceptible to injury by ovine progressive pneumonia virus (OPPV), nine of 15 experimentally infected sheep and all six naturally infected sheep had lympho-plasmacytic mastitis. Severity of the lesion increased with length of time after infection, nodules were seen in five sheep experimentally infected for 2.8 years or longer and in five naturally infected sheep that were 3.7 years old or older.

Caprine arthritis-encephalitis virus (CAEV), like other lenti-viruses, causes a persistent lifelong infection (Smith and Cutlip, 1988). The CAEV induced subclinical infection in most goats. Most of infected does are likely to have a viral infection of the mammary gland, which is a target organ for the virus (Kennedy-Stoskopf *et al*., 1985; Lerondelle *et al*., 1988). Signs of disease include progressive inflammation in one or more organs include udder. Lesions in the udder may arise even before puberty. Diffuse of nodules initially develop deep in the udder tissue and later become gradually more extensive (Bulgin, 1990)

1.6 Diagnosis of Clinical Mastitis

1.6.1 Physical examination

1.6.1.1 Visual examination and udder palpation

Clinical examination of the small ruminants' udder should be performed in order to palpate possible lesions on the skin of the udder and note visually colour changes, injuries and nodules as well as changes in the general shape of the udder for example, increase in size and atrophy. Also, the mammary glands should be palpated in order to fully complete udders' palpation, for example (shape, size, temperature and consistency of each gland should be assessed and recorded (Fthenakis, 1994). Pain is one of clinical symptoms of mastitic udder; therefore, any pain reaction during palpation should be noted. Furthermore the presence of nodules or hardness within the gland should be recorded. In severe cases there may be early gangrene, abscess may develop in the glandular tissues; diffused swelling accompanied by heat and pain is marked to acute inflammations. Local areas of fibrosis may occur in a quarter, varying in size from peak-like lesion to masses as large as a fist (Blood *et al*., 1983). Each teat was held between the thumb and the index finger, and palpated throughout its length to evaluate its shape, size and consistency; teats of the same udder were compared with each other (Mavrogianni *et al*., 2004). In another study in experimentally mastitic ewes, the examined teats represented changes in the mammary secretion (serous or sero-haemorrhagic appearance, with flakes) and the inoculated glands (enlarged, hot, with a red-colored skin). Finally, the supra-mammary lymph nodes of the examined animal should be palpated, in order to detect possible alterations in size or consistency (Mavrogianni *et al*., 2005).

1.6.1.2 Visual Examination of Mammary glands Secretion

The first few streams of milk are to be drawn from the teat onto a paddle or on the palm of the gloved handoff the investigator. Expression of milk can be evaluated; for example, expression in drops can be indicative of teats enosis. Then, presence of abnormal features in mammary secretion (e.g., clots, flakes, tints) should also be recorded (Mavrogianni *et al*., 2004).

1.6.2 Chemical Examination

1.6.2.1 Milk tests

Various tests were used to determine the type of the causative organisms and the infection level of the herd or even individual animal (Bodman and Rice, 1995). Wherefore making recommendations about a diagnostic test, it is important to consider the consequences of false-negative and false-positive results. In the case of mastitis, the costs of treatment or culling of positive animals are important factors, and the aim is generally to reduce the overall prevalence of infection without necessarily eliminating the disease; therefore, specificity takes priority. However, to eliminate a disease, the sensitivity would have a higher priority than specificity (Clements *et al*., 2003; Lafi, 2006). Appropriate tests include California Mastitis Test (CMT) and Somatic Cell Count (SCC) are regarded as the best indirect tests to diagnose intra-mammary infections (IMIs) in both sheep and goats, when they are interpreted correctly (McDougall *et al*., 2001; Bergonier and Berthelot, 2003; Luengo *et al*., 2004; Persson and Olofsson, 2011).

The use of CMT and White Side Test (WST) for diagnosis of ovine subclinical mastitis showed that CMT and WST were useful as quick diagnostic tests of subclinical mastitis. Score “1” for CMT or score "1 + "

for WST are recommended to be used as threshold values to differentiate subclinical mastitis (Schalm *et al*., 1971).

California Mastitis Test (CMT)

The California mastitis test (CMT) is a method used to detect mastitis. Agents in the test react with DNA present in the epithelial and inflammatory cells to form a gel. The more DNA in the milk, the more gel is formed and the higher a count of the bacteria. The results are essentially subjected to assessment of the amount of gel, the degree of precipitation and gel formation are graded according to Schalm and Noorlander, (1957). The grades were: 1) no precipitate; 2) trace precipitate; 3) distinct precipitate/weak gel formation; 4) distinct gel formation; 5) strong gel formation, but they generally correlate well with the SCC (Hueston *et al*., 1986; Menzies, 2001). In a study of Pampinta dairy sheep in Argentina, Suárez *et al*., (2002) found that the correlation between CMT and SCC was reasonably good. However, Keisler *et al*., (1992) and others found that the CMT was less reliable than the SCC for quantifying mastitis. Several studies evaluated the CMT as an indirect test for mastitis. In ewes, most of these studies suggested a cut-off of "1"(300- 1000 of SCC) for the CMT to identify infected glands (Hueston *et al*., 1986; González-Rodríguez and Carmenes, 1996; Las Heras *et al*., 1999; McDougall *et al*., 2001; Clements *et al*., 2003; Peixoto *et al*., 2010). The most of studies in goats proposed score "2" as the threshold for the detection of infected glands (Dossantos *et al*., 1995; Contreras *et al*., 1996; Haenlein, 2002; McDougall *et al*., 2010; Persson and Olofsson, 2011), Similarly, Peixoto *et al*., (2010), reported better agreement (81.4%) with bacteriological exams for the CMT scores ≥ 2 (Souza *et al*., 2012). On the other hand, in a wide array of scientific reports, authors have considered an increase in SCC as a marker of mastitis and the

identification of the bacterial species causing the mastitis was most often neglected.

Moreover a variety of factors (e.g., age of ewe/does, breed of ewe/does, stage of lactation, number of lactation, milk yield, time of the day at sampling, daily frequency of milking, number of lambs suckled) influence the results of somatic cell counting (Raynal-Ljutovac *et al.*, 2007). Therefore, the standard tool in the diagnosis of mastitis in small ruminants is provided by bacterial culture (Contreras *et al.*, 2007). The classification error of CMT can range between 25% and 50% (Ruegg and Reinemann, 2002; Pyörälä, 2003). No doubt, one of the disadvantages of the CMT is that occasionally, acute clinical mastitis milk will not score positive due to the destruction of leucocytes by toxins from the infecting organism (Rice, 1981). For, economic and practical reasons, only one sample of milk is taken to diagnose IMI (Contreras *et al.*, 1997) and the accurate identification of infected animals relies on the positive culture of pathogens from aseptically collected milk samples (Souza *et al.*, 2012).

Somatic Cells Count (SCC)

The most common method for the detection of high bacteria counts in milk is the measurement of the somatic cell count (SCC), which gives an indication of whether an immune response to mastitis has begun in an animal. The numbers of epithelial and inflammatory cells (per ml) are counted in a milk sample, usually taken on the ‘test’ days at regular intervals during lactation. The numbers of somatic cells indicate the degree to which an immune response has been mounted against organisms that cause intra-mammary infections. The SCC can be regarded as a surrogate measure of mastitis (Barillet *et al.*, 2001). It is important to know the threshold level of SCC because it has impact on the milk production and health of sheep in dairy sheep, current estimates

in the range of 600,000 to 800,000 cells per ml indicate presence of infection (Billon and Decremoux, 1998) and ewes with less than 500,000 cells per ml are considered to be 'healthy' (Barillet *et al.*, 2001). The current legal milk SCC limit for bulk tank milk for goats and sheep in the United States is 1000 and 750 × 1000/ml, respectively. Milk somatic cell counts for goats are higher than milk SCC for cows and sheep (Paape *et al.*, 2001)

Modified White Side Test (WST)

California Mastitis Test and Whiteside Test are recommended as threshold values for subclinical mastitis, offering at least 93% accuracy of the diagnostic methods. In the WST, nucleic acids of the leucocytes of milk form a sodium salt with NaOH producing a gelatinous mass to which serum solids and fat globules become absorbed to produce the characteristic precipitate of the reaction (Schalm *et al*., 1971). The test is performed by adding (1-2drops) of N sodium hydroxide solution 0.4% to (5drops) of cold milk on glass on black background and then stirring the mixture vigorously for 20 seconds. In positive reaction the milk was separate to water and shreds or flakes but in negative reaction the mixture remains uniformly opaque (Kelly, 1984).

1.6.2.2 Indication Papers tests

pH indication papers

The test strips detect the more alkaline pH in quarters with mastitis. Normal milk has a pH of approximately 6.5 to 6.7 where as mastitis milk often approaches plasma pH of 7.4 (Rebhun *et al*., 1995; George *et al*., 2008).

Mastitis Card Test

The test was carried out by adding drops of milk sample to the spot indicated on the indicator paper and observing for possible colour

changes. A change of colour from yellow to green or bluish green on the indicator paper was considered positive. Those weak reactions with light green colour were regarded as doubtful and not included in the result as positive (Megersa *et al*., 2010).

1.6.3 Ultrasonographic Examination

Ultrasonography is useful in monitoring changes in the examined udder and provides significant information about the udder status, whilst being non-invasive, non-ionising, rapid and painless. Ultrasonographic imaging of the teats can be carried out with ewes/does in the standing position, with an ultrasound scanner (Franz *et al*., 2001). The technique is useful for identification of structures, which, due to their size and location, cannot be identified during clinical examination, as well as for differentiation of structures identified in the parenchyma (e.g., haematoma, abscess, granuloma) (Lazaridis *et al*., 2012).

1.6.4 Endoscopic Examination

Endoscopic examination is effective and allows the evaluation of abnormalities of the teat duct, and teat cistern. Teat endoscopy is an easy, non-invasive and effective method for the diagnosis of teat lesion in dairy sheep. This method appeared to be objective and practical and can be applied in field conditions. Nodular proliferations were the most common findings followed by the diaphragms.The colour of the mucosa can help to distinguish between a recent and an old haemorrhage of the lamina propria. In case of a post-mortem examination, histopathological investigation ensures a more integrated picture of the lesion, especially concerning the aetiopathology in intensive dairy sheep breeding (Kiossis *et al*., 2009). Advantage of endoscopy is the detailed, real-time imaging of the teat and the lower part of the parenchyma. Nevertheless, the method requires extensive equipment and experienced personnel for

successful performance; moreover, there is always a risk of mastitis by introducing into the mammary parenchyma potential bacterial flora from the teat duct (Fragkou *et al*., 2007; Fragkou *et al*., 2014).

1.6.5 Bacteriological Examination of Milk Samples

Bacteriological examination of milk samples remains the 'gold standard' for aetiological diagnosis of the clinical mastitis in small ruminants. Isolation of bacteria in pure culture and sufficient growth from only one milk sample from a ewe/doe with mastitis suffices to demonstrate the aetiological role of these organisms in the disease (Contreras *et al*., 2007). Repeat samplings and bacteriological examinations are required only in following up efficacy of treatment regimes or monitoring control programs. The greatest advantage of bacteriological examination of milk samples is the possibility to demonstrate the aetiological role of the isolated organisms in mastitis. Nevertheless, the method requires some time for definitive results and cannot be performed onsite (Fragkou *et al*., 2014). Specificity and positive predictive value were higher for post-milking samples than for pre-milking samples and false positive diagnoses were more frequent for pre-milking samples. Post-milking samples should be used to diagnosis of goat intra-mammary infection (Sánchez *et al*., 2004).

1.6 Factors affecting Occurrence and timing of the Onset of Mastitis

Several factors have been associated with the occurrence of clinical mastitis in small ruminants such as parity, dystocia, breed, region and the number of lambs/kids born. Notable suckling two or three lambs is associated with a greater mastitis risk than suckling only one lamb per ewe (Larsgard and Vaabenoe, 1993; Arsenault *et al*., 2008; Waage and Vatn, 2008). However, (Arsenault *et al*., 2008), reported an increased risk

of mastitis in ewes suckling triplets, but found there was no difference in the risk of clinical mastitis between ewes with one or two lambs.

Factors related to the herd such as number of lactations, stage of lactation, and age, have been demonstrated to significantly influence prevalence of mastitis in goats; usually, infection rates are less frequent at the first kidding, and increase with age (Ameh and Tari, 1999; McDougall *et al*., 2002; Moroni *et al*., 2005b), goats aged 2 to 5 years are at higher risk of developing mastitis. Furthermore, differences in climate can lead to increase exposure udders to mastitis as shown in study by (Larsgard and Vaabenoe, 1993); they stated that most cases of mastitis occurred on spring pasture.

In addition, production forms and management practices may give rise to differences both in the epidemiology, bacteriology and clinical manifestations of mastitis. The timing of the onset of mastitis is important, in previous review of five studies, by Bergonier *et al*., 2003; they reported that the majority of cases of mastitis occurred from the beginning of machine milking and during the first third of lactation. Other studies have reported mastitis occurring from the first week postpartum to three weeks (Saratsis *et al*., 1998) and caused mainly by different pathogens related to poor environmental hygiene.

There is much information on the possible relationship between anatomical features of the udder and teat and susceptibility to mastitis. In sheep, udder depth and circumference correlate positively with milk production. Teat length is highly correlated with teat diameter. Sphincter patency and speed of milking may influence infection. Some of these characteristics may be related to resistance to mastitis but more stringent investigation is required to establish their possible significance (Jones and Watkins, 1998). Factors significantly associated with increased the

incidence rate of clinical mastitis in ewes were increasing percentage of the flock with poor udder conformation (Cooper *et al*., 2016). Dairy goats with longer teats are more likely to develop clinical mastitis than does with short teats, these observations agreed closely with study in lactating dairy goats in Ethiopia (Megersa *et al*., 2010), they mentioned that "the major risk factors for mastitis prevalence in the studied does were poor body condition, long teat, late lactation, and wet season". This type of teats and udders can easily be exposed to mechanical injuries; as a result, variety of environmental and opportunistic microorganisms gets access to the teat.

In ewes that are housed for a few days after lambing, or when there is repeated use of lambing pens, faecal contamination of teats may predispose ewes to the development of coli-form mastitis. It is for this reason that this form of mastitis is more common during the first week of lactation than at other times. When ewes lie on expanded metal floors, they are more prone to teat injuries and mastitis than when on straw. Other environmental factors have been cited but are of uncertain significance and include cold wind and rain, lush pastures, and the presence of vectors (Jones and Watkins, 1998).

1.7 Economic Impact of Mastitis

Mastitis is of importance from three perspectives (Bergonier and Berthelot, 2003): hygienic, legal, and economic, that represent in reduced growth of lambs and their mortality, as well as mortality of ewes, treatment costs, reduced milk production, milk prices dependent on cellular quality in certain areas.

The worldwide losses due to the disease are approximately $35 billion annually (Bilal *et al*., 2004; Abdullah, 2016). Mastitis has a major impact on both economy and animal welfare in small ruminants

production and associated with a reduction of milk yield and quality in ewe due both clinical and subclinical ewe's udder infection (Las Heras *et al.*, 1999; Albenzio *et al.*, 2002). In addition to culling of affected ewes, reduction in milk yield has been suspected to decrease pre-weaning growth of lambs, and to increase lamb mortality from starvation in meat-producing sheep flocks (Watson and Busswell, 1984; Menzies, 2001). Reductions in milk production have been reported to be between 20 and 37 per cent leading at weaning to lambs from ewes with mastitis weighing up to 4 kg less than lambs from healthy ewes (Menzies, 2001). Furthermore, Arsenault *et al.*, (2008), reported a significant association between lamb's weaning weight and clinical mastitis in the ewe, but only for multiple litter size at birth or older ewes (older than 4 years old), and attributed the reduction in weaning weight to a reduction of milk production by the ewe.

While, mastitis caused by microbial infections in dairy goats reduces milk yield, modifies milk composition, and potentially contributes to morbidity in herds (Gutierrez-Chavez *et al*., 2016). In this context, manufacture of products from the meat and milk of small ruminants presents an opportunity to improve the economics of farming worldwide, thus, economic losses due to mastitis are of major concern to the dairy industry. Loss of milk quality caused by subclinical infection in dairy sheep has a negative effect on cheese manufacture (Abdelgawad *et al.*, 2016).

1.8 Treatment

Treatment of clinical mastitis in small ruminant had been described by many hypotheses (Bergonier and Berthelot, 2003). Theoretically, the aim of 'in lactation' treatment is the clinical and bacteriological cure of infected halves of clinically affected ewes with

mastitis and functional recovery. In fact, for acute and per-acute mastitis, the aim is to avoid death and allow culling. In any case, affected ewes must be removed from the dairy flock until culling or complete recovery. Antimicrobials are used in treatment of mastitis predominantly during lactation and, less frequently, during the dry period (Onni *et al*., 2011).

