DISCUSSION
V. DISCUSSION

India, the largest producer of milk in the world accounts for more than 13 per cent of the total world output and 57 per cent of the Asia’s, with a production level of 104.9 million tons in the year 2007-08. The challenging task is the production of quality milk adhering to international standard to hold the first position in the global dairy industry. Bovine mastitis is the single most important factor contributing to the economic losses to the dairy industry, resulting in reduction in milk yield and quality of the milk. Mastitis is one of the most prevalent diseases of high yielding dairy animals. It is of great economic importance to the dairy industry (Sharma and Prasad, 2003; Oncel et al., 2004).

Worldwide, mastitis is the most common infectious disease affecting dairy cows and the most economically important disease of the dairy industry. Mastitis is defined as an inflammation of the mammary gland and usually occurs primarily in response to intramammary bacterial infection. The occurrence depends on the interaction of host, agent, and environmental factors. Mastitis is characterized as clinical or sub-clinical mastitis, depending on, if clinical signs are present or not. More than 130 microorganisms are related to bovine mastitis, with mastitis-causing bacteria broadly classified as contagious or environmental pathogens (Watts, 1988). In most countries, the major mastitis pathogens are Streptococcus agalactiae, Staphylococcus aureus (contagious pathogens), Streptococcus dysgalactiae, Streptococcus uberis and Escherichia coli (environmental pathogens). The word “major” reflects their considerable impact on cow health, milk quality, productivity and prevalence.
This thesis focuses on sub-clinical mastitis, which is defined as mastitis that cannot be diagnosed by clinical examination, instead analysis of inflammatory products in the milk is needed. In most cases, direct or indirect measurement of the milk SCC is used to diagnose sub-clinical mastitis. This is often combined with bacteriological examination of the milk to get an etiological diagnosis. Subclinical mastitis 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). The losses in sub-clinical mastitis are mainly due to loss of milk production, reduced payment caused by lower milk quality, culling and replacement of cows (Sandgren et al., 2008).

In India, subclinical mastitis was found more (varying from 10–50 per cent 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. The factors like herd size, agro climatic conditions of the region, variations in socio-cultural practices, milk marketing, literacy level of the animal owner, system of feeding and management were found important factors affecting the incidence of subclinical mastitis (Joshi and Gokhale, 2006). Sub-clinical mastitis is 3-40 times more common than the clinical mastitis and causes the greatest overall losses in most dairy herds (Schultz et al., 1978).

The suitability of a detection method for routine diagnosis depends on several factors, such as specificity, sensitivity, expense, amount of time and applicability to large number of milk samples. The most common but nonspecific method to identify potential intra mammary infections (IMI) are Somatic Cell Count (SCC), Electrical Conductivity
(EC) and the California Mastitis Test (CMT) in field conditions and the automated method in the diagnostic laboratory. However, conventional microbiological methods have been the gold standard for identification of bacteria from milk.

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. Identification of bacteria in most clinical laboratories is currently based on analysis of phenotypic characteristics utilizing biochemical tests, serotyping and enzymatic profiles. However, there are several disadvantages associated with current microbiological methods such as 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 and generally require more than 48 hr to complete. Inadequate pathogen detection or confirmation techniques have often delayed timely intervention in disease control intervention.

To circumvent some of the problems associated with conventional microbiological procedures, the DNA-based assays are being used which focus on the unique nucleic acid composition of the bacterial genome rather than on phenotypic expression of products that nucleic acids encode. Also, 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.

Hence, with this preamble, in the present study, along with the conventional methods like SCC, EC, isolation and biochemical characterization, the necessity to adopt rapid, accurate and sensitive molecular methods like PCR and multiplex PCR for the early and accurate detection of subclinical mastitis causing pathogens was undertaken. These methods may also be extrapolated to determine different strains of a causative bacterial species lurking in the area of study and also may aid as a useful tool for the comparison of the strains between the farms. Further, the outcome may be useful in establishing the effective control strategies.

5.1 Somatic Cell Count

Somatic cell count is the most frequently used indicator of subclinical mastitis in dairy cattle. The most important cause of increased SCC is bacterial infections of the mammary gland. Nonbacterial factors that affect SCC include age, stage of lactation, season, stress, management, day-to-day variation, and diurnal variation. These factors are considered less important than IMI status (Dohoo and Meek, 1982).

During mastitis, the SCC increases significantly due to an influx of somatic cells from the blood into the milk, indicating that the milk composition is adversely affected (Schukken et al., 2003). Unfortunately, the range of SCC observed in cows with IMI and those without IMI overlap, so it is impossible to select a single threshold which clearly separates infected from non-infected cows. However, the SCC is also influenced by factors apart from the udder health, and therefore, there is an interest in finding new
markers for milk quality (Pyorala, 2003). Further, SCC is also influenced by stages of lactation, especially immediately after parturition SCC is high, but decreases fast to the normal level within 4–5 days after calving. Towards the end of the lactation period, SCC increases slightly. According to the recent studies, the physiological effects affect very little the SCC of truly healthy cows (Akineden et al., 2001).

As far as the cut off values of SCC is concerned, different values are adopted across different countries to define SCM and the cut off value of SCC to declare SCM is not uniform throughout the world. According to European Union regulations, Australia and New Zealand, the SCC penalty limit for saleable milk is at 400,000, Canada at 500,000 and the United States at 750,000 cells / mL (Paape and Contreras, 1997). In Sweden, the cut off value is 200,000 cells / mL. International Dairy Federation defined a break point of 500,000 cells / mL for SCC. However, under Indian Scenario, there have been no such standards adopted.

