Review of Literature
II. REVIEW OF LITERATURE

Bovine Mastitis

Mastitis is the most economically important disease of dairy cows (Halasa et al., 2007). Mastitis negatively affects the quality of milk, milk production, farm economics and animal welfare. Calculations of economic losses resulting from mastitis vary among countries. In India, the overall economic loss due to mastitis is estimated to be Rs. 7165.51 crores (Bansal and Gupta, 2009). Average decrease in milk yield due to clinical and subclinical mastitis was estimated to be 50 % and 17.5 %, respectively. The prevalence of bovine mastitis continues to affect the dairy herds throughout the world inspite of continued research activity on the problem over the century (Sadana, 2006). Organisms as diverse as bacteria, mycoplasma, yeasts and algae have been implicated as causes of mastitis; over 150 different contagious or environmental micro-organisms are reported as etiological agents (Kuang et al., 2009). The major contagious pathogens comprise \textit{S. aureus}, \textit{S. dysgalactiae}, \textit{S. agalactiae}; the major environmental pathogens comprise \textit{E. coli} and \textit{S. uberis}.

However, there is now an increasing body of evidence, discussed later in this review to suggest that coagulase-negative staphylococci (CoNS) have become the most common mastitis pathogens in many countries. In many modern dairy herds, CoNS are a frequent cause of bovine mastitis and their importance as mastitis pathogens has been recently reviewed (Pyorala and Taponen, 2009; Taponen and Pyorala, 2009). CoNS mastitis mostly remains subclinical or shows only mild clinical signs. CoNS can cause persistent infections resulting in increased milk SCC affecting milk quality and may be
related to decreased milk production. The economical impact of the increase in bulk milk SCC depends on the regulatory limits for milk SCC and quality premiums for milk with low SCC in individual countries. CoNS mastitis responds well to antimicrobial therapy. *S. simulans* and *S. chromogenes* are probably the predominant CoNS species in bovine mastitis. The knowledge on CoNS species involved in mastitis is still very limited and benefits would accrue from having more reliable diagnostic methods for species identification. Efficient strategies for prevention of CoNS mastitis can then be designed (Pyorala and Taponen, 2009).

2.1 Tests for detection of subclinical mastitis (SCM)

2.1.1 Somatic cell count (SCC)

In a normal, non infected and non inflamed quarter, the somatic cell population consists of polymorphnuclear cells, macrophages, lymphocytes and epithelial cells (Concha, 1986). The majority of cells are macrophages (approximately 60 % of cells), lymphocytes are approximately 30 % of cells, and polymorphonuclear cells form approximately 10 % of the population, the epithelial cells are about 2 % (Sandholm, 1995).

Barkema *et al.* (1998) opined that bulk milk SCC was related to management practice. Herds, managed by farmers who worked precisely, paid more attention to individual cows and implemented measures to prevent mastitis, more often had a lower bulk milk SCC.
Ma et al. (2000) observed that mastitis significantly elevated raw milk SCC and raw milk quality was lower in high SCC milk than in low SCC milk.

Somatic cell counts (SCCs) were used as quality parameter for raw milk with the assumption that high cell counts are associated with poor quality raw milk and lower cell counts indicate better quality. The SCC penalty limits for saleable milk differ between countries within the EU, Australia and New Zealand at 400,000, Canada at 500,000 and the United States at 7,50,000 cells / ml of milk (Paape and Contreras, 1997).

Schukken et al. (2003a) opined that the somatic cells in milk play an important role in the innate immunity of the uninfected mammary gland. A complete absence of cells would put cows at risk for disease and a very low concentration of somatic cells increases the risk of clinical mastitis.

Rysanek et al. (2007) stated that a potential source of bulk tank milk contamination by relevant pathogens (the environment or the mammary gland) may be elucidated. The probability of the contamination of bulk tank milk samples with mastitis pathogens predicted by the analysis of relationship between the bulk milk tank SCCs and the number of mastitis pathogen, showed a strong correlation. Further, it was observed that not all contaminations with major mastitis pathogens cause a coincident elevated level of Bulk Tank Milk SCC (BTMSCC).

Kamphuis et al. (2008) explored the potential value of in-line composite SCC sensing as a sole criterion or in combination with quarter-based EC of milk, for automatic detection of clinical mastitis during automatic milking and the results suggested that that
the performance of a clinical mastitis detection system improved when in-line composite SCC information was added to a detection model using EC information.

Miranda-Morales et al. (2008) determined the prevalence of microorganisms and SCCs in 112 Holstein bovine herds in two bulk tank milk-screening assays. Prevalence of *Mycoplasma* was 55 %; of *S. aureus*, 30 %; of *S. uberis*, 37.5 %; and of CoNS, 38.3 %. The geometric mean of the SCC was 465,000 cells/ml and there was no significant difference in the SCCs between the positive and negative samples of pathogens isolated.

Pantoja et al. (2009) studied the relationship between SCC and IMI across the dry period and the risk of SCM at the first dairy herd improvement (DHI) test of the subsequent lactation. The study also involved to determine SCC test characteristics for diagnosis of IMI at both the cow and quarter levels. It was observed that the new IMI detected from post-calving milk samples were caused by CoNS (46.7 %), Streptococci (26.7 %) and Gram-negative bacteria (11 %). The most frequent microorganisms related to cured IMI were CoNS (33 %). The study revealed that the quarters with SCC ≥200,000 cells/ml at both dry-off and post-calving sampling periods were 20.4 times more likely to be subclinically infected by a major pathogen and 5.6 times more likely by a minor pathogen at the first DHI test than quarters with SCC <200,000 cells/ml at both periods. It was also observed that cows with SCC greater than 200,000 cells/ml at both the last and the first DHI test between lactations produced 9.1 kg less milk on the first DHI test day than the average milk production of cows with SCC less than 200,000 cells/ml at both periods.
Mollenhorst et al. (2010) investigated whether on-line SCC assessment, when combined with EC, should be implemented at the udder quarter or at the cow level. The data analyzed from three farms with automatic milking systems, resulting in 3,191 quarter milkings revealed that quarter level SCC assessment was superior to cow level assessment when combined with EC measurement at quarter level.

### 2.1.2 Electrical conductivity (EC)

Sheldrake and Hoare (1981) compared EC and SCC in three herds for their ability to correctly identify the infection status of quarters. For EC, thresholds of 6.0 and 6.8 milli Siemens (mS/cm) and for SCC a threshold of 5,00,000 cells/ml was used for comparison. They observed considerable variation between herds for sensitivity and specificity for both EC and SCC. The mean sensitivity and specificity of EC was 49 % and 79 % respectively, whereas for SCC, the means were 71 % and 81 % respectively. These variations in sensitivity and specificity of EC between herds were attributed to differences in the distribution of EC for quarters of similar infection status. It was concluded that the differences in herd EC precluded the use of pre-determined EC thresholds which were applicable for detecting mastitis in all herds.

Fernando et al. (1982) evaluated the potential value of EC of milk as a screening test for SCM and observed that conductivity of milk seems to hold promise as an indicator of SCM. In the study, conductivity of foremilk and of post-milking stripping was measured for 368 quarters of 92 cows. It was observed that the milk conductivity increased with infection and the conductivity of post-milking stripping was higher than that of foremilk in samples from quarters infected by primary pathogens. The study
revealed that the number of quarters with elevated conductivity of post-milking stripping tended to be higher when SCC was greater than 5,00,000/ml in both normal and infected groups.

While studying the efficacy of detecting SCM by EC of milk compared with that of other indirect methods including chloride, sodium, potassium, lactose, bovine serum albumin, and SCC of milk, Fernando et al. (1985) observed that the accuracy of the EC of milk for detection of SCM compared favourably with all indirect methods and has considerable potential as a screening test for SCM.

Hillerton and Watson (1991) examined a hand-held, commercially available instrument (Milk Checker) for measuring the EC of milk for its usefulness and accuracy in detecting subclinical and clinical mastitis. It was observed that the foremilk from uninfected quarters had an EC of 5.4 to 5.6 mS/cm whereas, milk from cows with subclinical *S. aureus* infections had higher milk conductivity (7.1 to 7.5 mS/cm), but, milk from cows with subclinical *S. uberis* infections showed no increase in conductivity (5.3 to 5.6 mS/cm). They opined that the equipment could be a useful veterinary tool.

Nielen *et al.* (1992) observed that with an overall sensitivity of 66 % and specificity of 94 %, the predictive value of a positive EC test remained low in a low prevalence population.

Lansbergen *et al.* (1994) evaluated a prototype on-line system for measurement of EC of quarter milk for accuracy in detection of SCM compared with that of
bacteriological culture and SCC of sampled quarters. It was observed that the prototype on-line system did not detect SCM with high accuracy.

Milner et al. (1996) observed that clinical mastitis caused by two major pathogens *Staph. aureus* and *S. uberis* could be detected earlier by measuring changes in EC of milk than by waiting for a herdsperson to detect visible changes in milk. The study also indicated that the changes in EC could detect all subclinical infections from *Staph. aureus* but not *S. uberis*.

Musser et al. (1998) assessed the utility of a hand-held EC meter to detect SCM under field conditions. Milk from each quarter of 425 lactating cows on 15 dairies in Costa Rica were tested using a hand-held EC meter and the results of bacteriologic culture were compared with highest absolute EC score for each cow. It was observed that the absolute EC score for cows with SCM was significantly higher than that for cows without SCM and was significantly associated with detection of SCM. The study revealed that, if absolute EC score of $\geq 7$ was considered indicative of SCM, then sensitivity was 0.43, specificity was 0.83, predictive value of a positive result was 0.39 and predictive value of a negative result was 0.85. Further, differential EC score for cows with mastitis was significantly higher than that for cows without SCM. If differential EC score of $\geq 2$ was considered indicative of SCM, then sensitivity was 0.53, specificity was 0.77, predictive value of a positive result was 0.37 and predictive value of a negative result was 0.87. They inferred that a hand-held EC meter may be used to screen cows for SCM.
Spakauskas et al. (2001) observed that the EC of milk samples taken from healthy cows (61.7%) was found to be 4.3-5.7 mS/cm and in cases of cows with SCM, EC of milk increased to 6.1-8.5 mS/cm.