1.8.1 Antimicrobial therapy of Mastitis

Bacterial infections are the predominant cause of infectious mastitis and antimicrobial agents remain a component of infectious mastitis treatment and control. The successful control relies much on antibiotic treatments. The use of antibiotics in food animals is under challenge, particularly use of broad spectrum, multi-component products and use of prophylactic treatment. Recent research has confirmed that antibiotic treatment is practical and cost-effective. Moreover, it remains essential for animal welfare (Hillerton and Berry, 2005; Oliver *et al*., 2011).

Antibacterial agents are described as narrow-spectrum if they inhibit only bacteria or broad-spectrum if they also inhibit mycoplasma, rickettsia, and Chlamydia and some antibacterial drugs are also considered narrow-spectrum in that they inhibit only Gram-positive or Gram-negative bacteria, whereas broad-spectrum drugs inhibit both Gram-positive and Gram-negative bacteria (Giguère *et al*., 2013).

Using antibiotic in the treatment of clinical infections needs selection of the most appropriate antibiotic and requires knowing the organism’s identity, the organism’s susceptibility to a particular antibiotic, the site of the infection (targeted tissue in present study is udder), individual factors of ewe/doe, the safety of the antibiotic in use and the cost of antibiotherapy (Richard *et al*., 2012)

An interesting classification of antibiotics that are therapeutically effective against the clinically important bacteria had been reported by Richard *et al*., (2012).

1.8.1.1 Antibiotherapy of Mastitis during Lactation period

The concept of administering intra-mammary infusions of antiseptic solutions as a treatment for mastitis caused by infectious agents has been present for at least a century. The major developmental thrust for antibacterial as a treatment for mastitis has been directed against gram-positive organisms, particularly staphylococci and streptococci (Erskine *et al*., 2003). In addition, application of management practices that decrease the prevalence of contagious pathogens such as *Str*. species and *S. aureus* has shifted the focus of mastitis control and economic losses to environmental pathogens that are associated with more frequent episodes of clinical mastitis. That way, antibiotherapy should start immediately after detection of the first signs of the disease on mastitic udder and should be performed using effective antimicrobial agents (Erskine *et al*., 2003). Progressing of clinical signs in mastitic udder and subsequent damage to the gland is rapid; histological lesions in the mammary gland are evident within a few days after infection. No doubt, early instigation of treatment is important, to minimize mammary lesions and to restore health of the affected ewes and does. Although clinical cure takes place, sometimes bacteriologic cure cannot be achieved. Subsequently, bacteria present in the mammary gland may cause decreased production, develop mammary abscesses, or cause a recrudescence of clinical disease (Mavrogianni *et al*., 2005).

1.8.1.2 Dry-off Treatment

Certain clinical situations require the use of antibiotics for the prevention rather than the treatment of infections. Because the

indiscriminate use of antimicrobial agents can result in bacterial resistance and super infection, prophylactic use of antibiotics is restricted to clinical situations in which the benefits outweigh the potential risks. The duration of prophylaxis should be closely observed to prevent unnecessary antibiotic exposure (Richard *et al.*, 2012). The program applied in previous study by Kiossis *et al.*, (2007) to test the effectiveness of a standard treatment for the control of subclinical mastitis during lactating period in a modern, commercial dairy sheep farm, resulted in limitation of subclinical mastitis during lactation and a better health status for udders entering the dry period. Additionally, more and better quality milk was produced. Systematic treatment of goats at drying-off (1 syringe by half) by a combination of Penicillin, Nafcillin, and dihydro Streptomycin labelled for bovines was an efficient method for the cure of subclinical mastitis and control of SCC at the beginning of the following lactation and that effectiveness of post milking teat disinfection remains to be demonstrated (Poutrel *et al.*, 1997). While the intra-mammary ampicillin dicloxacillin treatment had the best treatment rates, the combination of intramuscular amoxicillin clavulonic acid was also successful. Intramuscular amoxicillin clavulonic acid as the sole treatment was not as effective as intra-mammary therapy (Doğruer *et al.*, 2010).

1.8.1.3 Antimicrobial Resistance

Antibiotic resistance is one of the important problems encountered in the treatment and control of mastitis. Mastitis caused by resistant bacteria is difficult to cure and has severe consequences. Thus, determination of the antibiotic susceptibilities of pathogens causing mastitis is of crucial importance for the treatment and control of mastitis in dairy ewes (Tel *et al.*, 2012). Ovine milk represents a reservoir of

multi-drug resistant bacteria, which representing a potential risk for human health through direct contact and/or animal food consumption (Onni *et al.*, 2011). Judicious use of the antibiotics currently available and better infection control practices might help prolong the effectiveness of the drugs that are currently available. However, even if we improve these practices, resistant bacteria will continue to develop and new drugs will be needed (Giguère *et al*., 2013).

Microorganisms associated with mastitis in dairy goats are commonly controlled with antibiotics, but it is known that continued use of these chemical agents promotes antibiotic resistance among bacterial populations (Gutiérrez-Chávez *et al.*, 2016). It is clear that use of antibiotics in adult dairy animal such as cows, small ruminants and other food-producing animals does contribute to increased antimicrobial resistance. Although antimicrobial resistance does occur, we are of the opinion that the advantages of using antibiotics in adult dairy animals far outweigh the disadvantages (Oliver *et al*., 2011). In order to develop strategies to minimize the increasing of antimicrobial resistance many bacteriocins (antimicrobial peptides produced by certain bacteria), might warrant serious consideration as alternatives to traditional antibiotics, have been used, these bacteriocins have properties which suggest that they could be of value in clinical settings. However, to date, the primary focus for use of these bacteriocins has been on animal, the use of Nisin as the active agent in the mastitis prevention product (Cotter *et al*., 2013).

1.9 Vaccination

Inactivated vaccine against staphylococcal mastitis was carried out in ewes in Spain (Amorena *et al.*, 1994). The vaccine was tested on the ewes by route of two injections were administered, a month before and a month after lambing. The prevalence of IMI did not differ significantly

between the vaccinated and control groups of ewes; nevertheless, the frequency of clinical mastitis was reduced. The efficiency of auto vaccines has not been definitively proven in controlled trials, Stock vaccines exist, but a demonstration of their efficiency for the prophylaxis of IMIs remains to be performed. Currently, vaccination does not appear to be a decisive tool for the prevention of IMIs in the ewe on a large scale (Bergonier and Berthelot, 2003). Vaccines against clinical gangrenous mastitis, that are available on the market for small ruminants, are widely used when there is a high incidence of clinical gangrenous mastitis. However, owing to the reported different effectiveness of these vaccines for dairy cows and sheep, and their inability to prevent new infections (Contreras *et al.*, 2007), a study was conducted by Tollersrud *et al.*, (2002) to compare two vaccines using different adjuvant with regard to their ability to stimulate antibody production against the α- and β-toxins and the exopolysaccharide of *S. aureus*. The results indicated differences between the oil-adjuvant and Carbopol-adjuvanted vaccines with regard to their ability to stimulate antibody production against *S. aureus* protein antigens in sheep. A previous study (Kautz *et al.*, 2014) in Saaneen goat herd demonstrated that vaccination with Lysigin® (commercial bacterin) reduced the prevalence of mastitis and SCC over an 18-month period, it was concluded that vaccination was instrumental in reducing mastitis and lowering the SCC in that particular problem herd that might have otherwise been degraded due to unacceptable milk quality (Kautz *et al.*, 2014).

1.10 Control

The fight against mastitis should not be limited to treating isolated clinical episodes, but also requires the surveillance and control of both, clinical and subclinical mastitis (Paterna *et al.*, 2014). Prevention of

clinical mastitis in small ruminants can be described upon program that includes: vaccination, application of techniques of husbandry. Improved techniques depend on a better understanding of the nature of predisposing factors, and breeding for resistance. In some countries there is increasing interest in the possibility of breeding for resistance to mastitis, for example: Australia, Italy, Norway, Poland and the USA (Jones and Watkins, 1998). Prevention is the key to control clinical mastitis in small ruminants via controlling source of infection. Nevertheless, the factors predisposing to acute clinical mastitis in ewes/does are poorly understood. Teat damage and teat lesions are the most significant causes in that they enable pathogens to colonize and gain entry into the teat canal, thus prevention of acute mastitis entails a high standard of shepherding, cleanliness in lambing areas and good sound management to prevent trauma to udder tissue, particularly the teat ends (Watson and Buswell, 1984) .While Billon and Decremoux, (1998) demonstrated that the IMIs in dairy ewes have different characteristics from those observed in dairy cows. However, prevention and elimination of infections require various measures, for example: control and good maintenance of milking machines, good milking routine without over milking and removal of clusters without impacts; furthermore, hygiene after milking (disinfection of teats) (if possible) and good housing management. The knowledge of risk factors and aetiological agents involved are also important in order to recommend specific and efficient control measures (Arsenault *et al.*, 2008).

The most significant modern approach for controlling mastitis is developing long-term strategies to breed mastitis resistant-sheep; an approach that is considered as a sustainable method for its control (Conington *et al.*, 2008). Notable twelve countries round the world have

included mastitis in breeding programs alongside conventional traits to try to reduce the increases in genetic susceptibility to mastitis induced by selection for increased milk yield (Veerkamp *et al*., 1998). Thus genetic improvement might be a useful option for diminishing mastitis frequency (Bouvier-Muller *et al*., 2016).

1.11 Culling

Among health disorders involved in both culling and mortality, udder disorders appeared to be the most frequent. Clinical mastitis can lead to mortality or culling of up to 90% of mastitic ewes in the flock. Mastitic animals are not often immediately culled, and acute cases may become chronic for several months, therefore, culling of mastitic (even chronic) animals is highly recommended. In ewe flocks, culling for IMI is increasing up to 7% of total causes. In specialized goat herds, 18% of the animals culled or dead for disease reasons experienced mastitis. In the two species, mammary pathology is the first cause of culling for sanitary reasons; it is more frequent during the first months of lactation (Saratsis *et al*., 1998; Malher *et al*., 2001; Bergonier *et al*., 2003). Additional management strategies that may be helpful to control subclinical mastitis include the use of post-milking teat disinfection, culling of chronically infected ewes (Ruegg, 2011).

Chapter Two

Materials and Methods

2.1 Study area

In current study samples were collected from sheep and goats farms. Study zone located in diverse geographical areas of the region.

2.2 Schedule time of Study

Samples were collected during December 2014 and April 2015.

2.3 Animals sampled

Eleven flocks of sheep and goats were selected for the purpose of the current Study. The flocks included seven sheep flocks and four lactating goat flocks. Investigated farms were mainly of local breed in urban and peri-urban areas. Milk samples were collected from only adult lactating ewes/does in the second lactation period, notable lambing / kidding period between October and April. The total of fifty one mastitic milk samples was from (41 ewes and 10 does/duplicate/head), samples were obtained from clinical cases.

2.4 Clinical diagnosis of mastitis

Clinical examination of each ewe/doe with all or some symptoms of mastitis was carried out, at least one clinical sign of mastitis was detected (swelling, redness, heat and pain), the history of all cases was taken, (loss of milk production and inflammatory changes in milk secretions), beside other clinical observations for instance morphology of individual mastitic udder certainly teats. The milk samples collected were containing blood, clots or flakes which were accompanied by discoloration.

2.5 Collection of milk samples

Aseptic technique was adopted in all steps pertaining to milk samples collection. Contamination was avoided by following the procedures described by Harmon *et al.*, (1990). Sterile vials were utilized in samples collection. Cotton balls soaked in 70% alcohol were also used. Cooler with ice packs was used for storing samples. Paper towels and disinfectant were used for cleaning teats. Permanent ink pen was used for labelling. The first few drops of milk were discarded and about 10 ml were collected in the sterile universal bottles and transported on ice to the laboratory in not more than three hours.

2.6 Indirect Detection of Mastitis in sheep and goats

2.6.1 Rapid Test: California Mastitis Test (CMT)

In the present study CMT test was used. Suspected milk samples were mixed with the reagent, the CMT reagent reacted with leucocytes that were usually present in large numbers when an infection occurred (Rice, 1981). The greater of the gel formation indicated high CMT score (Table 2.1). CMT positive and negative milk samples were transported to microbiology laboratory.

2.7. Bacteriological Analysis

Aseptically the CMT positive milk samples were collected for isolation and identification of micro-organisms. Milk samples were stored immediately on cooler with ice-packs. Samples then activated by incubation for 12 hours at 37°C first, and then cultured within 24 hours after collection.

2.7.1. Sterilization

2.7.1.1 Equipment sterilization

All equipment were sterilized according to (Barrow and Feltham, 2004).

Table 2.1 : Interpretation of the CMT results

Scoring	CMT interpretation	Result Interpretation
Non	Negative (N)	Normal and healthy
Slight	Trace (T)	Any thickening, suspicious, test again
1	Weak (W)	Any thickening, suspicious, test again
2	Distinct (D)	Infected teat
3	Strong (S)	Heavily infected teat

Source: Kelly, (1984).

2.7.1.2 Culture, media and solutions sterilization

Peptone water, MacConkey's agar, blood agar and nutrient agar were sterilized in autoclave of 15 pound pressure for 15 minutes at 121°C (Stuart, 1959). The Liquid media had been clarified by filtration through paper sintered (Seitz filtration) Barrow and Feltham (2004).

2.7.1.3 Heat Sterilization (Flaming)

Metal wire and loops were sterilized by flame use of dry heat. For apparatus and glassware, sterilization was achieved by the using dry heat in a hot air oven provided that the temperature to be raised to and maintained at 160 °C for at least one hour; a higher temperature will char paper and cotton wool (Barrow and Feltham, 2004).

2.7.1.4 Laboratory safety and hygiene

Before and during any work in the laboratory benches were cleaned and disinfected using cotton dipped in ethyl alcohol (70%). Hands were also washed with soap and disinfectant. All cultures were sterilized by autoclaving, and associated apparatus and materials were sterilized or disinfected before disposal or reuse (Barrow and Feltham, 2004).

2.7.2 Preparation of Media

2.7.2.1 Nutrient agar

This medium was prepared with 10 gm of beef extract,10 gm of peptone and 5gm sodium chloride in 1liter of distilled water, the mixture was boiled while stirring to fully dissolve all components and sterilized at121°C for15 minutes then cooled to 50°C in water bath before dispended into Petri dishes .

2.7.2.2 Blood agar

Fifty ml of defibrinated sheep blood was added to 950 ml of Nutrient Agar (cooled to 50°C), then dispended the whole mixture into Petri dishes.

2.7.2.3 MacConkey agar

The mixture in this selective medium consists of peptone 20gm, lactose 10gm, sodium taurocholate 5gm, sodium chloride 5gm and Neutral red1%. The mixture was dissolved in 1 liter of water then boiled when PH was adjusted to 8.0 for 20 minutes. After that the mixture was cooled and filtered while PH adjusted to 7.4, then autoclaved at 121°C for 15 minutes, finally the mixture was poured aseptically in Petri dishes Barrow and Feltham, (2004).

2.7.3 Culture Procedure and Purification

Cultured samples were incubated at 37°C for 24-48 hours. Then sub-culture on plates was used to purify colonies on Nutrient Agar followed by incubation at 37°C for another 24 hours. Purified isolates were identified according to the reaction of Gram's stain, shape of the bacterial colonies, presence or absence of spores, motility, the colonial characteristics on different media, haemolytic of blood agar and biochemical tests as outlined by Barrow and Feltham, (2004).

2.7.4 Gram's staining

Bacterial colonies were described and characterized morphologically using Gram's stain according to the method described by Preston and Morrell (1962) modified Lillie's method by retarding decolonization with an acetone-iodine mixture (Preston and Morrell, 1962).

Films were made from purified cultures on clean glass slides, then dried by air and heated for fixation. The stain used was crystal violet (2min) and lugol's iodine (30sec) after washed by tap water. The slides were decolourized by alcohol and washed, then counter stained with dilute carbolfuchsin (15sec) (Barrow and Feltham, 2004).

2.7.5 Biochemical tests

All the biochemical tests were done according to the methods detailed in Cowan and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham, 2004). Tests performed included: Oxidation or Fermentation of glucose test (OF), acid from Carbohydrates test, Catalase test, Coagulase test, Oxidase test, Indole test, Citrate utilization, Decarboxylase test, Fermentation of sugars, Motility test, Urease Test, Starch hydrolysis Test, Mannitol Test, Temperature tolerance (60 °C).

2.7.5.1 Oxidation or fermentation of glucose Test (OF)

Two tubes of oxidative-fermentative medium were inoculated by stabbing 3cm from the bottom with the test organism. One of the two tubes was overlaid with 1 cm of melted soft paraffin. The colour of the medium was changed to yellow in opened and sealed tubes indicating fermentation. When the colour changed in the opened tubes only, indicated that the organism was oxidative and when the colour was not changed in both tubes indicated that the organism was negative (Barrow and Feltham, 2004).

