Discussion
V. DISCUSSION

Bovine Mastitis is economically the most significant disease of dairy cows and continues to be a persistent problem in the dairy industry at global level. It remains a major challenge to milk production in spite of wide spread implementation of mastitis control strategies both at individual cow as well as herd levels. Despite mastitis having been researched for decades, the problem it represents has not been solved and incidence of mastitis still remains high in most countries. Its impact on Indian economy is also remarkable. 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 half before (Singh and Singh, 1994).

In India, subclinical mastitis (SCM) 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 Holstein and Jersey 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). Further, SCM 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 losses in sub-clinical mastitis are mainly due to loss of milk production, reduced payment caused by lower milk quality, and culling and replacement of cows (Sandgren et al., 2008).
As for the etiology is concerned, mastitis is caused by over 150 different contagious or environmental micro-organisms (Kuang et al., 2009). Although a wide spectrum of bacterial species have been identified as causative agents of mastitis, staphylococci, several species of streptococci and coliforms are of economic and epidemiological importance. For decades, the genus Staphylococcus has been dominating in intra-mammary infections (IMI). To date, more than 50 Staphylococcus species and subspecies have been characterized. The genus is divided into coagulase-positive staphylococci (CoPS) (predominantly S. aureus) and coagulase negative staphylococci (CoNS) based on their ability to coagulate plasma. Although, S. aureus is commonly associated with bovine mastitis in many countries, of late, CoNS are being reported frequently (Bengtsson et al., 2005; Kuang et al., 2009). Traditionally, CoNS have been considered to be minor pathogens.

**Coagulase-negative staphylococci in bovine mastitis**

The last forty years have seen a change in the relative and absolute importance of different pathogens. In recent years, as group CoNS have become the most common bacteria associated with bovine mastitis in many countries (Tenhagen et al., 2006). Their importance has increased and they have become the predominant pathogens isolated from subclinical and clinical mastitis in several countries (Tenhagen et al., 2006; Koivula et al., 2007; Lim et al., 2007; Schukken et al., 2009) and could therefore be described as “emerging mastitis pathogens” (Pyorala and Taponen, 2009). But in Indian context, knowledge of CoNS species involved in bovine mastitis is still limited and there is a dearth of information about their prevalence (Rajeev et al., 2009; Shome et al., 2011) and
benefits would accrue from having more reliable diagnostic methods for species identification.

Hence, the present study was undertaken to give an insight into the profile of CoNS associated with clinical/subclinical mastitis in southern and northern districts of Karnataka. The overall aim of the thesis was to improve the knowledge on the association of different CoNS species, their identification and antimicrobial resistance patterns, in connection especially with SCM in dairy cows. The present study presents the results of the detailed investigation carried out on 149 field staphylococcal isolates obtained from cases of bovine mastitis.

CoNS can behave as contagious or environmental pathogens. More than ten different CoNS species have been isolated from mastitic bovine milk samples elsewhere, and the species most commonly reported are *S. chromogenes* and *S. simulans* (Trinidad *et al*., 1990; Matthews *et al*., 1992; Taponen *et al*., 2007). With subtle variation, frequently isolated CoNS species from bovine IMI included *S. epidermidis, S. haemolyticus, S. hyicus* and *S. xylosus* (Jarp, 1991; Birgersson *et al*., 1992; Matthews *et al*., 1992; Aarestrup, 1995; Myllys, 1995; Taponen *et al*., 2006; Thorberg *et al*., 2006). However, a number of other species have also been reported.

5.1 Screening of milk samples for subclinical mastitis

This investigation focuses mainly on sub-clinical mastitis that cannot be diagnosed by clinical examination; instead analysis of inflammatory cells or their products in the milk is needed. Hence, the detection of SCM cases by the appropriate tests is crucial. Traditionally, methods of detection include; direct or indirect estimation
of somatic cell counts - an indication of inflammation, measurement of conductance (EC test) and pH (BTB test) in milk, measurement of biomarkers associated with the onset of the disease (e.g. the enzymes N-acetyl-β-D-glucosaminidase and lactate dehydrogenase) and identification of the causative microorganisms, which often involves culturing (Viguier et al., 2009).

Early diagnosis of mastitis is of utmost importance as it enables early treatment and thereby could avoid high economic losses. Further, continuous monitoring of mastitis and its careful management, is essential for the sustained well-being of a dairy herd. This can be achieved through the detection of inflammation at its early stages and subsequently, the detection and treatment of the mastitis infection.

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 indirect methods to identify potential IMI are Somatic Cell Count (SCC), Electrical Conductivity (EC), California Mastitis Test (CMT) and Bromothymol blue (BTB) strip tests in field conditions and, the automated method in the diagnostic laboratory.

Although SCC is relatively more specific and sensitive, it is not user friendly and can not be performed on site. Rapid, ‘cow-side’ mastitis tests such as EC test, CMT and BTB strip tests could be used by farmers and veterinarians to diagnose and treat mastitis cases in its early stages, thus having the potential to stop the propagation of the disease in the herd (Viguier et al., 2009). Considering the fact that no single test can detect all SCM cases, all the four tests viz., SCC, EC test, CMT and BTB strip tests were used to detect
SCM cases in the present study. A comparative evaluation of all these with respect to the bacteriological analyses would also give an indication about the usefulness of these tests to detect the SCM cases.

5.1.1 Somatic Cell Count (SCC)

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; but 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., 2003b). 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 SCC of truly healthy cows is little affected by the physiological factors (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 4,00,000, Canada at 5,00,000 and the United States at 7,50,000 cells/ml (Paape and Contreras, 1997). In Sweden, the cut off value is 2,00,000 cells/ml, International Dairy Federation defined a break point of 5,00,000 cells/ml for SCC. However, under Indian Scenario there have been no such standards adopted (Hegde, 2011).

5.1.2 Effect of CoNS intra-mammary infection on milk SCC

In the present study, a total of 313 milk samples collected from seven organized and seven unorganized sectors were categorized under 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). Evaluation of these milk samples for SCC indicated SCM at 42.8% cent (134 out of 313 samples were positive for SCM) when the conventional criteria of SCC ≥ 5 lakhs was considered to declare positivity.

The analysis of the SCC revealed an overall SCM at 50 per cent in the organized sector which was higher than in the unorganized sector (35.5%) (Table 14.2). Organized farms in the dairy sector are supposed to be organized in a scientific way with respect to the management practices which would in turn reduce the incidence of IMI. On the contrary, the overall higher incidence of SCM diagnosed in organized farms in the present study probably reflect the lack of implementation of mastitis control strategies as well as hygienic and standard management practices adopted at farm level. It was observed that none of the farms adopted strategies for the clean milk production before, during and after milking.
Increased SCC as an indicator of IMI has been described earlier (Trinidad et al., 1990, Hayakawa et al., 2000). CoNS infection is generally seen as an increase in the SCC in milk of the infected quarter. Milk SCC usually remains below 500,000 cells/ml (Djabri et al., 2002). Even a transient CoNS infection causes a temporary increase in SCC (Taponen et al. 2007; Laevens et al. 1997).

It was observed in this study that CoNS isolates were recovered in large number in samples having SCC >5 lakh cells/ml (Table 13c). With some variation, in the range of 1-2 lakh cells/ml, there was correlation between the SCC values and the isolation rate of CoNS, i.e., there was correlation between the SCC counts and the recovery of the CoNS.

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 or nil SCC (0 – 20,000 cells /ml). Although, India is the largest producer of milk in the world, the challenging task is the production of quality milk adhering to international standards to hold the first position in the global dairy industry. In view of this, it is necessary to lay down regulatory limits in India with regard to SCC cut off value to declare SCM cases and make provision for the quality premium to be paid for the quality milk based on the SCC.

5.1.3 Electrical conductivity (EC)

Measuring EC of milk to detect mastitis is based on the ionic changes which occur during inflammation. This test measures the increase in conductance in milk caused by the elevation in levels of ions such as sodium, potassium, calcium, magnesium and chloride during inflammation. Also, the change in pH and decrease of fat affect EC.
While this test offers the advantage that it can be used ‘on-site’, non-mastitis-related variations in EC can present problems in diagnosis (Viguier et al., 2009). EC measurements in milk from healthy cows generally ranged from 5.5 to 6.5 mS/cm (Norberg et al., 2004).

In the current study, EC categorized the samples into two broad groups; 162 (51.8%) samples with EC value of < 6.5 mS and the remaining 151 samples (48.2%) with EC ≥ 6.5 mS. Thus, the prevalence of SCM was 48.2 % by EC test when a conventional criterion of > 6.5 mS was considered SCM positive.

Contrary to the SCC, 57.2 % of the CoNS isolates were recovered from the samples whose EC values were below 6.5 mS and this was still higher (71.9 %) in CoPS, i.e., the milk samples declared to be SCM negative by EC test yielded more no. of staphylococcal isolates.

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 (Hamann et al., 1998; Jensen and Knudsen, 1991). 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 SCM 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 wherein the EC values were less than 6.5 mS/cm.

