2.4 DISCUSSION

2.4.1 Identification of bacterial isolates from the bovine mastitis milk.

Bovine Mastitis is a result of interaction between three elements like bacteria, cow and environment (Mohammed et al., 2012). Mastitis, primarily results from invasion and colonization of the secretor tissue by several microbial organisms (Radostitis, 2001). In the present study based on biochemical test different bacteria were identified. The predominant species were Staphylococcus aureus followed by Coagulase Negative Staphylococcus, Bacillus spp., E.coli, Pseudomonas spp., Aerococcus spp., Cronobacter spp. and others bacteria. Gitau et al., (2011) reported Streptococcus species were predominant followed by Staphylococcus species, Escherichia coli, Klebsiella spp., Actinomyces and Pseudomonas spp. in the mastitis. Our results are also comparable with the results of Miltenburg et al., (1996); Barkema et al., (1999) and Erskine et al., (2002), where they reported the leading causes of mastitis are Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Staphylococcus dysagalactiae, Escherichia coli and Corynebacterium bovis. Similarly Firaol et al., (2013) reported that S. aureus was the predominant species and Serratia marcescens was the least frequently in the mastitis in their study. Mahantesh and Kaliwal, (2011) have also reported that the major pathogens isolated from the bovine mastitis milk samples were S.aureus, E.coli, coagulase negative Staphylococci (CNS), Streptococcus species, Bacillus subtilis, Serratia marcesens and other Bacillus species.

In the present study a total of 210 (23.91%) isolates of S. aureus were isolated. Staphylococci are normal inhabitants of the skin and mucous membranes of animals and humans. Pathogenic strains are usually coagulase-positive (Mahon et al., 1995)
and have been found to cause disease in their hosts throughout the world (Matsunaga et al., 1993, Larsen et al., 2000). Diseases in cattle caused by *Staphylococcus aureus* range from simple abscesses and mastitis to the more severe toxic shock syndrome (Matsunaga et al., 1993, Onasanya et al., 2003). Even though the prevalence of *S. aureus* has been reported to vary with the size and geographic region of the area sampled, a high proportion of these bacteria in milk relates to poor hygiene practices (Deresse et al., 2012). The possible reason for detection of this species of bacteria at high prevalence supports its contagious nature that gives the chance to circulate and infect the udder once introduced to a heard (Sharif et al., 2009).

The present study revealed that a total of 165 (18.79%) isolates of Coagulase Negative *Staphylococcus* were isolated. Coagulase Negative *Staphylococcus* (CNS) in cows has frequently been considered as minor udder pathogens, causing relatively less udder health problems. However, CNS infections may cause substantial herd problems due to high prevalence of subclinical and clinical mastitis (Wilson et al., 1997). The proportion of CNS among bacteria isolated from milk samples from clinical mastitis is very low in many countries (Pyorala et al., 2009). Coagulase negative *Staphylococcus* infections are associated with damage to milk secretory tissue of the mammary gland by increased connective tissue stroma, moderate increases in milk and significant production decreases (Timms et al., 1987).

The results showed that, a total of 221 (25.17%) isolates of *Bacillus* spp. were isolated. Mohammed et al., (2012) reported the presence of *Bacillus* group from Bovine mastitis. Nieminen et al., (2007) concluded the presence of *B. pumilus, B. licheniformis* and *B. cereus*. Parkinson et al., (1999) reported the Presence of *Bacillus cereus* from the mastitis milk. *Bacillus cereus* is recognized as being ubiquitous in the
farm environment and the numbers of *Bacillus cereus* spores in soil rises throughout the winter (Davies and Wray, 1996). It is a common contaminant of milk at all stages of processing (Crielly et al., 1994). The organism is not generally considered to be a primary mastitis pathogen, but causes mastitis after accidental introduction to the udder (Parkinson et al., 1999). The presence of *Bacillus* spp. in the study may be due to the environmental factors like soil, water and manure, these are the main source of bacteria and when animals are exposed to soil, water and manure these bacteria infect animals via teat canals (Mohammed et al., 2012). Therefore, the present study suggest that mastitis can be controlled by hygienic conditions, cleaning manure, keeping the animals away from the stagnant water, washing udder before milking with germicidal solution.