2.7.5.2 Acid from carbohydrates Test

Phenol-red broth sugar for (haemophili and streptococci) inoculated heavily with growth from blood agar medium, then incubated at 37°C and examined for acid production daily for up to 7 days (can normally be read after 1-2 days). For streptococci, used media was containing 0.1% agar. Yellow colouration indicated positive result and the negative one was coloured with orange-red. In case of equivocal results, pH was checked a decrease to pH 5.0-6.3 indicate fermentation Barrow and Feltham, (2004).

2.7.5.3 Catalase test

In this test Petri dish and sterile inoculating loop were used. Small amount of organism from colony which incubated overnight was collected and placed onto the microscope slide. Then -using a dropper– dropped hydrogen peroxide (3% H_2O_2) onto the organism on the microscope slide and observed for immediate bubble formation (O_2 + water = bubbles). Positive reactions were evidenced by immediate effervescence after 5 minutes (bubble formation), In contrast, No bubble formation represented a catalase negative reaction (Barrow and Feltham, 2004).

2.7.5.4 Coagulase test

Undiluted 0.5 ml rabbit plasma was mixed separately in two different test tubes containing an equal volume of 24 hours cultured broth and incubated at 37°C for 6 hours. The tubes were examined after 3-6 hours of mixing cultured broth for detecting the presence of clots of plasma and the result was recorded according to the standard method described by (Gillespie, 1943). The negative tubes were left at room temperature for overnight and then re-examined.

2.7.5.5 Oxidase test

A fresh solution of the reagent was prepared with 0.1 % ascorbic acid kept at -20°C and a loop-full of tetra methyl-p-phynyl diamine dihydrochloride to about 3 ml of sterile distilled water. Then a filter paper disc was placed in a sterile plastic Petri dish, wetted with a few drops of the indicator solution and smeared the test culture (grown on a medium free from glucose and nitrate) across the moist paper with a platinum. The appearance of a dark purple colour on the paper within 30 seconds denotes a positive reaction (Barrow and Feltham, 2004).

2.7.5.6 Indole test

Suspected colony was inoculated in peptone water and incubated for 24 hrs at 35°C. Test for Indole was done by adding 0.2- 0.3ml Kovacs' reagent, appearance of distinct red colour means positive result and yellow means negative result (Barrow and Feltham, 2004).

2.7.5.7 Citrate utilization

The tested organism was inoculated on Koser's citrate medium and examined daily for up to 7 days for turbidity. Positive results were confirmed by subculturing again to Koser's citrate medium. Appearance of turbidity was considered positive result (citrate utilized), no turbidity considered negative result (citrate not utilized) (Barrow and Feltham, 2004).

2.7.5.8 Decarboxylase Test

From a single isolated colony as inoculum and aseptically transfer it to the decarboxylase broth tube with a straight wire into tubes of the four media (arginine, lysine, ornithine, and control) through the paraffin layer. Alternatively, inoculated by adding a drop of a suspension of the organism above the paraffin layer and shaken to distribute the inoculum. After incubation, tubes were examined daily for up to 5 days. If the colour of the media changed to purple it indicated decarboxylation by the test organism meaning that the organism was decarboxylase positive. The lack of a purple colour (became yellow) indicated acid has not been decarboxylated which meant negative reaction (Barrow and Feltham, 2004).

2.7.5.9 Fermentation of sugars

Suspected isolated colonies were inoculated with the Carbohydrate media. Changing of the colour to pink was regarded as a positive result.

The cultures were monitored for 7days before they were discarded (Barrow and Feltham, 2004).

2.7.5.10 Motility test

The targeted organism was stab- inoculated in tubes of motility medium to a depth of about 5mm and incubated at 37°C for 24hrs. Motile organisms migrated throughout the medium which became turbid while the growth of non-motile organisms was confined to the stab inoculums (Barrow and Feltham, 2004).

2.7.5.11 Urease test

Christens urea medium was prepared. A wire loop with suspected colonies were streaked on a dry, fresh slope of Christens urea medium, incubated at 37°C and examined daily for up to 5 days. Positive organism changes the colour into red (Barrow and Feltham, 2004).

2.7.5.12 Starch hydrolysis test

Starch hydrolysis result was read after adding Lugols iodine to grown culture in starch medium. Positive organism showed a clear colorless zone around the grown bacteria (Barrow and Feltham, 2004).

2.7.5.13 Mannitol test

The test strains were cultured onto Mannitol sugar. Positive results give pink colour, which indicated Mannitol fermentation (Barrow and Feltham, 2004).

2.7.5.14 Temperature tolerance (45 and 60°C)

1 ml of a 24 hours Nutrient Broth culture in a small test tube, placed in a water bath at 60°Cfor 30 minutes, after that cooled under cold running water and incubated at 37°C for 24 hours. Subculture was done to a Serum Agar slope and incubated for 24 hours and examined for growth which indicated the ability of the organism to survive the temperature time conditions, this ability got as a positive, in contrast,

disability of the organism to survive made it negative (Barrow and Feltham, 2004).

2.7.6 Bacteriological Identification Using VITEK®2 Compact

2.7.6.1 Reagent Cards and Culture Requirements

A total of 16 isolates were subjected in parallel to direct identification for species and sub-species of the organism's and genera using VITEK®2 compact v510731-10EN1 (2008) 24262, BioMérieux, Inc. Durham, North Carolina 27704-0969 in USA, (BioMérieux, 06/2008).

2.7.6.2 Suspension Preparation and inoculation

A sterile swab was used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 ml sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. Identification cards were inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension was placed into cassette and the identification card was placed in the neighbouring slot while inserting the transfer tube into the corresponding suspension tube. Then the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells (Pincus, 2006).

2.7.6.3 Biochemical Tests by VITEK®2 System

Biochemical reactions was attempted for all isolates using GP and GN identification cards in a VITEK®2 system as indicated by the manufacture shown in Tables **(**2.2 and 2.3), respectivley.

Table 2.2 : Biochemical Test on GP card

Well	Test	Mnemonic	Amount/Well
2	D-AMYGDALIN	AMY	0.1875 mg
4	PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C	PIPLC	0.015 mg
5	D-XYLOSE	dXYL	0.3 mg
8	ARGININE DIHYDROLASE 1	ADH1	0.111 mg
9	BETA-GALACTOSIDASE	BGAL	0.036 mg
11	ALPHA-GLUCOSIDASE	AGLU	0.036 mg
13	Ala-Phe-Pro ARYLAMIDASE	APPA	0.0384 mg
14	CYCLODEXTRIN	CDEX	0.3 mg
15	L-Aspartate ARYLAMIDASE	AspA	0.024 mg
16	BETA GALACTOPYRANOSIDASE	BGAR	0.00204 mg
17	ALPHA-MANNOSIDASE	AMAN	0.036 mg
19	PHOSPHATASE	PHOS	0.0504 mg
20	Leucine ARYLAMIDASE	LeuA	0.0234 mg
23	L Proline ARYLAMIDASE	ProA	0.0234 mg
24	BETA GLUCURONIDASE	BGURr	0.0018 mg
25	ALPHA-GALACTOSIDASE	AGAL	0.036 mg
26	L-Pyrrolidonyl-ARYLAMIDASE	PyrA	0.018 mg
27	BETA-GLUCURONIDASE	BGUR	0.0370 mg
28	Alanine ARYLAMIDASE	AlaA	0.0216 mg
29	Tyrosine ARYLAMIDASE	TyrA	0.0276 mg
30	D-SORBITOL	dSOR	0.1875 mg
31	UREASE	URE	0.15 mg
32	POLYMIXIN B RESISTANCE	POLYB	0.00093 mg
37	D-GALACTOSE	dGAL	0.3 mg
38	D-RIBOSE	dRIB	0.3 mg
39	L-LACTATE alkalinization	ILATk	0.15 mg
42	LACTOSE	LAC	0.96 mg
44	N-ACETYL-D-GLUCOSAMINE	NAG	0.3 mg
45	D-MALTOSE	dMAL	0.3 mg
46	BACITRACIN RESISTANCE	BACI	0.0006 mg
47	NOVOBIOCIN RESISTANCE	NOVO	0.000075 mg
50	GROWTH IN 6.5% NaCl	NC6.5	1.68 mg
52	D-MANNITOL	dMAN	0.1875 mg
53	D-MANNOSE	dMNE	0.3 mg
54	METHYL-B-D-GLUCOPYRANOSIDE	MBdG	0.3 mg
56	PULLULAN	PUL	0.3 mg
57	D-RAFFINOSE	dRAF	0.3 mg
58	O/129 RESISTANCE (comp.vibrio.)	O129R	0.0084 mg
59	SALICIN	SAL	0.3 mg
60	SACCHAROSE/SUCROSE	SAC	0.3 mg
62	D-TREHALOSE	dTRE	0.3 mg
63	ARGININE DIHYDROLASE 2	ADH2s	0.27 mg
64	OPTOCHIN RESISTANCE	OPTO	0.000399 mg

Source: Pincus (2006).

Table 2.3 : Biochemical Test on GN card

Well	Test	Mnemonic	Amount/Well
2	Ala-Phe-Pro-ARYLAMIDASE	APPA	0.0384 mg
3	ADONITOL	ADO	0.1875 mg
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018 mg
5	L-ARABITOL	lARL	0.3 mg
7	D-CELLOBIOSE	dCEL	0.3 mg
9	BETA-GALACTOSIDASE	BGAL	0.036 mg
10	H2S PRODUCTION	H2S	0.0024 mg
11	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	0.0408 mg
12	Glutamyl Arylamidase pNA	AGLTp	0.0324 mg
13	D-GLUCOSE	dGLU	0.3 mg
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	0.0228 mg
15	FERMENTATION/ GLUCOSE	OFF	0.45 mg
17	BETA-GLUCOSIDASE	BGLU	0.036 mg
18	D-MALTOSE	dMAL	0.3 mg
19	D-MANNITOL	dMAN	0.1875 mg
20	D-MANNOSE	dMNE	0.3 mg
21	BETA-XYLOSIDASE	BXYL	0.0324 mg
22	BETA-Alanine arylamidase pNA	BAlap	0.0174 mg
23	L-Proline ARYLAMIDASE	ProA	0.0234 mg
26	LIPASE	LIP	0.0192 mg
27	PALATINOSE	PLE	0.3 mg
29	Tyrosine ARYLAMIDASE	TyrA	0.0276 mg
31	UREASE	URE	0.15 mg
32	D-SORBITOL	dSOR	0.1875 mg
33	SACCHAROSE/SUCROSE	SAC	0.3 mg
34	D-TAGATOSE	dTAG	0.3 mg
35	D-TREHALOSE	dTRE	0.3 mg
36	CITRATE (SODIUM)	CIT	0.054 mg
37	MALONATE	MNT	0.15 mg
39	5-KETO-D-GLUCONATE	5KG	0.3 mg
40	L-LACTATE alkalinisation	lLATk	0.15 mg
41	ALPHA-GLUCOSIDASE	AGLU	0.036 mg
42	SUCCINATE alkalinisation	SUCT	0.15 mg
43	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	0.0306 mg
44	ALPHA-GALACTOSIDASE	AGAL	0.036 mg
45	PHOSPHATASE	PHOS	0.0504 mg
46	Glycine ARYLAMIDASE	GlyA	0.012 mg
47	ORNITHINE DECARBOXYLASE	ODC	0.3 mg
48	LYSINE DECARBOXYLASE	LDC	0.15 mg
52	DECARBOXYLASE BASE	0DEC	N/A
53	L-HISTIDINE assimilation	lHISa	0.087 mg
56	COUMARATE	CMT	0.126 mg
57	BETA-GLUCORONIDASE	BGUR	0.0378 mg
58	O/129 RESISTANCE (comp.vibrio.)	O129R	0.0105 mg
59	Glu-Gly-Arg-ARYLAMIDASE	GGAA	0.0576 mg
61	L-MALATE assimilation	lMLTa	0.042 mg
62	ELLMAN	ELLM	0.03 mg
64	L-LACTATE assimilation	lLATa	0.186 mg

Source: Pincus (2006).

2.7.6.4 Card sealing, incubation and reading test's results

Inoculated cards were passed to loading into the carousel incubator, cards were incubated at 35.5 ±1.0ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings data were collected at 15 minute intervals during the entire incubation period. Then test reaction results appeared as "+"," –", "(–)" or "(+)", results were assigned based on the numerical probability calculation and shown in Table (2.4).

2.7.6.5 Test Card Incubation and Reading of Results

Once test cards were sealed, they were ready to be incubated and read. The test card transport system moves the boat and cassette into position for a mechanism, called the Card Loader, to place each test card into a slot on a carousel, where it remains throughout the reading cycle.

2.8 Antimicrobial Sensitivity test

Representative isolates were examined for their *in vitro* drug sensitivity. The isolates were first cultured in nutrient broth for 48 hrs. The sensitivity discs (DIFCO, OXIDO) were placed on the inoculated Mueller Hinton agar, A zone of inhibition was seen around the disc containing the antibiotic for which the organism was sensitive (Barrow and Feltham, 2004), inhibition zone were measured after 48-72 hrs aerobically incubation at 37°C. The zone was read based on average of diameter of growth inhibition zone in millimetres (ADIZ) shown in Table (2.5).

2.8.1 The Antibiotics Disc used in the Study

Commercially prepared discs were placed on the plates and lightly pressed down to ensure the antibiotics disc were in contact with the agar. The discs used were : Gentamicin (Gnt.10ug), Clindamycin (Clind.5ug), Penicillin G (PenG.10iu), Enrofloxacin (Enro.5ug), Cefoperazone

(Cefopraz.30ug), Streptomycin (Strep.10ug), Neomycin (Neo.30iu), Tetracycline (Tetr.30ug), Trimethoprim (Trim.25ug) and Ampicillin (Amp.10ug), For both Gram-negative isolates and Gram-positive isolates, respectively, which they were available in the market and subjected by owners and veterinarians in field.

Table 2.4: Results based on the Numerical Probability Calculation

ID Message Confidence level	Choices	Probability %	Comments
Excellent	1	96 – 99	N/A
Very Good	1	93 – 95	N/A
Good	1	89 – 92	N/A
Acceptable	1	85 – 88	N/A
Low Discrimination	2 to 3	Sum of choices=100: After resolution to one choice, percent probability reflects the number associated with the selected choice.	2 to 3 taxa exhibit the same biopattern separate by supplemental testing
Unidentified Organism	>3 or 0	N/A	Either > 3 taxa exhibit the same biopattern. Or Very a typical biopattern. Doesn't correspond to any taxon in the database. Check Gram satin and purity.

Table 2.5 : Average of Diameter of Growth Inhibition Zone (ADIZ)

Diameter of growth inhibition zone	The Effectiveness
20 – 18 mm	Sensitive
17 – 14 mm	Moderate
13– 10 mm	Low sensitivity
9 – 0 mm	Resistant

Chapter Three

Results

3.1 Occurrence of Clinical Mastitis according CMT

Milk samples of fifty one mastitic udders were tested by CMT kit, 39 (76.47%) samples were positive in CMT test, while the rest twelve (23.53%) were negative (Figure 3.1). On the other hand CMT positive cultured samples revealed 16 isolates out of 39 positive samples (41.03%), since no bacterial growth of each examined CMT negative sample (0%). The result of CMT was recorded into four categories i.e. trace, weak, distinct and strong based on the intensity of gel formation (Table 3.1).

3.2 Isolation and identification of Bacterial Causes

The bacteria were isolated from 41.03% of positive samples, while meaning, the rest of examined positive samples 58.97%. In addition, in the investigated goat flocks the occurrence of mastitis caused by bacterial isolates reach up to 40%, while the bacterial causative isolates among sheep flocks represent 29.26% (Table 3.3). The bacterial isolates revealed that 62.5% were Gram positive and 37.5% where as Gram negative bacteria as (Table 3.4).