5.1.4 California Mastitis Test (CMT)

This assay indirectly measures the SCC in milk samples. A bromocresol-purple-containing detergent is used to break down the cell membrane of somatic cells and, the subsequent release and aggregation of nucleic acid forms a gel-like matrix with a viscosity that is proportional to the leukocyte number. The test is cost effective, rapid, user friendly and can be used ‘on-site’ or in the laboratory (Schalm and Noorlander, 1957).

In all, CMT detected SCM in 21.4 % (67 / 313) of the samples screened; the least when compared with the other SCM tests employed in the study.

California mastitis test is one of the oldest and best known SCM tests, and has several advantages. However, the interpretation can be subjective and might result in false positives and negatives (Viguier et al., 2009), and it is reflected in the present study also.
5.1.5 Bromothymol Blue (BTB) strip test

The rise in milk pH, due to mastitis, can be detected using highly absorbent blotting paper impregnated with bromothymol blue. The test is user friendly, cost effective and rapid but not as sensitive as other tests (Viguier et al., 2009). The test can be used to screen herds with a relatively high incidence of mastitis or used in combination with cow cell counts to locate abnormal quarters (Marschke and Kitchen, 1985).

A total of 84 (26.8%) samples were found to be positive by BTB strip test in the present study. In the present study, the BTB test detected more number of SCM cases (5.4% higher) when compared to CMT test on the contrary to an earlier report that stated BTB test was less sensitive than the CMT (Marschke and Kitchen, 1985) but offered several practical advantages for use on farm.

Indicator colour scores (1 to 4) for quarter foremilks increased with somatic cell count and pH, although variability within each color score was large. Sensitivity of the BTB test ranged from 51 to 56% and specificity from 89 to 90% for most reference criteria used to classify normal and abnormal milk (Marschke and Kitchen, 1985).

5.2 Isolation of Staphylococcal species from milk samples

A preliminary evaluation of 313 milk samples indicated that 134 samples (42.8%) were positive for SCM when the conventional criteria of SCC $\geq$5 lakhs/ml of milk was considered to declare positivity. Since this SCC cut-off value may underestimate SCM
(Hegde et al., manuscript in preparation), all the samples were subjected for bacteriological isolation.

In comparison with the SCC, CoNS isolates were recovered in large number in samples having SCC >5 lakh cells/L (Table 14.1). In contrast, lesser number of CoNS isolates was obtained in the samples positive by EC test. *i.e.*, there was positive correlation between SCC and number of isolates recovered as against to the association of EC values with the isolation of CoNS.

In all, 152 CoNS isolates including 14 from clinical samples were obtained in this study (Table 13.1 and 13.2). All these isolates as well as seven staphylococcal MTCC strains were subjected to phenotype-based speciation using KB004 HiStaph™ Identification Kit (Hi-media).

### 5.3 Biochemical characterization and Speciation of CoNS isolates based on biochemical profile

The HiStaph™ Identification Kit used in the present study could differentiate as many as 43 different *Staphylococcus* species. CoNS were isolated in 44.09% of the milk samples and 24 different CoNS species were detected from the 313 bovine milk samples (Table 15). Although SCM status was shown in 42.8% of the samples, there was no difference in the variety of the CoNS species detected in SCM positive and negative samples. The predominant CoNS species were *S. arlettae*, *S. fleurettii*, *S. equorum*, *S. epidermidis*, *S. saprophyticus*, *S. sciuri* and *S. xylosus*. The staphylococcal species that were isolated less frequently included *S. muscae* and *S. sciuri subsp. carnaticus*. Rarely, *S. warneri*, *S. homini*, *S. vitulus* and *S. cohnii subsp cohnii* were also isolated. In clinical
cases of mastitis, the CoNS species identified were *S. fleurettii*, *S. muscae*, *S. aureus*, *S. chromogenes*, *S. felis* and *S. auricularis* (Table 16).

Interestingly, only four out of seven MTCC reference cultures viz., *S. aureus* 96, *S. sciuri*, *S. chromogenes* and *S. haemolyticus* were identified correctly by the Hi-Media identification system used in the study. *S. epidermidis* 3615 was identified as *S. schleiferi* subsp *coagulans* (Table 17).

In all, 30 strains were not assigned any species as they showed variable biochemical properties which did not match to any of the species listed in the index provided with the kit and they were merely considered as *Staphylococcus* species. This included 28, 4 and 2 strains from subclinical, clinical and MTCC reference cultures, respectively (Table 16 and 17).

Identification of bacteria in most clinical laboratories is currently based on analysis of phenotypic (biochemical) characteristics, serotyping and enzymatic profiles. There is only one other report (Rajeev et al., 2009) on phenotypic speciation of Indian staphylococcal isolates using the Hi-Media kit, and our results partially agree with this results. Their results revealed that *S. equorum*, *S. xylosus*, *S. sciuri* subsp *rodentium*, *S. intermedius* and *S. saprophyticus* were the predominant CoNS recovered in SCM, whereas, in clinical cases, *S. arlettae*, *S. capitis* subsp *ureolyticus*, *S. haemolyticus*, *S. felis* and *S. equorum*, were identified (Rajeev et al., 2009).

Another study conducted in China detected *S. epidermidis* (15%) and *S. saprophyticus* (10%), previously considered as naught pathogenic bacteria, in clinical
or sub-clinical bovine mastitic milk samples based on morphologic examination and biotyping (Cheng *et al*., 2010).

However, the variety of the predominant CoNS species detected in the present study is relatively different and appears to be conflicting to the majority of the reports published earlier in which the species such as *S. chromogenes*, *S. simulans*, *S. epidermidis*, *S. haemolyticus*, *S. hyicus* and *S. xylosus* were reported to be most prevalent in bovine IMI (Trinidad *et al*., 1990; Myllys, 1995; Rajala-Schulz *et al*., 2004; Luthje and Schwarz, 2006; Thorberg *et al*., 2006; Taponen *et al*., 2008; Thorberg *et al*., 2009). It is noteworthy that all these workers relied upon the molecular methods for the detection of CoNS species. A recent report from India also states that *S. chromogenes*, *S. epidermidis*, *S. sciuri* and *S. haemolyticus* are the predominant CoNS in bovine milk samples as detected by m-PCR (Shome *et al*., 2011).

This could be attributed mainly to the poor accuracy of the commercial identification systems in distinguishing different species of CoNS and/or the variability in expression of the phenotypic characters (Birnbaum *et al*., 1991; Bannerman *et al*., 1993; Grant *et al*., 1994; Ieven *et al*., 1995; Renneberg *et al*., 1995; Calvo *et al*., 2000). In addition, most commercial identification systems may vary in accuracy when used on animal strains (Langlois *et al*., 1983; Thorberg and Brandstrom, 2000).

Misidentification of CoNS species using conventional/commercial identification systems, in comparison with the molecular methods (Lee and Park, 2001; Becker *et al*., 2004; Sivadon *et al*., 2004; Ben-Ami *et al*., 2005; Fujita *et al*., 2005; Heikens *et al*., 2005; Skow *et al*., 2005; Shittu *et al*., 2006) have also been reported.
Of late, molecular detection methods are being used for identification of the CoNS species due to higher sensitivity, specificity and rapidity (Poyart et al., 2001; Devriese et al., 2002; Drancourt and Raoult, 2002; Heikens et al., 2005; Skow et al., 2005; Capurro et al., 2009; Thorberg et al., 2009 and Shome et al., 2011) and therefore, an effort was made in the present study to develop and standardize the PCR based methods to identify the CoNS to genus and species level.

5.4 Molecular Characterization of CoNS

5.4.1 PCR based confirmation of CoNS to genus level

*Tuf* gene encodes the elongation factor ‘*tu*’, 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).

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 adopted for the direct detection of staphylococci from contaminated blood or from normally sterile clinical specimens such as blood or urine (Martineau et al., 2001). Many authors reported the usefulness of *tuf* gene in the development of PCR assays to differentiate and identify *Staphylococcus* species (Martineau et al., 2001; Chen and Hwang, 2008 and Hegde, 2011). 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 earlier findings, wherein *tuf* gene based PCR was successfully used to identify *Staphylococcus* at genus level, prompted us to target the same gene. Thus, in the present study, genus level confirmation was done by the amplification of 235 bp product using the designed primers (Hegde, 2011) and the PCR was standardized using the Staphylococcal MTCC cultures (Plate 3.1). The primers were found to be genus specific covering all CoNS species as confirmed by the NCBI BLAST report and amplified all reference CoNS species as well *S. aureus* used in the study.

Of the 152 CoNS isolates subjected for PCR for genus level identification, 149 isolates were found belonging to *Staphylococcus* genus. The remaining three were found to be non-staphylococcal isolates, despite their similar cultural and morphological properties to that of *Staphylococcus* species. With the morphological features, they may be tentatively identified as micrococcal species.

### 5.4.2 Detection of CoNS species by m-PCR

Accurate species identification of bovine CoNS isolates is important for monitoring of species distribution in herds, studying potential species differences in virulence and detection of possible reservoirs involved in bovine mastitis. The valuable information about species-specific difference can be a vital data for effective mastitis therapy and management.

Because of the poor accuracy of commercially available phenotypic methods for identification of bovine CoNS, various DNA-based methods have been developed. Sequencing of housekeeping genes is often considered as the gold standard for identification purposes. However, for accurate species identification on a large scale,
alternative methods with similar accuracy but faster turnaround and lower costs are preferable. Multiplex PCR based methods relying on the usage of species specific primers targeting the variable region of the housekeeping genes have been proposed as valid alternatives.