Mansell and Seguya (2003) evaluated the accuracy of a hand-held EC meter for the detection of SCM in an Australian dairy herd in late lactation. Milk samples collected from 233 quarters from 59 cows revealed that the EC was higher in infected quarters than uninfected quarters. Further, the variability between individual quarters and between cows in this study was such that the use of this device to measure the conductivity of milk was not a reliable method to diagnose SCM in cows in late lactation. They concluded that the interpretation of 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.

Norberg et al. (2004) defined four EC traits as the inter-quarter ratio (IQR) between the highest and lowest quarter EC values, the maximum EC level for a cow, IQR between the highest and lowest quarter EC variation, and the maximum EC variation for a cow and observed that all EC traits increased significantly when cows were subclinically or clinically infected.

Claycomb et al. (2009) assessed a novel method for automated in-line clinical mastitis detection system using measurement of conductivity from foremilk of individual udder quarters. The data set of approximately 27,000 individual milking was tested against several published or potential alternative 'gold standards' for diagnosing clinical
mastitis. In the pilot trial, 12-14 clinical events were detected out of 19 true clinical quarters, with a false-alert rate of between three and five false EC alerts per 1,000 individual milking. Further, in the more rigorous field trial, sensitivity ranged from 68 to 88 %, and the false-alert rate ranged from 2.3 to 7.0. They concluded that the novel clinical mastitis detection system, based on separation of the flow and measurement of EC from foremilk of individual udder quarters, has the potential to provide a new tool for helping farmers to monitor clinical mastitis in herds milked with conventional clusters.

Juozaitiene et al., (2010) evaluated the electrical conductivity of milk during milking phases and determine its relationship with cow productivity and milk SCC. Fifty four of lactating cows after II-VI calving were selected. The average of electrical conductivity was 6.30 ± 0.09 mS/cm. These results demonstrate that highest electrical conductivity was at the beginning of milking 7.14 ± 0.11 mS/cm, of plateau phase was lower 0.82 ± 0.12 mS/cm (p=0.001), the main milking phase 6.71 ± 0.10 mS/cm and of the descending phase 6.21 ± 0.15 mS/cm electrical conductivity of milk was comparable and statistically not different. Results demonstrated the positive statistically significant correlation (p=0.0001) between somatic cell in the milk and the electrical conductivity, and statistically insignificant negative correlation between milk yield and electrical conductivity. The highest influence on somatic cell count was shown by the increment of the electrical conductivity of milk at the beginning of milking (p=0.048).

Shahid et al. (2011) in their study, subjected milk samples collected from 125 animals (25 buffaloes, 30 crossbred cows, 15 Sahiwal and 55 Achai breed) apparently mastitis free, to surf test, NaOH, pH meter and electronic detector. On the electronic
detector 65.2%, On Surf and NaOH tests animals, 56.8% (each) animals were found positive for SCM. While On pH meter, 40.8% animals were positive for SCM. In case of Sahiwal cattle, prevalence of SCM was more in left fore quarter (P<0.05). In buffaloes, it was more prevalent in both hind quarters. The results of the current study indicated that electronic detector is more sensitive than surf test, NaOH and pH detector.

2.1.3 California Mastitis Test (CMT)

Lazzari et al. (2002) isolated CoNS, S. uberis, S. dysgalactiae, alfa-haemolytic Streptococcus species, Enterococcus species, Rhodococcus equi, Corynebacterium species and Moraxella bovis from the buffalo milk samples. They compared cultural isolation with CMT and Whiteside Test (WS) and found 67 % of the CMT positive samples, and 40 % of WS, have been positive for the bacterial isolation.

One of the oldest and best known is the California mastitis test (CMT). It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and lead to the formation of a 'gel-like' matrix consistency. However, the interpretation can be subjective, and this might result in false positives and negatives (Viguier et al., 2009).

2.1.4 Bromothymol Blue (BTB) strip test (Indicator paper method)

Marschke and Kitchen, (1985) evaluated a simple bromothymol blue indicator test was for farm diagnosis of mastitis. The test required highly absorbent blotting paper impregnated with four spots of bromothymol blue. Indicator color 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 bromothymol blue test ranged from 51 to 56% and specificity from 89 to 90% for most reference criteria used to classify normal and abnormal milk. Predictability of a positive test ranged from 49 to 52% (false positives 51 to 48%) and predictability of a negative test from 90 to 97% (false negatives 10 to 3%) for the same criteria. Overall the bromothymol blue test incorrectly diagnosed 11 to 20% of 3772 quarters. By classifying colour score 2 as negative, predictability of a positive result was 70 to 75% and sensitivity was 26 to 30%. The test can be used by dairy producers to screen herds with a relatively high incidence of mastitis or used in combination with cow cell counts to locate abnormal quarters. The bromothymol blue test was less sensitive than the California Mastitis Test but offered several practical advantages for use on farm.

2.2 Coagulase negative Staphylococci in bovine intramammary infections

Fujikura and Shibata (1965) surveyed dairy herds, covering five rural areas near Tokyo by CMT and found that 84.1 % of herds were infected with mastitis and 43.8 % of the cows were affected. Bacteriological examination showed that S. aureus was present in 72.4 % of samples; CoNS, Str. agalactiae, Str. dysgalactiae, Str. uberis, Corynebacterium pyogenes, Pseudomonas aeruginosa, Bacillus cereus and Enterobacteriaceae were also present.

Misra et al. (1973) screened milk samples from 305 cows by indirect tests and cultural examination and found that nearly a quarter of all the cows screened had udder infection. The major pathogens isolated included S. aureus (33.1%), albus Staphylococcus (15.3 %) and Klebsiella species (8.4 %). Other pathogenic bacteria
isolated were *Corynebacterium pyogenes*, *Pseudomonas aeruginosa*, *E. coli* and *Str. agalactiae*.

Verma *et al.* (1978) reported that out of 562 quarters of 141 apparently healthy cows, 73 (13 %) quarters of 43 (30 %) cows were subclinically mastitic and culture yielded *S. aureus* (34 %), *E. coli* (27 %), *S. epidermidis* (23 %), *Alcaligenes fecalis* (7 %), *Pseudomonas pyocyanea* (7 %), *Str. dysgalactiae* (4 %), *Micrococcus* species (4 %) and *Proteus morganii* (4 %).

Gonalez *et al.* (1980) found that 2388 (57.3 %) of 4168 quarter milk samples examined from 30 dairy farms were positive for CMT reaction. The cultural examination of 300 samples giving CMT two or three reactions yielded *S. aureus* (43 %), *S. epidermidis* (21%), *Str. uberis* (19 %), *Str. agalactiae* (13 %), *Str. dysgalactiae* (9 %), *Corynebacterium. bovis* (7 %), *Corynebacterium pyogenes* (1.3 %), Coliforms (1.7 %) and mixed Streptococcal and Staphylococcal cultures.

A study conducted by Swartz *et al.* (1984) reported the prevalence and types of bacteria associated with SCM in Bloemfontein dairy herds. *S. aureus* was the dominant mastitis-associated organism, constituting 66.4 % of all bacteria isolated. Other bacterial strains isolated included *Corynebacterium bovis* (6.3 %) and the CoNS (11.0 %).

Ferriro *et al.* (1985) screened 4286 quarters of 1067 lactating cows and found 1046 (24.50 %) quarters produced suspect milk and obtained 896 cultures mainly from quarters with SCM. The organisms isolated included mainly the members of
Micrococcaceae (31.96 %), S. aureus (16.96 %), S. epidermidis (13.25 %), Str. agalactiae (12.05 %), Str. uberis (8.82 %) and Str. dysgalactiae (5.14 %).

Singh et al. (1988) reported that, of the 1373 isolates from the cases of SCM; 1011 (73.5 %) were Staphylococcus species; 72 (5.2 %) Streptococcus species; 37 (2.7 %) Pseudomonas species; 255 (18.6 %) E. coli and others.

Schukken et al. (1989a) examined a total of 1140 clinical cases of mastitis. E. coli (16.2 %) was the most frequently isolated organism, followed by CoNS (13.0 %), S. aureus (9.6 %) and Str. uberis (8.0 %). Only two isolates of Str. agalactiae were found.

Malinowski et al. (1992) examined milk samples from 2084 cows from 20 farms out of which 945 cows were found positive for SCM. The pathogens isolated were S. epidermidis (32.5 %), Micrococcus species (19.9 %), Str. agalactiae (15.3 %), S. aureus (11.0 %), Str. uberis (10.3 %), Str. dysgalactiae (8.1 %) and other microorganisms (2.0 %).

Tuteja et al. (1993) observed that among 307 isolates from apparently healthy quarters, Staphylococci were the predominant organisms (44.3 %) followed by Micrococci (30.0 %), Streptococci (18.9 %), Corynebacterium species (5.55 %), Bacillus species (1.0 %) and Enterococci (0.3 %).

Lafi et al. (1994) monitored 19 Jordanian dairy farms from July 1991 to August 1992 and concluded that the most prevalent bacterial pathogens in subclinical udder infections were CoNS (9.4 to 11.8 %), S. aureus (9.2 to 12.1 %) and Corynebacterium
bovis (5.8 to 7.5 %) and the Gram negative organism isolated were E. coli and Enterobacter species.

Mitra et al. (1995) screened 528 milk samples and 116 (21.96 %) were found to be positive for SCM by Bromothymolblue test. All 116 milk samples were found to be culturally positive. The organisms isolated were Staphylococci 48 (41.37 %), Streptococci 32 (27.58 %), E. coli 28 (24.13 %) and Corynebacterium 8 (6.89 %).

Simpson et al. (1995) investigated the prevalence of SCM in beef cows and milk production in primiparous cows. Milk samples from 32 % (8/25) of the cows and 18 % (18/100) of the quarters were culture-positive for bacteria (S. aureus, Str. dysgalactiae, Streptococcus species, Actinomyces pyogenes, and (or) CoNS) at one or more times. It was observed that the milk SCC concentrations in primiparous cows were highly variable and higher SCC concentrations were associated with lower milk production in cows.