In the present investigation 97 (11.04%) isolates of *E. coli* were isolated and identified. Many epidemiological studies established that cows are infected with *Escherichia coli* from their environment, as feces and straw, as *E. coli* enters the udder via teat canal (Lipman et al. 1995). Bacteria, hosts and environmental factors are interdependent and influence susceptibility to mastitis. Many studies indicate that the severity of *E. coli* mastitis is mainly determined by host’s factors, rather than by *E. coli* pathogenicity. Intramammary infection (IMI) caused by *E. coli* is commonly considered to be of short duration and is mostly spontaneously eliminated (Burvenich et al., 2003). By understanding the *E. coli* nature of infection, the present study advice to maintain hygienic condition surrounding the cow atmosphere to avoid mastitis.

The results suggest that, a total of 72 (8.20%) isolates of *Pseudomonas* spp. were isolated. Water and soil are the primary sources of *Pseudomonas* spp. (Frank, 1977; Jay, 2000). Hose nozzles and milking equipment can become colonized by
*Pseudomonas*. Under stressful conditions, such as the presence of low levels of iodine-based disinfectants, these organisms produce a slimy glycocalyx (Ombaka *et al.*, 1983). This slime favors adherence to and colonization of pipe and hose surfaces, and offers increased resistance to surfactants, phagocytes and certain antibiotics. *Pseudomonas* spp. contamination of hoses and water can be extremely difficult to eliminate (Erskine *et al.*, 1987). Therefore, the present study recommends disinfection of udders and milking equipment’s were essential in the control of outbreaks of *P. aeruginosa* mastitis outbreaks.

Bacterial contamination of milk usually occurs during the milking process and this depends on the sanitary condition of the environment, utensils used for milking and the milking personnel. It could also result from micro-organisms that enter the udder through the teat opening canal (Kalsoom *et al.*, 2004). In certain cases, untreated groundwater was used to wash the containers that were used for milking. This may have contributed to the high level of pathogens. Based on observations made during the collection of samples, present study suggest that, the improper hygiene and poor farm management practices contributed to the presence of many pathogenic bacteria in the milk. Improving the hygienic conditions of the milking environment and utensils may reduce the prevalence of many pathogens in milk and prevent its transmission to humans.

2.4.2 Molecular characterization of bacterial isolates from the bovine mastitis milk

Molecular methods being highly sensitive and selective currently used to identify microorganisms. Environmental conditions may have intense impact on morphological and physiological characteristics, hence the accurate identification of
isolates turned out to be more difficult (Bakri et al., 2010). The molecular techniques are more significant for the characterization of the new isolates, allowing grouping the strains. Furthermore, complex studies (microbiological, biochemical and molecular) are essential, when the identification of new isolate is the purpose of the investigation (Rahna et al., 2013). In the present study Staphylococcus aureus, Staphylococcus sciuri, Staphylococcus hominis, Staphylococcus saprophyticus, Staphylococcus capitis, Bacillus subtilis, Bacillus methylotrophicus, Bacillus gaemokensis, Bacillus cereus, Bacillus mycoides, Lysinibacillus sphaericus, Geobacillus stearothermophilus, E.coli, Pseudomonas thivervalensis, Aerococcus viridans, Cronobacter sakazakii and Kurthia gibsonii were identified by 16s rRNA sequencing.

The development of molecular biological methods, such as nucleic acid analysis, protein patterns or fatty acid profiles, has added possibilities for the rapid identification of bacteria (Busse et al., 1996). Species-specific DNA sequences can be used for the identification of bacterial species. The 16s-23s rRNA has proven useful for identification of strains and species (Gurtler and Stanisich, 1996). The 16s rRNA sequencing makes it possible to identify and distinguish closely related bacterial species. In the present study the characterization of the bacterial species have been done by using the 16s rRNA sequence. Similarly, many molecular tools like 16s-23s rRNA spacer regions used by Forsma et al., (1997) PCR–DGGE method used by Ying et al., (2009), 16s rRNA sequencing used by Shea Beasley, (2004) for the identification of the bacterial species. rRNA sequences and especially the 16S rRNA represent the most important current targets of study in bacterial evolution and ecology, including the determination of phylogenetic relationships among taxa, the exploration of bacterial diversity in the environment and the quantification of the relative abundance of taxa of various ranks (Hugenholtz et al., 1998). The 16S rRNA
is suitable for this purpose for several reasons. The gene is universally distributed, allowing the analysis of phylogenetic relationships among distant taxa. As a functionally indispensable part of the core gene set, the 16S rRNA gene is expected to be only weakly affected by horizontal gene transfer (Daubin et al., 2003), which further supports its use for phylogenetic studies. Therefore the present molecular identification work suggest that, the 16s rRNA sequencing is more accurate for the species identification.