5.4.2.1 Genes targeted

A number of PCR amplicon-sequencing-based methods for identification of CoNS have been reported. Several molecular targets have been exploited for the molecular identification of *Staphylococcus* species. Gene sequence-based identification of bacteria at the species level may require resolving the whole gene, yet in some cases, phylogenetically closely related bacterial species cannot be differentiated from each other targeting a single gene (Ghebremedhin *et al.*, 2008). For example, 16S rRNA sequence similarity has been shown to be very high, 90 to 99%, in 29 *Staphylococcus* species studied (Kwok *et al.*, 1999). In this regard, although the comparison of the 16S rRNA gene sequences has been useful in phylogenetic studies at the genus level, its use has been questioned in studies at the species level. Recently, Ghebremedhin *et al.* (2008) opined that the closely related species of *Staphylococcus* cannot always be distinguished from each other based on the analysis of 16S rRNA gene sequences and thus, new phylogenetic markers for *Staphylococcus* species are needed. Heikens *et al.* (2005) also opined that 16S rRNA gene sequencing suffered from the lack of high quality among sequences deposited in GenBank. Furthermore, it was shown that genotypic identification based on 16S rRNA sequences had limited discriminating power for closely related *Staphylococcus* species.
In the present study also, 16S rRNA gene was not targeted for species detection as a few sequences which were available did not offer variable region for targeting individual species.

Based on their results, Ghebremedhin et al. (2008) proposed the partial sequencing of the glyceraldehyde-3-phosphate dehydrogenase (gap) as an alternative molecular tool for the taxonomical analysis of Staphylococcus species and for decreasing the possibility of misidentification.

In another study, partial sequencing of the rpoB gene was proposed as a new tool for the accurate identification of Staphylococcus isolates (Drancourt and Raoult, 2002). The complete/partial sequences of rpoB, the gene encoding the β-subunit of RNA polymerase was determined for 32 Staphylococcus species and the staphylococcal rpoB sequence database was established.

By the way of sequence analysis of 429-bp-long DNA fragment internal to the sodA gene (sodAint) in 40 CoNS type strains Poyart et al. (2001) showed that the staphylococcal sodA genes exhibited a higher divergence than does the corresponding 16S rDNA. With the simple PCR and sequencing assays developed in their study, they confirmed that the sodA gene encoding the manganese-dependent superoxide dismutase constitutes a highly discriminative target sequence for differentiating closely related bacterial species.

In a recent study in which 5 different species of CoNS were targeted by m-PCR (Shome et al., 2011), gap/ rdr/ sodA genes were amplified for detection of CoNS species
and their primers were found to be specific when they were evaluated with 13 different staphylococcal species.

Therefore, considering the above findings reported by several workers, gap/ rpoB/ sodA genes were targeted for PCR based detection in the present study subsequent to the confirmation of the availability of variable region sufficient to design species specific primers.

However, the other genes/ regions; for instance, Cpn60 gene (Goh et al., 1997), 16S-23S rRNA spacer region (Forsman et al. 1997), hsp60 gene (Goh et al., 1996; Anita et al. 1999), tRNA intergenic spacer region (Devriese et al., 2002) or tuf gene sequence-based genotyping (Heikens et al., 2005; Capurro et al., 2009) reported to be suitable target for designing species-specific primers or probes for identification and phylogenetic analysis of staphylococci were not targeted in this study for two reasons; unavailability of sufficient sequences in GenBank and the available sequences did not offer variable region to design suitable primers.

In the present study, depending on the species, the genes such as gap, rpoB and sodA were determined to be targeted mainly based on the availability of sufficient sequence data and their descreminating power among different species of CoNS. We aligned the gap/ rpoB/ sodA gene sequences available in GenBank and designed species-specific primers for 10 different CoNS species that were commonly encountered in the mastitis. The gap/ rpoB/ sodA gene based PCR standardized for the identification of the CoNS at the species level amplified the desired product from 7 of the 10 CoNS species (Plates 4.1 to 4.4). For the remaining three species viz., S. fleurettii, S. simulans and S.
equorum, the positive controls were not available; yet they were included in m-PCR with the aim of detecting them among the isolates, if any. However, these primers showed high in silico specificity and their Tm values matched with the Tm values of the other primers.

5.4.2.2 Standardization of two-tube m-PCR

Even though the primers worked at all the annealing temperatures tested ranging from 51.8 to 60.1 °C with slight variation in the intensity of bands when the gradient PCR was set up for each set of primers, finally 60 °C was used in the two-tube multiplex PCR, to avoid non-specific amplification at lower temperatures. Finally, the primers to be used for two-tube multiplex PCR were first evaluated for their specificity by uniplex PCR using known positive and negative controls (Plates 5.1 to 5.7). All the primers were found to be specific and yielded amplicons of expected sizes when they were tested with the known reference cultures by uniplex PCR.

For S. arlettae, we did not have a positive reference strain to compare with. Hence, all the isolates identified to be S. arlettae based on biochemical properties were screened by the uniplex PCR using S. arlettae specific primers designed in this study. The isolate no. 5, the only isolate which was identified as S. arlettae by PCR and confirmed again by partial ‘gap’ sequencing, was used as positive control for further studies. Similar method was followed for S. xylosus even for which a positive reference was not available and, the isolate no. 28 identified and confirmed as S. xylosus was used as positive control.
For *S. arlettae* and *S. xylosus*, for which the positive references were not available, all the biochemically identified *S. arlettae* and *S. xylosus* isolates were screened by the uniplex PCR using respective species specific primers and, those which were confirmed as belonging to the corresponding species both by PCR and subsequent sequencing of the amplicon were used as positive reference strain.

The two-tube m-PCR was successfully standardized and the desired amplicons were obtained in both the tubes (Plates 6.1 and 6.2). However, when the ambiguity was noticed while screening the field isolates with respect to the outcome of PCR e.g., presence of additional band/s of different size or faint bands corresponding to the size specific to other species, the species of such isolates was confirmed either by repeating m-PCR or by sequencing of the amplicons of the expected size.

### 5.4.2.3 Screening of CoNS isolates by two-tube m-PCR

The two-tube m-PCR developed in this study was used to screen all the CoNS isolates for identification of species (Plate 7.1 and 7.2). Based on PCR results, *S. epidermidis*, *S. chromogenes* and *S. sciuri* were the major CoNS species detected in this study; followed by *S. xylosus*, *S. saprophyticus*, *S. haemolyticus* and *S. arlettae* (Table 19 & 20 and Fig. 1a).

Worldwide, several workers have reported similar variety of CoNS species *viz.*, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans* and *S. xylosus*, including *S. hyicus* and *S. warneri* (Trinidad *et al*., 1990; Matthews *et al*., 1992; Myllys, 1995; Forsman *et al*., 1997; Aarestrup and Jensen, 1997; De Vliegher *et al*., 2003; Rajala-Schulz *et al*., 2004; Luthje and Schwarz, 2006; Taponen *et al*., 2006; Taponen *et al*., 2007;
Thorberg et al., 2006; Thorberg et al., 2009; van den Borne et al., 2010; Sampimon et al., 2011; Shome et al., 2011; Supré et al., 2011). The only exception with respect to the variety of CoNS species detected in this study is that none of the isolates were identified to be S. simulans, a predominant CoNS species reported elsewhere. But our study is in accordance with another report from India (Shome et al., 2011) which stated that S. chromogenes, S. epidermidis, S. haemolyticus and S. sciuri as the predominant CoNS in bovine milk samples as detected by mPCR. S. simulans was detected in one sample only that was culture negative. Species distributions vary among countries and herds, but the reasons for this are not known.

*S. chromogenes* is a highly prevalent species in SCM (Sampimon et al., 2011; Shome et al., 2011; van den Borne et al., 2010; Supré et al., 2011) with a well established impact on SCC (Devriese et al., 2002). On the other hand, *S. epidermidis* is also found to be most common CoNS species isolated next to *S. chromogenes/ S. haemolyticus* in different studies (Sampimon et al., 2011; Shome et al., 2011; Waller et al., 2011). In this study also, these two species constituted 44.2 % of the total CoNS strains isolated.

The prevalence of CoNS in bovine mastitis varied from as low as 6.2 % as in case of acute clinical mastitis from Sweden (Ericsson Unnerstad et al., 2008) to as high as 58.4 % in case of SCM from Brazil (Medeiros et al., 2009). In the Finnish national mastitis survey, where all quarters of cows were sampled from a random sample of all dairy herds, 17% of all quarter milk samples were CoNS positive and the proportion of CoNS of all isolated pathogens was 50% (Pitkala et al., 2004). In the Netherlands, 11% of all quarters and 32% of cows were CoNS positive (Sampimon et al., 2009). A similar
prevalence was reported in Belgium, where 12.5% of the quarters and 41.1% of the cows were infected with CoNS (Piepers et al., 2007).