A study was conducted on clinical mastitis in Himachal Pradesh in which milk samples collected from 93 affected quarters (57 cows) for bacteriological examination revealed Staphylococcus species (68.83 %), Streptococcus species (16.88 %), E. coli (7.8 %), Klebsiella species (2.59 %), Proteus species and Corynebacterium species (1.3 %) (Wadhwa et al., 1996).

Mallikarjunaswamy and Murthy (1997) investigated microbial etiology of mastitis in 162 dairy cows from 6 dairy farms in and around Bangalore. They isolated 214 bacterial isolates from cases of bovine SCM which included S. epidermidis (80), S. aureus (66), S. intermedius (2), Str. agalactiae (34), Str. dysgalactiae (2), Str. uberis (1),
Actinomyces pyogenes (4), Bacillus subtilis (7), Bacillus cereus (1), Pseudomonas aeruginosa (8), E. coli (4), Enterobacter aerogenes (2), Proteus vulgaris (2) and Proteus mirabilis (1).

Shlke et al. (1998) screened quarter milk samples from 106 buffaloes for SCM and the organisms encountered predominantly were coagulase positive Staphylococci (58.38 %), CoNS (13.82 %) and Streptococcus species (12.19 %).

Umakanthan (1998) analyzed 20 mastitis milk samples randomly and isolated Staphylococcus species from 11 samples (55 %), non-hemolytic Streptococci and Gram positive rods each from three samples (15 %) and Coliforms, Bacillus species and E. coli each from one sample (5 %).

Mandial et al. (1999) examined milk samples collected from 303 quarters of 148 animals and found that 263 (86.8 %) samples were positive, with mixed bacterial infections. Staphylococcus species was the predominant isolate (52.9 %), followed by Streptococcus species (30.4 %) and E. coli (13.2 %).

Busato et al. (2000) examined the udder health and risk factors for SCM in organic dairy farms in Switzerland. They analyzed the data separately for cows from 7 to 100 and from 101 to 305 days post partum. Prevalence of SCM at the quarter level was 21.2 % for lactation period 7-100 days and 34.5 % for 101-305 days post partum. Samples at 7-100 and 101-305 days post partum were positive for S. aureus in 16.0 and 7.4 %, for CoNS in 51.5 and 50.6 %, for Str. agalactiae in 0.0 and 0.8 %, for other Streptococci in 19.4 and 15.6 %, for E. coli in 1.0 and 0.4 %, and for Corynebacterium
bovis in 25.7 and 45.1 %, respectively. Risks of SCM increased significantly with increasing days post partum and advancing age of cow. Cows that were sampled when staying in alpine dairies had considerably higher risks of subclinical mastitis than cows staying in home barns. Significantly lower risks of subclinical mastitis were observed in farms where CMT was performed regularly as a control measure.

Datta and Rangenkar (2001) tested 173 milk samples from 45 cows tested against mastitis, of which 46 samples from 29 cows (64.4 %) were found positive on CMT and cultural examination. Results revealed the predominance of Staphylococcus species (60.9 %), followed by Streptococcus species (23.9 %), Pseudomonas species (6.5 %) and Bacillus species (2.2 %).

Mohinikumari and Janakiram Guptha (2002) subjected a total of 81 milk samples to Strip cup test, CMT, WST and BTB indicator card. Of these, only 19 (23.45 %) were positive by strip cup test, while 71 (87.65 %) were found positive by CMT, BTB indicator card test and WST. The cultural examination revealed Staphylococcus species (35.21 %), E. coli (28.39 %), followed by Streptococcus species (18.5 %), Pseudomonas species (4.9 %), Corynebacterium species (2.46 %) and Klebsiella species (2.46 %).

Workineh et al. (2002) had undertaken the study to determine the etiology and prevalence of mastitis in hand-milked cows (n=186) in two major Ethiopian dairies. The CMT and culturing for bacteria revealed that 21.5 % of the cows were clinically infected and 38.2 % had SCM. Most mastitis pathogens isolated from milk samples testing positive by the CMT were Gram-positive cocci. Staphylococci constituted 57 % of the
isolates; other mastitis pathogens isolated included Streptococci (16.5%), Coliforms (9 %) and Corynebacteria (5 %).

Sharma and Prasad (2003) isolated a total of 111 bacterial isolates from 107 quarters while 12 quarters did not yield any microbial growth. The predominant isolates in their study were *Staphylococcus* species (54.05 %) followed by *Streptococcus* species (14.41 %), *E. coli* (11.71 %), *Bacillus* species (9.01 %) and *Corynebacterium* species (7.21 %).

Kerro and Tareke (2003) studied the aetiology and prevalence of IMI in southern Ethiopia. A total of 307 lactating and non-lactating cows, of which 162 were indigenous Zebu, 85 Jersey and 60 Holstein-Friesian, were examined by clinical examination, CMT and isolation studies. 40.4 % were positive by CMT and prevalence rates of clinical mastitis was 37.1 % and subclinical mastitis was 62.9 %. *Staphylococcus* accounted for 39.2 %, *Streptococcus* for 23.6 %, Coliforms for 14.1 %, *Micrococcus* and *Bacillus* species for 8.0 % each and *Actinomyces* or *Arcanobacterium* (Corynebacterium) for 7.0 % of the isolates.

Mdegela *et al.* (2004) carried out a study to establish the prevalence of mastitis in smallholder dairy farms in Kibaha and Morogoro districts of Tanzania. CMT and microbiological assessment of milk was carried out on samples collected from 210 milking cows from 105 herds. The cow based prevalence of SCM was 72.4 % based on CMT. Of the 919-quarter milk samples cultured, 8.2 % were positive for aerobic bacteria with predominant isolates being *S. epidermidis* (2.8 %), *S. aureus* (1.7 %), *Str. agalactiae*. 
(1.2 %) and *S. intermedius* (1.1 %). There was a strong association between CMT positivity and bacteriological isolation.

Rajeev *et al.* (2009) screened a total of 300 milk samples collected from four different farms for subclinical mastitis by CMT, White Side Test (WST) and Bromothymol blue (BTB) strips. A total of 149 isolates were recovered. Of these, 110 isolates (73.82 %) were Gram positive, 33 isolates (22.14 %) were Gram negative and six were yeasts (4.02 %).

A total of 145 milk samples were collected from cases of bovine mastitis in and around Puducherry. Out of 145 milk samples, 100 samples were positive by culture and 124 microbial isolates could be obtained from these 100 milk samples of which 116 were bacteria and eight were yeast like organisms (Amsaveni, 2007).

Piepers *et al.* (2007) studied the prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. A total of 178,668 quarter milk samples were collected at 1087 cross-sectional dairy herd screenings performed in three consecutive years. Of the dairy cows, 40 % had at least one culture-positive quarter. More than 50 % of all IMI were caused by non-aureus staphylococci. *S. agalactiae* was almost eradicated in Flanders, whereas *Staph. aureus* was isolated from 18 % of the culture-positive quarters. They also studied the distribution of mastitis pathogens in quarter milk samples from selected dairy cows with an elevated SCC. From 6390 cows with a geometric mean composite SCC 250,000 cells/ml, nearly 65 % had at least one culture-positive quarter. The majority of the IMI were caused by non-aureus staphylococci (41.1 %), whereas *Staph. aureus* and aesculin-positive cocci were found in
respectively 25% and 18% of the culture-positive milk samples. It was concluded that non-aureus staphylococci were the predominant cause of IMI, warranting more research regarding the epidemiology and pathogenicity of those species.

Taponen et al. (2007) studied persistence of CoNS in IMI during lactation in a research dairy herd of University of Helsinki. Milk samples from 328 udder quarters of 82 dairy cows (30 primiparous, 52 multiparous) were collected two weeks before calving, at calving, and every four weeks thereafter until the end of lactation or until the cow left the herd. The CoNS isolated from the milk samples were genotyped using amplified fragment length polymorphism (AFLP) analysis. In addition, the SCC of the milk samples was measured during lactation. In total, 63 CoNS infections were detected during lactation in 30 and 33 quarters in the first and later lactations, respectively. Twenty-nine of these infections persisted and 34 were transient. Most of the persistent infections lasted until the end of lactation. In 57 quarters, CoNS infection was detected before calving, at calving, or both, but only half of these quarters were infected by CoNS during subsequent lactation. The geometric mean of SCC in quarters during persistent CoNS infection was 6,57,600 cells/ml, and the mean of SCC in quarters with transient CoNS infection was 6,19,100 cells/ml. The median of SCC in quarters during persistent CoNS infection was 3,55,400 cells/ml, and the median of SCC in quarters with transient CoNS infection was 1,33,500 cells/ml. According to AFLP results *S. chromogenes* and *S. simulans* were the CoNS species isolated most often.

Karimuribo et al. (2008) conducted a cross-sectional study on 200 randomly selected farms in each of the Iringa and Tanga regions of Tanzania to estimate the
prevalence of SCM in dairy cows kept by smallholders. SCM was assessed using the CMT, and by the bacteriological culture of 1500 milk samples collected from 434 clinically normal cows. The percentages of the cows (and quarters) with SCM were 75.9 % (46.2 %) when assessed by the CMT and 43.8 % (24.3 %) when assessed by culture.

Miranda-Morales et al. (2008) studied the prevalence of pathogens associated with bovine mastitis in bulk tank milk in Mexico. Microorganism’s prevalence and SCCs were determined in 112 Holstein bovine herds in two bulk tank milk-screening assays. Prevalence of Mycoplasma was 55 %; of CoNS 38.3 %; of Str. uberis 37.5 %, and of S. aureus, 30 %. The geometric mean of the SCC was 4, 65,000 cells/ml. No significant differences were observed in the SCCs between the positive and negative samples of pathogens isolated.

