REVEIW OF LITERATURE
II. REVIEW OF LITERATURE

Bovine Mastitis

India is the world leader in milk production with the total milk production accounting to about 14 per cent of global production and 57 per cent of total Asia’s production. Mastitis is the costliest disease confronting the dairy industry and it is estimated that mastitis alone accounts to about 70 per cent of all avoidable losses incurred during milk production. Bovine mastitis is the most prevalent disease complex in dairy cattle and one of the most important diseases affecting the world’s dairy industry; inflicting a heavy economic burden on milk producers all over the world. Mastitis is known to be an economically devastating disease hampering desired progress in dairy industry. The overall national economic loss in India due to mastitis was to the tune of Rs.16,072 million (Singh and Singh, 1994) (that due to clinical mastitis to be Rs.2,856.4 million and Rs.2,345.9 million and due to subclinical mastitis Rs.6,038.7 million and Rs.4,831 million in cattle and buffaloes, respectively). Average decrease in milk yield due to clinical and subclinical mastitis was estimated to be 50 per cent and 17.5 per cent, respectively. 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).

2.1 Subclinical mastitis: prevalence and bacteriological examination

Fujikura and Shibata (1965) surveyed dairy herds, covering five rural areas near Tokyo by California mastitis test and found that 84.1 per cent of herds were infected with mastitis and 43.8 per cent of the cows were affected. Bacteriological examination showed
that *S. aureus* was present in 72.4 per cent of samples, coagulase negative Staphylococci (CoNS), *S. agalactiae, S. dysgalactiae, S. uberis, Corynebacterium pyogenes* (*C. pyogenes*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Bacillus cereus* (*B. 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 per cent), *Staphylococcus albus* (*S. albus*) (15.3 per cent) and *Klebsiella* species (8.4 per cent). Other pathogenic bacteria isolated were *C. pyogenes, P. aeruginosa, E. coli* and *S. agalactiae*

Havelka (1975) subjected 1,78,853 milk samples received from 72,454 dairy cows, during 1972 to 1974 for bacteriological examinations and observed that on an annual average, the number of examined samples was higher by 61 per cent compared to 1967-1971. Pathogens responsible for the inflammation of the mammary gland were isolated in the milk of 22.32 per cent of the dairy cows. The bacterial pathogens isolated were *S. agalactiae* (15.77 per cent), *S. aureus* (4.19 per cent), other Streptococci (1.49 per cent), *E. coli* (0.18 per cent), *Klebsiella* species (0.23 per cent), *C. pyogenes* (0.20 per cent), other germs (0.26 per cent). The study indicated that *S. agalactiae* isolated was higher by 3.29 per cent and that of *S. aureus* by 1.15 per cent than in the period from 1967 - 1971.

Filev (1977) studied the occurrence and etiology for subclinical mastitis in 16,571 cows on 89 farms by means of the Bernburg test. A total of 18,047 milk samples from the
positively reacting quarters of the udder were subjected for bacteriologic investigation and found that in 53.95 per cent of the cases there were secretory lesions and due to *S. agalactiae* (6.23 per cent), *S. dysgalactiae* (5.69 per cent), *S. uberis* (8.47 per cent), *S. aureus* (2.44 per cent), hemolytic streptococci of the C, G and L groups (0.28 per cent), *S. viridans* (0.03 per cent), *C. pyogenes* (0.41 per cent), and catarrhal mastitis (30.4 per cent).

Klastrup and Halliwell (1977) examined the prevalence of bovine subclinical mastitis in Malawi. The survey comprised 934 cows of a total of 1180 from 22 dairy herds and the examination of bulk milk samples from 273 small-holders. The results of the survey revealed that the major problems of IMI were due to the infections caused by *S. agalactiae* and *S. aureus*

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

Gonzalez *et al.* (1980) found that 2388 (57.3 per cent) 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 per cent), *S. epidermidis* (21 per cent), *S. uberis* (19 per cent), *S. agalactiae* (13 per cent), *S. dysgalactiae* (9 per cent), *Corynebacterium bovis* (*C. bovis*) (7 per cent), *C. pyogenes*
(1.3 per cent), Coliforms (1.7 per cent) and mixed Streptococcal and Staphylococcal cultures.

