INTRODUCTION
I. INTRODUCTION

According to the Food and Agricultural Organization (FAO), Asia, the world's largest milk production region in 2006 accounts for 34 per cent of global output. Within the Asian region, India’s milk production has grown by an average of nearly four per cent per year since 1970 and became the largest milk producing country in the world in 2006. India is endowed with the largest livestock population in the world. It accounts for 57 per cent of the world’s buffalo population and 14 per cent of the cattle population (Livestock Census, 2007). Animal Husbandry and Dairying plays a prominent role in the rural economy in supplementing the income of rural households, particularly, the landless, small and marginal farmers. Several measures initiated by the Government to increase the productivity of huge livestock has resulted in significant increase in the raw cow milk output from 8.74 million metric tonnes (MT) in 1970 to 44.1 million MT in 2008.

In India, improvisation in quality and quantity of milk produced is a prerequisite for export of milk and milk products. However, it is threatened by mastitis which continues to be a cause of significant economic loss to the dairy industry not only in India, but also internationally. It impacts on animal health and welfare, on market image, on profitability and critically, on the quality of life of dairy farmers. Despite significant advances in our understanding of the disease, clinical and sub-clinical mastitis remain a problem in dairy herds and prevalence rates in many countries remain similar to those published decades ago. In the complex milieu of the modern dairy farming, the interaction of production diseases like mastitis, their relationship with nutritional strategy, housing, environment and the fundamental influence of social and attitudinal factors
make prevention and control a challenge. Importantly, clinical mastitis represents only the ‘tip of the iceberg’ and it is the significance of the sub-clinical mastitis that is frequently underestimated.

The overall national economic loss in India due to mastitis was to the tune of Rs.16,072 million (that due to clinical mastitis to be Rs.2,856.4 million and Rs.2,345.9 million and due to subclinical mastitis Rs.6,038.7 million and Rs.4,831 million in cattle and buffaloes, respectively). Average decrease in milk yield due to clinical and subclinical mastitis was estimated to be 50 per cent and 17.5 per cent respectively (Singh and Singh, 1994).

Subclinical mastitis (SCM) is the main form of mastitis in modern dairy herds, exceeding 20 to 50 per cent of cows in given herds (Wilson et al., 1997; Pitkala et al., 2004). The cost of subclinical mastitis is very difficult to quantify, but most experts agree that subclinical mastitis costs the average dairy farmer more than does clinical mastitis. Assuming a 45 per cent prevalence of subclinical mastitis, the cost has been estimated in the range of $180 to $320 per case (Wilson et al., 1997). Approximately 70 per cent of this cost is associated with a reduction in milk production. A large portion of it results from irreversible damage to the mammary tissue (Oliver and Calvinho, 1995). Although antibiotics are very useful to treat the infection, they do not directly protect the gland from being damaged. Subclinical mastitis was found more important in India (varying from 10–50 percent in cows and 5–20 per cent in buffaloes) than clinical mastitis (1–10 per cent). The incidence was highest in Purebred Holsteins and Jerseys cows and lowest in local cattle and buffaloes (Joshi and Gokhale, 2006).
Mastitis, an inflammation of the mammary gland usually occurs primarily in response to intramammary bacterial infection, but also to intramammary mycoplasmal, fungal, viral or algal infections. Mechanical trauma, thermal trauma, and chemical insult also predispose the gland to intramammary infection (IMI). Occurrence of mastitis depends on the interaction of host, agent, and environmental factors. In most countries, the major mastitis pathogens are Streptococcus agalactiae (S. agalactiae) Staphylococcus aureus (S. aureus) (grouped as contagious pathogens) and Streptococcus dysgalactiae (S. dysgalactiae), Streptococcus uberis (S. uberis) and Escherichia coli (E. coli) (grouped as environmental pathogens). The word “major” reflects their considerable impact on cow health, milk quality and productivity. The gold standard to measure inflammation is the cytological investigation; milk somatic cell count (SCC) and other methods are compared with SCC (Hamann, 2002).

Somatic cell count (SCC) is the most frequently used indicator of subclinical mastitis in dairy cattle. 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. To make optimal interpretations of SCC tests, it is always necessary to collect the milk samples immediately before milking. The diagnosis of mastitis according to the International Dairy Federation (IDF) recommendations is based on the SCC and microbiological status of the quarter (Hillerton, 1999). At present, the only indirect mastitis tests which can be used as the “cow side” tests are the California Mastitis Test (CMT) and measuring the electrical conductivity (EC) of the milk using a
hand-held meter. Electrical conductivity of milk has been introduced as an indicator trait for mastitis over the last decade. Measuring EC of milk to detect mastitis is based on the ionic changes which occur during inflammation, since the sodium and chloride concentrations increase in milk (Kitchen, 1981). The use of EC as a diagnostic method for the identification of subclinical mastitis has been studied for a long time and a positive correlation between SCC and EC has been reported.

Identification of a bacterial pathogen in milk from a cow with mastitis is regarded as the definitive diagnosis of an IMI. Identification of mastitis pathogens is generally performed by traditional culture followed by biochemical tests on bacterial isolates (Oliver et al., 2004). Conventional microbiological methods have been the gold standard for identification of bacteria from milk. Identification of bacteria in most clinical laboratories is currently based on analysis of phenotypic characteristics utilizing biochemical tests, serotyping and enzymatic profiles. Advantages associated with conventional culture methods are that viable bacteria can be identified as the causative agent of mastitis and antimicrobial susceptibilities can be performed providing information for selection of appropriate antimicrobial therapy. However, 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). Current methods of mastitis pathogen identification are time consuming; identification of most pathogens by standard biochemical methods generally requires more than 48 hr to complete.
Inadequate pathogen detection or confirmation techniques have often delayed timely intervention in disease control.

Considering the limitations of conventional approaches, use of DNA-based assays have 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. The DNA-based identification systems are targeted for specific pathogens, allow for rapid screening of a large number of pathogens simultaneously and provide definitive confirmation of pathogens. The development of PCR based methods provides a promising option for the rapid identification of bacteria. With this method, rapid identification of bacterial species can be made in hours, rather than days required for conventional culture methods. PCR being highly sensitive and specific can improve the level of detection. Theoretically, only a few numbers of the pathogens are necessary to yield a positive PCR diagnosis. In view of this, with this method, the presence of pathogens can be shown at earlier stages of infection and in carrier animals, when the numbers of bacteria in milk may be very low. Moreover, PCR can detect bacteria in the presence of residua of therapeutic antibiotics and preservatives in milk and therefore there won't be false negative results because of lack of bacterial growth. Polymerase chain reaction protocols have been developed for identification of various mastitis pathogens (Jayarao et al., 1996; 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).
However, aforementioned methods are currently very 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 tested simultaneously thereby saving precious time and cost (Phuektes et al., 2003; Bottero et al., 2004).

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

1. Isolation and identification of major bacterial pathogens from the cases of subclinical bovine mastitis

2. Standardization of simplex and multiplex PCR for rapid identification of *Staphylococcus aureus, Escherichia coli* and predominant Streptococcal species

3. Direct, rapid and simultaneous detection of *Staphylococcus aureus, Escherichia coli* and predominant Streptococcal species pathogens by multiplex-PCR method in milk samples of subclinical cases of bovine mastitis