Mdegela et al. (2009) conducted a cross sectional study, to determine the prevalence of clinical and subclinical mastitis and to assess the milk quality on smallholder dairy farms in Tanzania. Overall prevalence of clinical mastitis at herd level was 21.7 % (n=69) where as the prevalence of SCM at animal level was 51.6 % (n=91). Prevalence of bacterial isolates at animal level was 35.2 % (n=91). The milk quality parameters for most of the milk samples were within acceptable levels. Findings in this study have demonstrated high prevalence of SCM that may contribute to low productivity of dairy cattle.

Thorberg et al. (2009) investigated the epidemiology of different CoNS species in dairy herds with problems caused by subclinical CoNS mastitis. In 11 herds, udder quarter samples were taken twice one month apart, and CoNS isolates were identified to
the species level by biochemical methods. The ability of different CNS species to induce a persistent infection, and their associations with milk production, cow milk SCC, lactation number, and month of lactation in cows with SCM were studied. Persistent IMI were common in quarters infected with *S. chromogenes*, *S. epidermidis* and *S. simulans*. The results did not indicate differences between these CoNS species in their association with daily milk production, cow milk SCC, and month of lactation in cows with SCM. In cows with SCM, *S. epidermidis* IMI were mainly found in multiparous cows, whereas *S. chromogenes* IMI were mainly found in primiparous cows.

Bal *et al.* (2010) studied the prevalence of Staphylococci and Streptococci from SCM cases and identified 100 CoNS and 34 Streptococci. The most frequently isolated species were *S. haemolyticus* (27 %) and *S. simulans* (24 %).

Botrel *et al.* (2010) studied the distribution of clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France. Out of 1631 bacterial isolates, *S. uberis* (22.1 %), *E. coli* (16 %), and CoNS (15.8 %) were identified as the major causative agents of CM, whereas CoPS (30.2 %), CoNS (13.7 %), and *S. dysgalactiae* (9.3 %) were predominantly implicated in SCM.

Shome *et al.*, (2011) from India detected *S. chromogenes* (n=44), *S. epidermidis* (n=37), *S. haemolyticus* (n=12) and *S. sciuri* (n=7) as the predominant CoNS in 110 bovine milk samples by mPCR. *S. simulans* was detected in one sample only that was culture negative.
2.3 Biochemical characterization and Speciation of CoNS isolates

In a German study conducted by Hummel and Lehmann (1994), *S. simulans*, *S. chromogenes*, and *S. epidermidis* were the species of CoNS most frequently involved in SCM as characterized by biochemical features. In cases of clinical mastitis of cows *S. xylosus*, *S. simulans*, *S. haemolyticus* and *S. chromogenes* predominated.

Rajeev *et al.* (2009) isolated and characterized 110 isolates obtained form a total of 300 milk samples using Hi-media Identification Kit. Speciation revealed, *S. equorum*, *S. xylosus*, *S. sciuri* subsp. *rodentium*, *S. intermedius* and *S. saprophyticus* were the predominant CoNS recovered in subclinical cases of mastitis, whereas, in clinical cases, *S. arlettae*, *S. capitis* subsp. *ureolyticus*, *S. haemolyticus*, *S. felis* and *S. equorum*, were identified.

Cheng *et al* (2010) investigated into the prevalence of the pathogens in 100 milk samples with clinical or sub-clinical bovine mastitis in 5 farms in Jiangsu Province of China based on morphologic examination and biotyping. *Staphylococcus epidermidis* (15%) and *Staphylococcus saprophyticus* (10%), previously considered as naught pathogenic bacteria, were also detected although *E. coli* was the commonest organism in mastitis cases, being implicated in 82% cases, followed by *Streptococcus uberis* (53%), *Staphylococcus aureus* (41%), *Streptococcus dysgalactiae* (29%) and *Streptococcus agalactiae* (27%). *S. epidermidis* and *S. saprophyticus* were similar in statistics between clinical and sub clinical bovine mastitis.
2.4 Molecular characterization of CoNS isolates by PCR

2.4.1 Extraction of genomic DNA

Christensen et al. (1993) introduced a simple technique for isolation of genomic DNA from bacterial cultures. In this method, bacterial cells were lysed with SDS at 56 °C for 30 min. The cell proteins were precipitated by addition of 7.5M ammonium acetate. The DNA was extracted by Phenol:Chloroform treatment and precipitated with isopropanol and the template DNA was used for amplification reaction.

Pheuktes et al. (2001) used the standard Phenol:Chloroform extraction method for preparation of DNA from spiked milk samples containing suspensions of standard reference strains *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis* from bovine mastitis for developing multiplex PCR. They also used a spin column (Axygen, USA) for extraction of DNA from milk samples and concluded that this method was easy to perform and required minimum sample manipulation.

Cremonesi et al. (2006) reported an improved method for rapid DNA extraction from Gram-positive pathogens associated with mastitis. In this approach, the DNA extraction method was based on lysing and nuclease inactivating properties of guanidine isothiocyanate and nucleic acid binding properties of silica particles. This technique was applicable to milk sample analysis without sample enrichment.

2.4.2 Polymerase chain reaction and sequencing (PCR)

Since the initial development of PCR in 1985, the basic principle of *in vitro* nucleic acid amplification has extensive applications in all aspects of fundamental and
applied clinical science. Modifications of sample preparations have allowed PCR analysis to be performed on clinical specimens, considerably reducing the time required for bacterial identification (Rapley et al., 1992).

Riffon et al. (2001) standardized a rapid and sensitive PCR assay for identification of major pathogens in bovine mastitis like *E. coli*, *S. aureus*, *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis*. The results indicated that PCR could avoid cumbersome cultivation steps, which could be performed within hours, and was sensitive and specific.

### 2.4.2.1 Confirmation of staphylococcal species to genus level by molecular methods

**‘Tu’ elongation factor (‘tuf’) gene**

Martineau et al. (2001) developed a PCR assay, targeting the ‘tuf’ gene for the detection of Staphylococci at the genus level and found to be specific. The analysis of the multiple sequence alignment demonstrated sufficient interspecies polymorphism to generate genus- and species-specific capture probes. The information allowed the development of *Staphylococcus*-specific and species-specific capture probes using ‘tuf’ gene.

Sakai et al. (2004) standardized a real-time PCR assay that uses two fluorescence resonance energy transfer probe sets and targets the ‘tuf’ gene of staphylococci, one probe set detect the *Staphylococcus* genus, whereas the other probe set was specific for *S. aureus*. In all, one hundred thirty-eight cultured isolates, which contained 41 isolates of staphylococci representing at least nine species, and 100 positive blood cultures that
contained gram-positive cocci in clusters were tested. The assay was 100% sensitive and 100% specific for the detection of the *Staphylococcus* genus and of *S. aureus*.

Chen and Hwang (2008) developed a PCR-denaturing gradient gel electrophoresis method targeting the ‘*tuf*’ gene to differentiate and identify *Staphylococcus* species. In the study, eleven tested species were differentiated into ten separated patterns. Variance of the patterns between strains within a species was analyzed using nine strains of *Staph. aureus* and several strains of other species. It was shown that strains within a species migrated the same distance in the denatured gradient gel electrophoresis (DGGE) assay. When mixed cultures of different *Staphylococcus* species in milk were subjected to DNA extraction and PCR-DGGE, the resulted patterns corresponded to the species of the mixed cultures, including those of potential to secret enterotoxins.

2.4.2.2 Detection of CoNS species by molecular methods

Goh *et al.* (1997) demonstrated that a 600-bp region of the chaperonin 60 (Cpn60) genes from various bacterial isolates could be amplified by PCR with a pair of degenerate primers and that the products could be used as species-specific probes in direct dot blot hybridization for *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. saprophyticus* and *S. schleiferi*.

Forsman *et al.* (1997) determined the sequences of the 16S-23S rRNA spacer regions for the nine species which cause mastitis: *S. aureus*, *S. chromogenes*, *S. epidermidis*, *S. hyicus*, *S. simulans*, *S. xylosus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. Significant variation was found between the spacer sequences of different species with the lengths of the spacers varying from 240 to
461 bp. The spacers shared only short conserved regions (8-9 bp) between genera and within genera the sequence identities varied from 53 to 85%. This variation made it possible to construct specific primer pairs for these species and genera. The specificities of these primers were tested with 25 bacterial species and 51 isolates from cattle with clinical mastitis.

Gribaldo et al. (1997) designed PCR identification assays for 8 major species of CoNS on the basis of three variable regions found in the 16s rRNA gene. The PCR assays were tested with 41 staphylococcal strains representing the diversity of staphylococci defined by classical biotyping schemes. Twenty-six of the 41 strains were identified by PCR as belonging to one of the eight species for which primers had been designed and none of the remaining strains was misidentified. They concluded that the PCR assays are suitable for rapid and definitive speciation of CoNS.

Anita et al. (1999) investigated the phylogenetic relationships among 36 validly described species or subspecies within the genus Staphylococcus were by cloning and sequencing their 60 kDa heat-shock protein (hsp60) genes using a set of universal degenerate hsp60 PCR primers. The cloned partial hsp60 DNA sequences from nine S. aureus strains were highly conserved (97–100% DNA sequence similarity; mean 98%), indicating that the hsp60 gene of multiple isolates within the same species have little microheterogeneity. At the subspecies level, DNA sequence similarity among members of S. aureus, S. schleiferi, S. cohnii and S. capitis ranged from 91 to 98%. At the interspecies level, sequence similarity among 23 distinct species of staphylococci ranged from 74 to 93% (mean 82%). Importantly, phylogenetic analysis based on the neighbour-
gene to distinguish between *S. aureus* and CoNS. The expected fragments were amplified from each of 60 staphylococcal isolates. The utility of the protocol for the analysis of clinical samples was verified. Of 77 blood cultures tested, only 7 yielded results inconsistent with those of conventional methods of diagnosis and susceptibility testing. Of those, one was identified as a CoNS species by PCR and *S. aureus* by conventional methods. The other four samples failed to yield any amplification product even with a control set of primers corresponding to a conserved region of the eubacterial 16S rRNA genes.