Rahman and Baxi (1982) tested 90 isolates of *S. aureus*, 63 of *S. epidermidis*, 37 of *S. agalactiae*, 10 *S. dysgalactiae*, 14 strains of other Streptococci and 38 strains of other bacteria from bovine subclinical mastitis 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.

Haggard *et al.* (1983) investigated the prevalence of subclinical mastitis of beef cows and evaluated the quarter samples obtained about 30 days after calving by the CMT and by direct microscopic cell count. It was observed that in one herd, seventeen quarters of twelve cows were infected with *S. aureus*, *Streptococcus* spp. or *Klebsiella* spp. whereas in the other herd, eight quarters of eight cows were infected with *S. aureus*

Swartz *et al.* (1984) studied the prevalence and types of bacteria associated with subclinical mastitis in Bloemfontein dairy herds. *Staphylococcus aureus* was the dominant mastitis-associated organism, constituting 66.4 per cent of all bacteria isolated. There was a low prevalence of classical mastitis caused by streptococci (0.7 per cent) and of gram-negative bacterial infections (6.3 per cent). The gram-negative bacteria were almost invariably isolated from neglected herds in which the cows were generally in poor
condition and the hygienic measures employed were totally inadequate. Other bacterial strains isolated were *C. bovis* (6.3 per cent) and the CoNS (11.0 per cent).

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

Oliver (1987) screened around 23,000 lactating cows over a period of eight years and found that mastitis organisms were present in twice as many cows and four times as many quarters in herds where *S. agalactiae* was present. In herds infected by *S. agalactiae*, the predominant pathogens were *S. agalactiae* and *S. aureus*, whereas *S. aureus* and Coliforms were the most common pathogens in herds infected with other Streptococci.

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

Schukken *et al.* (1989) examined a total of 1140 clinical cases of mastitis. *E. coli* (16.2 per cent) was the most frequently isolated organism, followed by CoNS (13.0 per cent), *S. aureus* (9.6 per cent) and *S. uberis* (8.0 per cent). Only two isolates of *S. agalactiae* were found. They observed that as the incidence of clinical mastitis increased,
the proportion of *S. aureus* also increased, whereas the proportions of *E. coli, S. uberis* and *S. dysgalactiae* remained same.

Zingeser et al. (1991) conducted a national survey of clinical and subclinical mastitis in Jamaican dairy herds between 1985-1986. The study involved examination of 1,645 lactating cows using the CMT test and 254 composite milk samples collected for bacteriological examination. Fifty-six per cent of all quarters were found to have CMT scores of one or higher, 0.8 per cent showed clinical mastitis and 3.2 per cent were blind. *Staphylococcus aureus* was the most common bacterial pathogen recovered from 31 per cent of sampled cows. The resultant milk loss from clinical and subclinical mastitis was estimated to be 20 per cent of the potential national production.

Malinowski et al. (1992) examined milk samples from 2084 cows belonging to 20 farms and found 945 cows positive for subclinical mastitis. The pathogens isolated were *S. epidermidis* (32.5 per cent), *Micrococcus* species (19.9 per cent), *S agalactiae* (15.3 per cent), *S. aureus* (11.0 per cent), *S. uberis* (10.3 per cent), *S. dysgalactiae* (8.1 per cent) and other microorganisms (2.0 per cent).

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

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 per cent), *S. aureus* (9.2 to 12.1 per cent), *Corynebacterium bovis* (5.8 to 7.5 per cent) and the Gram negative organism isolated were *E. coli* and *Enterobacter* species.

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

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

Over 135 different microorganisms have been isolated from bovine IMI, but Staphylococci, Streptococci and Gram-negative bacilli caused majority of the infections (Bramley *et al.*, 1996).