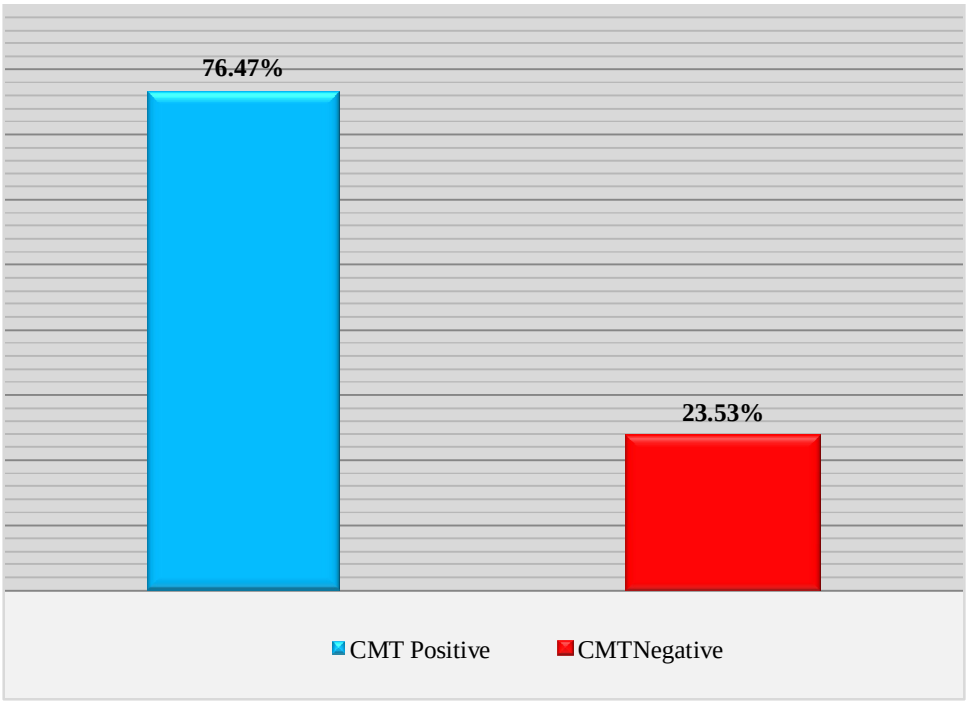

Figure 3.1 : Occuerrance of clinical mastitis (%) using CMT test in sheep and goats

Table 3.1: CMT interpretation based on gel formation

Species	Trace	Weak	Distinct	Strong	Total Positive	Total Negative
Sheep	5	7	11	8	31	10
Goat	1	2	3	2	8	2
Sub-total	6	9	14	10	39	12
				Total	51	

Table 3.2 : Type, number and percentage of the bacterial isolates

No.	Causative Bacteria	Type of Bacteria	No. of isolates	Percentage %
1	*Enterobacter ssp*	GN	5	31.25
2	*E. coli*	GN	1	6.25
3	*Streptococcus ssp*	GP	2	12.5
4	*Gardnella ssp*	GP	1	6.25
5	*Enterococcus ssp*	GP	1	6.25
6	*Lactococuss ssp*	GP	3	18.75
7	*Staphylococcu ssp*	GP	1	6.25
8	*Leuconostoc ssp*	GP	2	12.5
	Total		16	100

GN = Gram Negative

GP = Gram Positive

Table 3.3: Positive and negative cultured milk samples from 41 mastitic sheep and 10 mastitic goats

Species	No.	Positive (%)	Negative (%)
Sheep	41	12 (29.26%)	29 (70.73%)
Goat	10	4 (40%)	6 (60%)
Total	51	16 (31.37%)	35 (68.63%)

Table 3.4 : Gram positive and Gram negative bacteria isolated from mastitic milk samples

Type of Bacteria	Sheep	Goat	Percentage
Gram+ve	4	6	62.5%
Gram-ve	6	0	37.5%
Total	10	6	100%

3.2.1 Gram-positive bacteria

Lactococcus ssp, *Streptococcus ssp*, and *Leuconostoc ssp* represented the highest percentage of isolated Gram positive organisms (Table 3.2). The total percentage of isolated Gram positive organism represented 62.5% (Table 3.4).

3.2.1.1 *Staphylococcus* species

Morphological and cultural characterization of the isolates:

The organism appeared, in stained smears as Gam positive cocci arranged in clusters. *Staphylococci* were non-motile and irregular in clusters like bunches of grapes (Figure 3.2). Colonies produced were circular, smooth, opaque, low convex, soft and easily to be emulsified when touch by the wire loop.

Biochemical reactions:

The main characteristics of examined isolate were: catalase, coagulase, urease, and oxidase, all biochemical tests conducted for identification of *staphylococcus ssp* are shown in Table (3.5).

Identification of the bacterial causes using VITEK®2 system:

In the current study, *S.aureus* was detected in 6.25% of samples from doe mastitic udder using VITEK®2 with biochemical substrate of GP card with high probability range reach to 99% at 4.15 hours' time analysis (Tables 3.9 and 3.13).

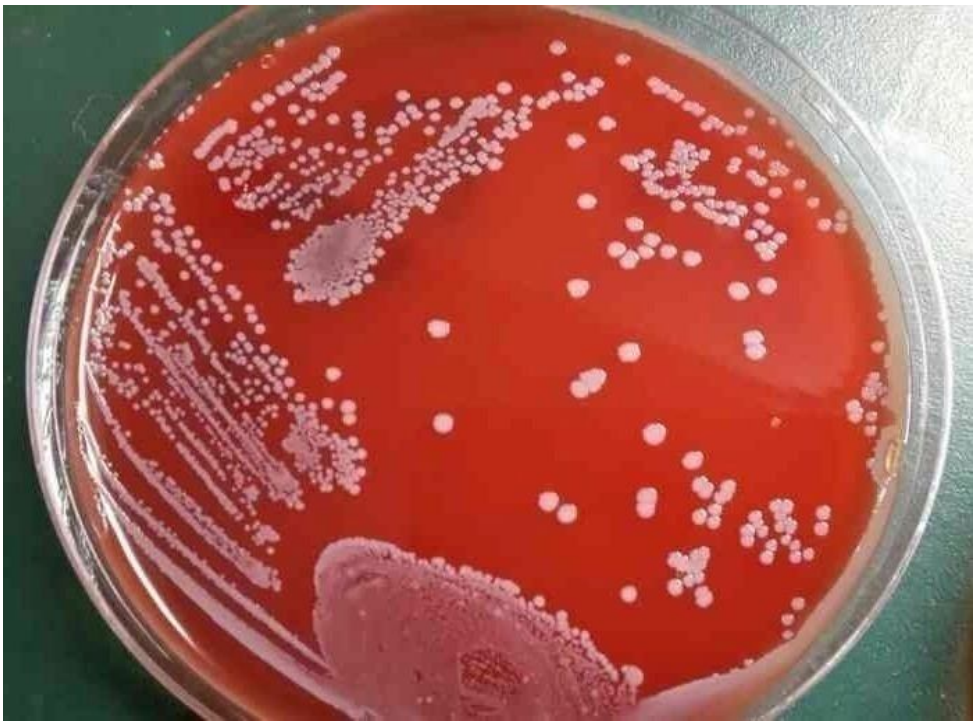

Figure 3.2 : The growth of *Staphylococcus aureus* on Blood Agar medium

Table 3.5: Biochemical tests used for identification of *Staphylococcus ssp*

Biochemical tests	***Staphylococcus ssp***
Gram stain	+
Haemolysis	+
Motility	Non motile
Catalase	+
Oxidase	-
OF	+
Glucose with (VP) production	+
Lactose	+
Sucrose	+
Fructose	+
Maltose	+
Xylose	-

3.2.1.2 *Streptococcus* species

Morphological and cultural characterization of the isolates:

After 24 hours incubation at 37°C, *Streptococcus.ssp* colonies were detected as dewdrop like surrounded with a narrow zone of complete haemolysis.

Biochemical reactions:

The isolates of Streptococci were identified using catalase, oxidase and indole tests (Table 3.6).

Identification using VITEK®2 system:

GP Card with biochemical substrates were used for automatic identification of *streptococcus ssp*., in approximately 6 hours with probability (95%) as shown in Tables (3.9 and 3.13). The *Streptococcuss oralis/mitis* had been isolated from 12.5% of clinical cases of mastitis in ewes from mastitic udders.

Table 3.6: Biochemical Tests used for identification of Streptococcus ssp

Biochemical tests	***Streptococcus ssp***
Gram stain	+
Haemolysis	+
Motility	-
Catalase	-
Oxidase	-
OF	+
Glucose with (VP) production	+
Lactose	+
Sucrose	+
Indole	-
Maltose	+
Growth at45°C	-
H_2O_2 production	+

3.2.1.3 *Enterococus* species

Morphological and cultural characterization of the isolates:

The organism appeared, in stained smears as Gram-positive cocci and non-motile. On primary isolation, the cultures were incubated at 37°C for 48 hours. The colonies occur of short chains in pairs like shape of streptococci colonies **(**difficult to distinguish them morphologically**).**

Biochemical reactions:

In order to differentiate *Enterococuss* species from other cocci (*Streptococcus* species), two biochemical tests: growth in 6.5% Salt Broth and growth at 45°C (temperature tolerance 45°C: survival after heating at 45°C for 30 minutes) were done and resulted in positive for *Enterococuss* colonies.

Identification using VITEK®2 system

As in Tables (3.9 and 3.13) automated identification of *Enterococcus* spp using GP card was completed in 6 hours time analysis. *Enterococus hirae* was identified in 6.25% of clinical mastitis samples from ewes with 95% probability from mastitic udder.

3.2.1.4 *Leuconostoc species*

Morphological and cultural characterization of the isolates:

Isolates of this organism were Gram-positive, coccobacilli, the organism appeared in stained smears as Gram-positive small sticks in short chains. The Growth was obtained after 48 hours incubation at 37°Cand the colonies appeared small and watery in sucrose Agar.

Biochemical reactions:

In order to differentiate the organism from other Gram-positive cocci catalase and starch with other biochemical tests were used (Table 3.7).

Table 3.7: Biochemical Tests used for identification of *Leuconostoc ssp*

Biochemical tests	***Leuconosto ssp***
Gram stain	+
Haemolysis	+
Motility	-
Catalase	-
Oxidase	-
OF	+
Glucose with (VP) production	+
Lactose	+
Sucrose	+
Fructose	+
Starch	-

Identification using VITEK® 2 system

Leuconostoc spp were identified using VITEK®2 utilizing growth-based technology, GP card contained different biochemical substrates for many organisms included *Leuconostoc* spp. In 6hours and 8hours automated identification was completed for two organisms, *Leuconostoc mesenteroides ssp* with probability 90%, and very low discrimination between *Leuconostoc mesenteroides ssp dextranicum* and *Leuconostoc mesenteroides ssp cremoris* (Tables 3.9 and 3.13). In the current study, the two organisms were detected in 12.5% of the clinical mastitis in ewes.

3.2.1.5 *Lactococcus* species

Morphological and cultural characterization of the isolates:

The isolated organism colonies appeared as a Gram-positive cocci bacterium in pairs and short chains and ovoid, non-spore forming and non-motile.

Biochemical reactions:

After culture incubated for 24 hours at 37°C, two tests were used to identify organism and differentiate them from other Gram-positive cocci. Colonies grew in salt broth in 4% but not in 6.5% and the growing colonies were detected in plates at 40°C whereas no growing colonies were detected at 45°C.

Identification using VITEK®2 system

The GP identification card was used to identify sub-species genera of Lactococcus species, which contained many biochemical substrates for different gram-positive organisms (primarily cocci). The result of identification completed at 5hours and 5:15hours time analysis as shown in (Table 3.9) respectively, and revealed that *lactococcus lactis ssp lactis* is one of the causative agents of clinical mastitis in small ruminants,

especially in does in present study with percentage of (18.75%) from the total number of the causative isolates with 90% probability (Table 3.13).

3.2.1.6 *Gardnerella* ssp

Morphological and cultural characterization of the isolates:

Stained organism appeared as very small coccobacilli, scattered on plate, non-sporing and non-motile. The growing colonies circular in shape and convex.

Biochemical reactions:

Gardnerella produced diffuse haemolysis on blood agar (Table 3.8).

Identification using VITEK®2 system

Significant automated identification was conducted for *Gardnerella ssp* organism using GP card contained different substrates for 115 different gram-positive organisms and *Gardnerella ssp*. As the results were obtained 8hours (Table 3.9), slash line identification was done with low discrimination of mixed colonies for two organisms and *Gardnerella vaginalis* was the first one. *Gardnerella vaginalis* represented a pathogenic organism of clinical mastitis in ewes in rare cases with percentage of 6.25% in the current study (Table 3.13).

Table 3.8: Biochemical Tests used for identification of Gardnerella ssp

Biochemical tests	***Gardnella ssp***
Gram stain	+
Haemolysis	+
Motility	-
Catalase	-
Oxidase	-
Urease	+
Glucose with (VP) production	-
Lactose	+
Sucrose	+
Maltose	+

Table 3.9: Biochemical Profile of Gram-positive Isolates from 39 mastitic milk samples (Vitek 2 system)

Code	Reagent	Identification of Gram-positive Isolates using GP card								
		STY	SRP Mi	SRP Or	EN	LE Dx	LE Cr	LT all	LT 1	GRD
5	dXYL	-	-	-	-	-	-	+	+	-
8	ADH1	+	-	-	+	-	-	+	+	-
9	BGAL	-	-	-	+	-	+	-	-	-
11	AGLU	+	+	+	-	+	-	-	-	+
13	APPA	-	+	+	-	-	-	+	-	-
15	AspA	-	-	-	+	-	-	+	+	-
23	ProA	-	-	-	-	-	+	+	+	+
28	AlaA	-	+	+	+	-	-	-	-	-
45	dMAL	+	+	-	+	-	+	+	+	+
47	NOVO	-	-	-	+	+	-	+	+	-
50	NC6.5	+	-	-	+	-	+	+	+	+
53	dMNE	+	-	-	+	+	-	+	+	-
62	dTRE	+	-	+	+	+	-	-	-	-
64	OPTO	+	+	+	+	+	-	-	+	-
Time	Analysis(Hr)	4:15	5:45	5:45	6	6	8	5	5:15	8

3.2.2 Gram-Negative bacteria

3.2.2.1 *Enterobacter* ssp

Morphological and cultural characterization of the isolates:

Isolates of *Enterobacter ssp* are Gram-negative small and thin rods. On MacConkey agar the Enterobacter produce of mucoid and pink colonies (Lactose fermenting) (Figure 3.3), in blood agar they were mucoid and light pinkish-grey

Biochemical reactions:

All examined colonies were catalase-positive, oxidase-negative and produced acid when attacked sugars, in order to differentiate them from others Gram-negatives organisms biochemical tests were done (Table 3.10).

Identification using VITEK®2 system

GN card was used to identify *Enterobacter* organisms from other Gram-negative bacterium. The card contained different substrates. *Enterobacter aerogenes* was isolated at 4hours time analysis with high probability (98%) (Table 3.9**).** The five isolates of *Enterobacter aerogenes* (Table 3.13) were detected in 31.25% of the causative organisms in clinical mastitis cases in ewes.

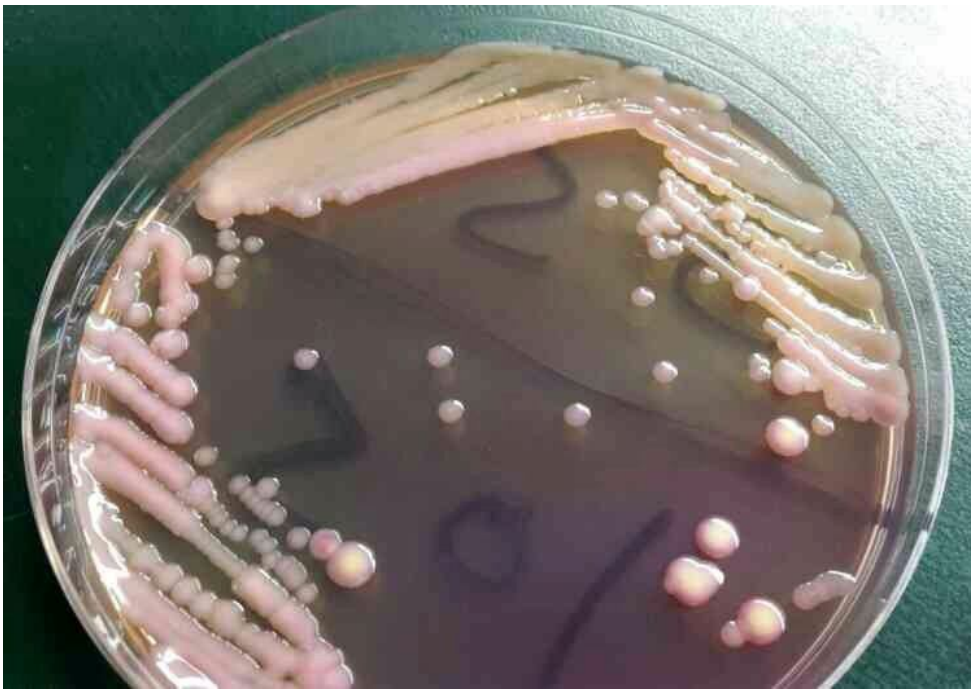

Figure 3.3: The growth of ***Enterobacter aerogenes*** **on MacConkey Agar medium**

Table 3.10: The results of biochemical tests used for identification of *Enterobacter ssp*

Character	***Enterobacter ssp***
Shape	Rod
Motility	+
Oxidase	-
Catalase	+
OF	F
Yellow pigment	-
Red pigment	-
MacConkey's growth	+
Urease	-
H_2S Production (pb Ac) paper	-
Acid from:	
Glucose (gas)	+
Lactose	+
Maltose	+
Mannitol	+
Sorbitol	+
Sucrose	-
Xylose	+
Starch	-
Indole	-

3.2.2.2 *E. coli*

Morphological and cultural characterization of the isolates:

Isolates of *E.coli* were Gram-negative rods, occurred singly, long and thin. They were motile and non-spore forming. Coloning in MacConkey after 24 hours incubation at 37ºC Colonies were smooth, shiny, large (2-4 mm in diameter) and pink in colour due to Lactose fermenting, but in blood agar medium appeared white to yellowish, moist, glistening, opaque and circular with an entire edge.