Devriese *et al*. (2002) carried out identifications on 73 strains collected from quarter-milk samples in Belgian dairy herds during routine diagnostic testing/surveys using *tRNA intergenic spacer* PCR analysis and identified 70 strains as *S. chromogenes* and three as *S. sciuri* using *tRNA*-PCR.

Drancourt and Raoult (2002) proposed partial sequencing of the *rpoB* gene as a new tool for the accurate identification of *Staphylococcus* isolates. The complete sequence of *rpoB*, the gene encoding the beta-subunit of RNA polymerase was determined for *S. saccharolyticus*, *S. lugdunensis*, *S. caprae* and *S. intermedius*, and partial sequences were obtained for an additional 27 *Staphylococcus* species. The complete *rpoB* sequences varied in length from 3,452 to 3,845 bp and had a 36.8 to 39.2% GC content. The partial sequences had 71.6 to 93.6% interspecies homology and exhibited a 0.08 to 0.8% intraspecific divergence. With a few exceptions, the phylogenetic relationships inferred from the partial *rpoB* sequences were in agreement with those previously derived from DNA-DNA hybridization studies and analyses of 16S
ribosomal DNA gene sequences and partial \textit{hsp60} gene sequences. The staphylococcal \textit{rpoB} sequence database that were established enabled to develop a molecular method for identifying \textit{Staphylococcus} isolates by PCR followed by direct sequencing of the 751-bp amplicon. In blind tests, this method correctly identified 10 \textit{Staphylococcus} isolates and no positive results were obtained with 10 non-\textit{Staphylococcus} gram-positive and gram-negative bacterial isolates.

Heikens \textit{et al.} (2005) showed that although genotypic methods are clearly superior to phenotypic identifications, \textit{16S rRNA} gene sequencing suffered from the lack of high quality among sequences deposited in GenBank and genotypic identification based on \textit{16S rRNA} sequences had limited discriminating power for closely related \textit{Staphylococcus} species.

Taponen \textit{et al.} (2007) studied persistence of CoNS in IMI during lactation in a research dairy herd of University of Helsinki. Milk samples from 328 udder quarters of 82 dairy cows (30 primiparous, 52 multiparous) were collected two weeks before calving, at calving, and every four weeks thereafter until the end of lactation or until the cow left the herd. The CoNS isolated from the milk samples were genotyped using amplified fragment length polymorphism (AFLP) analysis. The AFLP patterns were used for similarity analysis between CoNS isolates and for species identification. According to AFLP results, \textit{S. chromogenes} and \textit{S. simulans} were the CoNS species isolated most often.

Ghebremedhin \textit{et al.} (2008) opined that the closely related species of \textit{Staphylococcus} cannot always be distinguished from each other based on the analysis of
16S rRNA gene sequences and thus, new phylogenetic markers for *Staphylococcus* spp. are needed. They partially sequenced the *gap* gene (~931 bp), which encodes the glyceraldehyde-3-phosphate dehydrogenase, for 27 *Staphylococcus* species. The partial sequences had 24.3 to 96% interspecies homology and were useful in the identification of staphylococcal species. The DNA sequence similarities of the partial staphylococcal *gap* sequences were found to be lower than those of 16S rRNA (~97%), rpoB (~86%), hsp60 (~82%), and sodA (~78%). Phylogenetically derived trees revealed four statistically supported groups: *S. hyicus/ S. intermedius, S. sciuri, S. haemolyticus/ S. simulans* and *S. aureus/ epidermidis*. Thus, the phylogenetic analysis based on the *gap* gene sequences revealed similarities between the dendrograms based on other gene sequences as well as differences. Based on their results, they proposed the partial sequencing of the *gap* gene as an alternative molecular tool for the taxonomical analysis of *Staphylococcus* species and for decreasing the possibility of misidentification.

Shome *et al.* (2011) developed and evaluated a two-tube multiplex PCR (mPCR) assay for simultaneous detection of 10 bacterial species causing bovine mastitis namely, *S. aureus, S. chromogenes, S. epidermidis, S. sciuri, S. haemolyticus, S. simulans, S. agalactiae, S. dysgalactiae, S. uberis* and *E. coli* in milk. The genes targeted were *gap/rdr/sodA* for CoNS species and 23S rRNA for *S. aureus*. The accuracy of the mPCR was evaluated using 56 standard reference strains and 705 strains comprising of *E. coli* (n = 99), staphylococci (n = 522) and streptococci (n = 84). The threshold of detection of the mPCR assay was 10 fg of genomic DNA and <10^3 CFU ml^{-1}). A comparative evaluation of mPCR with culture method using 115 milk samples from subclinical mastitis showed
mPCR to be more efficacious. Subsequently, the mPCR showed successful detection of target bacteria, when applied directly for the assessment of 36 bulk milk samples.

### 2.4.2.3 Coagulase negative variants of S. aureus in bovine milk

A study conducted by Heltberg and Bruun (2009) to evaluate the usefulness of polymyxin susceptibility testing revealed coagulase-negative S. aureus strains during a six months study period. Fourteen staphylococcal isolates from four patients were initially found to lack coagulase activity and to be polymyxin resistant; in comparison, approximately 1500 ordinary, coagulase-positive, polymyxin resistant S. aureus isolates were found during the same period. One isolate from each of the four patients together with a previously isolated coagulase-negative S. aureus strain from our own collection were further characterized. Two of the strains turned out to show delayed coagulase activity on retesting, while the other three strains failed to produce clotting in any coagulase assay. Judged by thermostable nuclease activity, phage typing and biochemical profiles these three coagulase-negative strains are bonafide S. aureus strains. The polymyxin test thus appears to be useful in disclosing S. aureus variants not identified as S. aureus by routinely performed coagulase assays.

Bacteriological analysis of milk samples by Akineden et al. (2011) 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. Both isolates were also clumping factor and thermonuclease negative, and gave a negative reaction in the Staphaurex® test. The isolates were identified by using commercial biochemical systems, and by PCR analysis of different staphylococcal cell surface protein and exoprotein genes. Further molecular
identification of the isolates, which included sequencing of the \textit{16S rRNA} gene and RT-PCR of coagulase (\textit{coa}), clumping-factor (\textit{clfA}) and thermonuclease (\textit{nuc}) genes, was consistent with the diagnosis phenotypically 'coagulase-negative variant of \textit{S. aureus}'. They opined that the coagulase-negative \textit{S. aureus} variants can occur in the context of IMI in cattle and may result in incorrect diagnosis of CoNS in routine mastitis diagnosis, at least in rare cases. To fully ensure correct species diagnosis, sequencing of the \textit{16S rRNA} gene and amplification of specific genes such as \textit{coa} was necessary in these cases.

\textbf{2.5 Comparison of phenotyping and genotyping methods for species level identification of CoNS}

Gribaldo \textit{et al.} (1997) designed PCR identification assays for 8 major species of CoNS on the basis of three variable regions found in the \textit{16s rRNA} gene. The PCR assays were tested with 41 staphylococcal strains representing the diversity of staphylococci defined by classical biotyping schemes. Each PCR result was compared with species-specific polymorphism in and around the 16s rRNA gene (i.e., 16s ribotype) and the phenotypic identification of the strain in a miniaturised biochemical test gallery (bioMérieux ATB 32 Staph). Twenty-six of the 41 strains were identified by PCR as belonging to one of the 8 species for which primers had been designed and none of the remaining strains was misidentified. For 22 of the 26 strains, there was complete agreement between the PCR identification, 16s ribotype and ATB identification. For the remaining four strains there was agreement between PCR identification and 16S ribotype. Two NCTC strains were re-assigned to different species and 10 previously unassigned strains were formally speciated for the first time. They concluded that PCR assays are suitable for rapid and definitive speciation of CoNS.
Devriese et al. (2002) concluded that *S. chromogenes* can be identified reliably in routine mastitis bacteriology by comparing simple identification tests with a genomic method. Seventy-three staphylococcal strains, all collected on different dairy farms, were tentatively identified as *S. chromogenes* based on their lack of hemolysis and their characteristic intermediate DNase activity. The identification of 70 strains was confirmed as *S. chromogenes* by *tRNA* intergenic spacer PCR (*tRNA* PCR). Three strains were identified as *S. sciuri*, a species that is naturally cloxacillin- and lincomycin-resistant.

Heikens et al. (2005) compared commonly used phenotypic methods with genotypic identification methods and they identified 47 clinical isolates of CoNS, 10 CoNS ATCC strains, and a *S. aureus* clinical isolate using the API Staph ID test, BD Phoenix Automated Microbiology System, and sequencing of ‘16S rRNA’ gene, ‘*tuf*’ gene and, when necessary part of the *sodA* gene for definitive identification. The results showed that ‘*tuf*’ gene sequencing was the best method for identification of CoNS, but the API Staph ID test is a reasonably reliable phenotypic alternative. The performance of the BD Phoenix Automated Microbiology System for identification of CoNS was poor.

Shittu et al. (2006) observed in their study that the API STAPH system classified the 15 CoNS isolates into 6 species, whereas the *16S–23S rRNA* intergenic spacer length polymorphism analysis (ITS-PCR) identified only 2 species. The overall agreement of the API STAPH system and ITS-PCR was 60% (9 out of 15 isolates). However, four isolates identified as *S. haemolyticus* by ITS-PCR were classified by the API STAPH system as *S. epidermidis* (2), *S. hominis* (1) and *S. warneri* (1). Similarly, two *S. saprophyticus*
isolates (ITS-PCR identification) were grouped as *S. cohnii* and *S. hominis* by the API STAPH system.

Taponen *et al.* (2006) analyzed CoNS isolated from IMI during lactation in a research dairy herd of University of Helsinki with the API Staph ID 32 (bioMe´rieux, Marcy l’Etoile, France) test (API) and amplified fragment length polymorphism (AFLP) analysis. The AFLP patterns were used for similarity analysis between CoNS isolates and for species identification. In total, 63 CoNS infections were detected during lactation in 30 and 33 quarters in the first and later lactations, respectively. According to the results of API test and AFLP, *S. chromogenes* and *S. simulans* were the CoNS species isolated most often. Identification results for API and AFLP corresponded in 71.9% of the isolates.