Wadhwa *et al.* (1996) conducted a study of clinical mastitis in Himachal Pradesh. Milk samples collected from 93 affected quarters (57 cows) subjected for bacteriological examination revealed *Staphylococcus* species (68.83 per cent), *Streptococcus* species
(16.88 per cent), *E. coli* (7.8 per cent), *Klebsiella* species (2.59 per cent), *Proteus* species and *Corynebacterium* species (1.3 per cent).

Mallikarjunaswamy and Krishnamurthy (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 subclinical bovine mastitis which included *S. epidermidis* (80), *S. aureus* (66), *S. intermedius* (2), *S. agalactiae* (34), *S. dysgalactiae* (2), *S. uberis* (1), *A. pyogenes* (4), *Bacillus subtilis* (*B. subtilis*) (7), *B. creus* (1), *P. aeruginosa* (8), *E. coli* (4), *Enterobacter aerogenes* (*E. aerogenes*) (2), *Proteus vulgaris* (*P. vulgaris*) (2) and *Proteus mirabilis* (*P. mirabilis*) (1). The antibiotic sensitivity of these isolates was tested *in vitro*. They found that most isolates were sensitive to chloramphenicol, but resistant to streptomycin, neomycin and penicillins. The *S. aureus* isolates were highly sensitive to ciprofloxacin (100 per cent), ceftizoxime (100 per cent), gentamicin (98.4 per cent), cloxacillin (98.48 per cent), tetracycline (93.9 per cent) and co-trimoxazole (92.42 per cent) and moderately sensitive to neomycin (86.36 per cent). Most of the isolates showed resistance to penicillin (68.18 per cent) and streptomycin (57.57 per cent).

It has been observed that over 75 per cent of the intramammary infections were caused by *S. agalactiae*, *Streptococcus* species other than *S. agalactiae*, *S. aureus*, and CoNS (Wilson *et al.*, 1997).

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

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

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

In a study conducted over a period of 9 years (1987-1995), 51.51 per cent of 728 mastitis milk samples were cultured positive for bacteria. The mastitis due to *S. aureus* was the highest (61.76 per cent) followed by *S. pyogenes* (12.03 per cent) and 2.4 per cent of *S. epidermidis* (Nagal *et al.*, 1999).

Busato *et al.* (2000) examined the udder health and risk factors for subclinical mastitis 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 subclinical mastitis at the quarter level was 21.2 per cent for lactation period 7-100 days and 34.5 per cent 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 per cent, for CoNS in 51.5 and 50.6 per cent, for *S. agalactiae* in 0.0 and 0.8 per cent, for other Streptococci in 19.4 and 15.6 per cent, for *E. coli*.
coli in 1.0 and 0.4 per cent, and for C. bovis in 25.7 and 45.1 per cent, respectively. Risks of subclinical mastitis 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 for mastitis, of which 46 samples from 29 cows (64.4 per cent) were found positive on CMT and cultural examination. Results revealed the predominance of Staphylococcus species (60.9 per cent), followed by Streptococcus species (23.9 per cent), Pseudomonas species (6.5 per cent) and Bacillus species (2.2 per cent). In vitro drug sensitivity showed that all the isolates were highly sensitive to enrofloxacin. Majority of the isolates were moderately sensitive to gentamicin and oxytetracycline and resistant to ampicillin and streptomycin.

A total of 107 bacterial isolates including S. aureus (30), S. agalactiae (16), S. dysgalactiae (12), B. subtilis (14), P. aeruginosa (13) and E. coli (22) were isolated from cases of bovine mastitis in Idukki district of Kerala (Ross et al., 2001).