In the present study, a total of 246 milk samples collected from four organized and three unorganized sectors, were affiliated to different groups based on SCC as <1 lakh SCC (78 milk samples); 1 to 2 lakh SCC (19 milk samples); 2 to 5 lakh SCC (38 milk samples) and SCC> 5 lakh (111 milk samples). A preliminary evaluation of SCC of these milk samples indicated SCM at 45 per cent (111 out of 246 samples were positive for SCM), if the conventional criteria of SCC ≥5 lakhs is considered to declare positivity. However, a total of 186 milk samples were subjected for bacterial isolation based on high SCC> 500,000 cells / mL (111samples), EC of > 6.5 mS/cm and remaining samples from other groups.
The analysis of the SCC of the organized farms revealed an overall SCM at 61 per cent, and varied from 33- 90 per cent between individual farms. Increased SCC as an indicator of IMI has been described earlier (Trinidad et al., 1990; Hayakawa et al., 2000). However, in the present study, the cut off value of 5,00,000 cells / mL to declare as SCM does not appear to be an absolute one since the mastitis pathogens could be isolated from milk samples with very low SCC (10,000 – 20,000 cells /mL). In view of this it is necessary to fix SCC cut off value to declare SCM cases in India.

5.2 Electrical conductivity

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. Also, the change in pH and decrease of fat affect EC. Mastitis is not the only factor affecting the ionic content of milk, and non-mastitis related variation in EC is a major drawback to the diagnostic value of EC (Hamann et al., 1998). As many other mastitis parameters, conductivity is also influenced by the age of the cow and the stage of lactation (Nielen et al., 1992). The use of EC as a diagnostic method for the identification of subclinical mastitis has been studied for a long time with variable results (Jensen and Knudsen, 1991; Hamann et al., 1998). EC measurements in milk from healthy cows generally ranged from 5.5 to 6.5 mS/cm (Norberg et al., 2004). Spakauskas et al. (2001) observed that the EC of milk samples taken from healthy cows (61.7 per cent) was found to be 4.3-5.7 mS/cm and in cases of cows with subclinical mastitis, EC of milk increased to 6.1-8.5 mS/cm. While evaluating the accuracy of a hand-held EC meter for the detection of subclinical mastitis, Mansell and Seguya (2003) observed that the EC was higher in infected quarters than uninfected quarters. However, the results from handheld
conductivity meters when used for the diagnosis of subclinical mastitis in dairy cows needs to be made with care and in some circumstances the results obtained do not accurately reflect the bacteriological status of either individual quarters or cows. This also reveals that measuring EC values would not give the actual picture of the SCM and udder health.

The present study supported this fact that mastitis pathogens were even isolated from milk samples where, the EC values were less than 6.5 mS/cm. Screening of organized farms by EC indicated overall SCM at 62 per cent (150/246) when a conventional criteria of > 6.5 mS/cm was considered to declare SCM positivity. As for individual organized farms are concerned, screening of 10 milk samples from Farm A showed SCM at 80 per cent (8/10). Whereas, at Farm B, screening of 17 milk samples showed SCM at 47 per cent (8 /17). Further, SCM at 89 per cent (54 /61) was observed in case of samples from Farm C and it was 85 per cent in Farm D (23/27). In the present study, the highest prevalence of SCM at 89 per cent was observed in farm C which comprised of pure Holstein Friesian animals when compared to the least prevalence at 38 per cent (28/71) was noticed in the unorganized sector dominated by Hallikar, a local indigenous breed. This indicates Holstein Friesian breed predisposition associated with high prevalence of SCM.

5.3 Isolation and characterization

Currently, the method of detection of pathogen is by in vitro culture which is considered to be the “gold standard” based on plethora of biochemical tests. However, this approach is labour intensive, at times non specific and the results are confusing.
Furthermore, approximately 20 per cent of the clinical cases of coliform mastitis were culture negative due to high SCC (Gonzalez et al., 1980).

5.3.1 Streptococci

In the present study, 246 milk samples from different regions in and around Bangalore screened for SCM revealed prevalence of 45 per cent SCM. On conventional isolation and characterization, 26.5 per cent (85 of 323 isolates) of isolates were found to be Streptococci, 29.40 per cent (95 of 323 isolates) were found to be Coagulase positive staphylococci (S. aureus), and equal number of CoNS and 14.90 per cent (48 of 323 isolates) found to be E. coli. The results, obtained in this isolation studies were very much in accordance with the earlier workers from India, who have isolated about 27.58 per cent (Mitra et al., 1995), 22.83 per cent (Mallikarjunaswamy and Krishnamurthy, 1997), 23.9 per cent (Datta and Rangenkar, 2001), 18.51 per cent (Mohinikumari and Janakiramgupta, 2002) and 14.01 per cent (Rajeev, 2006) Streptococci.

Despite thorough biochemical characterization of Streptococcal isolates, the present study could not lead to precise identification of these isolates upto the species level, may be due to variability in their biochemical profiles (Table 10). Furthermore, since the conventional biochemical tests identify not only typical but also atypical Streptococcal isolates, the application of all these biochemical tests for identification of Streptococcus at species level are considered confusing. Our observations are supported by the report of Odierno et al. (2006). In earlier studies on the isolation of the mastitis pathogens, S.uberis, S. dysgalactiae, S. bovis, S. faecalis, S. equinus were frequently isolated and described as esculin positive / variable (King, 1981; Calvinho et al., 1991;
Khan et al., 2003). Further, *S. uberis* was also found to be CAMP positive (McDonald and McDonald, 2002) in contrast to it’s CAMP negativity described in standard books/manuals (Barrow and Feltham 1993; FDA-Bacteriological Analytical Manual, 2001; Collee et al., 2008). In yet another study, interestingly, seventy per cent of the Streptococcal reference strains demonstrated variability in the biochemical properties (Odierno et al., 2006). While comparing the conventional and molecular methods for identification of aerobic, catalase negative gram positive cocci, Bosshard et al. (2004) could not identify majority of the strains to the species level by the API 20 Strep system or the species identification was doubtful.