The prevalence of CoNS is high especially in SCM. CoNS are also more commonly isolated in clinical mastitis. In England and Wales, the prevalence of CoNS mastitis was 8.1% in clinical mastitis and 14.9% in SCM (Bradley et al., 2007). In Finnish routine mastitis sampling, the prevalences were 17.6% and 23.5%, respectively (Koivula et al., 2007). Incidence rate of clinical CoNS mastitis in Canada is 1.15 per 100 cow years, which is lower than that for three major mastitis pathogens (Olde Riekerink et al., 2008).

In the present study, 44.08% of the milk samples were positive for CoNS, which is relatively towards higher side compared to the above findings. Subclinical mastitis caused by IMI with CoNS is common in dairy cows and may cause herd problems. Control of CoNS mastitis is complicated by the fact that CoNS contain a large number of different species.

5.4.2.4 CoNS in clinical mastitis

In clinical mastitis cases, CoNS were isolated in 33% (14/42) of the milk samples, but the incidence rate recorded in this study may not represent the actual status for clinical mastitis as only a limited number of samples (n=42) were analyzed. Regarding the CoNS species isolated from the clinical cases in the present study, S. epidermidis was the most frequent (4/14) and together with the S. chromogenes, it constituted nearly half of the total strains (44.85%).
The proportion of CoNS among bacteria isolated from clinical mastitis (CM) cases remains very low in many countries. In a recent study from Canada, CoNS were isolated from 6% of quarters with CM (Olde Riekerink et al., 2007) and in Sweden; they comprised only 6% of bacteria isolated from CM (Ekman and Østeras, 2004). In Switzerland, the respective figure was 17% (Schallibaum, 2001) and in Israel, it was 9% (Shpigel et al., 1998). Among 77,051 routine mastitis samples submitted to laboratories in Finland during 2004 – 2006, CoNS were the most frequently isolated bacteria in samples from clinical (18%) and subclinical (24%) mastitis cases (Koivula et al., 2007). In Germany, the prevalence of CM in primiparous cows was 27.4% and in multiparous cows 16.4% (Tenhagen et al., 2009). In the practice area of the Faculty of Veterinary Medicine, University of Helsinki, Finland, more than 20% of bacterial isolates from milk samples from CM were CoNS (Nevala et al., 2004). In a study on CM carried out in the same area about 30 years ago, the proportion of CoNS was only 6.5% (Pyorala and Syvajarvi, 1987). The proportion of CoNS is generally high in samples collected from animals with SCM but low in samples from animals with clinical mastitis. In countries where the biggest udder health problems are caused by major environmental mastitis pathogens, CoNS infections have often been ignored (Pyorala and Taponen, 2009).

Under the field conditions, CoNS infections probably progress more slowly and mostly without clinical signs. However, spontaneous CoNS infections can result in CM (Olde Riekerink et al., 2008; Tenhagen et al., 2009; Wenz et al., 2010). Mastitis represents a dynamic process; clinical mastitis typically turns to SCM during the course of the disease (Grönlund et al., 2003).
CoNS are important pathogens in cattle of all ages, but the predominant CoNS species causing infection seems to differ between age groups. *S. chromogenes* was the major CoNS species in pre-calving heifers and primiparous cows (Trinidad *et al*., 1990; Rajala-Schultz *et al*., 2004; Taponen *et al*., 2006), whereas *S. simulans* and *S. epidermidis* were mostly isolated from cows in later lactations (Taponen *et al*., 2006; Thorberg *et al*., 2009). Multiparous cows generally become infected with CoNS during later lactation whereas primiparous cows usually already have the infection at the beginning of lactation (Grohn *et al*., 2004; Taponen *et al*., 2007).

5.4.2.5 CoNS detected in normal bovine milk samples

It is interesting and worth mentioning that fairly good number of isolates (21%, 29/138 isolates) were obtained from the milk samples with very less or nil SCC also (0-1 lakh cells/ml) in this study (Table 14.1). The possible explanation is that intramammary CoNS infection can be found in a quarter with a normal milk SCC (<100,000 cells/ml) (Piepers *et al*., 2010) and SCC may fluctuate (Taponen *et al*., 2007). Infection can be transient and be eliminated spontaneously or remain persistently in the mammary gland (Chaffer *et al*., 1999; Taponen *et al*., 2007; Gillespie *et al*., 2009). It has been shown that half of CoNS IMIs persist in the udder for long periods (Taponen *et al*., 2006; Thorberg *et al*., 2009; Supré *et al*., 2011). However, the mention of persistence of CoNS infection and the fluctuation of SCC is beyond the scope of this study as the sequential monitoring of neither SCC pattern nor the CoNS persistence was made.

Nevertheless, infection with CoNS induces an immunological reaction in the udder and should not be considered merely teat canal colonization or a normal situation
for the udder (Taponen et al., 2007). Some authors have suggested that infection with minor pathogens like *S. chromogenes* or other CoNS and *Corynebacterium* species would be beneficial because it might protect the quarter from mastitis with major pathogens like *S. aureus* (Schukken et al., 1989b; Matthews et al., 1990). Possible mechanisms for this effect could be increased SCC or bacteriocins produced by the bacteria (Matthews et al., 1990; De Vliegher et al., 2004). However, the possible protective effect of CoNS IMI remains theoretical and CoNS infections should be dealt with as any IMI (Taponen et al., 2007).

Finally, 11 CoNS isolates in this study were negative by both two-tube m-PCR specific for CoNS and triplex PCR specific for *S. aureus*. These can be any species that were not targeted by PCR in this study.

### 5.2.4.6 Limitations

According to the observations made in the current study, the following limitations were encountered while designing the primers for PCR for the identification of CoNS species.

Lack of whole genome sequence data deposited in GenBank for most of the CoNS species

Lack of sequence data in GenBank for a particular gene *i.e.*, whole gene is not resolved

Lack of high quality among sequences deposited in GenBank.
Limited discriminating power of especially the partial sequences of CoNS for closely related *Staphylococcus* species

Misidentification of the CoNS species even with the amplicon- sequence based method cannot be ruled out due to non-availability of a particular targeted gene sequences for all CoNS species.

5.4.3 Detection of coagulase negative variants of *S. aureus* in bovine milk

In the present study, 18 of 19 *S. aureus* strains were misjudged by HiStaph™ as non-aureus staphylococcal strains which were later identified as *S. aureus* by PCR amplification of the *fib*, *nuc* and 23S rRNA genes and, subsequent sequencing of the representative strains. In all, 19 of the 20 thermonuclease positive CoNS isolates (Table 15) were found to *S. aureus* (19/149; 12.75 %) by m-PCR (Plate 8).

These strains failed to coagulate rabbit plasma on retesting them with the tube coagulase assay for the second time. Whilst the strains of *S. aureus* failing to produce coagulase are very rare, the finding of coagulase negative strains of *S. aureus* in clinical mastitic samples in the present study supports the fact that these strains could be pathogenic and show most of the biochemical properties of their species (Collee *et al.*, 1989). Such variants of *S. aureus* have been described previously as a cause of IMI in a herd of dairy cattle (Fox *et al.*, 1996) as well as in human infections (Heltberg and Bruun, 2009).

Recently, Akineden *et al.* (2011) reported last year that bacteriological analysis of milk samples from quarters of a dairy cow suffering from SCM yielded two isolates
of *S. aureus* which gave a negative reaction in the standard coagulase test as well as for clumping factor and thermonuclease. The isolates were identified phenotypically by using commercial biochemical systems (Staphaurex® test) as *S. aureus* and the diagnosis was consistent with PCR analysis of different staphylococcal cell surface protein and exoprotein genes, sequencing of the 16S rRNA gene and RT-PCR of coagulase (*coa*), clumping-factor (*clfA*) and thermonuclease (*nuc*) genes.

The fact that coagulase-negative *S. aureus* variants can occur in the context of IMI in cattle may result in the incorrect identification as CoNS, occasionally. Proper identification of CoNS species and *S. aureus* is very important in this aspect which can be partly accomplished by conducting all three phenotypic assays; coagulase, clumping factor and thermonuclease and, to fully ensure accurate species identification, sequencing of the 16S rRNA gene and amplification of specific genes such as *coa* is necessary in these cases (Akineden *et al*., 2011).

### 5.5 Comparison of commercial phenotyping system (HiStaph™ Identification Kit) and genotyping methods for species level identification of CoNS

A number of commercial identification kits for species identification of CoNS have been evaluated by mastitis researchers. Most phenotypic systems are considered to accurately identify >80% of staphylococci but have not been designed to detect all taxa that have been associated with bovine mastitis (Ruegg, 2009).

The overall agreement between phenotypic and genotypic methods in the present study was only 11%. Of the total (n=152) strains examined, 71% strains were misjudged and the remaining 18% of the strains had no match in the list provided by the
manufacturer when the results of m-PCR and subsequent sequencing based speciation of CoNS were compared with that of phenotyping using HiStaph™ Identification Kit and supplementary tests (Table 21).

Misidentification of CoNS species by conventional/ commercial identification systems, in comparison with the molecular methods have been reported earlier also (Lee and Park, 2001; Becker et al., 2004; Sivadon et al., 2004; Ben-Ami et al., 2005; Fujita et al., 2005; Heikens et al., 2005; Skow et al., 2005; Shittu et al., 2006; Capurro et al., 2009).