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 (WST) and found 67 per cent of the CMT and 40 per cent of WST positive samples were positive for the bacterial isolation.
Mohinikumari and Janakiramguptha (2002) subjected a total of 81 milk samples to Strip cup test, CMT, WST and BTB indicator card. Of these, only 19 (23.45 per cent) were positive by strip cup test, while 71 (87.65 per cent) were found positive by CMT, BTB indicator card test and WST. The cultural examination revealed *Staphylococcus* species (35.21 per cent), *E. coli* (28.39 per cent), followed by *Streptococcus* species (18.5 per cent), *Pseudomonas* species (4.9 per cent), *Corynebacterium* species (2.46 per cent) and *Klebsiella* species (2.46 per cent). Antibiotic sensitivity test revealed all the isolates were sensitive to gentamicin, followed by chloramphenical, kanamycin, nalidixic acid and erythromycin.

Workineh *et al.* (2002) had undertaken the study to determine the aetiology 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 per cent of the cows were clinically infected and 38.2 per cent had subclinical mastitis. Most mastitis pathogens isolated from milk samples testing positive by the CMT were Gram-positive cocci. Staphylococci constituted 57 per cent of the isolates, while the predominant cause of bovine mastitis was *S. aureus* (40.5 per cent). Other mastitis pathogens isolated included Streptococci (16.5 per cent), Coliforms (9 per cent) and Corynebacteria (5 per cent).

Aasmae *et al.* (2003) conducted a study to investigate the antibacterial resistance of the pathogens causing clinical mastitis in dairy cows from Estonia. The bacteria most frequently isolated were *S. aureus* (169 out of 543) and *S. agalactiae* (81 out of 543).

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 per cent) followed by *Streptococcus* species (14.41 per cent), *E. coli* (11.71 per cent), *Bacillus* species (9.01 per cent) and *Corynebacterium* species (7.21 per cent).

Kerrodego 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. California mastitis test revealed 40.4 per cent positivity and the prevalence rate of clinical mastitis was 37.1 per cent and subclinical mastitis was 62.9 per cent. *Staphylococcus* accounted for 39.2 per cent, *Streptococcus* for 23.6 per cent, Coliforms for 14.1 per cent, *Micrococcus* and *Bacillus* species for 8.0 per cent each and *Actinomyces* or *Arcanobacterium* (*Corynebacterium*) for 7.0 per cent of the isolates. It was concluded that there was a high prevalence of clinical and subclinical mastitis, mainly caused by *S. aureus*, *S. agalactiae* and *E. coli*.

Balakrishnan et al. (2004) isolated 40 bacterial isolates from 65 milk samples. The spectrum comprised of *S. aureus* (35 per cent), *E. coli* (27.5 per cent), *S. agalactiae* (17.5 per cent), *P. aeruginosa* (12.5 per cent), *S. dysgalactae* (2.5 per cent), *Pasteurella haemolytica* (2.5 per cent) and *Actinobacillus capsulatus* (2.5 per cent).

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. A total of 57 herds comprising 114 milking cows in Kibaha and 48 herds consisting of 96 milking animals in Morogoro were included in the study. California mastitis test and microbiological assessment of milk was carried out to establish the status of mastitis and
responsible etiological agents. Based on CMT, the cow based prevalence of subclinical mastitis was 82.4 per cent in Kibaha and 62.4 per cent in Morogoro. Of the 919-quarter milk samples cultured, 8.2 per cent were positive for aerobic bacteria with predominant isolates being *S. epidermidis* (2.8 per cent), *S. aureus* (1.7 per cent), *S. agalactiae* (1.2 per cent) and *S. intermedius* (1.1 per cent). There was a strong association between CMT positivity and bacteriological isolation. Fungal growth was observed in 21.8 per cent of the samples and the isolates were yeast (19.2 per cent), *Mucor* (2.5 per cent) and *Aspergillus* (0.1 per cent).

Palinivel *et al.* (2005) obtained 12 bacterial isolates from 80 mastitis positive milk samples and the bacterial agents isolated were *S. aureus*, *S. uberis*, *S. dysgalactiae*, *E. coli*, *Corynebacterium* species and *Pseudomonas* species.