Ruegg (2009) evaluated the utility of phenotypic identification systems used for species identification of CoNS relative to use of genotypic identification. Typical results were observed in the evaluation of agreement between 2 systems used for identification of staphylococci (*n* = 54) isolated from cases of mastitis. Satisfactory agreement (Kappa > 0.87) was achieved for API Staph but low agreement at the species level was seen for the BBL Crystal Gram-Positive system (Kappa = 0.25). They opined that in spite of the limited precision of some phenotypic identification systems, their consistent use with an adequate number of isolates in the diagnostic algorithm is probably sufficient for most mastitis control programs, which are currently not based on species level identification. Results of this small study are typical of similar studies and confirm that differences occur among phenotypic identification systems. However, they concluded that genotypic
identification will be useful for advancing knowledge of the role of CoNS in bovine mastitis.

2.6 Antimicrobial resistance of CoNS

Rahman and Baxi (1982) tested 90 isolates of *S. aureus*, 63 of *S. epidermidis*, 37 of *S. agalactiae*, 10 of *S. dysgalactiae*, 14 strains of other Streptococci and 38 strains of other bacteria from bovine SCM against eight antibiotics and found that Staphylococcal strains were highly sensitive to neomycin, chloramphenicol and nitrofurantoin, while the Streptococci were uniformly sensitive to all the drugs, 20 strains of Corynebacteria were sensitive to neomycin and erythromycin, while 13 representatives of *Enterobacteriaceae* were most sensitive to neomycin, followed by chloramphenicol and streptomycin.

Dickgiesser and Kreiswirth (1986) described a method for identification of the genes conferring aminoglycoside resistance in *S. aureus* by dot-blot and Southern blot techniques. As radioactive probes, fragments of plasmids pAT48, pUBH2, and pH13, carrying the genes for an aminocyclitol-3'-phosphotransferase, an aminocyclitol-4'-adenyllyltransferase, and an aminocyclitol-2"'-phosphotransferase-aminocyclitol-6'-acetyltransferase, respectively, were used.

Schmitz *et al.* (1999) studied the prevalence of aminoglycoside resistance in Europe by testing 699 and 249 consecutive unrelated clinical isolates of *S. aureus* and CoNS, respectively, isolated obtained from 19 hospitals in 12 different European countries as part of antimicrobial surveillance program, for susceptibility to gentamicin, tobramycin, kanamycin and streptomycin, and examined the relationship between susceptibility to these antimicrobials and susceptibility to methicillin. Three hundred and
sixtythree staphylococcal isolates demonstrated resistance to at least one of the aminoglycosides tested; all of these isolates were screened for the presence of aac(6')-Ie aph(2'), ant(4')-Ia and aph(3')-IIIa, the genes encoding the most clinically relevant aminoglycoside-modifying enzymes. In S. aureus, resistance to aminoglycosides was closely associated with methicillin resistance. Susceptibility of S. aureus to gentamicin has decreased by 9% from previous European studies to a current level of 77%, while susceptibility of CoNS, currently at 67%, has increased by 21%. Geographical variation occurred, correlating with methicillin resistance, although intra-country variation was considerable. aac(6')-Ie aph(2'), ant(4')-Ia and aph(3')-IIIa were found throughout Europe in 68%, 48% and 14% respectively of staphylococci resistant to at least one aminoglycoside. and aph(3')-IIIa was considerably more common in methicillin-susceptible S. aureus and CoNS isolates; the reverse was true for the other two resistance genes.

By using amultiplex PCR strategy, 310- and 686-bp regions of the mecA and femA genes, respectively, were coamplified by Vannuffel et al. (1995) to identify susceptible (lacking mecA) and resistant (mecA+) staphylococci and to differentiate S. aureus (femA+) from CoNS (lacking femA). One hundred sixty-five staphylococcal strains were tested. All 72 methicillin-resistant strains were found to be mecA+, and 92 of the 93 susceptible isolates lacked mecA. Only one CoNS isolate carrying the mecA gene was highly susceptible to oxacillin (MIC<0.016 mg/ml). According to the National Committee for Clinical Laboratory Standards, 15 intermediately resistant S. aureus strains did not express the mecA determinant; conversely, none of the mecA-negative strains expressed methicillin resistance. The femA determinant, a unique feature of S.
joining distance method revealed remarkable concordance between the tree derived from partial hsp60 gene sequences and that based on genomic DNA–DNA hybridization, while 16S rRNA gene sequences correlated less well. The results demonstrated that DNA sequences from the highly conserved and ubiquitous hsp60 gene offer a convenient and accurate tool for species-specific identification and phylogenetic analysis of staphylococci.

Poyart et al. (2001) developed simple PCR and sequencing assays that utilize a single pair of degenerate primers were used to characterize a 429-bp-long DNA fragment internal (sodAint) to the sodA gene encoding the manganese-dependent superoxide dismutase in 40 CoNS type strains. 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 gene sequences. Sequence analysis revealed that the staphylococcal sodA genes exhibit a higher divergence than does the corresponding 16S ribosomal DNA. These results confirm that the sodA gene constitutes a highly discriminative target sequence for differentiating closely related bacterial species. Clinical isolates that could not be identified at the species level by phenotypical tests were identified by use of this database. These results demonstrated the usefulness of this method for rapid and accurate species identification of CoNS isolates, although it does not allow discrimination of subspecies.

Mason et al. (2001) reported the development of a multiplex PCR protocol for the diagnosis of staphylococcal infection. Staphylococcus-specific regions of the 16S rRNA gene was targeted to detect any staphylococcal species, similarly S. aureus specific clfA
aureus was found in 100% of the S. aureus strains tested but was undetectable in all of the CoNS tested. Coamplification of mecA and femA determinants proved to be very reliable both for rapid detection of methicillin resistance and differential diagnosis between S. aureus and other staphylococci.

Mallikarjunaswamy and Murthy (1997) studied the in vitro antibiotic sensitivity of 214 bacterial isolates isolated from cases of bovine SCM from 162 dairy cows from 6 dairy farms in and around Bangalore. The major bacteria identified were S. epidermidis (80), S. aureus (66), S. intermedius (2), Str. agalactiae (34) and others. They found that most isolates were sensitive to chloramphenicol, but resistant to streptomycin, neomycin and the penicillins. The S. aureus isolates were highly sensitive to ciprofloxacin (100 %), ceftizoxime (100 %), gentamicin (98.4 %), cloxacin (98.48 %), ciprofloxacin (93.93 %), tetracycline (93.9 %) and co-trimoxazole (92.42 %) and moderately sensitive to neomycin (86.36 %). Most of the isolates showed resistance to penicillin (68.18 %) and streptomycin (57.57 %).

In vitro drug sensitivity, tested on 46 isolates from 29 CMT positive cows in which Staphylococcus species were predominant (60.9 %), showed that all the isolates were highly sensitive to enrofloxacin. Majority of the isolates were moderately sensitive to gentamicin and oxytetracycline, whereas resistant to ampicillin and streptomycin (Datta and Rangenkar, 2001).

Mason et al. (2001) reported the development of a multiplex PCR protocol for the diagnosis of staphylococcal infection. Staphylococcus-specific regions of the 16S rRNA gene was targeted to detect any staphylococcal species, similarly S. aureus specific clfA
gene to distinguish between *S. aureus* and CoNS and *mecA* gene to detect resistance to oxacillin. The expected fragments were amplified from each of 60 staphylococcal isolates (13 oxacillin-resistant *S. aureus* isolates, 23 oxacillin-sensitive *S. aureus* isolates, 17 oxacillin-resistant CoNS, and 7 oxacillin-sensitive CoNS). The utility of the protocol for the analysis of clinical samples was verified. Of 77 blood cultures tested, only 7 yielded results inconsistent with those of conventional methods of diagnosis and susceptibility testing. They identified two isolates that were *mecA* positive but were oxacillin sensitive according to conventional methods.

Devriese *et al.* (2002) determined minimal inhibitory concentrations (MICs) of different antibiotics for *Staphylococcus chromogenes*, a highly prevalent species in SCM and searched for the presence of important resistance mechanisms and resistance determining genes. Concentrations of 0.06–128 mg/l were tested for penicillin G, cloxacillin, oxytetracycline and erythromycin. Despite the low penicillin MICs (<0.06–0.5 mg/l) recorded, 27 of the 70 *S. chromogenes* strains (38%) were found to produce penicillinase. This characteristic was more often found (P=0.003, Fischer’s exact test) in non-pigmented strains (10/13) than in pigmented strains (17/57). None of the strains tested proved to be *mecA*-positive, and all strains proved susceptible to penicillinase-stable penicillins and cephalosporins, represented in this study by cloxacillin (MIC=0.06–0.25 mg/l). Two strains were resistant to tetracycline (MIC ≥128 mg/l). Four strains showed resistance to lincomycin (MIC=8–16 mg/l), while remaining susceptible to the related antibiotic erythromycin. Three lincomycin-resistant *S. chromogenes* strains were found to carry the *linA* gene. No resistance was found against gentamicin, neomycin, erythromycin and enrofloxacin. The three *S. sciuri* strains all showed low-level resistance
to cloxacillin (MIC=8–16 mg/l), lincomycin (8 mg/l) and they were tetracycline-resistant (MIC= 32 mg/l). It was concluded that the only resistance of importance is against penicillinase-susceptible penicillins.

Gentilini et al. (2002) investigated a total of 123 isolates of CoNS isolated from bovine clinical and SCM in Argentina from March 1998 to March 2000 was for in vitro susceptibility to several antimicrobial agents. MIC that inhibit 90% of the isolates tested (reported in mg per ml) were: 4.40, 0.38, 4.00, 0.75, 0.75, 3.60, and 2.00 for penicillin, oxacillin, cephalothin, gentamicin, erythromycin, clindamycin, and ampicillin sulbactam, respectively. Resistance was detected in 34 (27.6%), 4 (3.2%), 7 (5.7%), and 6 (4.8%) isolates for penicillin, oxacillin, erythromycin, and pirlimycin, respectively. No resistance was detected for gentamicin, cephalothin, or ampicillin-sulbactam. Results indicated that CoNS isolates in Argentina exhibited the highest degree of resistance to penicillin of all antimicrobial agents tested.