By comparing commonly used phenotypic methods, the API Staph ID test and the BD Phoenix Automated Microbiology System, with PCR amplicon-sequencing based methods, targeting the 16S rRNA, tuf and sodA genes, Heikens et al. (2005) demonstrated the superiority of genotypic methods over phenotypic assays for identification of CoNS species. This was supported by another study which indicated that the phenotypic methods (using the Staph-Zym™ test) are unreliable for identification of CoNS from bovine IMI and sequence-based genotyping is a better alternative for species-level identification (Capurro et al., 2009). In comparison with results obtained by tuf gene sequencing, they observed that only 61% of the isolates were correctly identified using Staph-Zym™ and 34% of these identifications needed supplementary tests. Among the remaining isolates, 11% had no match in the Staph-Zym™ code list.

These studies also contradict the fact that the conventional biochemical assays are the gold standard in the isolation and identification of the bacterial pathogens and support the statements about the inconsistency of the results of these tests which are also time
consuming (Freney et al., 1992; Phuektes et al., 2001, 2003; Oliver et al., 2004; Picard et al., 2004).

The results of the present study indicates that micro-methods such as HiStaph™ Identification Kit based phenotypic identification may not reliably distinguish between different CoNS species, and that genotypic methods are more reliable for diagnostic purposes in the identification of CoNS from bovine IMI. It is remarkable that the accuracy of HiStaph™ Identification Kit was low (11%) and the test had low specificity in identification of important udder pathogens such as *S. epidermidis* and *S. chromogenes*. There was also a tendency to give an incorrect species name rather than a ‘No match’. Additionally, a drawback of HiStaph™ was that the coagulase negative *S. aureus* strains were not identified. The observations made in current study based on the outcome of results of genotyping compelled to conclude that phenotypic assay employed in this study has low precision for identification of CoNS species.

5.6 Nucleotide sequence and Phylogenetic analysis of CoNS isolates

The genetic analysis of organism is very important to establish the genetic relatedness and evolutionary variation among the closely related micro-organisms. Since CoNS species are very closely related, the study of their nucleotide sequence is important to understand the evolution of new types, its importance in the development of diagnostic, prophylactic and molecular epidemiological investigations in identifying the source of spread of infection. Further, this technique is beneficial in identification of even single nucleotide deletion/ addition or substitution, which cannot be detected in any other methods.
In principle, the phylogenetic analysis based on alignment of nucleotide sequence of specific gene provided an insight into the genetic makeup of the organisms and its sharing with the other members of the group. This approach is perhaps the most realistic and presents a quick bird’s eye view of the complex inter-relatedness.

5.6.1 Analysis of *S. epidermidis* based on *rpoB* gene sequence

The analysis involved nucleotide sequences from 21 reference strains and 6 isolates of *S. epidermidis* obtained in this study. Of the entire gene sequences of CoNS species analyzed, maximum number of nucleotide substitutions was identified in *rpoB* gene sequence of *S. epidermidis* isolates. Out of the 460 nucleotides in the sequence analyzed with the reference strain (EF173659), a total of 6 nucleotides were substituted in two isolates (nos. 108 and 147) with 4 transitions and 2 transversions.

All the *S. epidermidis* isolates obtained in this study were placed in the same cluster and formed a major cluster along with other reference strains of the same species isolated elsewhere in the world. Two isolates (nos. 108 and 147) which had 6 nucleotide substitutions formed a sub cluster in the same group. This reveals the genetic homology of the present *S. epidermidis* isolates with the other isolates isolated elsewhere. All other strains from different CoNS species were placed in entirely different clusters (Fig. 2).

5.6.2 Analysis of *S. chromogens* strains based on *sodA* gene sequence

Analysis of the 296 nucleotides with the reference strain (AJ343901) revealed transversion at only one position in isolate no.23 and the remaining isolates showed 100% sequence similarity.
The analysis involved nucleotide sequences from 11 reference strains and 5 isolates of *S. chromogenes* obtained in this study. The *S. chromogenes* isolates obtained in this study were placed in two different clusters; 4 isolates formed one group with the reference strain isolated from France (AJ343901) and the remaining isolate (no. 23) which had one nucleotide substituted formed a separate group with the two reference strains from the same country (AJ343944 and AJ343945). Whereas, the strains from other species such as *S. simulans* and *S. capitis* sharing homology formed two different sub clusters and, *S. intermedius* strain formed a separate cluster. This reveals the genetic homology of the present *S. chromogenes* isolates with the other isolates isolated elsewhere. All other strains from different CoNS species were placed in entirely different clusters (Fig. 3).

**5.6.3 Analysis of *S. sciuri* strains based on gap gene sequence**

The analysis involved nucleotide sequences from 16 reference strains and 2 isolates of *S. sciuri* obtained in this study. In spite of 2-3 nucleotide substitutions in a total of 348 positions compared with the reference strain (FJ578004), the isolates (nos.2 and 52) together formed a different sub cluster which was affiliated to a major cluster exclusively of *S. sciuri* strains. All 5 sub clusters had only *S. sciuri* strains isolated from France, USA and China. All other CoNS species in the tree like *S. vitulus*, *S. fleurettii* and *S. lentus* etc., were genetically diverse and identified in relatively distant groups/subgroups (Fig. 4).
5.6.4 Analysis of S. xylosus strains based on rpoB gene sequence

The analysis involved nucleotide sequences from 23 reference strains and 2 strains of S. xylosus obtained in this study. Analysis of the 358 nucleotides with the reference strain (FJ906727) revealed transversion in 2 and 3 positions in the isolates nos.28 and 57 of the present study, respectively. These two isolates shared the same but distinct sub cluster and were genetically closer to the strains isolated from Poland. Although some of the CoNS species like were S. nepalensis, S. cohnii, S. aureus and S. warneri were affiliated to the nearest cluster, all the S. xylosus strains in the tree were placed closely in the same major group. However, S. haemolyticus, S. saprophyticus and S. lugdunensis were genetically more diverse from the others (Fig. 5).

5.6.5 Analysis of S. saprophyticus strains based on gap gene sequence

Analysis of the 602 nucleotides with the reference strain (DQ321695) revealed nucleotide substitution in only one position (nos. 15 and 90) or 2 positions (no. 87) in isolates of the present study. Whereas, the isolate no. 85 showed 100% sequence similarity with the reference sequence.

The analysis involving nucleotide sequences from 27 reference strains and 4 strains of S. saprophyticus obtained in this study revealed one cluster shared by the present isolate nos. 15 and 90 with the other two reference strains isolated from France and another very closely placed but distinct sub cluster in which another isolate (no. 87) with 2 nucleotides substituted was found. The remaining isolate of this study (no. 85) was found in a separate group along with the other strains isolated from Japan, Germany, China and France. However, all the S. saprophyticus strains were affiliated to the same
main group. Interestingly, all other CoNS species found in the tree were identified in different sub clusters and none of the species shared the same cluster signifying wide genetic diversity among the CoNS with respect to gap gene sequence (Fig. 6).

5.6.6 Analysis of *S. haemolyticus* strains based on sodA gene sequence

Analysis of the 281 nucleotides with the reference strain (EU652775) revealed transitions at 4-5 different positions in two isolates (nos. 75 and 102) of the present study. Whereas, the other isolate (no. 29) was 100% identical with the reference sequence.

The analysis involved nucleotide sequences from 18 reference strains and 3 strains of *S. saprophyticus* obtained in this study revealed; a distinct sub cluster formed by the isolates nos. 75 and 102 (in which 4-5 nucleotides were substituted) but shared a common branch with other strains of *S. haemolyticus* and another isolate no. 29 (without any nucleotide substitution) was affiliated to a group with most of the strains isolated from France. All other species were comparatively placed separately and none of the species was identified close to *S. haemolyticus* (Fig. 7).

5.6.7 Analysis of *S. arlettae* strains based on gap gene sequence

The analysis involved nucleotide sequences from 15 reference strains and one isolate of *S. arlettae* (JQ764624) obtained in this study. It was observed that the present *S. arlettae* isolate (no.5) showed 100% sequence similarity with the reference strain (DQ321674) and was identified in a distinct cluster along with other two reference strains belonging to the same species isolated from Germany and China. The other species of CoNS viz., *S. capitis*, *S. aureus*, *haemolyticus*, *S. equorum* and *S. saprophyticus* exhibited
a wide genetic diversity between species leading to the formation of exclusively species-wise clusters (Fig. 8).

5.6.8 Analysis of coagulase negative S. aureus strains based on 23S rRNA, nuc and fib genes

There was 100 % sequence similarity observed between the nucleotide sequences of coagulase negative S. aureus isolates (nos. 107, 109 and 145) obtained in this study and the reference strains with respect to 23S rRNA (CP003033) and fib sequences (CP003033).

Whereas, analysis of the 461 nucleotides of nuc with the reference strain (FR821779) divided the isolates into two groups; two isolates (nos. 107 and 109) with 5 nucleotide substitutions (resulting in substitution of 2 amino acids at different positions) and isolate no. 145 with one nucleotide substitution (that resulted in substitution of one amino acid).

Phylogenetic analysis based on the 23S rRNA revealed a major cluster shared by all other reference S. aureus strains isolated from all over the world. This cluster was relatively closer to S. lugdunensis and S. haemolyticus strains isolated from United Kingdom and Japan, respectively, and the CoNS species were distantly related (Fig. 21).