Schroder *et al.* (2005) analyzed the data of 1692 susceptibility tests acquired from April 2003 through March 2004 in the mastitis laboratory of the Institute for Food Quality and Safety. Two third of the milk samples were infected with Gram-positive cocci. One third of these were identified as *S. uberis*, one fourth as *S. aureus*

Bacteria such as Coliforms (*Escherichia, Klebsiella* species, *Enterobacter* species, *Citrobacter* species), Streptococci (*S. uberis, S. agalactiae, S. dysgalactiae*) and Staphylococci (*S. aureus* and CoNS) were the frequently reported organisms from cases of bovine mastitis (Dembele *et al.*, 2006).

Rajeev (2006) screened a total of 300 milk samples collected from four different farms for subclinical mastitis by CMT, White Side Test (WST) and Bromo thymol blue
(BTB) strips. A total of 149 isolates were recovered. Of these, 110 isolates (73.82 per cent) were Gram positive, 33 isolates (22.14 per cent) were Gram negative and six were yeasts (4.02 per cent).

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 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 1,78,668 quarter milk samples were collected at 1087 cross-sectional dairy herd screenings performed in three consecutive years. Of the dairy cows, 40 per cent had at least one culture-positive quarter. More than 50 per cent of all IMI were caused by non-aureus staphylococci. S. agalactiae was almost eradicated in Flanders, whereas S. aureus was isolated from 18 per cent 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 2,50,000 cells/ml, nearly 65 per cent had at least one culture-positive quarter. The majority of the IMI were caused by non-aureus staphylococci (41.1 per cent), whereas S. aureus and aesculin-positive cocci were found in respectively 25 per cent and 18 per cent 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.
Roesch et al. (2007) compared the prevalence of subclinical mastitis in dairy cows in Swiss organic and conventional production systems. A total of 970 cows were studied for subclinical mastitis prevalence and udder pathogens. Cows showing more than or equal to 1+ (>or=1+) positive California Mastitis Test (CMT) in at least one quarter were considered to have subclinical mastitis. It was observed that quarter-level prevalence were higher in OP than CP (15 per cent and 18 per cent in OP and 12 per cent and 15 per cent in CP). In milk samples from quarters showing a CMT reaction >or=2+, the prevalence of CoNS were lower, whereas prevalence of non-agalactiae streptococci were higher in OP than in CP cows. It was concluded that under Swiss conditions, subclinical mastitis was a greater problem in organic than in conventional production systems.

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 subclinical mastitis in dairy cows kept by smallholders. Subclinical mastitis was assessed using the CMT and by bacteriological culture of 1500 milk samples collected from 434 clinically normal cows. The percentages of cows (and quarters) with subclinical mastitis were 75.9 per cent (46.2 per cent) when assessed by the CMT and 43.8 per cent (24.3 per cent) when assessed by culture.

Miranda-Morales et al. (2008) studied the prevalence of pathogens associated with bovine mastitis in bulk tank milk in Mexico. Microorganisms prevalence and SCCs were determined in 112 Holstein bovine herds in two bulk tank milk-screening assays. Prevalence of Mycoplasma was 55 per cent; of S. aureus, 30 per cent; of S. uberis, 37.5
per cent and of CoNS 38.3 per cent. 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 per cent (n=69) where as the prevalence of subclinical mastitis at animal level was 51.6 per cent (n=91). Prevalence of bacterial isolates at animal level was 35.2 per cent (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 subclinical mastitis that may contribute to low productivity of dairy cattle.

Nithinprabhu (2010) screened 163 milk samples collected from organized and unorganized sectors for SCM by SCC and EC and observed that 47 per cent of the samples were positive for SCM. The cultural examination of the SCM milk samples revealed 40 streptococcal species and four isolates were confirmed as S. agalactiae biochemically and by PCR.