However, there are reports on specific identification of a particular species of *Streptococcus* from bovine mastitis cases, based on their biochemical characters. Anshusharma and Bharadwaj (1992) isolated *S. agalactiae* (8.13 per cent), *S. dysgalactiae*, *S. uberis* (0.54 per cent) from a total of 390 quarter milk samples in an organized farm. Further, Mallikarjunaswamy and Krishnamurthy (1997) have reported thirty four isolates of *S. agalactiae*, two isolates of *S. dysagalactiae* and a single isolate of *S. uberis* from bovine mastitis merely based on Gram staining, Catalase, Esculin hydrolysis and VP test as against plethora of prescribed biochemical tests in the aforementioned standard books / manuals. Gianneechini et al. (2002) recovered two isolates of *S. agalactiae* and single isolate of *S. uberis* and Balakrishnan et al. (2004) reported 2.5 per cent *S. dysgalactiae* among streptococcal pathogens from the SCM cases. These studies in general indicated the high prevalence of *S. agalactiae* among Streptococci isolated from bovine mastitis cases based on their biochemical properties.
The findings in the present study emphasize that the conventional biochemical tests like Esulin hydrolysis, hydrolysis of Sodium hippurate, VP, Sugar fermentation for speciation of streptococci are confusing. Furthermore, these findings also contradict the fact that the conventional biochemical assays are the gold standard in the isolation and identification of the bacterial pathogens and streptococci in particular and support the debate stating about the inconsistency of these test profiles which are labor intensive and time consuming (Freney et al., 1992; Phuektes et al., 2001; Phuektes et al., 2003; Picard et al., 2004). Gonano and Winter (2008) also observed that a reliable biochemical differentiation of *Streptococcus* species was difficult and conventional routine diagnostics have to be improved to give precise information on species level for establishing farm specific mastitis prevention programs. Hence, these findings emphasize the need for development of molecular methods based on 16S rRNA gene for precise identification of Streptococci since this is one of the most useful tools applied to the revision of the bacterial classification system (Facklam, 2002).

### 5.3.2 Staphylococci

The economic importance of the *S. aureus* causing clinical and subclinical bovine mastitis is largely recognized. *S. aureus* is a contagious pathogen commonly transmitted among the cows by contact with infected milk and the infection reach up to 32 per cent of the herd (Pitkälä et al, 2004). This pathogen is particularly important because it causes mainly subclinical forms of infectious mastitis that are often difficult to detect by the herdsman. In the present study, 29.40 per cent of the isolates were *S. aureus* based on mannitol fermentation, catalase, coagulase and thermonuclease tests. Several workers also found that *Staphylococcus* species were the predominant isolates in subclinical
mastitis cases (Schukken et al., 1989; Bartlett et al., 1992; Aarestrup et al., 1995; Wadhwa et al., 1996; Mallikarjunaswamy and Krishnamurthy, 1997 and Nagal et al., 1999).

Prior to the 1970’s, clinicians and microbiologists generally regarded Coagulase negative staphylococci (CoNS) as contaminants in clinical specimens and *S. aureus* as the only pathogenic *Staphylococcus* species. Today 41 different staphylococcal species have been described. CoNS have often been considered as minor udder pathogens, causing relatively small udder health problems. However, CoNS infections may cause substantial herd problems due to high prevalence of sub-clinical and/or clinical mastitis (Wilson et al., 1997). CoNS are now, a frequent cause of bovine mastitis in modern dairy herds in many countries. They have even become the predominant pathogen isolated from milk samples from cows with mastitis. CoNS are most frequently isolated from quarters with subclinical or clinical mastitis with only mild clinical signs. In a nationwide survey in Finland, CoNS were isolated from 17 per cent of the quarters and from 50 per cent of the quarters positive for bacterial growth (Pitkälä et al., 2004). In a similar survey in Norway, CoNS were isolated from 3 percent of all sampled quarters and from 14 per cent of the quarters positive for bacterial growth. Intramammary infections caused by CoNS have also become common in the USA and Canada. In two dairy research herds in Ontario, Canada, CoNS were the most common bacteria (51 per cent) causing intramammary infection (IMI) at drying off (Lim et al., 2007). In a study carried out a decade ago in New York and Pennsylvania in the USA, prevalence of CoNS infections among all sampled cows was 11 per cent, and 23 per cent of the bacteria isolated from the milk samples were CoNS (Wilson et al., 1997).
In the present study, 29.40 per cent of the isolates were CoNS. Identification of CoNS isolates was based on colony morphology, Grams staining, catalase reaction and coagulase test. The finding is in accordance with the earlier reports wherein the udder quarter prevalence of CoNS intramammary infections varied between 4 to 50 per cent (Trinidad et al., 1990) and it was 16.6 per cent in Finland (Pitkälä et al., 2004).

5.3.3 *Escherichia coli*

Bovine mastitis produced by gram negative bacteria, usually called coliform mastitis may range in severity from fatal peracute cases to chronic and subclinical infections. *E. coli* is the most prevalent organisms involved in coliform mastitis. These microorganisms are widely disseminated in the environment of the dairy cow especially in bedding material, manure and water. It is a multifactorial disease for which no program of prevention or control has proved to be entirely successful.

In the present study, a total of 48 isolates were confirmed as *E. coli* by Gram’s staining, growth on Mac Konkey’s and EMB agar (metallic sheen) and biochemical tests such as Indole, MR, VP and Citrate. The per cent prevalence of *E. coli* was found to be 14.86 per cent. The prevalence of *E. coli* as a major pathogen along with *Streptococci* and *Staphylococci* has been reported by several workers (Schukken et al., 1989; Mitra et al., 1995; Mandial et al., 1999; Ross et al., 2001; Rajeev, 2006; Botrel et al., 2010). The prevalence reported by earlier work ranged from 13.2 to 24.13 per cent. The finding of the present study was also in accordance with other reports.
5.3.4 Statistical analysis

The data generated from the present study was tabulated and analysis of variance (two way ANOVA) done as per the method described by Snedecor and Cochran (1980). It was analyzed by using Graph pad prism software version - 5.

Distribution of the organisms was analyzed against various groups of somatic cell count by two way ANOVA. It was found that there was a significant difference (P<0.0003) in the distribution of coagulase negative Staphylococcus between the groups. Whereas distribution of other organisms did not show any significant difference.

Interestingly in the organized farms, the occurrence of CoNS was significant (P<0.004) and there was no significant difference in the occurrence of other bacteria. Whereas, in unorganized farms there was a random distribution of organisms.

There was no significant difference in the distribution of S. aureus and CoNS in milk samples from sub clinically affected animals of both organized and unorganized farms.