2.4.3 Antibacterial susceptibility pattern to the bacterial isolates from the bovine mastitis milk

In the present study antibiotics belong to the groups Aminoglycosides, Beta lactams, Cephalosporins, Quinolones, Macrolides, Glycopeptides and Tetracyclines were used. A total of 20 antibiotics i.e Amikacin, Amoxyclav, Ampicillin, Methicillin, Oxacillin, Penicillin G, Cefaclor, Cefixime, Cefpodoxime, Ceftriaxone, Ciprofloxacin, Norfloxacin, Ofloxacin, Gentamicin, Azithromycin, Erythromycin, Streptomycin, Vancomycin, Tetracycline and Chloramphenicol were used.

The antibiotic susceptibility pattern revealed that all isolated strains were showed higher susceptibility to Ofloxacin (96.69%), Ciprofloxacin (94.41%), Chloramphenicol (91.57%), Azithromycin (91.34%), Tetracycline (90.54%), Norfloxacin (84.85%), Gentamicin (83.59%), Amikacin (80.18%), Amoxyclav (76.76%), Erythromycin (74.03%), Streptomycin (61.95%), Ceftriaxone (53.75%), Cefaclor (42.14%), Ampicillin and Cefixime (41.79%), Cefpodoxime (40.27%), Oxacillin (18.22%) and least to Penicillin G (8.93%). Most of the isolates were susceptible to Quinolones followed by Macrolides, Cephalosporins, Tetracyclines and least to Beta lactams.
These results were contradictory to the reports of Mahantesh and Kaliwal, (2011) where they have reported that, tetracycline was found to be more effective antibiotic among all the tested antibiotics followed by cloxacillin, chloramphenicol, ampicillin, streptomycin, gentamicin, rifampicin, cephalothin, amikacin, trimethoprim, penicillin-G, carbencillin and least was polymyxin-B. Mihaela and Herman, (2010) have also reported highest sensitivity was with cephalothin followed by Oxacillin, gentamicin, tetracycline, ampicillin and erythromycin. Whereas Magdalena et al., (2012) concluded that the strains were susceptible to amoxicillin with clavulanic acid, cephalexin, cloxacillin and Oxacillin. Sudhakar et al., (2010) reported that ciprofloxacin was the most effective antibiotic against bacteria isolated from mastitic milk. Whereas Nadeem et al., (2013) showed that enrofloxacin proved to be the most effective antibiotic followed by norfloxacin and gentamycin.

Antibacterial therapy is an important part of a mastitis control program in dairy cattle. Antimicrobial susceptibility tests can guide the veterinarian in selecting the most appropriate antimicrobial agent for treatment of the disease caused by pathogens. Antibiogram studies of mastitis pathogens are important to suggest suitable antibiotic treatment to provide quality milk to the consumers and to prevent antibiotic resistance, potential health risk for humans. Therefore the present study recommends frequently use of an in vitro antimicrobial sensitivity test of clinical or subclinical mastitis pathogens by bovine practitioners to guide cow or herd level treatment decisions.
2.4.4 Antibiotic resistance pattern to the bacterial isolates from the bovine mastitis milk.

In the Bovine mastitis the incorrect or incomplete treatment of animals also contributes significantly to the development of bacterial resistance against them. In the present study the overall antibiotic resistance pattern revealed that all isolated strains were showed higher resistance to Methicillin (100%) followed by Penicillin G (93.16%), Oxacillin (81.77%), Cefpodoxime (59.20%), Ampicillin and Cefixime (58.20%), Cefaclor (57.85%), Ceftriaxone (46.24%), Streptomycin (33.71%), Erythromycin (28.47%), Amoxyclyav (23.23%), Amikacin (19.81%), Gentamicin (16.40%), Norfloxacin (15.14%), Tetracycline (9.56%), Azithromycin (8.65%), Chloramphenicol (8.42%), Ciprofloxacin (5.58%) and least to Ofloxacin (3.30%).

The present study suggest that, a large number of the isolates were found to be resistant to earlier and established antibiotics compared to the newer developed antibiotics. Appearance of resistance against a particular antibiotic in a specific region may be due to its frequent and long-term use (Moon et al., 2007; Kumar et al., 2010). The most commonly used antibiotics on conventional dairies were Penicillin, Cephalosporin and Tetracycline’s. For mastitis, Penicillin, Ampicillin and Tetracycline’s were commonly used (Mohammed et al., 2012).