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 per cent) of the 118 S. aureus isolates, indicating that nearly 10 per cent of the Belgian farms suffering from S. aureus mastitis have an MRSA problem. The
in-herd prevalence varied between zero per cent to 7.4 per cent. 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 belonged 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 might cause future treatment problems.

Bal et al. (2010) studied the prevalence and antimicrobial susceptibilities of Staphylococci and Streptococci from subclinical mastitis cases and identified one hundred CoNS and thirty four Streptococci. The most frequently isolated species were S. haemolyticus (27 per cent) and S. simulans (24 per cent). Susceptible CoNS species revealed the highest resistance to penicillin G (58 per cent), ampicillin (48 per cent), neomycin (20 per cent), and oleandomycin (14 per cent). The most frequently isolated Streptococcus species were S. uberis (52 per cent) and S. agalactiae (15 per cent). Oleandomycin was the least effective antimicrobial agent on these isolates with 59 per cent susceptibility.

Botrel et al. (2010) studied the distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France. Out of 1631 bacterial isolates, S. uberis (22.1 per cent), E. coli (16 per cent), and CoNS (15.8 per cent) were identified as the major causative agents of clinical mastitis, whereas coagulase-positive staphylococci (30.2 per cent), CoNS (13.7 per cent), and S.
*dysgalactiae* (9.3 per cent) were predominantly implicated in subclinical mastitis. The overall proportion of antibiotic resistance was low, except for penicillin G in staphylococci, as well as for macrolides and tetracycline in streptococci.

### 2.2 Electrical conductivity

Sheldrake and Hoare (1981) compared electrical conductivity (EC) and somatic cell concentration 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 500 x 10^3 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 per cent and 79 per cent respectively, whereas for SCC, the means were 71 per cent and 81 per cent 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 subclinical mastitis and observed that conductivity of milk seems to hold promise as an indicator of subclinical mastitis. In the study, conductivity of foremilk and of postmilking strippings was measured from 368 quarters of 92 cows. It was observed that the milk conductivity increased with infection and the conductivity of postmilking strippings 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
postmilking strippings tended to be higher when SCC was greater than 5,00,000 cells/mL in both normal and infected groups.

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

Hillerton and Watson (1991) examined a hand-held, commercially available instrument for measuring the EC of milk (Milk Checker) 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 per cent and specificity of 94 per cent, 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 subclinical mastitis compared with that of
bacteriological culture and SCC of sampled quarters. It was observed that the prototype on-line system did not detect subclinical mastitis with high accuracy.

Nielen et al. (1995) developed a logistic regression model based on automatically collected data for detection of subclinical mastitis with the variables such as milk EC, milk production, parity, and DIM. The model had a sensitivity of 55 per cent and specificity of 90 per cent for individual milkings. They opined that the model could be used as an initial screening tool in a herd with a high incidence of subclinical mastitis.

Milner et al. (1996) observed that clinical mastitis caused by two major pathogens S. 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 S. aureus but not S. uberis.

Musser et al. (1998) assessed the utility of a hand-held EC meter to detect subclinical mastitis 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 subclinical mastitis was significantly higher than that for cows without subclinical mastitis and was significantly associated with detection of subclinical mastitis. The study revealed that, if absolute EC score of more than or equal to seven (> or = 7) was considered indicative of subclinical mastitis, 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 subclinical mastitis. If differential EC score of more than or equal to two (≥ 2) was considered indicative of subclinical mastitis, 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 subclinical mastitis.

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.

Mansell and Seguya (2003) evaluated the accuracy of a hand-held EC meter for the detection of subclinical mastitis in an Australian dairy herd in late lactation that had a high prevalence of *S. agalactiae* and *S. aureus* infection. 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 subclinical mastitis in cows in late lactation. They concluded that the interpretation of results from handheld conductivity meters when used for the diagnosis of subclinical mastitis in dairy cows needs to be made with care and in some circumstances the results obtained do not accurately reflect the bacteriological status of either individual quarters or cows.

Norberg et al. (2004) defined four EC traits as the interquarter 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.