5.4 Polymerase chain reaction

Diagnostic microbiology is in the midst of a new era. Rapid nucleic acid amplification and detection technologies are quickly displacing the traditional assays based on pathogen phenotype rather than genotype. The polymerase chain reaction (PCR) has increasingly been described as the latest gold standard for detecting some microbes, but such claims can only be taken seriously when each newly described assay is suitably compared to its characterised predecessors. PCR is the most commonly used nucleic acid
amplification technique for the diagnosis of infectious disease, surpassing the probe and signal amplification methods. Polymerase chain reaction protocols have been developed for identification of various mastitis pathogens (Kim et al., 2001; Riffon et al., 2001; Phuektes et al., 2001). In the present study, PCR protocol has been standardized to detect major bacterial pathogens such as *S. agalactiae, S. dysgalactiae, S. uberis, S. aureus* and *E. coli*.

**5.4.1 Streptococcus and Staphylococcus genus specific PCR**

In the present study, PCR was standardized for the identification of the *Streptococcus* and *Staphylococcus* at genus level and major mastitis pathogens *S. agalactiae, S. dysgalactiae* and *S. uberis, S. aureus* and *E. coli*.

Many authors reported the usefulness of *tuf* gene in the development of PCR assays as *tuf* gene encodes the elongation factor *Tu* (EF-*Tu*) (Ke et al. 1999; Martineau et al., 2001; Picard et al., 2004). Elongation factor -*Tu* is a GTP-binding protein playing a central role in the protein synthesis. In the bacterial genome, there are up to three *tuf*-genes present in various copy numbers, when only one is present in the majority of gram-positive bacteria with the low GC content (Ke et al., 1999). Nevertheless, 16S rRNA may also be exploited for the genus identification of the Streptococcal agents. However, the *tuf* gene provided a better discrimination over the 16S rRNA at the *Streptococcus* genus level that should be particularly useful for the identification of very closely related species. Thus, this peculiarity of the streptococcal *tuf* gene was used in the present study.

In the present study, the *tuf* gene based PCR has been standardized for the identification of the *Streptococcus* isolates at the genus level by the amplification of 110
bp product from all the 85 Streptococcal isolates. Further, the specificity of the designed primer was confirmed by sequencing and BLAST analysis. Picard et al. (2004) utilized the same gene to draw the phylogenetic analysis. The tuf gene was also used to identify the enterococcal species and has been found to be specific and suitable for the genus confirmation of enterococci isolated from foods and environmental samples (Cupakova et al., 2005).

In the present study, we aligned the tuf gene sequences available in GenBank and designed broad-range staphylococcal primers that were expected to amplify the members of this genus, particularly the members that are most commonly encountered in the mastitis. The tuf gene based PCR standardized for the identification of the Staphylococcus isolates at the genus level amplified 235 bp product from all the Staphylococcus isolates. In earlier studies, it has been shown that the tuf-based PCR assay for staphylococcal species identification has shown excellent sensitivity and specificity, and could be adapted for the direct detection of staphylococci from contaminated blood or from normally sterile clinical specimens such as blood or urine (Martineau et al., 2001). Chen and Hwang (2008) also developed PCR targeting the tuf gene to differentiate and identify Staphylococcus species. Sakai et al. (2004) standardized a real-time PCR assay targeting tuf gene based probes one to identify Staphylococcus at genus level and other to differentiate Staphylococcus at species level. The result of the present study is also in accordance with the earlier findings where in tuf gene based PCR can be used to identify Staphylococcus at genus level.
5.4.2 Detection of *Streptococcus agalactiae* by PCR

Bovine mastitis caused by *S. agalactiae* is mainly subclinical and therefore can be diagnosed only in the laboratory. *S. agalactiae* is a highly infectious bovine mastitis pathogen that can rapidly spread throughout the herd from an infected animal. Consequently, early diagnosis of the presence of the infection in a herd is important for effective control. Many workers targeted 16S rRNA for the specific identification of *S. agalactiae*. 16S rRNA has been a reliable site for the development of rapid and accurate detection methodologies (Hall *et al.*, 1995; Ahmet *et al.*, 1999; Backman *et al.*, 1999; Meiri-Bendek *et al.*, 2002).

In the present study, 16S rRNA gene was targeted in designing the primers for the identification of *S. agalactiae*. The primers designed in the present study amplified all the seven *S. agalactiae* isolates yielding 329 bp amplicons. The sequencing and BLAST analysis of these amplicons exemplified the fact that the primer designed were specific and can be used reliably to identify *S. agalactiae* isolates without any ambiguity. Thus the present study is in accordance with the earlier findings by many workers in identifying *S. agalactiae* based on the 16S rRNA sequences (Martinez *et al.*, 2001; Phuektes *et al.*, 2001; Riffon *et al.*, 2001; Meiri-Bendek *et al.*, 2002; Gillespie and Oliver, 2005; Chotar *et al.*, 2006; Moatamedi *et al.*, 2007).

The presence of the surface immunogenic protein (*sip*) gene in bovine Group B Streptococci (GBS), described previously only in human beings, has been confirmed (Chotar *et al.*, 2006). In our study, the *sip* gene was targeted for the designing of the *sip* specific primer pair which would confirm and justify the specificity of the 16S rRNA
gene based primers. The sip gene based primers amplified all seven isolates of *S. agalactiae* and a reference *S. agalactiae* (AD1) precisely, yielding 266 bp amplicon. Furthermore, sequence BLAST analysis of these amplicons did not uncover any other microbial sequences.

### 5.4.3 Detection of *Streptococcus dysgalactiae* by PCR

The identification of *S. dysgalactiae* was accomplished by designing the primers based on 16S rRNA sequence. The designed primers amplified the specific region of *S. dysgalactiae* 16S rRNA gene yielding 549 bp amplicon as expected. Earlier, 16S rRNA based PCR has been standardized by many workers (Forsman et al., 1997; Gillespie and Oliver, 2005; Moatamedi *et al.*, 2007) for identification of *S. dysgalactiae*. Interestingly, none of the Streptococcal isolates obtained in the present study were identified as *S. dysgalactiae* by PCR in contrast to the biochemical test based observations. This provided a clear-cut indication about the ambiguous outcome of the conventional biochemical assays. Reference isolate *S. dysgalactiae* (AD3) was precisely identified by same set of primers as well.