Phylogenetic analysis based on the nuc gene sequence revealed two different subclusters which originated from different branches in which the present isolates were grouped in. Two isolates which had 5 nucleotide substitutions (nos. 107 and 109) were placed in a distinct and separate cluster very close to a strain isolated from Denmark.
(CP003134). The other isolate no. 145 having one nucleotide substitution was affiliated to a group shared by strains isolated from UK (FR812779) and China (DQ399678 and DQ507377), and genetically it was distantly related to the other two isolates of the present study (Fig. 22).

With a few exceptions, the phylogenetic relationships inferred from the partial gap/ rpoB and sodA sequences were in agreement with those previously derived from DNA-DNA hybridization studies and analyses of 16S rDNA gene sequences and partial hsp60 gene sequences. The topology of the phylogenetic tree obtained was in general agreement with that which was inferred from an analysis of their 16S rRNA or hsp60 (Goh et al., 1996) gene sequences.

Sequence analysis revealed that the staphylococcal gap and sodA genes exhibit a higher divergence than does the corresponding rpoB. These results confirm that the gap/ sodA gene constitutes a highly discriminative target sequence for differentiating closely related bacterial species. Alternatively, rpoB gene sequences can also be used. Clinical isolates that could not be identified at the species level by phenotypical tests were identified by use of this database. These results demonstrate the usefulness of this method for rapid and accurate species identification of CoNS isolates, although it does not allow discrimination of subspecies.

These preliminary data suggest the presence of species-specific sequence variation within the conserved genes of staphylococci viz., gap, rpoB and sodA. Further work regarding validation is required to determine the specificity of the primers designed in the study using all available CoNS reference cultures for species-specific microbial
identification and phylogenetic investigation of staphylococci and perhaps other microorganisms in general.

5.7 Antimicrobial resistance of CoNS

Antimicrobial resistance has been recognized since the earliest days of chemotherapy. 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. CoNS species may differ in antimicrobial susceptibility, virulence factors, host response to infection and transmissibility. Hence, studies on species specific antimicrobial sensitivity are needed to adopt appropriate antimicrobial therapy and to develop species-specific management practices (Sampimon et al., 2009).

CoNS 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 horizontally to *S. aureus* and other Staphylococci, including those pathogenic to humans (Archer and Climo, 1994). Hence, there is need to obtain vital data with respect to antibiotic resistance patterns of CoNS at species level which provides a valuable information for effective mastitis therapy and management.
5.7.1 In-vitro antimicrobial sensitivity of CoNS isolates

Antimicrobial agents are commonly applied to dairy cattle either to control or to prevent bacterial infections in lactating and dry cows. Thus, the results of *in vitro* sensitivity testing are an important tool to guide the veterinarian in selecting the most efficacious antimicrobial agent(s) for therapeutic and prophylactic interventions.

The antibiogram profile of the CoNS isolates was studied by employing disc diffusion method using 12 different antibiotics that are commonly used for the treatment of mastitis in the study area.

Most of the CoNS isolates (127 of 149, 85.23%) were found to be multi-drug resistant (Plate 9) *i.e.*, resistant to more than 3 class of antibacterials used in the study. Four isolates (nos. 44, 93, 125 and 173) were found to be resistant to all the antibacterials used in the study.

5.7.1.1 Resistance pattern – Antibacterial-wise

Among the 12 different antibacterials used in the study enrofloxacin represented florquinolones group and, streptomycin and gentamicin together represented aminoglycosides group. The remaining eight antibacterials except chlorphenicol belonged to β-lactams group.

The present study revealed differences in antimicrobial senstivity among the CoNS species evaluated. Highest resistance was detected to oxacillin and methicillin in CoNS as a whole, followed by ceftraixone with sulbactum/ tazobactum penicillin G (68.46%), ceftriaxone, amoxycillin with sulbactum, ampicillin and streptomycin. Highest
sensitivity was recorded for chloramphenicol followed by enrofloxacin and gentamicin (Fig. 21). In general, the sensitivity pattern for any antibacterial used in the study was not much variable among different species of CoNS (Table 23).

The statistical analyses of the antibiogram data by the Pearson chisquare as well as Liklihood ratio chisquare revealed the significant (p<0.001) association of CoNS species with antimicrobial resistance.

The present study revealed differences in antimicrobial sensitivity among the CoNS species evaluated. The majority of CoNS were resistant to β-lactam antibiotics that are commonly used for mastitis treatment and prevention. Highest resistance was noticed to oxacillin to the extent of 92.86 to 100 % among CoNS and 89.47 % in coagulase negative S. aureus; followed by methicillin. Next group of antibacterials was cephalosporins which comprised ceftriaxone either alone or in combination with β-lactamase inhibitors such as tazobactum or sulbactum to which CoNS exhibited resistance to 62.42–72.48 % extent. Similarly, about 50 % of the CoNS were resistant to aminopenicillins represented in this study by ampicillin and amoxicillin with sulbactum, whereas more than 50% were resistant to natural penicillins such as penicillin G. The most common resistance mechanism is β-lactamase production, which results in resistance to penicillin G and aminopenicillins (Chambers, 1997).

As a group, majority of CoNS in this study were susceptible to chloramphenicol and enrofloxacin.
5.7.1.2 Resistance pattern of predominant CoNS

Among the predominant CoNS species, *S. chromogenes* showed relatively higher sensitivity to the antibacterials used in the assay and *S. sciuri* exhibited uniformly high resistance to all the antibacterials tested. Highest resistance was observed in *S. saprophyticus* to all antibiotics except for enrofloxacin. The resistance/sensitivity patterns for the remaining species of CoNS detected in this study may not represent the pattern that can be generalized for the entire species as they were found in very small number. Hence, it is not discussed in detail in this thesis.

The oxacillin/ methicillin resistance demonstrated in all predominant CoNS species like *S. epidermidis* (94.22%), *S. chromogenes* (96.97%) and *S. sciuri* (100%).

β-Lactams are important antimicrobial agents used for the prevention and treatment of mastitis in dairy cows. They are frequently used in intramammary infusion therapy also. Mechanisms of β-lactam resistance in staphylococci include production of β-lactamases and low-affinity penicillin-binding protein 2a (PBP2a). β-lactamases hydrolyze the β-lactam ring of the antibiotic, whereas, low-affinity PBP2a substitutes the activities of essential PBPs and thus confers the resistance to β-lactamase resistant penicillins/ β-lactam antibiotics. The latter, designated for methicillin resistance, precludes therapy with any of the currently available β-lactam antibiotics, and may predict resistance to several classes of antibiotics other than β-lactams (Odd and Maeland, 1997; CLSI, 2002).

The presence of β-lactamases in CoNS has been observed both in human and veterinary isolates. Archer and Scott (1991) reported that 89% of CoNS isolated from
humans harboured inducible β-lactamase. Aarestrup et al. (1995) reported that 75% of the strains isolated from bovine mastitis in Denmark were resistant to penicillin. β-lactamase production has been reported in 21–84% of CoNS isolated from dairy cows with mastitis (Thornsberry et al., 1997; Myllys et al., 1998; Gentilini et al., 2002). More recently, Taponen et al. (2006) reported presence of β-lactamases in 19% of CoNS that caused mastitis in lactating dairy cows. Strains with an MIC value of 0.06 mg/ml are known to carry β-lactamase encoding gene blaZ, which mediates resistance to penicillin (Suominen et al., 2002). But in the majority of these studies, isolates were reported as CoNS and were not further identified to the species level.

In this study, the high resistance of CoNS to penicillins (51.02 - 68.42%) is in agreement with the results reported in other studies (Archer and Scott, 1991; Aarestrup et al., 1995). The increasing resistance of staphylococcal strains involved in bovine mastitis to penicillin might be related to improper use of this drug in mastitis treatment and the routine use of β-lactam antibiotics for intramammary infusion in Indian herds.

5.7.1.3 Methicillin (oxacillin)-resistant staphylococci (MRS)

Staphylococcal isolates are frequently resistant to penicillinase-resistant penicillins. Organisms exhibiting this type of resistance are referred to as methicillin (oxacillin)-resistant staphylococci (MRS) (Mandell et al., 1995). MRS show an intrinsic resistance to penicillinase-resistant β-lactam antibiotics. This resistance is based on “mecA” gene encoding PBP2a, an inducible and an altered PBP that has low affinity for binding β-lactam antibiotics. The gene ‘mecA’ is located on the chromosome of MRS and methicillin resistance is not due to destruction of the antibiotic by β-lactamase. MRS
strains also show a heterogeneous character with the level of resistance varying according to the culture conditions and β-lactam antibiotic being used. Because of this heterogeneous resistance, the detection of MRS by phenotypic methods becomes problematic. Furthermore, certain laboratory conditions (e.g. prolonged incubation, incubation at low temperatures, higher inoculum) also can cause the sensitivity test results for susceptible strains to shift toward or above the breakpoint for resistance (Gerberding et al., 1991).

Methicillin-resistant staphylococcal isolates were frequently resistant to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicols, tetracyclines and fluorquinolones (Mandell et al., 1995). Additionally, MRS strains should be considered resistant to all cephalosporins, cephems and other β-lactams and carbapenems, regardless of the in vitro test results obtained with those agents (CLSI, 2002).

