Introduction
I. INTRODUCTION

Mastitis, a multi-etiological disease, continues to be the most important disease economically in modern dairy industry. It remains a major global challenge to milk production even in the face of widespread implementation of mastitis control strategies. Despite the significant advances in understanding the disease, both clinical and sub-clinical mastitis remain a problem in dairy herds and prevalence rates in many countries remain similar to those observed decades ago. The prevalence of bovine mastitis continues to affect the dairy herds throughout the world in spite of continued research activity on the problem over the century (Sadana, 2006).

Mastitis negatively affects the quality of milk, milk production, farm economics and animal welfare. It is estimated that mastitis alone accounts to about 70 per cent of all avoidable losses incurred during milk production. In India, the overall economic loss due to mastitis is estimated to be Rs. 7165.51 crores (Bansal and Gupta, 2009), a number which increased from 1607.2 crores a decade and a half before (Singh and Singh, 1994).

Subclinical mastitis is considered economically the most important in modern dairy herds, affecting 20% to 50% of cows in given herds (Wilson et al., 1997; Pitkala et al., 2004). Although the loss due to subclinical mastitis (SCM) is difficult to quantify, most experts agree that it costs the average dairy farmer more than the clinical mastitis does (Hegde, 2011).

According to the International Dairy Federation, the diagnosis of mastitis is based on the somatic cell count (SCC) and microbiological status of the quarter (Hillerton, 1999). Although several tests are being employed for diagnosis of SCM, SCC is the most
frequently used indicator of SCM in dairy cattle and is considered the gold standard to detect SCM status (Hamann, 2002). The most important cause of increased SCC is a bacterial infection of the mammary gland (Dohoo and Meek, 1982; Harmon, 1994). Even though the nonbacterial factors such as age, stage of lactation, season, stress, management, day-to-day variation, and diurnal variation affect SCC values, they were considered less important than IMI status.

The indirect mastitis tests such as the California Mastitis Test (CMT), measuring the electrical conductivity (EC) of the milk and bromothymol blue (BTB) strip test can also be used as the “cow side” tests to detect SCM and are relatively quick, simple and easy to perform. While the CMT indirectly predicts the somatic cell count of milk, the BTB strip test detects the increased alkalinity of the milk due to inflammation of the udder. Measuring EC of milk as an indicator of mastitis has been used widely over the last decade. Increased EC of milk is due to the increased sodium and chloride concentrations in milk which occur during inflammation (Viguier et al., 2009). Although no single test can detect all SCM cases, a comparative evaluation of all these with respect to the bacteriological analyses would give us an indication about the usefulness of these tests to detect the SCM cases.

In most countries, the major mastitis pathogens are *Staphylococcus aureus, Streptococcus agalactiae* (contagious pathogens), *Escherichia coli, Streptococcus dysgalactiae* and *Streptococcus uberis* (environmental pathogens). Staphylococcal species are the most commonly associated bacteria with intra-mammary infections (IMI). Coagulase production is considered to be one of the virulence properties of
*Staphylococcus.* Based on this property of ability to coagulate to coagulate plasma, more than 50 *Staphylococcus* species have been characterized and divided into two groups; viz., coagulase positive staphylococci (predominantly *S. aureus*) and coagulase negative staphylococci (CoNS). Although, *S. aureus* is commonly associated with bovine mastitis in many countries, CoNS are also frequently found (Bengtsson *et al.*, 2005).

In contrast to *S. aureus*, coliforms and streptococci which cause severe mastitis, CoNS have been often considered opportunistic minor pathogens, causing only subclinical or mild clinical mastitis, with only a marginal increase in SCC (Honkanen-Buzalski *et al.*, 1994; Taponen *et al.*, 2006). However, in recent years, as a group, CoNS have become the most common bacteria associated with bovine mastitis in many countries (Tenhagen *et al.*, 2006) and could therefore be described as “emerging mastitis pathogens”. CoNS can behave as contagious or environmental pathogens (Taponen and Pyörälä, 2009). The understanding and control of CoNS mastitis is complicated by the heterogeneity of this group of bacteria. So far, 16 CoNS species have been isolated from mastitic bovine milk samples, and despite some variation between herds and countries, *S. simulans*, *S. chromogenes*, *S. haemolyticus* and *S. epidermidis* seem to be the most common (Luthje and Schwarz, 2006; Taponen *et al.*, 2006).

In routine mastitis diagnostics, CoNS are normally not identified to species level and are treated as a group, but in reality, they consist of many different species. Because of the increasing clinical significance of CoNS, accurate species identification of CoNS is the need of the hour to permit a more precise determination of host-pathogen relationship of CoNS.
Definitive diagnosis of an IMI is based on the identification of a bacterial pathogen/s in milk from a cow with mastitis and it is generally performed by traditional culture followed by the analysis of phenotypic characteristics utilizing biochemical tests, serotyping, and enzymatic profiles of the bacterial isolates (Oliver et al., 2004). Conventional microbiological methods, often considered to be the gold standard for identification of bacteria from milk, permit the identification of viable bacteria as the causative agent of mastitis as well as the study of antimicrobial susceptibilities which helps for adopting appropriate antimicrobial therapy.