Biochemical reactions

All examined isolates were catalase and indole positive, they were oxidase negative. The rest of biochemical tests were used to differentiate *E. coli* from other isolates shown in (Table 3.11).

Identification using VITEK®2 system:

GN card was used to identify *Escherichia ssp* and it contains other different substrates for 135 different Gram-negative organisms. The result of identification of *E. coli* using VITEK 2 system completed at 3:30 hours' time analysis (Table 3.12). *E. coli* was one of the causative organisms of acute clinical mastitis (Table 3.5), in ewes reached in a percentage of 6.25% of the total isolates with high probability reach 99% (Table 3.13).

Table 3.11: The results of biochemical tests used for identification of *E. coli*

Character	***E. coli ssp***
Shape	Rod
Motility	+
Oxidase	-
Catalase	+
OF	F
Yellow pigment	-
Red pigment	-
MacConkey's growth	+
Urease	-
H_2S Production (pb Ac) paper	-
Acid from:	
Glucose (gas)	+
Lactose	+
Maltose	+
Mannitol	+
Sorbitol	+
Sucrose	-
Xylose	+
Starch	+
Indole	+

Table 3.12: Biochemical Profile of Gram-negative Isolates from mastitic milk samples using Vitek 2 system

Code	Reagent	Identification of Gram-negative isolates using GN card		
		Enterobacter ssp (all)	*Enterobacter (1)*	*E. Coli*
18	dMAL	+	+	+
33	SAC	+	+	+
34	dTAG	+	+	-
39	5KG	-	+	-
47	ODC	+	-	+
59	GGAA	+	+	-
Time	Analysis (hr)	3:45	4	3:30

Table 3.13: Probability in percentage and Confidence Level of Identified Causitive Isolates using VITEK®2 system

Causative Isolate	Number and percentage of isolates	Probability in Percentage	Confidence level (VITEK®2 Description)
Enterobacter ssp	5 = 31.25%	98%, 98%, 94%, 93%, 89%	2 Excellent, 2 very good, good
E. coli	1 = 6.25%	99%	Excellent
Staphylococcu ssp	1 = 6.25%	99%	Excellent
Streptococcus ssp	2 = 12.5%	95%,95%	2 Very good
Enterococcus ssp	1 = 6.25%	95%	Very good
Lactococuss ssp	3 = 18.75%	90%,86%,86%	Good, 2 acceptable
Leuconostoc ssp	2 = 12.5%	90%,low	Good , low
Gardnerlla ssp	1 = 6.25%	Low	Low
Total isolates	16 = 100%	**Probability range** : 93%	**confidence rate** : very good

3.3 Antibacterial Sensitivity

Sensitivity of the causative bacteria isolated from the fifty-one mastitic milk samples to ten different antibiotics using the disk diffusion method are shown in (Table 3.14). Fourteen isolates (81.8%) from the all sixteen isolated bacteria were sensitive to Gentamicin and the rest two isolates were moderate, while the majority of isolates 63.6% (Figure3.7) were resistant to Ampicillin and represented resistance also to Tetracycline(45.5%). Thirteen isolates of causative bacteria were sensitive to Trimethoprim (Table 3.14), while another three isolated organisms appeared resistant. Cefoprazone and Clindamycin were regarded quietly high efficiency against isolated bacteria, with resistance for Clindamycin antibiotic. Four isolates (Table 3.14 and Figure 3.4) were resistant to Penicillin G, in contrast another four organisms were found sensitive to Penicillin G. The susceptibility of isolated bacteria to Enrofloxacin, Streptomycin and Neomycin revealed same result of low efficiency reached 36.4% (Figure 3.4) with no resistant.

All five isolated strains of *Enterobacter aerogenes* (Table 3.15) were sensitive to Gentamicin and Trimethoprim but the majority (Four isolates) of them showed high resistance to Ampicillin. According to the result of sensitivity test of *Leuconostoc mesentroids ssp dextranicum* and *Leuconostoc mesentroids ssp cremoris* in (Table 3.16) it was found that two isolates were sensitive to Gentamicin, Clindamycin, Penicillin G and Ampicillin, in contrast *L.mesentroids dexatrnicum* was resistant to Tetracycline and Trimethoprim.

The three isolates of *Lactococcus lactis ssp. lactis* were sensitive to Gentamicin, Enrofloxacin, Cefoprazone and Neomycin (Table 3.17), but they were resistant to Ampicillin, in addition, one isolate appeared

resistant to Clindamycin, Penicillin G, Tetracycline, Trimethoprim and Ampicillin.

Table 3.14: The Sensitivity Tests Results of isolated bacteria obtained from mastitic milk samples

Isolated Bacteria	Samples No. and %	Sensitive to									
		Gnt	Clnd	PenG	Enro	Cefopraz	Sterp	Neo	Tetr	Trim	Amp
Enterobacter aerogenes (all)	4= 25%	S	-	-	S	S	S	S	S	S	R
Enterobacter aerogenes (1)	1= 6.25%	S	M	M	-	-	-	-	M	S	M
E. coli	1= 6.25%	S	-	-	S	S	S	S	M	S	R
Staphylococcus aureus	1= 6.25%	S	S	S	-	-	-	-	R	S	R
Streptococcus oralis/mitis	2= 12.5%	S	S	R	-	-	-	-	S	S	R
Enterococcus hirae	1= 6.25%	M	S	R	-	S	-	-	R	R	R
Leuco. Mesentroids dextranicum	1= 6.25%	S	S	S	-	-	-	-	R	R	S
Leuco. mesentroids cremoris	1= 6.25%	S	S	S	-	-	S	-	S	S	S
Lctococcus lactis spp lactis(all)	2= 12.5%	S	-	-	S	S	S	S	S	S	R
Lctococcus lactis spp lactis(1)	1= 6.25%	S	R	R	S	S	-	S	R	R	R
Gardnerella vaginalis	1= 6.25%	M	S	S	-	S	-	-	R	S	S

Strep = Streptomycin **Gnt =** Gentamycin **Trim** = Trimethoprim **Clind** = Clindamycin

PenG = Penicillin G **Enro =** Enrofloxacin **Cifopraz** = Cifoprazone **Neo** = Neomycin

Tetra = Tetracycline **Amp =** Ampicillin

S = Sensitive **M** = Moderate **R:** Resistant

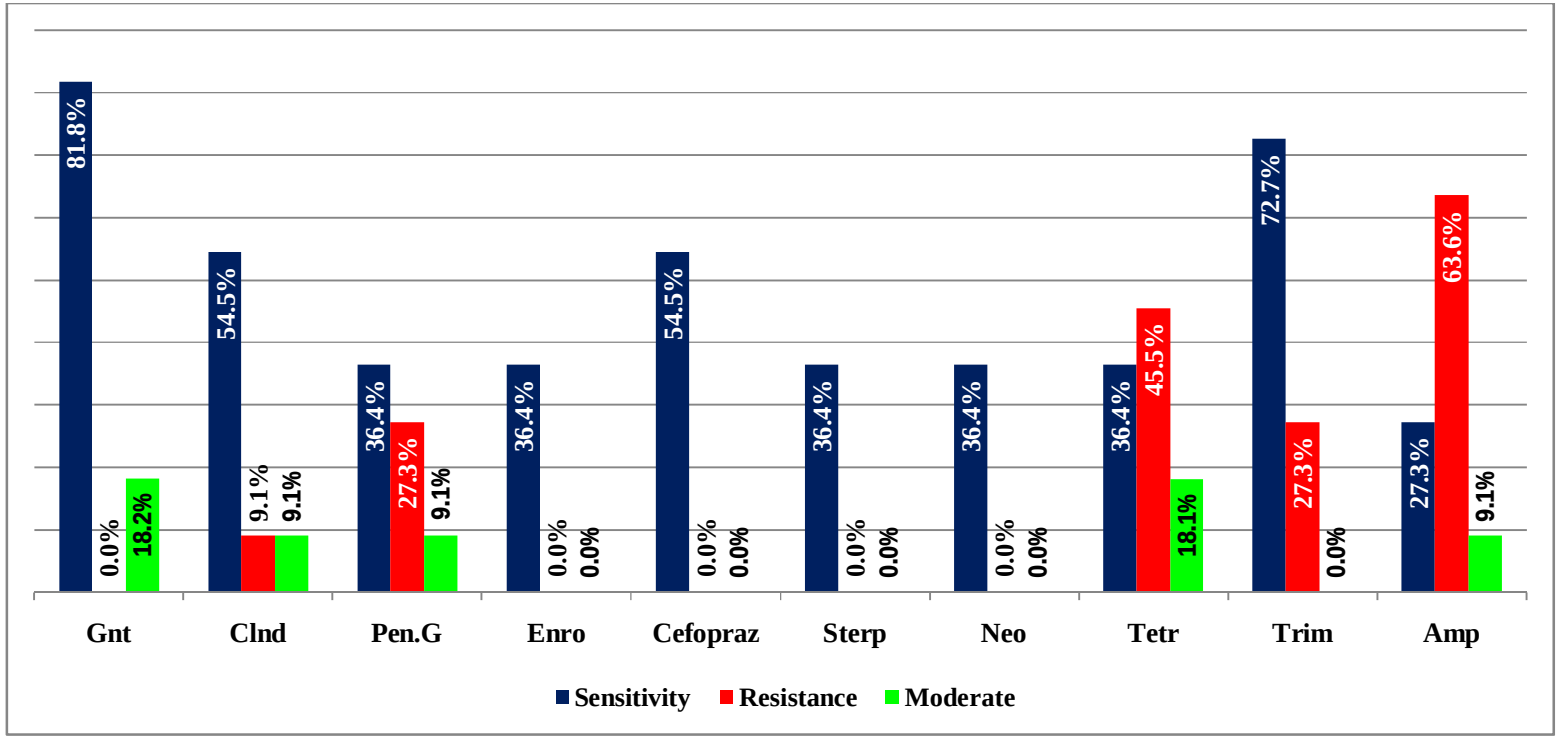

Figure 3.4: The percentage of the Sensitivity Tests for the isolated causitive agents

Table 3.15: The percentag of the Sensitivity Tests of *Enterobacter* strains

Isolated Bacteria	Sensitivity to									
	Gnt	**Clnd**	**PenG**	**Enro**	**Cefoprazon**	**Sterp**	**Neo**	**Tetr**	**Trim**	**Amp**
Enterobacter aerogenes (all)	S	-	-	S	S	S	S	S	S	R
Enterobacter aerogenes (1	S	M	M	-	-	-	-	M	S	M
Sensitivity Percentage	100%	25%	25%	50%	50%	50%	50%	50%	100%	0%
Resistanc Percentage	0%	0%	0%	0%	0%	0%	0%	0%	0%	50%
Moderate percentage	0%	50%	50%	0%	0%	0%	0%	50%	0%	50%

Table 3.16: The percentage of the Sensitivity Tests for *Leuoconostoc* strains

Isolated Bacteria	Sensitivity to									
	Gnt	**Clnd**	**PenG**	**Enro**	**Cefoprazon**	**Sterp**	**Neo**	**Tetr**	**Trim**	**Amp**
L.mesentroidsdextranicum	S	S	S	-	-	-	-	R	R	S
L.mesentroids. cremoris	S	S	S	-	-	S	-	S	S	S
Sensitivity Percentage	100%	100%	100%	-	-	50%	-	50%	50%	100%
Resistance Percentage	0%	0%	0%	-	-	0%	-	50%	50%	0%

Table 3.17: The percentage of the Sensitivity Tests for *Lactococcus* strains

Isolated Bacteria	Sensitivity to									
	Gnt	**Clnd**	**PenG**	**Enro**	**Cefoprazon**	**Sterp**	**Neo**	**Tetr**	**Trim**	**Amp**
L. lactis. spp lactis (all)	S	-	-	S	S	S	S	S	S	R
L. lactis. Spp lactis (1)	S	R	R	S	S	-	S	R	R	R
Sensitivity Percentage	100%	0%	0%%	100%	100%	50%	100%	50%	50%	0%
Resistance percentage	0%	50%	50%	0%	0%	0%	0%	50%	50%	100%

Chapter Four

Discussion

Results obtained in the current study showed that 76.47% of the collected samples were clinically positive and 31.4% were positive to bacterial culture, while meaning the rest of examined positive samples must be were due to other causes such as yeast, Mycoplasma sp. and viral organisms. It is worth to mention that all the negative samples to CMT (12 samples were) were also negative in bacteriological examination. In contrast, the number of clinically negative sheep with positivity to bacterial culture was relatively low in previous studies performed on Spanish flocks, whereas the bacterial positivity was found to be 35% (Bergonier *et al*., 2003). In another report Arsenault *et al*., (2008), reported isolation of potentially pathogenic bacteria in 28.8% of subjects with clinically normal udders.

Beside less common isolated causative agents *Lactococcus ssp*, *Leuconostoc ssp* and *Gardnerella ssp*., dominant isolated organisms in current study were *Enterobacter aerogenes*, *Staphylococcus aureus*, *Streptococcus ssp*., *E.coli* and *Enterococcus hirae* a result that is in line with previous findings (Quinlivan, 1968; Lafi *et al*., 1998; Onnasch *et al*., 2002; Bergonier *et al*., 2003).

The present results revealed that it is important to note that certain microbes, host and/or environmental factors, like *Streptococcus* other than agalactiae, *Enterococcus* ssp and coliform bacteria including *Escherichia coli* and *Enterobacter* ssp may induce environmental agents to behave as contagious pathogens and this supported by previous studies (Bergonier *et al.*, 2003; Mørk *et al.*, 2007; Arsenault *et al.*, 2008). Several previous studies indicated that in sheep and goats environmental

pathogens, are important etiological agents of clinical mastitis (Zadoks *et al.*, 2001; Quinn and Markey, 2003; Pyörälä and Taponen, 2009).

During clinical and microbiological examination of milk samples of selected lactating goats and sheep within flocks suffering repeated mastitis in present study, opportunistic *Enterobacter aerogenes* and *E.coli* were found to be the main isolated causative agent from mastitic milk samples (37.5% of isolates). Such finding seems to be associated with the changes occurred in the breeding style, which is semi-extensive and intensive. Increased susceptibility to environmental pathogens may be linked to their ability to thrive in the microclimate generated into the sheep/goat fold, where is usually warm, humid at the end of Autumn to cold and dry in winter (period of kidding/lambing in small ruminants between October and April in the area), and protected by the direct exposure to ultraviolet radiations. Inside the sheep/goat fold, the crowded conditions and the recumbence of ewes/does during rumination and sleep increase exposure of the udder to contact with the environmental microbial flora (Gebrewahid *et al.*, 2012).

In the present study, *Enterobacter aerogenes* was the single bacterial species showing the highest incidence (31.25%), this result is in line with a study reported by Albenzio *et al.*, (2002). Infections caused by *Enterobacter aerogenes* have been underestimated and under rated in the sanitary management of small animals, although serious damages to the mammary gland may result from the infection by this species. Thus, these results are opening up new possibilities in the understanding of the balance and direct relation between infection and commensally lifestyle. Moreover, they provide preventive new strategies against this opportunistic pathogens.

No isolation of Gram-negative bacteria during this study for lactating dairy goats (0% of isolates),this result is similar to previous report by Ryan and Greenwood, (1990) and Contreras *et al*., (1995), they found that Gram-negative bacteria rarely induced mastitis in goats.

In this study *E. coli* was isolated from 6.25% of sheep samples and nearly the same percentage was reported in a study in Qassim region in the middle of Saudi Arabia Kingdom (Alharbi, 2014). This low incidence might be referred to the lower diffusion of Gram negative bacteria in sheep than in cattle, where the most “classical” forms of mastitis is caused by *Escherichia coli* and considered a "major" pathogen in cattle (Quinn and Markey, 2003).

During the study period, several bacterial strains induced mastitis in the clinical picture were isolated such as: *Lactococcus lactis ssp lactis* (18.75%), *Leuconostoc ssp* (12.5%) : *(Leuconostoc mesenteroides ssp dextranicum* (6.25%) and *Leuconostoc mesenteroides ssp cremoris* (6.25%), *Enterococcus hirae* (6.25%), and *Gardnerella vaginalis* (6.25%), However in this investigation, *S. aureus* represented low percentage (6.25%) of the total isolates, and this finding is in agreement with results in other studies (González-RodríGuez *et al*., 1995; Gonzalo *et al*., 2002; Bergonier and Berthelot, 2003). It has been mentioned that *S. aureus* is responsible for up to 40% of clinical mastitis cases in ewes and its prevalence is linked with high somatic cell counts (Maisi and Riipinen, 1991; Mavrogianni *et al*., 2011). However this prevalence is only found in sporadic cases for sheep and goats, and the incidence is usually below 5% per year (Bergonier *et al*., 2003; Contreras *et al*., 2007). IMIs caused by *S. aureus* require special attention because, this bacterium is responsible for both clinical and subclinical mastitis in small ruminants (Bergonier *et al*., 2003; Moroni *et al*., 2005b; Contreras *et al*., 2007;

Mørk *et al*., 2007; Arsenault *et al*., 2008; de Sousa Guaraná *et al*., 2011; Seyffert *et al*., 2012) . From previous published work it has been known that *S. aureus* is a pathogen with little prevalence and little capacity of transmission among goats. The herds in which *S. aureus* is present usually have only a few infected animals with the exception of outbreaks due to milking machine alterations or other exceptional circumstances. This low prevalence is also frequent despite the fact that early detection of infected animals, by regular SCC or CMT and milking order are not habitual practices. It still has to be proof whether this low prevalence is due to the goat's resistance or to the characteristics of the caprine strains (Contreras *et al*., 2003).