The antibiotic resistant of this study revealed that the isolated Staphylococcus aureus showed resistant to multi drugs. These results were compared to the reports of Hulya et al., (2006), where the Staphylococcus aureus isolates were similar to Methicillin and higher to Penicillin G, Ampicillin, Amoxicillin, Cloxacillin and Gentamicin. The findings were similar to the findings of Mahantesh and Kaliwal, (2011) where they reports resistance to Penicillin and Ampicillin. The results were higher to the reports of Unakal and Kaliwal, (2010) were showed resistance to

The antibiotic resistant of the present study revealed that the isolated Bacillus spp. showed resistant to multi drugs. These results were in line with the reports of Mohammed et al., (2012), were the resistance of Ampicillin, Cefotaxime, Ceftizoxime, Amikacin, Ofloxacin and Tetracycline. The reports were higher to the reports of Firaol et al., (2013) to the Penicillin G, lower to Chloramphenicol and Gentamycin.

The usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Shitandi et al., 2004). These traits are coded for by particular genes that may be carried on the bacterial chromosome, plasmids (Rychlik et al., 2006), hence these are easily transferred among isolates. From the study, a large number of isolates were found to be resistant to previous and established antibiotics compared to the newer developed antibiotics. Appearance of resistance against a particular antibiotic in a specific region may be due to its frequent and long-term use (Moon et al., 2007; Kumar et al., 2010). The genetic flexibility of bacteria has contributed to their survival in altered environments, because of their capacity to acquire and transfer resistant genes. Bacteria have developed resistance to all different classes of antibiotics discovered to date. The most frequent type of resistance is acquired and transmitted horizontally either through conjugation, transformation or transduction of plasmid (Frost et al., 2005).

It has been also reported that MRSA isolates that are resistant to beta-lactam antibiotics may develop induced resistance to Vancomycin (Gundogan et al., 2005).
But in the present study all the isolates were shown susceptible to Vancomycin but these results were contradictory to the reports of Deresse et al., 2012, where they have reported that, in Ethiopia *Staphylococcus aureus* were resistance to Vancomycin and also to the reports of Unakal and Kaliwal, (2008) in India, showed resistant to Vancomycin isolated from human clinical specimens. The presence of antibiotic-resistant *Staphylococcus aureus* has been reported to negatively affect the treatment of its associated infections in humans and animals (Brouillette et al., 2005; Moneoang et al., 2009).

It is possible that mastitogenic bacteria can lose the sensitivity to antibiotics over the time or even acquire sometimes this feature (Edward et al., 2008). It is necessary to monitor mastitis pathogens to assess any changes in their antibiotic resistance patterns. Careful use of antibiotics can avoid the increase and dissemination in antimicrobial resistance arising from the use of antimicrobial drugs in animals (Fluit et al., 2006; Schwarz et al., 2001). Also previous investigations of Malinowski et al., (2006) showed that recovery rates from acute mastitis equals “zero” if the pathogens were resistant to antibiotics used to intrammary treatment. Lack of stringent regulations and monitoring in the dispensing and use of antimicrobials in veterinary establishments and mass inoculation of herds of animals by some farmers has risen as a contributory factor to increase antimicrobial resistance (Alemu et al., 2012). Some natural products like Bacteriocin may be used as an alternative type of antibiotic (Kaur et al., 2012). These natural agents which may inhibit growth of huge number of microbes. They exist in various ecological niches. (Suneel and Kaliwal, 2014) have Reported the bacteriocins produced by the *Lactococcus garvieae* showed antibacterial effect against the *B. cereus* and *B. subtilis*. 
The present study demonstrated the presence of alarming level of resistance of frequently and commonly used antimicrobial agents to the isolated bacteria from Bovine mastitis. Important reasons for the failure of treatment of mastitis are the indiscriminate use of antibiotics without *in vitro* sensitivity of causal organisms (Kulkarni and Kaliwal, 2013). Therefore, an examination of the antibiotic resistance profiles of the isolates may serve as a major tool in evaluating both the hygienic conditions employed during milking and the health hazards that humans may encounter when infected by antibiotic resistant strains (Deresse *et al.*, 2012) and also the study recommends implementation of a systematic application of an *in vitro* antibiotic susceptibility test, earlier to the use of antibiotics in both treatment and prevention of Bovine mastitis.