### 5.4.4 Detection of *Streptococcus uberis* by PCR

Earlier *S. uberis* epidemiological studies utilized species specific 16S and 23S rRNA gene PCRs (Hassan *et al.*, 2001) for primary identification following aesculin and hippurate hydrolysis (Field *et al.*, 2003; Zadoks *et al.*, 2005; Coffey *et al.*, 2006; Pullinger *et al*. 2006). Prior to the adoption of 16S rRNA gene sequence analysis for species identification and confirmation for *S. uberis* (Pullinger *et al.*, 2006), it is highly likely that it was difficult to differentiate *Streptococci* closely related to *S. uberis* from it
and were included in previous *S. uberis* epidemiological studies (Jayarao *et al.*, 1991; Jayarao *et al.*, 1993; Baseggio *et al.*, 1997; Douglas *et al.*, 2000; Phuektes *et al.*, 2001; Khan *et al.*, 2003).

In the present study, 16S rRNA was again targeted to design the *S. uberis* specific primers. Further, these primers amplified an 854 bp amplicon and sequence BLAST analysis of the same confirmed its specificity.

Alternatively, it was also shown that *pauA* gene based PCR (Zadoks *et al.*, 2005) could be used for rapid species identification, since *pauA* is *S. uberis* species-specific and absent in other *Streptococcus* species or other bacteria commonly associated with bovine mastitis (Ward and Leigh, 2004). In the present study, the primers were designed by targeting *pauA* gene for the identification of *S. uberis* by PCR which precisely identified the reference *S. uberis* isolates (AD2 and AD6). Surprisingly, none of the Streptococcal isolates obtained in our study were identified as *S. uberis* by PCR in contrast to the biochemical assays. Further, *pauA* gene primer based PCR revalidated the earlier identification process by using 16S rRNA gene based PCR.

### 5.4.5 Identification of other *Streptococcal* species in subclinical mastitis

Majority of the isolates in the present study could not be speciated based on biochemical tests, nor they were either *S. uberis* or *S. dysgalactiae*. Sequence specific primer for identification of these isolates was designed at lead centre, NAIP Subproject on Bovine mastitis: Unraveling molecular details of host-pathogen interaction and development of molecular diagnostic methods, PD_ADMAS, Bangalore and used for
PCR amplification. The amplified products were sequenced and NCBI blast results indicated that these isolates belong to *S. bovis-equinus* complex.

There are many reports of unidentified *Streptococcal* species such as *S. canis* (Hassan et al., 2004), *S. bovis, S. equinus, S. equi, S. pleurianimalium* etc. from the cases of SCM. These workers basically identified the above mentioned organisms based on 16S rRNA based PCR (King, 1981; Calvinho et al., 1991; Khan et al., 2003). Further, *S. bovis* was identified based on the amplification of *groEL* gene (Chen et al., 2008).

**5.4.6 Detection of *Staphylococcus aureus* by PCR**

*S. aureus* is recognized worldwide as a frequent cause of subclinical intramammary infections in dairy cows. The main reservoir of *S. aureus* seems to be the infected quarter, and transmission between cows usually occurs during milking. *S. aureus* produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism.

*S. aureus* strains produce an extracellular thermostable nuclease, thermonuclease (TNase) with a frequency similar to that at which they produce coagulase. The TNase protein has been well characterized and its gene, the *nuc* gene, has been cloned and sequenced. An enzymatic test for TNase production is used in many laboratories for the identification of *S. aureus* isolates.

In the present study, *nuc* gene and *sodA* gene based PCR were standardized to detect *S. aureus*. The *nuc* gene based PCR was very specific to detect *S. aureus* and screening of all the 95 *S. aureus* isolates revealed the presence of *nuc* gene with a specific
amplification of 181bp, where as, only 87 isolates amplified the sodA gene with specific amplification of 159 bp. In earlier studies also it was observed that PCR for amplification of the nuc gene has potential for the rapid diagnosis of S. aureus infections by direct testing of clinical specimens, including specimens from patients with ongoing antimicrobial therapy (Brakstad et al., 1992). The nuc gene based PCR assay for the detection of S. aureus was 100 per cent specific and sensitive and the assay was particularly suitable for analysis of samples shipped or stored without refrigeration (Khan et al. 1998). nuc gene based PCR to detect S. aureus from IMI has also been studied and found to be very specific (Kim et al., 2001; Kuzma et al., 2003; Kalorey et al., 2007). The manganese-dependent superoxide dismutase (SOD) is encoded by sodA gene. The enzyme SOD allows for intracellular survival of S. aureus within macrophages in the presence of superoxide and hydrogen peroxide. SodA has been found to have a greater degree of interspecies divergence amongst staphylococci than 16S rRNA, making this gene an ideal discriminative target (Poyart et al., 2001). Species-specific sodA PCR developed to identify staphylococci of veterinary interest, including S. hyicus, S. intermedius and S. aureus (Baron et al., 2004) as well as select species of CoNS have been reported. Schissle (2009) observed that sodA based primer was the most sensitive primer pair for detection of S. aureus with 100 percent sensitivity.

5.4.7 Detection of Escherichia coli by PCR

In the present study, a total of 48 isolates were confirmed as E. coli by biochemical tests and four isolates suspected as E. coli were not confirmed by these tests. Alanine racemase (alr) gene based PCR had amplified all the 48 confirmed E. coli by biochemical tests with a specific amplification of 366 bp and four suspected
isolates as *E. coli* were not amplified. Alanine racemase is produced by all *E. coli* strains, including O157 and is essential for cell-wall biosynthesis by providing D-alanine to peptidoglycan. Thus, detection of the alanine racemase (*alr*) gene may be useful for the detection of *E. coli* in foods (Yokoigawa et al. 1999). It should be noted that the *alr* primers used in this study detect a wide range of *E. coli* strains but also lead to the detection of *Shigella* spp. (Yokoigawa et al., 1999). However, the incidence level of *Shigella* in milk is ordinarily very low and therefore would not be expected to complicate the detection limits of the PCR. To overcome this, a specific multiplex PCR to identify *E. coli* which has been standardized at Lead centre, NAIP Subproject on Bovine mastitis: Unraveling molecular details of host-pathogen interaction and development of molecular diagnostic methods, PD_ADMAS, Bangalore was also used in the present study to confirm the specificity of the *alr* gene based PCR. The multiplex-PCR also precisely identified 48 isolates as *E. coli* and four were negative for *E. coli*.