Goats are less frequently affected by contagious intra-mammary infections than cows (Radostits *et al*., 2000). In the investigation *S. aureus* was detected in 6.25% of tested samples. However a study in Norwegian dairy cows 22.1% of the animals shed *S. aureus* from at least one quarter (Sølverød and Østerås, 2001). In another reports that agrees with the present findings, *S. aureus* was detected in udder secretions from 3-7% of dairy goats (Menzies and Ramanoon, 2001; Jørgensen *et al*., 2005).

In current study *Streptococcus ssp* were significantly identified with high probability (95%), very good confidence results using VITEK®2, utilizing growth-based technology, has been carried to identify *Streptococcus mitis* and *Streptococcus oralis*. This result (12.5%) indicates that these two bacteria cause clinical mastitis in does and ewes. *Streptococcus ssp* were the common mastitis causing microbes in the dairy goats (Quinlivan, 1968; Watson *et al*., 1990; Onnasch *et al*., 2002, Rizwan *et al*., 2016), but in contrast to the previous studies that showed; Streptococcal mastitis are less common in goats and ewes (Bergonier *et*

al., 2003; Contreras *et al*., 2007). The detection of novel streptococcal species in this study and other recent studies represented the importance of submitting streptococci taxonomical rearrangements; therefore, the implication of streptococci in mastitis should be carefully re-evaluated (Romero *et al*., 2011).

Accurate identifications for less common bacterial causative agents of clinical mastitis in small ruminants such as *Lactococcus* ssp, and *Enterococcus* ssp. could be achieved by using conventional diagnostic procedures. Using VITEK®2 system in the present study enabled the identification of these species of bacteria. Using GP card reagents a "good and very good" confidence results with high probability 90% and 95% *Lactococcus lactis ssp lactis* and *Enterococcus hirae* with percentage of 18.75% and 6.25% of causative isolates at 5 hours and 7 hours time analysis, respect these findings supported by previous study (Devriese *et al*., 1999). Worth to mention, the isolation of these Gram-positive bacteria in mastitis in sheep and goats is usually associated with poor bedding and inadequate hygiene practices. Base of current finding *Leuconostoc mesenteroides ssp dextranicum*, *Leuconostoc mesenteroides ssp cremoris* and *Gardnerella vaginalis* were from 6.25% of clinical mastitis in does, which agrees with results of study by Špaková *et a*l., (2012).

In the present study the isolation of *Gardnerella vaginalis* from mastitic goat with clinical symptoms resulted in one isolate in a percentage 6.25% of the total isolates, the result is similar to study in Iraq using VITEK®2 system in isolation and identification by Zainab Oun Ali Al-Zainy, (2015), *Gardnerella vaginalis* was first described by Gardner and Dukes (1955) and *Gardnerella vaginalis* could cause mastitis (Fleming *et al*., 2005).

Considering the efficacy of antimicrobial agents on the basis of sensitive, moderate sensitivity and resistance nature, nine out of eleven examined isolates (81.8%) were sensitive to Gentamicin with moderate sensitivity to only two isolates (18.2%) and no resistance detected to all examined isolates, The isolates were second sensitive to Trimethoprim (72.7%). Clindamycin, Cefoperazone, Enrofloxacin, Streptomycin, Neomycin and Tetracycline showed morerate sensitivity, while Ampicillin and Penicillin G. (27.3%), revealed low efficacy and accompanied with high resistance to Ampicillin (63.6%).

This result is similar to previous findings of Islam *et al.*, (2012), and Alharbi, (2014). High resistance to the antibiotics especially Tetracycline seems to occur in Saudi Arabia because they are used extensively in the treatment of most of infectious diseases.

High resistance levels against specific antimicrobials may be associated with their frequent and long-term use on the farm (Pitkälä *et al*., 2004; Kumar *et al*., 2010).

Notable, in the area of our survey Ampicillin, Penicillin and Oxytetracycline are commonly used antibiotics in ovine mastitis, thus mastitic bacteria showed resistance to these antibiotics, where as Streptomycin represented 36.4% of sensitivity to the isolates, also have been used extensively with Penicillin (in synergism as a compound drug) for treating mastitis, it may have led to the development of low effectiveness in bacteria against these antibiotics. On the other hand, Enrofloxacin represent high-moderate efficiency with no resistance similar to result reported by (Attili *et al*., 2016).

In the present study, Cefoperazone, Streptomycin and Neomycin showed 54.5% and 36.4% of sensitivity to isolates, as we considered Cefoprazone (drug of Cephalosporin's) has a moderate-effectiveness

performance and both Streptomycin and Neomycin have low efficacy, with no resistance (0%) detected to all against isolates, and this finding is in line with Aydin *et al.*, (2009). In contrast, Cefoperazone has been included as first and third-generation drugs against isolates, and performed poorly (Moroni *et al.*, 2005a). It is worth mentioning that, previous study in *Awassi* sheep flocks in Jordon (Lafi and Hailat, 1998), reported that Neomycin represented high susceptibility against isolates. As well as in monitoring study of antimicrobial susceptibility revealed similar result to current study that Clindamycin represented moderate to high performance against causative isolated bacteria in a range of percentage 54.5 – 96.5% (Vyletělová *et al.*, 2014).

Conclusion and Recommendations

Conclusion

This study concludes that CMT together with bacteriological examination are important to prescribe treatment for mastitis in sheep and goats. In that sense, bacterial sensitivity test is an important step for mastitis treatment, the study observed the following point:

The main bacterial causative agents isolated from fifty one mastitic milk samples, are: five isolates of *Enterobacter aerogenes*, one isolate of *Staphylococcus aureus*, two isolates of *Streptococcus ssp*, one isolate of *E. coli*, one isolate of *Gardnella ssp*, one isolate of *Enterococcus ssp*, one isolate of *Lactococuss ssp* and two isolate of *Leuconostoc ssp*.

Recommendations

Present study recommends that:

1. Application of VITEK® system could be practiced at selected samples from time to time to confirm identification of causative organisms.
2. Particular attention should be paid to *Enterobacter aerogenes* that cause the highest incidence in small ruminants.
3. As the breeding style and environments are the source of infection therefore, improvement of hygiene, reduction of exposure to potential pathogens, hygienic housing and milking practices are necessary and play a crucial role to minimize the impact of clinical mastitis.
4. Present study suggests that Gentamicin could be recommended as the antibiotic of choice against bacterial causative agents, while majority of causative isolates expressed high resistance against Ampicillin.

5. Manufacturing specifically intramammary products for ewes and does and the antimicrobial products should include antibiotics with high performance against common pathogenic agents such as Gentamicin.

References

Abdelgawad, A.R., Rovai, M., Caja, G., Leitner, G., and Castillo, M., (2016). Evaluating coagulation properties of milk from dairy sheep with subclinical intramammary infection using near infrared light scatter. A preliminary study. *Journal of Food Engineering*, **168**(2016): 180-190.

Abdullah, A.H., (2016). Study the Inhibitory Effect of Aqueous Extract of *Punica granatum L*. on Resistant Staphylococcus aureus Isolate from Mastitic milk. *Kufa Journal For Veterinary Medical Sciences* **5**(2):1- 8.

Ahmad, G., Timms, L., Morrical, D., and Brackelsberg, P., (1992a). Dynamics and significance of ovine subclinical intramammary infections and their effects on lamb performance. *Sheep Research Journal,* **8:** 25-29.

Ahmad, G., Timms, L., Morrical, D., and Brackelsberg, P., (1992b). Ovine subclinical mastitis: Efficacy of dry treatment as a therapeutic and prophylactic measure. *Sheep Research Journal,* **8:** 30-33.

Albenzio, M., Taibi, L., Muscio, A., and Sevi, A., (2002). Prevalence and etiology of subclinical mastitis in intensively managed flocks and related changes in the yield and quality of ewe milk. *Small Ruminant Research,* **43**(3): 219-226.

Alharbi, K.B., (2014). Detection of Antimicrobial Resistance of Staphylococcus aureus Isolates at Qassim Region central of Saudi Arabia. *International Journal of Food, Agriculture and Veterinary Sciences,* **4**(1): 81-92.

Ameh, J.A., Addo, P.B., Adekeye, J.O., and Gyang, E.O., (1993). Prevalence of clinical mastitis and of intramammary infections in Nigerian goats. *Preventive Veterinary Medicine,* **17**(1): 41-46.

Ameh, J.A., and Tari, I.S., (1999). Observations on the prevalence of caprine mastitis in relation to predisposing factors in Maiduguri. *Small Ruminant Research,* **35**(1): 1-5.

Amorena, B., Baselga, R., and Albizu, I., (1994). Use of liposome-immunopotentiated exopolysaccharide as a component of an ovine mastitis staphylococcal vaccine. *Vaccine,* **12**(3): 243-249.

Arranz, J., and de Heredia, B., (1989). Testing of the CMT method in the detection of subclinical mastitis in sheep, In: *International Symposium on Machine Milking of Small Ruminants*, pp. 369-380.

Arroyo, P., Mediano, P., Martin, V., Jimenez, E., Delgado, S., Fernandez, L., (2011). Etiological diagnosis of infectious mastitis:proposal of a protocol for the culture of human milk samples. *Acta Pediatrica Espanola,* **69**(6): 276–281

Arsenault, J., Dubreuil, P., Higgins, R., and Bélanger, D., (2008). Risk factors and impacts of clinical and subclinical mastitis in commercial meat-producing sheep flocks in Quebec, Canada. *Preventive Veterinary Medicine,* **87**(3–4): 373-393.

Attili, A.R., Preziuso, S., Ngu Ngwa, V., Cantalamessa, A., Moriconi, M., and Cuteri, V., (2016). Clinical evaluation of the use of enrofloxacin against Staphylococcus aureus clinical mastitis in sheep. *Small Ruminant Research,* **136**: 72-77.

Aydin, I., Kav, K., and Celik, H.A., (2009). Identification and antimicrobial susceptibility of subclinical mastitis pathogens isolated from hair goats' milk. *Journal of Animal and Veterinary Advances,* **8**(6): 1086-1090.

Balows, A., Hausler Jr., W.J., Herrmann, K.l., and Isenberg, H.D., (1991). Manual of Clinical Microbiology. *Revista do Instituto de Medicina Tropical de São Paulo,* **33:** 434-434.

Barber, S., Allen, J., Mansell, P., Browning, G., (2006). Mastitis in the ewe, In: P*roceedings of the Australian Sheep Veterinarians 2006 Conferences*, pp. 127-132.

Barillet, F., Rupp, R., Mignon-Grasteau, S., Astruc, J.-M., and Jacquin, M., (2001). Genetic analysis for mastitis resistance and milk somatic cell score in French Lacaune dairy sheep. *Genetics Selection Evolution*, **33**(4): 397-416.

Barrow, G., and Feltham, R.K.A., (2004). *Cowan and Steel's Manual for the Identification of Medical Bacteria*. Cambridge University Press,UK.

Bergonier, D., and Berthelot, X., (2003). New advances in epizootiology and control of ewe mastitis. *Livestock Production Science*, **79**(1):1-16.

Bergonier, D., de Cremoux, R., Rupp, R., Lagriffoul, G., Berthelot, X., (2003). Mastitis of dairy small ruminants. *Veterinary Research* **34**(5): 689-716.

Bilal, M., Iqbal, M., Muhammad, G., Avais, M., and Sajid, M., (2004). Factors affecting the prevalence of clinical mastitis in buffaloes around Faisalabad district (Pakistan). *International Journal of Agriculture & Biology*, **6**(1): 185 - 187.

Billon, P., and Decremoux, R., (1998). Mastitis of dairy ewes: Etiology, Detection, and Control, In: *Dairy Sheep Symposium*, Athens, Greece, Wageningen Pers, The Netherlands, p. 49.

BioMérieux, S., (06/2008). Biomerieux VITEK® 2 Instrument User Manual i 510731-10EN1. *Durham, North Carolina 27704-0969 / USA.http://www.biomerieux.com*.

Blood, D., Radostits, O., Henderson, J., Arundel, J., and Gay, C., (1983). *Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Goats and Horses*. London: Bailliere Tindall.

Bocklisch, H., and Wetzstein, D., (1994). Clinical, diagnostic laboratory and therapeutic studies of mastitis in a large sheep breeding flock. *Tierarztliche Praxis,* **22**(6): 524-528.

Bodman, G.R., and Rice, D.N., (1995). Mastitis is a Disease-control is an Everyday Task. Cooperative Extension, Institute of Agriculture and Natural Resources, University of Nebraska-Lincoln.

Bor, A., Winkler, M., and Gootwine, E., (1989). Non-clinical intramammary infection in lactating ewes and its association with clinical mastitis. *British Veterinary Journal,* **145**(2): 178-184.

Bourabah, A., Ayad, A., Boukraa, L., Hammoudi, S., Benbarek, H., (2013). Prevalence and etiology of subclinical mastitis in goats of the Tiaret region, Algeria. *Global Veterinary,* **11:** 604-608.

Bouvier-Muller, J., Allain, C., Enjalbert, F., Tabouret, G., Portes, D., Caubet, C., Tasca, C., Foucras, G., and Rupp, R., (2016). Response to dietary-induced energy restriction in dairy sheep divergently selected for resistance or susceptibility to mastitis. *Journal of Dairy Science,* **99**(1): 480-492.

Bulgin, M., (1990). Ovine progressive pneumonia, caprine arthritis-encephalitis, and related lentiviral diseases of sheep and goats. *The Veterinary Clinics of North America. Food Animal Practice,* **6**(3): 691-704.

Burriel, A., (1997). Udder orf infection and its role in ovine clinical mastitis caused by Pasteurella haemolytica. *Journal of Trace Elements in Medicine and Biology,* **11**(1): 28-31.

Clements, A.C., Taylor, D.J., and Fitzpatrick, J.L., (2003). Evaluation of diagnostic procedures for subclinical mastitis in meat-producing sheep. *Journal of Dairy Research,* **70**(02): 139-148.

CLSI, (2009). Clinical Laboratory Institute Standards:standards for antimicrobial disk and dilution susceptibility tests for bacteria

isolated from animals. *Methods for Dilution Antimicrobial Susceptibility;Tests for BacteriaThat Grow Aerobically; Approved Standard—Eighth Edition,* **28**(7)15-20.

Conington, J., Cao, G., Stott, A., and Bünger, L., (2008). Breeding for resistance to mastitis in United Kingdom sheep, a review and economic appraisal. *The Veterinary Record,* **162**(12): 369-376.

Contreras, A., Corrales, J., Sanchez, A., and Sierra, D., (1997). Persistence of Subclinical Intrammary Pathogens in Goats Throughout Lactation. *Journal of Dairy Science,* **80**(11): 2815-2819.

Contreras, A., Corrales, J.C., Sierra, D., and Marco, J., (1995). Prevalence and aetiology of non-clinical intramammary infection in Murciano-Granadina goats. *Small Ruminant Research,* **17**(1): 71-78.

Contreras, A., Luengo, C., Sanchez, A., and Corrales, J., (2003). The role of intramammary pathogens in dairy goats. *Livestock Production Science,* **79**(2): 273-283.

Contreras, A., Sierra, D., Corrales, J., Sanchez, A., and Marco, J., (1996). Physiological threshold of somatic cell count and California Mastitis Test for diagnosis of caprine subclinical mastitis. *Small Ruminant Research,* **21**(3):259-264.

Contreras, A., Sierra, D., Sánchez, A., Corrales, J., Marco, J., Paape, M., and Gonzalo, C., (2007). Mastitis in small ruminants. *Small Ruminant Research.* **68**(1): 145-153.

Contreras, G.A., and Rodríguez, J.M., (2011). Mastitis: comparative etiology and epidemiology. *Journal of Mammary Gland Biology and Neoplasi,a* **16**(4): 339-356.

Cooper, S., Huntley, S.J., Crump, R., Lovatt, F., and Green, L.E., (2016). A cross-sectional study of 329 farms in England to identify risk

factors for ovine clinical mastitis. *Preventive Veterinary Medicine*, **125**: 89-98.