Methicillin resistant staphylococci are considered resistant to penicillins, penems, carbapenems, and cephalosporins making it difficult to treat if associated with infections (Chambers, 1997). Appropriate therapy of methicillin-resistant staphylococcal infection requires knowledge of antimicrobial resistance profile. Gentilini et al. (2002) recommended that animals that carry methicillin-resistant CoNS isolates should be culled. Moreover, the prudent use of antibiotics and, rapid and continuous screening for resistant microorganisms should be more focused to prevent the emergence and spread of MRCoNS.
5.8 Amplification of antibiotic resistant genes and coa gene of CoNS isolates by mPCR

5.8.1 Detection of mecA gene

Methicillin resistance in staphylococci is caused by expression of PBP2a, encoded by mecA and femA genes, which are highly conserved in staphylococcal species, so it is possible to detect methicillin resistance by detection of these genes (Hartman and Tomasz, 1984). As methicillin resistance mediated by PBP2a is often heterogeneously expressed in staphylococci (Tomasz et al., 1991; Bignardi, et al., 1996) PCR detection of mecA gene is the “gold standard” for the detection of methicillin resistance (Bignardi et al., 1996; Salisbury et al., 1997).

Methicillin resistant S. aureus (MRSA) is one of the most important MDR pathogens around the world. MRSA was generated when methicillin-sensitive S. aureus (MSSA) exogenously acquires a methicillin resistance gene, mecA, carried by a mobile genetic element, staphylococcal cassette chromosome mec (SCC-mec), which is speculated to be transmissible across staphylococcal species. One of the animal-related (commensal bacterium of animals) Staphylococcus species, S. fleurettii, is reported to be the highly probable origin of the mecA gene (Tsubakishita et al., 2010).

In this study, the presence of the mecA gene was investigated by multiplex PCR method (Plate 10). Of all the strains tested for genotypic methicillin resistance in this study, only 10 strains were mecA positive and none of the strains possessed novel mecA gene (Table 24). The positive detection rate of mecA in MRCoNS was only 7.7%. Seven of the ten (isolate nos. 149, 189 and CLM 101110) mecA positive MRCoNS strains
showed multi-resistance patterns (resistant to 3 or more class of antibiotics) on the basis of the multi-resistance definition by Coombs *et al.* (2004). Resistance to oxacillin in CoNS has become a problem, as CoNS express resistance to all β-lactam antibiotics, and leads to a significant limitation in therapeutic options (York *et al.*, 1996).

It was interesting to note that *mecA* gene was found only in *S. epidermidis* strains (n=8) and *S. saprophyticus* (n=2). This finding is of public health importance since *S. epidermidis* could be transmitted to the associated human beings and thereby responsible for acquisition of methicillin resistance by other *Staphylococcus* of human origin.

Despite the standardized recommendations for the sensitivity testing of MRS given by the CLSI (2010), many of the isolates in this study that did not carry *mecA* were phenotypically resistant to methicillin/oxacillin by disc diffusion method. This could be related to a type of resistance due to either overproduction of β-lactamase or the presence of altered penicillin binding protein (PBP) not related to 2a or 2’ (Georgopapadakou, 1993). These strains appeared to be β-lactamase hyper-producing strains because most of them remained sensitive to amoxicillin-sulbactum. The phenotypic expression of resistance can vary depending on the growth conditions (e.g., the temperature or osmolarity of the medium), making sensitivity testing of MRS by standard microbiological methods potentially difficult (Chambers, 1997).

Moon *et al.*, 2007 observed that three isolates did not amplify the *mecA* gene among MRSA isolates. The isolates appeared to show poor expression of *mecA* genes or production of methicillinase (alteration of PBP subtypes) or seem to overproducing β-
lactamase, as they remained sensitive to amoxicillin-clavulanate and growth conditions may be involved in the expression of methicillin resistance (Moon et al., 2007). The phenotypic expression of resistance could vary due to growth conditions or there might be limitations in detection in microbiological methods (Chambers et al., 1997; Ravinder Kumar et al., 2010).

Shittu et al. (2006) observed in their study that five oxacillin-resistant S. haemolyticus isolates were mecA positive, while the two S. saprophyticus isolates, which exhibited resistance to oxacillin using the disc diffusion method, lacked the mecA gene. York et al. (1996) and Bogado et al. (2001) have also observed this trend, and suggested that a mechanism other than the production of PBP2a may be involved in methicillin resistance in S. saprophyticus. It also reaffirms the problematic nature in screening for methicillin resistance and the need for reliable methods for its detection in this species. The hyperproduction of β-lactamase has been suggested as a major factor in false-positive detection of oxacillin resistance in CoNS (Ghoshal et al., 2004).

Two of the CoNS isolates were mecA-positive but sensitive to methicillin (no. 189) or to both oxacillin and methicillin (CLM 180311). There are several possible explanations for this discrepancy. For instance, the detection of oxacillin-sensitive strains that carry mecA is not particularly surprising given the multifactorial nature of oxacillin resistance (Berger-Bachi, 1997).

Ciftci et al. (2009) found only 4 of 59 (6.7%) methicillin resistant (by oxacillin disc diffusion method) S. aureus isolates to be MRSA in triplex PCR. Similarly, some investigators (Unal et al., 1994: Araj et al., 1998) have also reported the discrepant
results between disc diffusion methods and PCR for detection of methicillin resistance. It is reported that conventional sensitivity tests such as agar disc diffusion and broth dilution methods may not give reliable results in detecting MRSA because of heterogenic expression of resistance (Unal et al., 1994). In this study, MRSA lacking mecA gene are classified as false resistant by the oxacillin disc diffusion method and it was considered that it may be due to another resistance mechanism such as hyperproduction of β-lactamase.

Of late, oxacillin/ methicillin disk diffusion test is eliminated to detect methicillin resistance as too many false resistances were observed with oxacillin for CoNS (CLSI M100 - S19. Table 2C. pp. 54). Alternatively CLSI (2010) recommends performing of phenotypic confirmation of mecA-mediated oxacillin/ methicillin resistance using cefoxitin discs. Here, cefoxitin serves as surrogate to report resistance for oxacillin/ methicillin.

The use of cefoxitin to report resistance for methicillin is recommended very recently (CLSI, 2010) before which the assay was carried out in this study using oxacillin and methicillin. The fact that cefoxitin disc, a preferred test, was not included in the present study might have resulted in the overestimation of the phenotypic methicillin resistance.

5.8.2 Novel mecA gene

Animals can act as a reservoir and source for the emergence of novel MRSA clones in human beings. Very recently, a strain of S. aureus (LGA251) isolated from bulk milk that was phenotypically resistant to methicillin but tested negative for the mecA
gene was reported from UK during an investigation on such strains in bovine and human populations (Garcia-Alvarez et al., 2011). A divergent mecA homologue (mecALGA251) was discovered in the genome of LGA251 strain of *S. aureus* located in a novel staphylococcal cassette chromosome mec element (designated type-XI SCCmec) while searching for mecA PCR-negative bovine *S. aureus* isolates showing phenotypic methicillin resistance. The mecALGA251 was 70% identical to *S. aureus* mecA homologues. The mecALGA251 homologue was identified in human mecA-negative MRSA isolates also. Garcia-Alvarez et al. (2011) opined that new diagnostic guidelines for the detection of MRSA should consider the inclusion of tests for mecALGA251. Hence, PCR assay in the current study included the amplification of the novel homologue mecALGA251 region also using published primers.

Although none of the isolates were found to carry novel mecA, the possibility of such divergent mecA homologues cannot be ruled out which might be operative in mecA negative strains of the present study.

**Aminoglycoside Resistance**

Aminoglycosides are antibiotics of major importance in the treatment of IMI infections caused by Gram-positive and Gram negative pathogens in bovines. They still play an important role in antistaphylococcal therapies, although emerging resistance amongst staphylococci is widespread. To further the understanding of the prevalence of aminoglycoside resistance in the study area, all CoNS and coagulase negative *S. aureus* isolates of this study were studied for the same. The main mechanism of aminoglycoside resistance in staphylococci is drug inactivation by cellular aminoglycoside- modifying
(AME) enzymes. Several distinct gene loci encoding such modifying enzymes have been characterized in staphylococci. Clinically, the most important of these encode aminoglycoside acetyltransferase (\textit{aac}), aminoglycoside adenylyltransferase (\textit{ant}) or aminoglycoside phosphotransferase (\textit{aph}) activity. Aminoglycosides modified at amino groups by \textit{aac} enzymes or at hydroxyl groups by \textit{ant} or \textit{aph} enzymes, lose their ribosome-binding ability and thus no longer inhibit protein synthesis (Paulsen \textit{et al.}, 1997).

\subsection*{5.8.3 Detection of \textit{aacA-D} and \textit{aph(3')-IIIa} genes}

Among the aminoglycoside resistance strains tested in this study, only 24 \% of the phenotypically positive strains were positive for \textit{aacA-D} gene or \textit{aph(3')-IIIa} or both (Plate 10 and Table 24). Of the 16 strains tested positive to either of the aminoglycoside resistance genes, 7 (43.75 \%) strains belonged to \textit{S. epidermidis} and 5 (31.5 \%) strains to \textit{S. saprophyticus} (Table 24).