Mohinikumari and Janakiram Guptha (2002) subjected a total of 81 isolates to antibiotic sensitivity test in which Staphylococcus species were predominant (35.21 %). All the isolates were sensitive to gentamicin, followed by chloramphenical, kanamycin, nalidixic acid and erythromycin.

Ghoshal et al. (2004) observed that detection of oxacillin resistance in CoNS by phenotypic methods is often difficult. The present study compared the National Committee for Clinical Laboratory Standards (NCCLS) revised guidelines of phenotypic methods with a meca-based polymerase chain reaction (PCR) for C-NS. Ninety clinical CoNS isolates were tested for oxacillin resistance by disk diffusion (1-µg disk), minimum
inhibitory concentration (MIC) breakpoint (0.5 µg/ml) after 24 h, and mecA-based PCR. The sensitivity and specificity of disk diffusion was 80% and 93%, and the sensitivity and specificity of the MIC breakpoint after 24 h was 84% and 91%, respectively, against PCR as gold standard. Eleven strains (7 mecA-positive and 4 mecA-negative) showed discordant results between MIC breakpoint after 24 h and PCR. Six of the 7 mecA-positive and all 4 mecA-negative discordant strains had inducible oxacillin resistance and β-lactamase hyperproduction, respectively. The study concluded that inducible oxacillin resistance and β-lactamase hyperproduction are the major causes of discordant results between phenotypic methods and mecA-based PCR, and need special attention.

Luthje and Schwarz (2006) analyzed CoNS for their resistance to antimicrobial agents approved for the control of pathogens involved in bovine mastitis, with particular reference to macrolide and/or lincosamide (ML) resistance and the resistance genes involved. A total of 298 CoNS collected between 2003 and 2005 in Germany from cases of subclinical mastitis in dairy cows were identified to the species level and investigated for their MICs by broth microdilution. The ML resistance genes were detected using PCR and hybridization. Selected PCR products were cloned and sequenced. The CoNS isolates showed a low level of resistance to all antimicrobial agents tested (0–7.4%) except ampicillin (18.1%). In the erythromycin-resistant and/or pirlimycin-resistant isolates, The ML resistance genes erm(B), erm(C), msr(A), mph(C) and lnu(A) were present, either alone or in different combinations. Isolates carrying erm methylase genes or the exporter gene msr(A) showed higher MICs than those harbouring only the genes mph(C) or lnu(A) coding for inactivating enzymes. Most of the ML resistance genes were found on plasmids. The finding that five different resistance genes—present in various
combinations were responsible for ML resistance underlines the heterogeneous character of this resistance trait.

Turutoglu et al. (2006) carried out the antibiotic susceptibility test on 103 S. aureus and 136 CoNS strains. Only 35 of the isolates were susceptible to all antibiotics tested, while the remaining 204 isolates were resistant at least to one of the antibiotics. Among staphylococci, 18 isolates of S. aureus and 31 isolates of CoNS were found phenotypically resistant to methicillin and these isolates were also resistant to penicillin G, ampicillin, amoxycillin and cloxacillin. Out of 68 S. aureus strains, 55.9% were β-lactamase producers. β-Lactamase producer isolates were 21.1% resistant to methicillin, but were 100% susceptible to amoxycillin/clavulanic acid and 97.4% susceptible to ampicillin/sulbactam. The differences observed in the efficacy of the antibiotics tested against staphylococci show that the antibiotic susceptibility tests should be performed together with the identification of the bacterial agents.

Moon et al. (2007) determined the relationship between phenotypic antibiogram and the polymorphism of coagulase gene based on specific coagulase genotyping by PCR-RFLP used for discriminating among S. aureus strains. The staphylococci strains (835 S. aureus and 763 CoNS) were isolated from 3,047 bovine mastitic milk samples from 153 dairy farms in 8 provinces from 1997 to 2004 in the Republic of Korea. Twenty-one (2.5%) S. aureus and 19 (2.4%) CoNS strains were resistant to methicillin (oxacillin MIC ≥4μg/ml). The mecA gene was also found in 13 methicillin-resistant S. aureus (MRSA) and 12 methicillin-resistant CoNS (MRCoNS) isolates with a significantly higher detection rate of the mecA gene in MRSA with high MIC (≥16
µg/ml) compared with those with MIC ≤ 8µg/ml. MRSA and MRCoNS were also more resistant to other antibiotics (ampicillin, cephalothin, kanamycin, and gentamicin) than methicillin-susceptible staphylococci. Among 10 different coa PCR-RFLP patterns (A to J) in 706 S. aureus strains, the main types were A (26.9%), B (17.0%), G (10.5%), and H (15.4%), with the frequent observation of the A and H types (6 and 10 isolates) in MRSA.

Chandrakanth et al. (2008) evaluated aminoglycoside resistance in six clinically isolated and epidemiologically unrelated MDR strains randomly chosen from 40 clinical isolates of S. aureus from diabetic patients. Genotypical examination revealed that three isolates (HLGR-10, HLGR-12, and MSSA-21) have aminoglycoside-modifying enzyme (AME) coding genes and another three (GRSA-2, GRSA-4, and GRSA-6) lacked these genes in their genome. Whereas, isolates HLGR-10 and HLGR-14 possessed bifunctional AME coding gene aac(6′)-aph(2″), and aph(3′)-III and showed high-level resistance to gentamycin and streptomycin, MSSA-21 possessed aph(3′)-III and exhibited low resistance to gentamycin, streptomycin, and kanamycin. The remaining three isolates (GRSA-2, GRSA-4, and GRSA-6) exhibited low resistance to all the aminoglycosides because they lack AME coding genes in their genome. The transmission electron microscopy of the three isolates revealed changes in cell size, shape, and septa formation, supporting the view that the phenomenon of adaptive resistance is operative in these isolates.

Ciftci et al. (2009) optimized a triplex PCR was targetting 16S rRNA, nuc and mecA genes for detection of Staphylococcus species, S. aureus and methicillin resistance,
erythromycin (≥64 mg/ml) and pirlimycin (≥64 mg/ml). A total of 17 S. epidermidis, 11 S. chromogenes and one S. hyicus exhibited phenotypic resistance to ampicillin (≥0.5 mg/ml). CoNstitutive β-lactamase production was observed in all ampicillin resistant isolates except 4 S.epidermidis that exhibited inducible β-lactamase production. Induced β-lactamase production was also observed in 13 S. epidermidis that were phenotypically susceptible to the entire MIC panel. All isolates that produced β -lactamase either constitutively or by induction carried blaZ. S. epidermidis (n = 12, 32%) that were resistant to methicillin (oxacillin ≥0.5 mg/ml) carried low affinity penicillin-binding protein encoded by mecA. Most multi-drug resistant (MDR) S. epidermidis (≥2 resistance genes) were resistant to ampicillin, erythromycin and methicillin. All except one MDR S. epidermidis had icaAB, which encodes for polysaccharide intercellular adhesion. Based on pulsed field gel electrophoresis, MDR S. epidermidis were closely related genotypically, and were isolated from different cows on the same farm suggesting clonal dissemination. Bovine S. epidermidis share antimicrobial resistance patterns and virulence determinants of strains observed in human infections.

Bal et al. (2010) studied the prevalence and antimicrobial susceptibilities of Staphylococci from SCM cases and identified 100 CoNS. The most frequently isolated species were S. haemolyticus (27 %) and S. simulans (24 %). Study revealed the highest resistance to penicillin G (58 %), ampicillin (48 %), neomycin (20 %), and oleandomycin (14 %).

Botrel et al. (2010) studied the distribution and antimicrobial resistance on 1631 bacterial isolates from clinical and SCM cases in dairy cows in Rhône-Alpes, France. The
proportion of CoNS implicated in clinical and subclinical mastitis were (15.8 %) and (13.7 %), respectively. The overall proportion of antibiotic resistance was low, except for penicillin G in staphylococci, as well as for macrolides and tetracycline in streptococci.

Ravinder Kumar et al. (2010) carried out investigation to detect the distribution of antibiotic-resistant genes of S. aureus isolates. Isolates (n=128) of S. aureus from mastitic milk were collected, tested for antibiotics with disc-diffusion method, and resistant genes meca, linA, msra msrb, vatA, vatB, vatC ermA, ermC tetK, tetM and aacA-D were detected by PCR. The phenotypic antibiotics resistance percent in S. aureus isolates was classified as tetracycline (36.7), gentamycin (30.5), streptomycin (26.6), kanamycin (25.8) and penicillin G (22.7). All the isolates were susceptible to vancomycin. Among isolates, 10.2% were observed as methicillin-resistant. The distribution of antibiotic-resistant genes was linA (51.6) followed by msrb (46.1), tetK+M (34.4), msra and aacA-D (26.6%). Different antibiotic resistant genes combinations (meca/linA-2; meca/aacAD/tetK/linA/msrb-3; meca/linA/msra/msrb-3; aacA-D/linA/msra/msrb-4; aacA-D/linA/msrb-7; linA/msra/msrb-10; tetK/linA/msra/msrb-11; aacA/tetK/linA/msrb-12 isolates) were observed. All the isolates lacked amplification of vatA, vatB, ermA and ermC genes. Molecular typing resulted genetic variation in protein A (6–12 repeats) and coagulase genes (A–E patterns) were observed. Coagulase A and D genotypes were more prevalent in antibiotic-resistant isolates, while E, B and C in susceptible ones. The significant observation was the prevalence of methicillin-resistant S. aureus, which were resistant to multiple antibiotics. Findings revealed the status of resistant isolates in herd that might be helpful in treatment, controlling of resistant strains and culling of cows for mastitis reduction.
Tsubakishita et al. (2010) opined that MRSA is one of the most important multidrug-resistant pathogens around the world. MRSA was generated when methicillin-susceptible S. aureus (MSSA) exogenously acquires a methicillin resistance gene, mecA, carried by a mobile genetic element, staphylococcal cassette chromosome mec (SCCmec), which is speculated to be transmissible across staphylococcal species. However, the origin/reservoir of the mecA gene has remained unclear. Finding the origin/reservoir of the mecA gene is important for understanding the evolution of MRSA. Moreover, it may contribute to more effective control measures for MRSA. Here we report on one of the animal-related Staphylococcus species, S. fleurettii, as the highly probable origin of the mecA gene. The mecA gene of S. fleurettii was found on the chromosome linked with the essential genes for the growth of staphylococci and was not associated with SCCmec. The mecA locus of the S. fleurettii chromosome has a sequence practically identical to that of the mecA-containing region (~12 kbp long) of SCCmec. Furthermore, by analyzing the corresponding gene loci (over 20 kbp in size) of S. sciuri and S. vitulinus, which evolved from a common ancestor with that of S. fleurettii, the speciation-related mecA gene homologues were identified, indicating that mecA of S. fleurettii descended from its ancestor and was not recently acquired. It is speculated that SCCmec came into form by adopting the S. fleurettii mecA gene and its surrounding chromosomal region. Their finding suggests that SCCmec was generated in Staphylococcus cells living in animals by acquiring the intrinsic mecA region of S. fleurettii, which is a commensal bacterium of animals.