Corrales, J.C., Sanchez, A., Luengo, C., Poveda, J.B., and Contreras, A., (2004). Effect of clinical contagious agalactia on the bulk tank milk somatic cell count in Murciano-Granadina goat herds. *Journal of Dairy Science*, **87**(10): 3165-3171.

Cotter, P.D., Ross, R.P., and Hill, C., (2013). Bacteriocins-a viable alternative to antibiotics ? *Nature Reviews. Microbiology*, **11**(2): 95-105.

de Sousa Guaraná, E.L., dos Santos, R.A., Grace, A., Campos, S.S., da Silva, N., Silva, J.A.B.A., and de Mendonça, C.L., (2011). Dinâmica celular e microbiológica do leite de ovelhas Santa Inês acompanhadas durante a lactação1. *Pesquisa Veterinária Brasileira*, **31**(10): 851-858.

Deng, P., Cutlip, R., Lehmkuhl, H., and Brogden, K., (1986). Ultrastructure and frequency of mastitis caused by ovine progressive pneumonia virus infection in sheep. *Veterinary Pathology Online*, **23**(2): 184-189.

Devriese, L.A., Hommez, J., Laevens, H., Pot, B., Vandamme, P., and Haesebrouck, F., (1999). Identification of aesculin-hydrolyzing streptococci, lactococci, aerococci and enterococci from subclinical intramammary infections in dairy cows. *Veterinary Microbiology*, **70**(1–2): 87-94.

Doğruer, G., Saribay, M.K., Ergün, Y., Aslantaş, Ö., Demir, C., Ateş, C.T., (2010). Treatment of subclinical mastitis in Damascus goats during lactation. *Small Ruminant Research*, **90**(1–3): 153-155.

Dossantos, L., Castro, R., and Dacosta, E., (1995). California-Mastitis and Modified-Whiteside test as Screening to Caprine Mastitis. *Pesquisa Agropecuaria Brasileira*, **30**(2): 295-298.

Eberhart, R., Harmon, R., Jasper, D., Natzke, R., Nickerson, S., Reneau, J., Row, E., Smith, K., and Spencer, S., (1987). *Current Concepts of Bovine Mastitis*. Natl. Mastitis Council. *Inc., Arlington, VA.*

EFSA, (2015). Scientific opinion on the welfare risks related to the farming of sheep for wool, meat and milk production. *European Food Safety Authority (EFSA), Parma, Italy Rev 18 version 22-09-2014*.

Erskine, R.J., Wagner, S., and DeGraves, F.J., (2003). Mastitis therapy and pharmacology. *Veterinary Clinics of North America: Food Animal Practice*, **19**(1): 109-138.

Farah, I., (1992). A comprehensive review on bovine mastitis with special reference to Sudan, In: *Proc. 5th Conf. Arab Veterinary Assoc*. Khartoum, Sudan.

Fleming, J., Somes, J., Bernards, S., and Pierre, T.S., (2005). Methods, compositions, devices, and kits for detecting mastitis. https://www.google.com/patents/US20050260695 #backward.

Fragkou, I.A., Boscos, C.M., and Fthenakis, G.C., (2014). Diagnosis of clinical or subclinical mastitis in ewes. *Small Ruminant Research*, **118**(1–3): 86-92.

Fragkou, I.A., Mavrogianni, V.S., Cripps, P.J., Gougoulis, D.A., and Fthenakis, G.C., (2007). The bacterial flora in the teat duct of ewes can protect against and can cause mastitis. *Veterinary Research*, **38**(4): 525-545.

Franz, S., Hofmann-Parisot, M., Baumgartner, W., Windischbauer, G., Suchy, A., and Bauder, B., (2001). Ultrasonography of the teat canal in. *The Veterinary Record,* **149:**109-112.

Fthenakis, G., (1994). Prevalence and aetiology of subclinical mastitis in ewes of Southern Greece. *Small Ruminant Research,* **13**(3): 293-300.

Fthenakis, G.C., and Jones, J.E.T., (1990). The effect of experimentally induced subclinical mastitis on milk yield of ewes and on the growth of lambs. *British Veterinary Journal*, **146**(1): 43-49.

Gardner, H.L., and Dukes, C.D., (1955). *Haemophilus vaginalis vaginitis*. *American Journal of Obstetrics & Gynecology,* **69**(5): 962-976.

Gebrewahid, T.T., Abera, B.H., and Menghistu, H.T., (2012). Prevalence and Etiology of Subclinical Mastitis in Small Ruminants of Tigray Regional State, North Ethiopia. *Vet World*, **5**(2): 103-109.

Gelasakis, A., Mavrogianni, V., Petridis, I., Vasileiou, N., and Fthenakis, G., (2015). Mastitis in sheep–The last 10 years and the future of research. *Veterinary Microbiology* **181**(1):136-146.

George, L.W., Divers, T.J., Ducharme, N., and Welcome, F.L., (2008). Diseases of the Teats and Udder, In: *Rebhun's Diseases of Dairy Cattle* (Second Edition). W.B. Saunders, Saint Louis, pp. 327-394.

Giadinis, N.D., Arsenos, G., Tsakos, P., Psychas, V., Dovas, C.I., Papadopoulos, E., Karatzias, H., Fthenakis, G.C., (2012). "Milk-drop syndrome of ewes": Investigation of the causes in dairy sheep in Greece. *Small Ruminant Research,* **106**(1): 33-35.

Giguère, S., Prescott, J.F., and Dowling, P.M., (2013). *Antimicrobial therapy in Veterinary Medicine.* John Wiley & Sons, Oxford , UK.

Gil, M., De Mendoza, M.H., Rey, J., Alonso, J., de Mendoza, J.H., and Poveda, J., (1999). Aetiology of caprine contagious agalactica syndrome in Extreadura, Spain. *Veterinary Record,* **144**(1): 24-25.

Gillespie, E., (1943). The Routine Use of the Coagulase Test for Staphylocoeei. *Monthly Bull. Emergency Pub. Health Lab. Service. Medical Research Council,* **2:** 19-22.

González-Rodríguez, M., and Carmenes, P., (1996). Evaluation of the California mastitis test as a discriminant method to detect

subclinical mastitis in ewes. *Small Ruminant Research,* **21**(3): 245-250.

González-RodríGuez, M.C., Gonzalo, C., San Primitivo, F., and Carmenes, P., (1995). Relationship Between Somatic Cell Count and lntramammary Infection of the Half Udder in Dairy Ewes. *Journal of Dairy Science,* **78**(12): 2753-2759.

Gonzalo, C., Ariznabarreta, A., Carriedo, J., and San Primitivo, F., (2002). Mammary pathogens and their relationship to somatic cell count and milk yield losses in dairy ewes. *Journal of Dairy Science* **85**(6): 1460-1467.

Goodridge, L., Hill, A., Lencki, R., (2004). A review of international standards and the scientific literature on farm milk bulk-tank sampling protocols. *Journal of Dairy Science,* **87**(9): 3099-3104.

Gutiérrez-Chávez, A., Martínez-Ortega, E., Valencia-Posadas, M., León-Galván, M., de la Fuente-Salcido, N., Bideshi, D., Barboza-and Corona, J., (2016). Potential use of *Bacillus thuringiensis* bacteriocins to control antibiotic-resistant bacteria associated with mastitis in dairy goats. *Folia Microbiologica,* **61**(1):11-19.

Haenlein, G.F.W., (2002). Relationship of somatic cell counts in goat milk to mastitis and productivity. *Small Ruminant Research,* **45**(2): 163-178.

Harmon, R., Eberhart, R., Jasper, D., Langlois, B., and Wilson, R., (1990). Microbiological procedures for the diagnosis of bovine udder infection and Determination of Milk Quality . [NMC publication, 2004]. *Arlington (VA): National Mastitis Council.*

Hillerton, J., and Berry, E., (2005). Treating mastitis in the cow–a tradition or an archaism. *Journal of Applied Microbiology,* **98**(6): 1250-1255.

Hogan, J.S., Gonzalez, R. N., Harmon, R. J., Nickerson, S. C., Oliver, S. P., Pankey, J. W., and Smith, K. L. , (1999). *Laboratory Handbook on Bovine Mastitis*, National Mastitis Council. Inc., Madison, WI, USA.

Hueston, W., Hartwig, N., and Judy, J., (1986). Detection of ovine intramammary infection with the California mastitis test. *Journal of the American Veterinary Medical Association,* **188**(5): 522-524.

Islam, M.R., Ahamed, M.S., Alam, M.S., Rahman, M.M., Sultana, T., Roh, Y.-S., and Kim, B., (2012). Identification and antibiotic sensitivity of the causative organisms of subclinical mastitis in sheep and goats. *Pakistan Veterinary Journal,* **32:** 179-182.

Iturrlde, M., Aguilar, B., Baselga, R., and Amorena, B., (1993). Adherence of ruminant mastitis *Staphylococcus aureus* strains to epithelial cells from ovine mammary gland primary cultures and from a rat intestinal cell line. *Veterinary Microbiology,* **38**(1): 115-127.

Jones, J., and Watkins, G., (1998). Studies on mastitis in sheep at the Royal Veterinary College, In: Proceedings of Meetings-Sheep Veterinary Society. Scarborough, Yorkshire , England, pp. 83-92.

Jørgensen, H.J., Mørk, T., Høgåsen, H.R., and Rørvik, L.M., (2005). Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. *Journal of Applied Microbiology,* **99**(1): 158-166.

Kautz, F.M., Nickerson, S.C., and Ely, L.O., (2014). Use of a staphylococcal vaccine to reduce prevalence of mastitis and lower somatic cell counts in a registered Saanen dairy goat herd. *Research in Veterinary Science,* **97**(1): 18-19.

Keisler, D., Andrews, M., and Moffatt, R., (1992). Subclinical mastitis in ewes and its effect on lamb performance. *Journal of Animal Science,* **70**(6): 1677-1681.

Kelly, W., (1984). *Veterinary Clinical Diagnosis.* 3rd Edition, Bailler Tindall, London, UK .

Kennedy-Stoskopf, S., Narayan, O., and Strandberg, J., (1985). The mammary gland as a target organ for infection with caprine arthritis-encephalitis virus. *Journal of Comparative Pathology*, **95**(4): 609-617.

Kiossis, E., Brozos, C., Petridou, E., and Boscos, C., (2007). Program for the control of subclinical mastitis in dairy Chios breed ewes during lactation. *Small Ruminant Research,* **73**(1): 194-199.

Kiossis, E., Brozos, C.N., Papaioannou, N., Tzanidakis, N., and ,Boscos, C., (2009). Endoscopic and histopathological findings of teats in dairy ewes. *Small Ruminant Research* **87**(1–3): 70-75.

Kirk, J.H., Huffman, E.M., and Anderson, B.C., (1980). Mastitis and Udder Abnormalities as Related to Neonatal Lamb Mortality in Shed-Lambed Range Ewes. *Journal of Animal Science*, **50**(4): 610-616.

Koop, G., Rietman, J., and Pieterse, M., (2010). *Staphylococcus aureus*. *Veterinary Record*, **167:** 868-869.

Kretschmer, E.R., (2007). Efficacy of the PortaSCC Milk Test to Estimate Somatic Cell Count (SCC) and Detect Subclinical Mastitis in Sheep, and the Effect of Cell Counting Method, Sampling Day, and Udder Health Status on SCC and Constituents in Sheep Milk. ProQuest, University of Nevada, Reno, ProQuest Dissertations Publishing, 2007.

Kumar, R., Yadav, B., and Singh, R., (2010). Genetic determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. *Current Microbiology*, **60**(5): 379-386.

Lafi, S., (2006). Use of somatic cell counts and California Mastitis Test results from udder halves milk samples to detect subclinical

intramammary infection in Awassi sheep. *Small Ruminant Research*, **62**(1): 83-86.

Lafi, S., Al-Majali, A., Rousan, M., and Alawneh, J., (1998). Epidemiological studies of clinical and subclinical ovine mastitis in Awassi sheep in northern Jordan. *Preventive Veterinary Medicine*, **33**(1): 171-181.

Lafi, S., and Hailat, N., (1998). Incidence and antibiotic sensitivity of bacteria causing bovine and ovine clinical mastitis in Jordan. *Pakistan Veterinary Journal*, **18**(2): 88-94.

Larsgard, A., and Vaabenoe, A., (1993). Genetic and environmental causes of variation in mastitis in sheep. *Small Ruminant Research*, **12**(3): 339-347.

Las Heras, A., Domınguez, L., and Fernandez-Garayzabal, J., (1999). Prevalence and aetiology of subclinical mastitis in dairy ewes of the Madrid region. *Small Ruminant Research*, **32**(1): 21-29.

Las Heras, A., Vela, A.I., Fernández, E., Legaz, E., Domínguez, L., and Fernández-Garayzábal, J.F., (2002). Unusual outbreak of clinical mastitis in dairy sheep caused by Streptococcus equi subsp. zooepidemicus. *Journal of Clinical Microbiology*, **40**(3): 1106-1108.

Lazaridis, L., Brozos, C., Kiossis, E., A.,(2012). Applications of ultrasonography in ruminants (III): udder andgenital system of the male–A review. *Journal of the Hellenic Veterinary Medical Society*, **63**(3): 217-226.

Lerondelle, C., Fleury, C., and Vialard, J., (1988). [The mammary gland: target organ for infection with the caprine arthritis and encephalitis virus]. *Annales de Recherches Veterinaires [Annals of Veterinary Research]*, **20**(1): 57-63.

Luengo, C., Sánchez, A., Corrales, J.C., Fernández, C., and Contreras, A., (2004). Influence of intramammary infection and non-infection factors on somatic cell counts in dairy goats. *Journal of Dairy Research*, **71**(02): 169-174.

Maisi, P., and Riipinen, I., (1991). Pathogenicity of different species of staphylococci in caprine udder. *British Veterinary Journal*, **147**(2): 126-132.

Malher, X., Seegers, H., and Beaudeau, F., (2001). Culling and mortality in large dairy goat herds managed under intensive conditions in western France. *Livestock Production Science*, **71**(1): 75-86.

Mavrogianni, V., Fthenakis, G., Burriel, A., Gouletsou, P., Papaioannou, N., and Taitzoglou, I., (2004). Experimentally induced teat stenosis in dairy ewes: clinical, pathological and ultrasonographic features. *Journal of Comparative Pathology*, **130**(1): 70-74.

Mavrogianni, V.S., Fthenakis, G.C., Brooks, H., Papaioannou, N., Cripps, P.J., Taitzoglou, I., Brellou, G., and Saratsis, P., (2005). The effects of inoculation of Mannheimia haemolytica into the teat of lactating ewes. *Veterinary Research*, **36**(1): 13-25.

Mavrogianni, V.S., Menzies, P.I., Fragkou, I.A., and Fthenakis, G.C., (2011). Principles of mastitis treatment in sheep and goats. *Veterinary Clinics of North America: Food Animal Practice*, **27**(1): 115-120.

McCarthy, F., Lindsey, J., Gore, M., and Notter, D., (1988). Incidence and control of subclinical mastitis in intensively managed ewes. *Journal of Animal Science*, **66**(11): 2715-2721.

McDougall, S., Murdough, P., Pankey, W., Delaney, C., Barlow, J., and Scruton, D., (2001). Relationships among somatic cell count, California mastitis test, impedance and bacteriological status of

milk in goats and sheep in early lactation. *Small Ruminant Research*, **40**(3): 245-254.

McDougall, S., Pankey, W., Delaney, C., Barlow, J., Murdough, P.A., and Scruton, D., (2002). Prevalence and incidence of subclinical mastitis in goats and dairy ewes in Vermont, USA. *Small Ruminant Research*, **46**(2–3): 115-121.

McDougall, S., Supré, K., De Vliegher, S., Haesebrouck, F., Hussein, H., Clausen, L., and Prosser, C., (2010). Diagnosis and treatment of subclinical mastitis in early lactation in dairy goats. *Journal of Dairy Science*, **93**(10): 4710-4721.

McFarland, M., Holcombe, D., Redelman, D., Garner, D., Allen, J., Surian, M., and King, D., (2000). Quantification of subclinical mastitis using flow cytometry in sheep, In: *Proceedings-American Society of Animal Science Western Section*, pp. 380-383.

Megersa, B., Tadesse, C., Abunna, F., Regassa, A., Mekibib, B., and Debela, E., (2010). Occurrence of mastitis and associated risk factors in lactating goats under pastoral management in Borana, Southern Ethiopia. *Tropical Animal Health and Production*, **42**(6): 1249-1255.

Menzies, P.I., and Ramanoon, S.Z., (2001). Mastitis of sheep and goats. *The Veterinary Clinics of North America. Food Animal Practice* **17**(2): 333-358.

Minguijón, E., Reina, R., Pérez, M., Polledo, L., Villoria, M., Ramírez, H., Leginagoikoa, I., Badiola, J., García-Marín, J., de Andrés, D., (2015). Small ruminant lentivirus infections and diseases. *Veterinary Microbiology* **181**(1): 75-89.