*TraT* is a cell surface-exposed lipoprotein, which is assumed to inhibit the correct assembly or membrane insertion of the membrane attack complex of complement. *TraT* is encoded by the *TraT* gene carried on large conjugative plasmids. *TraT* was the most common virulent gene detected in the *E. coli* isolates from mastitis.

In the present study, the *E. coli* isolates from subclinical mastitis were also screened for the presence of virulence gene *TraT*. Out of 48 *E. coli* isolate, *TraT* gene was present in 40 isolates. These findings were in accordance with the earlier studies where it has been observed that the *TraT* gene was the major virulent gene present in the subclinical mastitis milk samples (Acik *et al.*, 2004; Altalhi and Hassan, 2009). In one
study, it was observed that the most common gene in both Finnish and Israeli isolates was TraT which was detected alone in 52 per cent of Finnish and 88 per cent of Israeli isolates. The detection of TraT gene in a high proportion of isolates may indicate a role for the gene in the pathogenesis of mastitis caused by E. coli, because this gene has been linked with serum resistance and more than 60 per cent of strains isolated from mastitis have been reported to be serum resistant. However, large scale studies are necessary to improve our understanding of the potential role of E. coli and relevant virulence factors in the aetiology of both clinical and subclinical mastitis in cows and to provide quantitative data on the economical significance of this disease complex which may help develop effective control and eradication strategies.

5.5 Sensitivity and detection limits of PCR

Recent development of PCR technology has provided a new detection platform for the identification of bacteria. Different PCR-based methods have been developed for specific and sensitive detection of mastitis pathogens in milk. In the present study, DNA from milk was directly extracted following the protocol described by Cremonesi et al. (2006) with modifications. The sensitivity of our extraction method was examined using culture negative bovine milk and normal saline inoculated with dilutions of reference cultures of S. agalactiae (AD1), S. dysgalactiae (AD-3), S. uberis (AD-6), S. aureus (SAU-3) and E. coli (EC-11) strains, starting from 10^7 cfu/mL to 1cfu/mL and PCR was carried out using these extracted DNA as template from 10^7 cfu/mL to 1cfu/mL for each strains.
Sensitivity of the extraction procedure followed by PCR was found to achieve a detection of 1 cfu/mL for *S. agalactiae* (AD1), *S. dysgalactiae* (AD-3) and *S. aureus* (SAU-3) with the DNA extracted from both normal saline and culture negative milk, whereas, it was $10^3$ cfu/mL for *E. coli* in normal saline and $10^4$ in milk and $10^3$ cfu/mL for *S. uberis* in both normal saline and milk. Even though the sensitivity was achieved to detect upto 1 cfu/mL, there was a quantitative decrease in the intensity of the amplicons. This reflected a corresponding decrease in cell numbers.

Detection limit of PCR for the overnight culture has been studied by several workers. Chotar *et al.* (2006) observed a detection limit of 3000 cfu/mL for GBS and *E. coli* whereas, it was 8000 cfu/mL for *S. aureus*. However, the sensitivity was diminished to 6000 cfu/mL for GBS and by two fold to *S. aureus* and *E. coli* when cultures were resuspended in milk before washing. Meiri-Bendek *et al.* (2002) while studying on *S. agalactiae* observed that sensitivity of detection by PCR was $10^4$-$10^5$ cfu/mL when PCR was performed on the DNA extracted directly from milk, where as overnight enrichment of the samples increased the sensitivity to 1cfu/mL. Phuektes *et al.* (2001) observed that the overnight enrichment has increase the sensitivity of PCR to detect upto 1cfu/mL. In the present study, rapid DNA extraction was carried out directly from bovine raw milk to obtain material for PCR detection of gram-positive bacteria such as *S. aureus, S. agalactiae, S. uberis*, and *S. dysgalactiae* and Gram negative *E. coli*. This method was based on the ability of silica resin to bind DNA in the presence of high concentrations of guanidine thiocyanate which guarantees excellent disruption of bacterial cells (Cremonesi *et al.*, 2006). The detection level in the present study eliminates the need for bacterial enrichment culturing of *Staphylococcus* and
Streptococcus species. This DNA extraction method was further extrapolated for the subclinical mastitis milk samples for simultaneous detection of these five major pathogens

5.6 Multiplex PCR

In the present study, a total of 186 milk samples categorized based on SCC and EC as subclinical mastitis milk samples were processed for bacteriological examination. A total of 313 isolates recovered from these milk samples were phenotypically identified as Streptococci spp. (85 isolates, including seven S. agalactiae isolates), S. aureus (95 isolates), E. coli (48 isolates) and CoNS (95 isolates). Although, the identification of a bacterial pathogen in milk from a cow with mastitis following the conventional microbiological procedure is regarded as the definitive diagnosis of an IMI, the process is time consuming and generally require more than 48 hr to complete. To overcome this, sensitive molecular methods like PCR and multiplex PCR for the early, rapid and accurate detection of subclinical mastitis causing major bacterial pathogens was undertaken.