Vanderhaeghen et al. (2010) studied the methicillin-resistant S. aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. The presence of
meca was investigated in 118 S. aureus strains originating from diagnostic mastitis milk samples from 118 different farms experiencing S. aureus mastitis. The mecA gene was detected in 11 (9.3%) of the 118 Staph. aureus isolates, indicating that nearly 10% of the Belgian farms suffering from Staph. aureus mastitis have an MRSA problem. The in-herd prevalence varied between 0% to 7.4%. Characterization of the MRSA strains showed that they were all resistant to tetracycline. Additional resistances to macrolides, lincosamides and aminoglycosides were frequently detected. The strains were ST398, spa-types t011 or t567 and had SCC mec-type IVa or V, proving that they belong to the emerging livestock-associated MRSA (LA-MRSA) strains of CC398. The study showed that after detection in Belgian pigs, horses and poultry, LA-MRSA has also attained Belgian cattle, and the multi-resistance of LA-MRSA strains may cause future treatment problems.

Basappa et al. (2011) investigated the prevalence and antimicrobial susceptibility of CoNS isolated from bovine mastitis in and around Dharwad region. A total of 310 samples were screened and 180 confirmed CoNS were obtained. The antimicrobial susceptibility of CoNS against 10 antimicrobial agents was tested using the disc diffusion method. The highest numbers of CoNS were susceptible to ceftriaxone 83.88% followed by cefotaxime 79.41%, methicillin 76.47%, ciprofloxacin 73.52%, erythromycin 70.05%, amikacin 66.11%, gentamycin 42.94%, amoxicillin 36.76%, ampicillin 29.41%, and the lowest susceptibility was shown in penicillin 23.23%. The results indicated that the increase in prevalence and antibiotic resistance pattern of the CoNS isolated from bovine mastitis exhibited the highest degree of susceptible to ceftriaxone of all the tested antimicrobial agents.
Garcia-Alvarez et al. (2011) reported the discovery of a strain of *S. aureus* (LGA251) isolated from bulk milk that was phenotypically resistant to meticillin but tested negative for the *mecA* gene and investigated the extent to which such strains are present in bovine and human populations. They searched for *mecA* PCR-negative bovine and human *S. aureus* isolates showing phenotypic meticillin resistance and whole-genome sequencing was done to establish the genetic basis for the observed antibiotic resistance. A divergent *mecA* homologue (*mecA_{LGA251}* ) was discovered in the LGA251 genome located in a novel staphylococcal cassette chromosome *mec* element, designated type-XI SCC*mec*. The *mecA_{LGA251}* was 70% identical to *S. aureus* *mecA* homologues and was initially detected in 15 *S. aureus* isolates from dairy cattle in England. These isolates were from three different multilocus sequence type lineages (CC130, CC705 and ST425); spa type t843 (associated with CC130) was identified in 60% of bovine isolates. When human *mecA*-negative MRSA isolates were tested, the *mecA_{LGA251}* homologue was identified in 12 of 16 isolates from Scotland, 15 of 26 from England, and 24 of 32 from Denmark. They opined that new diagnostic guidelines for the detection of MRSA should consider the inclusion of tests for *mecA_{LGA251}* as meticillin resistant, present confirmatory methods will not identify them as MRSA, although routine culture and antimicrobial susceptibility testing will identify *S. aureus* isolates with this novel *mecA* homologue.

Haran et al. (2011), in their study, estimated herd prevalence of *S. aureus* including MRSA from bulk tank milk (BTM) from Minnesota farms. A total of 150 pooled BTM samples from 50 farms, collected over 3 seasons (spring, summer, and fall of 2009), were assessed. Herd prevalence of meticillin susceptible *S. aureus* (MSSA)
was 92%, while MRSA herd prevalence was 4%. A total of 93 MSSA isolates and 2 MRSA isolates were recovered from 150 BTM samples. Antibiotic susceptibility testing of *S. aureus* isolates were pan susceptible (n=54), resistant to a single antibiotic class (n=21), resistant to two antibiotic classes (13) and resistant to ≥ 3 antibiotics classes and thus multidrug resistant (n=5). The two MRSA isolates displayed resistance to β-lactams, cephalosporins and lincosamides and were multiresistant. Staphylococcal protein A (spa) typing identified spa types t529 and t034 most frequently among methicillin susceptible isolates while t121 was observed in MRSAs. Seven isolates including the two MRSA isolates produced staphylococcal enterotoxins B, C, D and E on overnight culture. MRSA isolates were further genotyped using MLST and PFGE. Of the 2 MRSA, one isolate had a composite genotype profile of MLST:ST 5-PFGE:USA 100- spa 2 type (ridom unknown) which has been reported among hospital-associated MRSA isolates, while the second isolate carried – MLST:ST 8-PFGE:USA300-spa type t121 genotype commonly identified amongst community-associated MRSA isolates. These results suggest that MRSA genotypes associated with hospitals and community can be isolated from milk at very low rates.

### 2.6.1 Mechanism of Methicillin Resistance

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).
MRS show an intrinsic resistance to penicillinase-resistant beta 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. mecA gene 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. Although laboratory conditions has been changed (e.g. additional NaCl to medium, prolonged incubation, incubation at low temperatures, higher inoculum) to enhance the phenotypic expression, all strains cannot be classified. Furthermore, the conditions that are used to enhance expression of methicillin resistance also can cause the susceptibility test results for susceptible strains to shift toward or above the breakpoint for resistance (Gerberding et al., 1991).

Methicillin resistant staphylococci are considered resistant to penicillins, penems, carbapenems and cephalosporins making it difficult to treat if associated with infections. Appropriate therapy of methicillin-resistant staphylococcal infection requires knowledge of antimicrobial resistance profile (Chambers, 1997).

β-Lactams are important antimicrobial agents used for the prevention and treatment of mastitis in dairy cows. Bacterial β-lactam resistance mechanisms include production of β-lactamases and low-affinity penicillin-binding protein 2a (PBP2a). 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).

As methicillin resistance mediated by PBP 2a is often heterogeneously expressed in staphylococci, PCR detection of mecA gene is the “gold standard” for the detection of methicillin resistance (Salisbury, et al., 1997).

Gentilini et al. (2002) recommended that animals that carry methicillin-resistant CoNS isolates should be culled and opined that 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.

2.6.2 Mechanism of Resistance to aminoglycosides

Resistance to aminoglycosides has been known for some time; reports from the 1960s highlight the general resistance patterns of Staphylococcus aureus clinical isolates to all aminoglycosides. The mechanisms of aminoglycoside resistance are; (1) aminoglycoside-modifying enzyme (AME) mediated or AME-dependent resistance and (2) adaptive (AME-independent) resistance to aminoglycosides (Benveniste and Davies, 1973).

Clinical staphylococcal isolates exhibit resistance to aminoglycosides by producing three types of AME (Ubukata et al., 1984), namely aminoglycoside-3”-O-phosphoryltransferase-III (aph(3’)-III) (Rouch et al., 1987), aminoglycoside-4’-adenyltransferase-I (ant(4’)-I) and aminoglycoside-6’-N-acetyltransferase/2”-O-phosphoryltransferase (aac(6’)/aph(2’)) (Hancock, 1981), which are of particular
significance because they modify aminoglycosides like kanamycin, tobramycin, and gentamycin, respectively.

AME-independent/ adaptive aminoglycoside resistance occurs due to membrane transport defect or cell wall impermeability. Although enzymatic inactivation is the major mechanism in many clinical isolates of *S. aureus*, some strains have developed AME independent resistance to aminoglycosides (Mandel et al., 1984).

The AACs can be grouped into four classes; AAC(1’), AAC(2’), AAC(3’) and AAC(6’), based on the acetylation sites of the aminoglycosides. N-acetylation at the 6’ position catalyzed by AAC(6’) is one of the most prevalent forms of modification of aminoglycosides (Miller et al., 1997).
BRIEF METHODOLOGY

Milk Samples

Apparently Healthy Animals

Clinical Mastitis Cases

Screening for Subclinical Mastitis by SCC, EC, CMT and BTB strip tests

Isolation of *Staphylococcus*

Coagulase test

CoPS

Molecular Characterization

Confirmation at genus level by ‘tuf’ gene based uniplex PCR

Confirmation at species level by m-PCR (Genes targeted: sodA/ gap/ rpoB)

Positive by CoNS specific m-PCR

Negative by CoNS specific m-PCR

Nucleotide sequencing & Phylogenetic analyses

Subjected to *S. aureus* specific triplex PCR

Phenotype-based Speciation

Genotype-based Speciation

Comparison of phenotype- and genotype-based speciation

Antibiogram studies

Amplification of antibiotic resistant genes (*mecA/ novel mecA/ aacA-D/ aph3’-IIIa*) by m-PCR and Determination of MIC