There are several disadvantages associated with current microbiological methods. A negative culture may result from residual antibiotics following antibiotic therapy or from low numbers of pathogens in the sample. Presence of leukocytes in milk from cases of clinical mastitis may also result in negative culture results (Phuektes et al., 2001). Further, current methods of mastitis pathogen identification are time consuming; as currently employed identification of most pathogens by standard biochemical methods generally requires more than 48 hr to complete. Inadequate pathogen detection or confirmation techniques may delay timely intervention in disease control.

Considering the above limitations, the use of DNA-based assays has become popular recently. Perhaps the greatest single advantage of DNA-based diagnostic assays is that these methods focus on the unique nucleic acid composition of the bacterial genome rather than on various phenotypic expressions of products that nucleic acids encode. Therefore, DNA-based identification assays are subject to less variability compared with diagnostic methods based on phenotypic characterization. They allow
definitive confirmation of pathogens and at the same time permit rapid screening of a large number of pathogens, simultaneously. The development of polymerase chain reaction (PCR) based methods provide a promising option for the rapid identification of bacteria. With this method, identification of bacterial species can be made in hours, rather than days required for conventional culture methods. Polymerase chain reaction, being highly sensitive and specific, can improve the level of detection and only a few numbers of the pathogens are enough to detect a positive case. Hence, with this method, the diagnosis can be made at earlier stages of infection and also in carrier animals, when the numbers of bacteria in milk may be very low. Moreover, PCR can detect bacteria even in the presence of residual antibiotics and preservatives in milk which otherwise may inhibit bacterial growth in culture methods leading to false negative results. PCR protocols have been developed for identification of various mastitis pathogens (Forsman et al., 1997; Kim et al., 2001; Riffon et al., 2001; Daly et al., 2002; Meiri-Bendek et al., 2002; Phuektes et al., 2001, 2003). Polymerase chain reaction based detection of CoNS needs to be developed which can be adopted at different levels of diagnostic labs for routine diagnosis and herd diagnosis.

However, aforementioned methods are currently labour-intensive and it is expensive to do a separate PCR test for every possible mastitis pathogen. Hence, the significance of multiplex PCR tests is of interest, in which several pathogens can be detected simultaneously thereby saving precious time and cost (Phuektes et al., 2003; Bottero et al., 2004).
Further, there are findings which contradict the fact that the conventional biochemical assays are the gold standard in the isolation and identification of the bacterial pathogens and support the debate stating about the inconsistency of these test profiles (Freney et al., 1992; Phuektes et al., 2001, 2003; Oliver et al., 2004; Picard et al., 2004). In a few studies, the commonly used phenotypic methods were compared with PCR amplicon-sequencing based methods targeting different genes demonstrated the superiority of genotypic methods over phenotypic assays for identification of CoNS species (Heikens et al., 2005) and sequence-based genotyping were reported to be better alternative for species-level identification (Capurro et al. 2009). In view of this, phenotypic methods for identification of CoNS need to be compared with molecular methods regarding the accuracy and specificity in identification of CoNS.

The rampant use of antibiotics has created a scenario where antibiogram studies may need to be performed in addition to microbiological investigation for mitigating the disease by the selection of appropriate antibiotics. While the antimicrobial resistance has been recognized since the earliest days of chemotherapy, the problems appear to be accelerating, accumulating and global. Coagulase negative staphylococcal species may differ in antimicrobial susceptibility, virulence factors, host response to infection, and transmissibility, and thus studies on species specific antimicrobial susceptibility are needed to select appropriate antimicrobial therapy and to develop species-specific management practices (Sampimon et al., 2009). Coagulase negative staphylococci are reported to be more resistant than S. aureus and they easily develop multi drug resistance (Machado et al., 2008). Besides this, CoNS may also represent a reservoir of antibiotic resistant genes that could be transmitted to S. aureus and other Staphylococci, including
those pathogenic to humans (Archer and Climo, 1994). Hence, there is a need to obtain vital data with respect to antibiotic resistance patterns of CoNS at species level which provides valuable information for effective mastitis therapy and management.

As far as India is concerned, knowledge of CoNS species involved in bovine mastitis is limited. The literature about their prevalence is scanty in India (Rajeev et al., 2009; Shome et al., 2011) and insufficient to draw a conclusion on their association with bovine mastitis. Hence, the studies in this aspect are needed to update our knowledge of CoNS involved in bovine mastitis, and for this reason, there is a need to develop a rapid, sensitive and specific assay for detection of CoNS in mastitis cases in the country. Further, the dairy industry would benefit from more research on the epidemiology of CoNS in mastitis, more reliable methods for species identification and their pathogenic potential including antimicrobial sensitivity.

Keeping the above in view, the present study was undertaken with the following objectives:

1. Isolation of Coagulase Negative Staphylococci (CoNS) from clinical and subclinical cases of bovine mastitis
2. Identification of CoNS to species level based on cultural and biochemical properties.
3. Development and application of PCR for molecular characterization of predominant CoNS species
4. Study of antibiogram profile of CoNS isolates and detection of antibiotic resistant genes by PCR