Initially, uniplex PCR was standardized to identify Streptococcus and Staphylococcus at genus level using reference strains and validated by screening the isolates recovered from SCM cases. Uniplex-PCR was also standardized to identify S. agalatiae, S. dysgalactiae, S. uberis, S. aureus and E. coli at the species level and the standardized protocols had precisely identified the isolates recovered from SCM milk samples.
Polymerase chain reaction protocols have been developed for identification of various mastitis pathogens (Forsman et al., 1997; Riffon et al., 2001; Meiri-Bendek et al., 2002). These PCR methods allow identification of bacteria within hours. Since many bacteria are implicated in mastitis, it is expensive to do a separate PCR test for every possible mastitis pathogen and the test becomes very labour intensive. Many modifications to the basic PCR process have been described. Of particular relevance to diagnosis of mastitis is multiplex PCR, which, in a single assay, allows simultaneous screening for multiple pathogens that might be causing the disease. There are several reports on multiplex-PCR that have been developed to detect several mastitis pathogens (Phuektes et al., 2001; Phuektes et al., 2003; Gillespie and Oliver, 2005).

In the present study, a two tube multiplex-PCR has been standardized to simultaneously detect five major mastitis pathogens viz., *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *S. aureus* and *E. coli*. The two tube multiplex PCR developed had allowed the simultaneous detection of major mastitis pathogens using smaller amounts of reagents and less time to set up and analyze than uniplex PCR, thus making it more applicable to routine diagnostic use. In one tube, amplification of *sip* and *pauA* gene to detect *S. agalactiae* and *S. uberis* respectively, and in other tube amplification of 16S rRNA, *alr* and *nuc* gene to detect *S. dysgalactiae*, *E. coli* and *S. aureus* respectively was carried out. While optimizing the two tube multiplex PCR, the criteria such as primer and DNA polymerase concentrations were taken into consideration. The primer concentrations were titrated to achieve the simultaneous amplification of the all the target sites in the two-tube reaction. The primers used in the study were designed to have similar melting temperature. Earlier, while optimizing the multiplex PCR for simultaneous detection of *S.
agalactiae, S. dysgalactiae, S. uberis and S. aureus from bovine mastitis cases, Phuektes et al. (2001) also considered the above factors into consideration. The relative concentrations of the primers found to be an important factor in determining the approximately equal yields of amplification products from each of the organism was also in agreement with the findings of Henegariu et al. (1991). In the present study, both the reactions were standardized with a uniform annealing temperature in a 30 cycle PCR.

In most countries, the major mastitis pathogens are S. agalactiae, S. aureus (contagious pathogens), S. dysgalactiae, S. uberis, and E. coli (environmental pathogens), hence the standardization of multiplex PCR for simultaneous detection of these pathogens overcomes the cost effectiveness of conventional uniplex PCR. It reduces the amount of reagents, such as TaqDNA polymerase, used for each diagnosis. Moreover, it requires less preparation and analysis time than systems in which several tubes of simplex PCR are used. After standardizing the multiplex-PCR using reference strains, the protocol was validated by detecting the isolates recovered from the SCM cases in the present study.

Even though different PCR based methods have been developed for specific and sensitive detection of mastitis pathogens in milk, the direct isolation of high quality DNA from the target bacteria found in milk however was often problematic and might require overnight selective enrichment procedures (Phuektes et al., 2001; Meiri-Bendek et al., 2002; Gillespie and Oliver., 2005). These difficulties were may due to small concentrations of the pathogenic DNA present in a typical sample or various factors such as the degree of cellular lysis, binding of DNA to particulate material, and degradation or
shearing of DNA might affect the recovery of DNA. Furthermore, in the case of Gram positive bacteria such as *S. aureus* and Streptococci, an optimal sample processing method should efficiently lyse resistant bacterial cell walls without damaging target DNA which necessitated the use of enzymes such as lysozyme, lysostaphin, pronase etc. (Riffon *et al.*, 2001; Meiri-Bendek *et al.*, 2002). The presence of PCR-inhibitory substances which were frequently associated with the food matrix, particularly in milk, hampered the direct detection of pathogenic bacteria in food samples (Ramesh *et al.*, 2002). Components such as Ca$^{2+}$ proteinase, fats and milk proteins might block DNA and shield it from access by polymerase (Wilson, 1997). Hence, the development of a sample preparation strategy that can effectively sequester high-quality DNA of the pathogenic bacteria from food samples before PCR amplification was needed. Recently, Cremonesi *et al.* (2006) described a method for rapid DNA extraction directly from bovine and caprine raw milk to obtain material for PCR detection of gram-positive bacteria such as *S. aureus*, *S. agalactiae*, *S. uberis*, and *S. dysgalactiae*. The method was based on the ability of silica resin to bind DNA in the presence of high concentrations of guanidine thiocyanate. The efficient lysis of cells and removal of inhibitors were accomplished by increasing the concentration of guanidine thiocyanate and lysis buffer solution, both of which increased the disruption of bacterial cells, resulting in stronger and more reproducible amplification, avoiding the combination of enzymes and incubation conditions, and maintaining good characteristics of the method without time-consuming procedures. In the present study, DNA from milk samples was extracted following the procedure described by Cremonesi *et al.* (2006). Initially, two tube multiplex-PCR was standardized by using the DNA extracted from pasteurized and
mastitis milk samples and further applied for screening of subclinical mastitis milk samples.

The two tube multiplex-PCR was carried out on all the 186 subclinical mastitis milk samples processed for isolation. The milk samples after processing were frozen and DNA was extracted from these frozen milk samples after thawing following the procedure of Cremonesi et al. (2006). All the samples extracted were successfully amplified using target bacteria-specific primers. Identification of pathogens obtained by PCR assays was in full agreement with that obtained by microbiological methods. The results of the present study indicated that *S. agalactiae* and *S. aureus* could be detected and was in accordance with the species isolated from culturing. However, only 44 isolates of *E. coli* could be detected. This may be due to the fact that freezing and thawing had reduced the detection level of *E. coli*. In the present study, it was also observed that DNA extracted from second and subsequent freezing and thawing did not detect these pathogens. Reasons for non DNA recoveries were unknown, but might include lost DNA template through degradation. This indicates the necessity of using fresh milk samples for detection of mastitis pathogens.

The present study indicated that the two tube multiplex-PCR can be a useful tool for monitoring the SCM caused by *S. agalactiae, S. dysgalactiae, S. uberis, S. aureus* and *E. coli* at the herd level.