Morand-Fehr, P., Boutonnet, J.P., Devendra, C., Dubeuf, J.P., Haenlein, G.F.W., Holst, P., Mowlem, L., and Capote, J., (2004). Strategy for

goat farming in the 21st century. *Small Ruminant Research,* **51**(2): 175-183.

Mørk, T., Waage, S., Tollersrud, T., Kvitle, B., and Sviland, S., (2007). Clinical mastitis in ewes; bacteriology, epidemiology and clinical features. *Acta Veterinaria Scandinavica,* **49**(1)23.

Moroni, P., Pisoni, G., Antonini, M., Ruffo, G., Carli, S., Varisco, G., and Boettcher, P., (2005a). Subclinical Mastitis and Antimicrobial Susceptibility of Staphylococcus caprae and Staphylococcus epidermidis Isolated from Two Italian Goat Herds. *Journal of Dairy Science,* **88**(5): 1694-1704.

Moroni, P., Pisoni, G., Ruffo, G., Cortinovis, I., and Casazza, G., (2005b). Study of intramammary infections in dairy goats from mountainous regions in Italy. *New Zealand Veterinary Journal,* **53**(5): 375-376.

Moroni, P., Rossi, C.S., Pisoni, G., Bronzo, V., Castiglioni, B., and Boettcher, P., (2006). Relationships between somatic cell count and intramammary infection in buffaloes. *Journal of Dairy Science,* **89**(3): 998-1003.

Moulk, G., (1954). Observations on Mortality amongst Lambs in Queensland. *Australian Veterinary Journal,* **30**(6): 153-171.

Murray, R., Doetsch, R.N., and Robinow, C., (1994). Determinative and cytological light microscopy. *Methods for General and Molecular Bacteriology,* **1:** 22-41.

Olechnowicz, J., and Jaśkowski, J.M., (2014). Mastitis in small ruminants. *Medycyna Weterynaryjna* **70**(02): 67-72.

Oliver, S.P., Murinda, S.E., and Jayarao, B.M., (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of Veterinary and human pathogens: a comprehensive review. *Foodborne Pathogens and Disease,* **8**(3): 337-355.

Omaleki, L., Browning, G.F., Allen, J.L., Barber, S.R., (2011). The role of *Mannheimia* species in ovine mastitis. *Veterinary Microbiology* **153**(1): 67-72.

Onnasch, H., Healy, A., Brophy, P., Kinsella, A., and Doherty, M., (2002). A study of mastitis in Irish sheep. *Research in Veterinary Science,* 72: 42.

Onni, T., Sanna, G., Larsen, J., and Tola, S., (2011). Antimicrobial susceptibilities and population structure of *Staphylococcus epidermidis* associated with ovine mastitis. *Veterinary Microbiology,* **148**(1): 45-50.

Paape, M.J., Poutrel, B., Contreras, A., Marco, J.C., and Capuco, A.V., (2001). Milk Somatic Cells and Lactation in Small Ruminants. *Journal of Dairy Science,* **84**(Supplement): E237-E244.

Paterna, A., Contreras, A., Gómez-Martín, A., Amores, J., Tatay-Dualde, J., Prats-van der Ham, M., Corrales, J., Sánchez, A., and De la Fe, C., (2014). The diagnosis of mastitis and contagious agalactia in dairy goats. *Small Ruminant Research,* **121**(1): 36-41.

Peixoto, R.D.M., França, C.A.D., Souza Júnior, A.F.D., Veschi, J.L.A., and Costa, M.M.D., (2010). Etiology and profile of antimicrobial sensitivity of bacteria from small ruminant mastitis and relationship of diagnostic techniques. *Pesquisa Veterinária Brasileira,* **30**(9): 735-740.

Peris, C., Molina, P., Fernandez, N., Rodriguez, M., and Torres, A., (1991). Variation in Somatic Cell Count, California Mastitis Test, and Electrical Conductivity Among Various Fractions of Ewe's Milk1. *Journal of Dairy Science,* **74**(5): 1553-1560.

Persson, Y., and Olofsson, I., (2011). Direct and indirect measurement of somatic cell count as indicator of intramammary infection in dairy goats. *Acta Veterinaria Scandinavica,* **53**(15): 1-5.

Piessens, V., Van-Coillie, E., Verbist, B., Supré, K., Braem, G., VanNuffel, A., De-Vuyst, L., Heyndrickx, M. and De-Vliegher, S. (2011). Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. *Journal of Dairy Science,* **94**(6): 2933-2944.

Pincus, D.H., (2006). Microbial identification using the bioMerieux VITEK®2 System. *Encyclopedia of Rapid Microbiological Methods. Bethesda, MD: Parenteral Drug Association*.

Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V., and Honkanen-Buzalski, T., (2004). Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science,* **87**(8): 2433-2441.

Poutrel, B., de Crémoux, R., Ducelliez, M., and Verneau, D., (1997). Control of intramammary infections in goats: impact on somatic cell counts. *Journal of Animal Science,* **75**(2): 566-570.

Preston, N.W., and Morrell, A., (1962). Reproducible results with the Gram stain. *The Journal of Pathology and Bacteriology*, **84:** 241-243.

Pyörälä, S., (2003). Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research,* **34**(5): 565-578.

Pyörälä, S., and Taponen, S., (2009). Coagulase-negative staphylococci—Emerging mastitis pathogens. *Veterinary Microbiology,* **134**(1–2): 3-8.

Quinlivan, T., (1968). Survey observations on ovine mastitis in New Zealand stud Romney flocks: 2. The bacteriology of ovine mastitis. *New Zealand Veterinary Journal,* **16**(10-11): 153-160.

Quinn, P.J., and Markey, B.K., (2003). *Concise Review of Veterinary Microbiology*. Blackwell Publishing Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, Oxford, UK.

Radostits, O., Gay, C., Blood, D., and Hinchcliff, K., (2000). Veterinary Medicine. A textbook of the diseases of cattle, sheep, pigs, goats and horses. WB Saunders Company Ltd. Edinburgh, London, New York. Oxford, Philadelphia, St. Louis, Sydney, Toronto.

Rainard, P., Corrales, J.-C., Barrio, M.B., Cochard, T., and Poutrel, B., (2003). Leucotoxic activities of Staphylococcus aureus strains isolated from cows, ewes, and goats with mastitis: importance of LukM/LukF′-PV leukotoxin. *Clinical and Vaccine Immunology*, **10**(2): 272-277.

Raynal-Ljutovac, K., Pirisi, A., de Crémoux, R., and Gonzalo, C., (2007). Somatic cells of goat and sheep milk: Analytical, sanitary, productive and technological aspects. *Small Ruminant Research*, **68**(1–2): 126-144.

Rebhun, W.C., Guard, C., and Richards, C.M., (1995). *Diseases of Dairy Cattle*. Lippincott Williams & Wilkins, Baltimore, Maryland, USA.

Rice, D.N., (1981). G81-556 Using the California Mastitis Test (CMT) to detect subclinical mastitis. *Historical Materials from University of Nebraska-Lincoln Extension,* 483.

Richard A.H., Michelle A. Clark, Finkel, R., Jose A. Rey, Karen, W., and Champe, R., (2012). *Lippincott's Illustrated Reviews Pharmacology* (5^{th} edition). Philadelphia: Lippincott-Raven,2012.

Rizwan, M., Durrani, A.Z., Ijaz, M., Kashif, M., Firyal, S. (2016). Clinio-Bacterialogical Investigation of Sub-Clinical and Clinical Mastitis in Dairy Goats. *Veterinaria,* **4**: 4-6.

Roberson, J., (1999). Epidemiology of *Staphylococcus aureus* on dairy farms, In: *Annual Meeting-National Mastitis Council* pp. 38-47.

Romero, B., Morosini, M.-I., Loza, E., Rodríguez-Baños, M., Navas, E., Cantón, R., and del Campo, R., (2011). Reidentification of

Streptococcus bovis isolates causing bacteremia according to the new taxonomy criteria: still an issue? *Journal of Clinical Microbiology,* **49**(9): 3228-3233.

Rovai, M., Caja, G., Salama, A., Jubert, A., Lázaro, B., Lázaro, M., Leitner, G., (2014). Identifying the major bacteria causing intramammary infections in individual milk samples of sheep and goats using traditional bacteria culturing and real-time polymerase chain reaction. *Journal of Dairy Science,* **97**(9): 5393-5400.

Rowan, L., Morin, D., Hurley, W., Shanks, R., Kakoma, I., Hoffmann, W., Goetz, T., and Cullor, J., (1996). Evaluation of udder health and mastitis in llamas. *Journal of the American Veterinary Medical Association,* **209**(8): 1457-1463.

Ruegg, P.L., (2011). Mastitis-in-small-ruminants. *44th Annual Conference of the American Association of Bovine Practitioners, Small Ruminant Session: Sept 22-25, 2011, St. Louis MO: 1-26.*

Ruegg, P.L., Reinemann, D.J., (2002). Milk quality and mastitis tests. *Bovine Practitioner,* **36**(1): 41-55.

Ryan, D., and Greenwood, P., (1990). Prevalence of udder bacteria in milk samples from four dairy goat herds. *Australian Veterinary Journal,* **67**(10): 362-363.

Saini, V., Riekerink, R.G., McClure, J.T., and Barkema, H.W., (2011). Diagnostic accuracy assessment of Sensititre and agar disk diffusion for determining antimicrobial resistance profiles of bovine clinical mastitis pathogens. *Journal Clinical Microbiology,* **49**(4): 1568-1577.

Sánchez, A., Contreras, A., Corrales, J.C., and Muñoz, P., (2004). Influence of sampling time on bacteriological diagnosis of goat intramammary infection. *Veterinary Microbiology,* **98**(3–4): 329-332.

Sánchez, A., Contreras, A., Jiménez, J., Luengo, C., Corrales, J.C., and Fernández, C., (2003). Effect of freezing goat milk samples on recovery of intramammary bacterial pathogens. *Veterinary Microbiology,* **94**(1): 71-77.

Saratsis, P., Leontides, L., Tzora, A., Alexopoulos, C., and Fthenakis, G.C., (1998). Incidence risk and aetiology of mammary abnormalities in dry ewes in 10 flocks in Southern Greece. *Preventive Veterinary Medicine,* **37**(1–4): 173-183.

Schalm, O.W., and Noorlander, D.O., (1957). Experiments and observations leading to development of the California mastitis test. *Journal of American Veterinary Medical Association* **130**(5):199-204.

Schalm, O.W., Carroll, E.J., and Jain, N.C., (1971). Bovine Mastitis. A Symposium, Philadelphia, USA: Lea & Febiger, In: Bovine Mastitis.

Schukken, Y.H., Smit, J.A.H., Grommers, F.J., Vandegeer, D., Brand, A., (1989). Effect of Freezing on Bacteriologic Culturing of Mastitis Milk Samples. *Journal of Dairy Science,* **72**(7): 1900-1906.

Scott, M., and Jones, J., (1998). The carriage of *Pasteurella haemolytica* in sheep and its transfer between ewes and lambs in relation to mastitis. *Journal of Comparative Pathology,* **118**(4): 359.

Seyffert, N., Le Maréchal, C., Jardin, J., McCulloch, J.A., Rosado, F.R., Miyoshi, A., Even, S., Jan, G., Berkova, N., Vautor, E., Thiéry, R., Azevedo, V., and Le Loir, Y., (2012). *Staphylococcus aureus* proteins differentially recognized by the ovine immune response in mastitis or nasal carriage. *Veterinary Microbiology,* **157**(3–4): 439-447.

Shoop, D., and Myers, L., (1984). Serologic analysis of isolates of Pasteurella haemolytica and Staphylococcus aureus from mastitic

ewes. *American Journal of Veterinary Research,* **45**(10): 1944-1946.

Smith, M., (1990). Exclusion of infectious diseases from sheep and goat farms. *The Veterinary clinics of North America. Food animal Practice,* **6**(3): 705-720.

Smith, M., and Cutlip, R., (1988). Effects of infection with caprine arthritis-encephalitis virus on milk production in goats. *Journal of the American Veterinary Medical Association,* **193**(1): 63-67.

Sølverød, L., and Østerås, O., (2001). The Norwegian survey of subclinical mastitis during 2000, In: Proceedings of the 2nd International Symposium on Mastitis and Milk Quality. American Association of Bovine Practitioners, Rome, Ga., and National Mastitis Council, Madison, Wis, pp. 126-130.

Souza, F., Blagitz, M., Penna, C., Della Libera, A., Heinemann, M., and Cerqueira, M., (2012). Somatic cell count in small ruminants: Friend or foe? *Small Ruminant Research,* **107**(2): 65-75.

Špaková, T., Elečko, J., Vasil, M., Legáth, J., Pristaš, P., and Javorský, P., (2012). Limited genetic diversity of Aerococcus viridans strains isolated from clinical and subclinical cases of bovine mastitis in Slovakia. *Polish Journal of Veterinary Sciences,* **15**(2): 329-335.

Stuart, R., (1959). Transport medium for specimens in public health bacteriology. *Public Health Reports,* **74**(5): 431.

Suárez, V.H., Busetti, M.R., Miranda, A.O., Calvinho, L.F., Bedotti, D.O., and Canavesio, V.R., (2002). Effect of infectious status and parity on somatic cell count and California Mastitis test in Pampinta dairy ewes. *Journal of Veterinary Medicine, Series B* **49**(5): 230-234.

Tel, O., Aslantaş, Ö., Keskin, O., Yilmaz, E., and Demir, C., (2012). Investigation of the antibiotic resistance and biofilm formation of

Staphylococcus aureus strains isolated from gangrenous mastitis of ewes. *Acta Veterinaria Hungarica,* **60**(2): 189-197.

Tollersrud, T., Nørstebø, P.E., Engvik, J.P., Andersen, S.R., Reitan, L.J., and Lund, A., (2002). Antibody Responses in Sheep Vaccinated against Staphylococcus aureus Mastitis: A Comparison of Two Experimental Vaccines Containing Different Adjuvants. *Veterinary Research Communication,* **26**(8): 587-600.

Tolone, M., Mastrangelo, S., Di Gerlando, R., Sutera, A.M., Monteleone, G., Sardina, M.T., Portolano, B., (2016). Association study between β-defensin gene polymorphisms and mastitis resistance in Valle del Belice dairy sheep breed. *Small Ruminant Research* **136:** 18-21.

Veerkamp, R.F., Stott, A.W., Hill, W.G., Brotherstone, S., (1998). The economic value of somatic cell count payment schemes for UK dairy cattle breeding programmes. *Animal Science,* **66**(02): 293-298.

Vyletělová, M., Hanuš, O., Karpíšková, R., and Šťástková, Z., (2014). Occurrence and antimicrobial sensitivity in staphylococci isolated from goat, sheep and cow's milk. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* **59**(3): 209-214.

Waage, S., and Vatn, S., (2008). Individual animal risk factors for clinical mastitis in meat sheep in Norway. *Preventive Veterinary Medicine,* **87**(3–4): 229-243.

Watkins, G.H., Burriel, A.R., and Jones, J.E.T., (1991). A field investigation of subclinical mastitis in sheep in southern England. *British Veterinary Journal,* **147**(5): 413-420.

Watson, D., and Buswell, J., (1984). Modern aspects of sheep mastitis. *British Veterinary Journal,* **140**(6): 529-534.

Watson, D.L., Franklin, N.A., Davies, H.I., Kettlewell, P., and Frost, A.J., (1990). Survey of intramammary infections in ewes on the New England Tableland of New South Wales. *Australian Veterinary Journal,* **67**(1): 6-8.

Watts, J.L., (1988). Etiological agents of bovine mastitis. *Veterinary Microbiology,* **16**(1): 41-66.

White, E.C., Hinckley, L.S., (1999). Prevalence of mastitis pathogens in goat milk. *Small Ruminant Research,* **33**(2): 117-121.

Wikler, M.A., (2003). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth Edition, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

Windria, S., Widianingrum, D.C., Salasia, S.I.O., (2016). Identification of *Staphylococcus aureus* and Coagulase Negative Staphylococci isolates from Mastitis Milk of Etawa Crossbred Goat. *Research Journal of Microbiology* **11**(1):11.

Zadoks, R.N., Allore, H.G., Barkema, H.W., Sampimon, O.C., Gröhn, Y.T., and Schukken, Y.H., (2001). Analysis of an Outbreak of Streptococcus uberis Mastitis. *Journal of Dairy Science,* **84**(3): 590-599.

Zainab, O., Al-Zainy, O., Kefah**,** S., (2015). Prevalence of Gram positive Bacteria in Iraq Buffaloes Dairy Products. *International Journal of Advanced Research,* **3**(4): 216-255.

Zaninelli, M., Rossi, L., Costa, A., Tangorra, F.M., Agazzi, A., Savoini, G., (2015). Signal spectral analysis to characterize gland milk electrical conductivity in dairy goats. *Italian Journal of Animal Science* **14**(3):362-367.