Despite the absence of any AME genes in their genome, remaining 76 \% of the strains exhibited varying resistance to the aminoglycosides in general. Hence, the adaptive resistance to aminoglycosides appears to be operative in these strains. Similar reports have been made with \textit{Pseudomonas aeruginosa} and \textit{Enterobacter} species (Bryan, 1990; Poole, 2005). It has been held that AME-independent aminoglycoside resistance is an adaptive feature developed under stress conditions (Asseray \textit{et al.}, 2002) and, as such, the same can be ascribed to the AME-deficient but phenotypically resistant strains in the present study.
Resistance to gentamicin and concomitant resistance to tobramycin and kanamycin in staphylococci are mediated by a bifunctional enzyme displaying \( aac(6') \) and \( aph(2') \) activity (Ubukata et al., 1984). The \( aac(6')-aph(2') \) gene encodes this bifunctional enzyme and is encoded on composite transposon Tn4001. Tn4001-like elements are widely distributed in both \textit{Staphylococcus aureus} and CoNS. Tn4001 has been found conjugative plasmids such as pSK41, occasionally on \( \beta \)-lactamase/heavy metal resistance plasmids such as pSK23 and also in various chromosomal locations (Paulsen et al., 1997).

Resistance to neomycin, kanamycin, tobramycin and amikacin in staphylococci is mediated by an \( ant(4')-I \) enzyme encoded by \( ant(4')-Ia \). This gene is often carried on small plasmids, and then integrated into larger conjugative plasmids such as pSK41 (Paulsen et al., 1997).

Resistance to neomycin and kanamycin conferred by an \( aph(3')-III \) enzyme has also been described for staphylococci. The \( aph(3')-IIIa \) gene responsible for this phenotype is carried on the transposon Tn5405, which may be located on both the chromosome and plasmids (Derbise et al., 1996). The genetics of streptomycin resistance is somewhat more complex, being associated with an \( ant(6')-Ia \) gene, a resistance gene called \textit{str}, chromosomal mutations (\textit{strA}), an \( aph(3')-III \) gene and an \( ant(4')-Ia \) gene (Phillips and Shannon, 1984).

\section*{5.8.4 Coa gene of coagulase negative \textit{S. aureus}}

Upon detection of phenotypically coagulase negative \textit{S. aureus} in the present study, the \textit{coa} gene encoding the enzyme coagulase, was also targeted and included in the
multiplex PCR. The enzyme coagulase helps in formation of fibrin clots around the organism through which the organism shields itself from the harmful external substances and from host immune responses.

Of 19 coagulase negative *S. aureus* isolates identified in this study; 14 isolates yielded 850 bp amplicon specific for *coa* gene, one isolate (no. 63) yielded an amplicon of higher size (~1100 bp) and the remaining four isolates (nos. 68, 112, 163 and CLM 290311) did not yield any amplicon.

The reason for the difference in the size of the amplicons of *coa* gene may be similar to an observation of the *coa* gene polymorphism and its distinct size in different isolates (Phonimdaeng *et al.*, 1990; Hookey *et al.*, 1998).

Until recently, single banded *coa* PCR products were reported in *S. aureus* strains isolated from bovine milk samples; however, double-banded amplification products have also been reported recently from *S. aureus* strains derived from bovine mastitis in Brazil (Rodrigues da Silva *et al.*, 2005; Aslantas *et al.*, 2007). In the current study, double-banded amplification product was detected only in one *coa* positive isolate. Goh *et al.* (1992) explained the presence of double-banded amplification products with different allelic forms of the *coa* gene.

The possible explanation for some of the *S. aureus strains* not giving positive reaction in tube coagulase assay inspite of possessing the gene might be a mutation or deletion in the *coa* gene or in its regulatory sequences, but confirmation requires further investigation.
5.8.5 Analysis of partial nucleotide sequence of mecA, aacA-aphD and aph3′-IIIa genes of S. epidermidis

Nucleotide BLAST analysis of partial nucleotide sequences of mecA, aacA-aphD and aph3′-IIIa genes of S. epidermidis isolates (nos. CLM 160311, 111 and 47, respectively) revealed 100% identity with their respective reference strains with GenBank accession nos. CP003194, M18086 and JF909978 (Figs. 13-19).

Protein BLAST report of the partial protein sequence of aph3′-IIIa gene amplified product (isolate no. 47) proved that it produced significant alignment with the chain A, crystal structure of 3′,5′-aminoglycoside phosphotransferase (Protein Bank accession no.pdb|1L8T|A) encoded by aph3′-IIIa gene (Figs. 20-22).

5.8.6 Phylogenetic analysis of coagulase negative and coagulase positive S. aureus isolates based on coa gene nucleotide sequence

Nucleotide analysis of 692 positions in the sequence of the present isolates displayed two nucleotide substitution in one isolate (no. 107) and one transition in another isolate (no. 109). It also identified the deletion of 5 nucleotides in both the isolates.

The analysis of nucleotide sequences from 13 reference strains and 2 strains of S. aureus obtained in this study revealed the present isolates grouped in two different but sub clusters with very close evolutionary relationship which originated from the same branch shared by the strains isolated from mainly Japan and USA. Newman strain of
S. aureus subspecies aureus was placed in distinct branch altogether different from the remaining ones in the tree (Fig. 23).

5.9 Determination of minimum inhibitory concentration (MIC) values

MIC interpretation/ sensitivity categorization was done as per CLSI (2010) guidelines based on human interpretative criteria. Accordingly, the MIC break point values for CoNS considered were ≥0.5µg/ml, 8µg/ml and 1µg/ml for oxacillin, methicillin and gentamicin, respectively.

The MIC values with regard to oxacillin, methicillin and gentamicin were determined for all genotypically positive strains as well as for few strains that were genotypically negative but phenotypically positive (Table 25 and Plate 11).

The MIC values of oxacillin in the mecA positive isolates were more than reference break point (≥0.5µg/ml) except in one isolate (no. 29; MIC=0.256µg/ml). Similarly, seven of the ten isolates possessing mecA gene showed MIC values of methicillin more than the reference break point (Table 25). Vannuffel et al. (1995) compared standard sensitivity methods with resistance to methicillin as predicted by the PCR assay; only one oxacillin-susceptible (MIC<0.016 mg/ml) CoNS strain expressed mecA. Similar results have been reported by others (Murakami et al., 1991). A mutation or deletion in the mecA gene or in its regulatory sequences may explain such a discrepancy, but confirmation requires further investigation.

In 16 mecA negative strains assayed, oxacillin MIC was more than break point values for CoNS (≥0.5µg/ml) except for two isolates (nos. 135 and 148). On the
contrary, the MIC values of methicillin in the \textit{mecA} negative strains were far below the cut off value (8µg/ml) except in one isolate (no. 92). Barring one or two isolates, this observation suggests the need for the use of both methicillin and oxacillin for determination of MIC and it can be inferred that MIC of either methicillin or oxacillin indicate the the methicillin resistance. CoNS isolates that were phenotypically resistant to oxacillin ($\geq 0.5$ mg/ml) may be β-lactamase producers. Resistance to methicillin should be reported as resistant to all β-lactam antimicrobial agents, including cephalosporins and β-lactamase inhibitor combinations despite apparent \textit{in vitro} sensitivity (Clinical Laboratory Standard Institute, 2002).

The MIC values of gentamicin in \textit{aacA-D/aph3’-IIIa} positive strains were in the range of 0.064 to 8, whereas, in genotypically negative strains the least values were recorded \textit{i.e.}, either <0.064 or 0.064 except for three isolates (isolate nos. 44, 142 and CLM 2011010) in which it was slightly higher (0.128). Of the 16 strains genotypically positive to aminoglycoside resistance, 7 of them showed MIC values more than the reference break point (1µg/ml as per EUCAST guidelines) and the remaining 9 showed less than the break point.

However, the MIC values in all the negative strains were far below the break point which ranged from $\leq 0.064 – 0.128$. 
Conclusions

1. The high incidence of the CoNS in milk samples in the study area signifies their possible role as emerging mastitis pathogens both in clinical and subclinical mastitis.

2. Phenotypic methods for identification of CoNS appear to be unsatisfactory, less reliable and reproducible and molecular methods which may soon replace them are necessary to ascertain the correct identification of CoNS.

3. The two-tube multiplex PCR assay developed in the present study is an easy and rapid method to simultaneously detect the ten major CoNS prevailing in bovine mastitis and can be applied for direct detection of predominant CoNS species in milk samples. Hence, the regular analysis of milk by m-PCR may be a useful tool for determining the herd status with regards to the CoNS mastitis.

4. The study indicated the existence of methicillin resistance in CoNS isolates of bovine origin and the possibility of the CoNS acting as reservoir of the antibiotic resistant genes including aminoglycosides resistant genes for the horizontal transfer to other species.

5. The vital data obtained in this study regarding antimicrobial resistance patterns and the prevalence of CoNS at species level under Indian conditions provide valuable information about species-specific differences that can be used for effective mastitis therapy and management.

6. Interpretations made from our results are tentative and need to be confirmed by more systematic studies with appropriate methodology.