### 5.7 Screening of bulk milk samples by two tube multiplex PCR

Regular monitoring of bulk tank milk is a vital part of dairy herd health programmes to control mastitis. Analysis of bulk milk can provide information on the
extent of subclinical infection, the pathogens involved, and the level of milking hygiene and equipment sanitation in a herd (Guterbock and Blackmer, 1984). In the present study, a total of 147 bulk milk samples were collected from four milk chilling centres and screened for SCC and EC. Each sample represented milk collected from one Milk Producers Cooperative Society (MPCS). The results of SCC indicated prevalence of SCM at 78 per cent since, 115 out of 147 samples were positive for SCM, if the conventional criteria of SCC $\geq 5$ lakh cells / mL is considered to declare positivity. Bulk tank SCC levels are a good indicator of udder health in the herd and can be used to estimate production losses due to infections. It was estimated that herds with SCC higher than 500,000 cells /mL could be producing 6 per cent or more below potential (Eberhart et al., 1982). Although a useful tool in estimating infection level and production losses, bulk tank SCCs are merely a guideline with general indications of overall udder health status. Bulk milk SCC has limitations as it is impossible to determine an individual cow’s or groups infection status. It is also important to remember that bulk milk SCC is determined by individual cow’s SCC and production level. Hence it has been observed that herd size and management practices had considerable influence on SCC in bulk milk and the bulk milk SCC could serve as indicator and facilitate monitoring of herd udder health and milk quality (Jayarao et al., 2004). The values obtained in the present study are representative of the state of the herd on one particular day of the month when the test was performed. Observing the BMSCC is a readily available and inexpensive way of monitoring the mastitis trend in the herd and estimating SCM in the herd based on BMSCC can be the initial step in identifying mastitis problems in the herd and should lead to subsequent individual cow SCC testing. Single test-day SCM estimates are
representative of the state of the herd on one particular day and are sensitive to single-day upward or downward shifts. Therefore, an attempt to correlate the BMSCC data with single test-day SCM as in our study would not yield satisfactory results and thus, a different approach needs to be undertaken. The findings of the earlier studies suggest that in the case of individual cows, an average of several test-day results is more accurate at identifying cows with high SCC. Similarly, an average of multiple test days estimates of SCM will give a better estimate of herds SCM prevalence, less sensitive to single-day variation. Moreover, it is well established that BMSCC is subject to sustained seasonal changes that might partly reflect the changes of the SCM in the herd. From this standpoint, an estimate of SCM based on a monthly mean of BMSCC based on 15 to 30 readings should be representative of the average SCM status during that month if every milk shipment is tested. On the other hand, in herds that have their milk tested only once or twice a month, yearly data would have to be used to estimate the SCM in the herd. Future studies should try correlating BMSCC with more frequent estimates based on daily individual cow SCC testing.

The screening of the bulk milk samples for detection of five major mastitis pathogens (S. agalactiae, S. dysgalactiae, S. uberis, S. aureus and E. coli) by two tube m-PCR revealed the presence of pathogens in 81 bulk milk samples. S. aureus was the predominant pathogen detected, followed by E. coli, S. dysgalactiae and S. uberis.

In the present study, it was observed that majority (115/147) of the bulk milk samples had BMSCC of >5 lakh cells / mL. In earlier reports, a considerable high prevalence of S. aureus has been observed by analysis of quarter milk samples in herds
with high BMSCC (Barkema et al., 1998; Phuektes et al., 2003), however, did not find any significant association between presence of *S. aureus* and BMSCC. Although the presence of *S. aureus* in bulk milk is indicator of infection of the herd due to this organism, the interpretation is not reliable (Godkin and Leslie, 1993). However, *S. aureus* has been implicated as major contagious pathogen causing IMI and detection of the *S. aureus* indicate a definite IMI. There was also possibility of *S. aureus* entering bulk milk from non milk sources such as milkers, contaminating of both composite and bulk milk (Adesiyun et al., 1997). In the present study, the bulk milk samples were collected from the chilling centres where the milk samples were procured from different MPCs and the collection method did not allow the origin of the *S. aureus* to be ascertained and thus the possibility of *S. aureus* from environment rather than from the infected cows cannot be excluded.

*E. coli* is one of the major environmental pathogen implicated in IMI. In the present study, 42 per cent of the pathogens detected were *E. coli*. While the origin of these pathogens cannot be precisely determined, the two primary sources will be the milk from infected cows and from contaminates that enter the milk off the udder and teats where wet milking is permitted. Usually during coliform mastitis, the coliform bacteria are only shed in the milk for a short period of time suggesting that finding these bacteria in the milk reflects milking cows with wet, dirty udders and teats. Potential environmental mastitis pathogens can occur in milk as a result of such other contributing factors as dirty cows, poor cleaning of equipment, and/or inadequate cooling of raw milk. An accompanying elevated somatic cell count, however, can sometimes serve as
supportive evidence that mastitis may, especially SCM have caused an increase in the bulk milk bacteria count (Rysanek et al., 2007).

*S. dysgalactiae and S. uberis* were the most prevalent species in bulk milk samples (Bramley *et al.*, 1996). But in the present study, the prevalence of these pathogens was only at 8 and 6 per cent. As these species are ubiquitous in cow’s environment, it is more likely that these organisms enter the bulk milk from non specific contamination rather than milk from infected cows.

The multiplex PCR assay developed in the present study was an easy and rapid method to simultaneously detect the five major mastitis pathogens, *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. uberis* and *E. coli* in bulk milk. PCR required less time to process and results were easier to interpret. Hence, the regular analysis of bulk milk by m-PCR may be a useful tool for determining the herd status with regard to the detection of contagious and environmental mastitis pathogens. The result of the bulk milk study indicated the presence of both contagious and environmental mastitis pathogens. This necessitates continuing need to concentrate on control of *S. aureus* by following the proper managemental practices especially the mastitis control programme. It is also necessary to train the dairymen to ensure that their milkers are prepared to deal with the increased extent of wet, dirty udders and teats in order to control environmental pathogens. As with all areas of dairy management, attention to deal these aspects will provide great benefits. Mastitis control is no different and will require leadership and dedication of the dairy management to the production of high quality milk.