Povinelli (2007) stated that given the moderate heritability value and the high correlation with SCC, EC might be considered as an useful trait in selection schemes for Brown Swiss cows.

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 per cent, 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.

2.3 Somatic cell count

Meek and Barnum (1982) investigated the feasibility of developing a system so that measurements taken on bulk tank milk samples could be used to monitor the level of subclinical mastitis in dairy herds. The variables examined were the logarithmically transformed total SCCs and percentages of cell volume in channel eight, the presence or
absence of *S. agalactiae* and various husbandry/management factors including herd size and the use of teat dips. It was found that the inclusion of all variables resulted in a correct classification of approximately 85 per cent of herds and that no improvement was achieved by the use of rolling as opposed to actual monthly values. The use of both total cell counts and percentages of cell volume in channel eight did not improve the overall predictive value over that achieved by the sole use of percentage of cell volume in channel eight in the case of the quarter infection rate groupings but did to some extent in the case of the cow infection rate groupings. It was concluded that the system had limited application as a basis for selecting problem herds.

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

Shuster and McDaniel (1996) performed an *E. coli* challenge experiment and observed a negative correlation between pre-infection SCCs and severity of inflammation. Pre challenge cell counts were correlated to CD18 expression and inversely correlated to bacterial growth after challenge. Also, cytokine production was lower in cows with lower pre-challenge cell counts leading to significantly lower leukocyte recruitment in the early phase of the inflammation.
Somatic cell counts 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 somatic cell count penalty limits for saleable milk differ between countries within the European Union, Australia and New Zealand at 4,00,000, Canada at 5,00,000 and the United States at 7,50,000 cells / mL of milk (Paape and Contreras, 1997).

Barkema et al. (1998) opined that management practice was related to bulk milk SCC, wherein, 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.

Kelly et al. (1999) observed a seasonal variation in the SCC and PMN. There was a significant seasonal influence on milk PMN content, with milk from cows calving in the spring, having SCC > 160,000 cells/mL, had higher proportions of PMN in the total milk SCC than milk from autumn calving cows. They opined that concentration of PMN might be a useful indicator of herd status in bulk tank monitoring schemes.

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. They also observed that mastitis also adversely affected the quality of pasteurized milk and decreased its shelf life.

Schukken et al. (2003) 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.

Jayarao et al. (2004) conducted a study to establish guidelines for monitoring bulk tank milk SCC and bacterial counts, and also to understand the relationship between different bacterial groups that occur in bulk tank milk. One hundred twenty-six dairy farms in 14 counties of Pennsylvania participated, each providing one bulk tank milk sample every 15 days for 2 months. The study revealed that an increase in the frequency of isolation of *S. aureus* and *S. agalactiae* was significantly associated with an increased bulk tank SCC. Bulk tank milk with low (<5000 cfu/mL) standard plate count also had a significantly low level of mean bulk tank SCC (<2,000,000 cells/mL), preliminary incubation count (<10,000 cfu/mL), laboratory pasteurization count (<100 cfu/mL), CoNS and environmental streptococcal counts (<500 cfu/mL), and noncoliform count (<200 cfu/mL). It was observed that herd size and farm management practices had considerable influence on somatic cell and bacterial counts in bulk tank milk. It was concluded that categorized bulk tank somatic cell and bacterial counts could serve as indicators and facilitate monitoring of herd udder health and milk quality.

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).

Tancin et al. (2007) studied the milk flow parameters at udder and quarter levels in relation to SCC and other risk factors for mastitis such as bimodality, duration of decline, and over milking phase. The study indicated that both at the udder and quarter levels milk yield was reduced in groups with increased SCC. Quarters with high SCC had lower peak flow rate and longer over milking phases compared with quarters with low SCC. There was a tendency for a longer duration of the decline phase in quarters with high SCC and longer declines in bimodal milk flows was also observed at the quarter, but not at the udder, level. The duration of the decline phases at the quarter level influenced all measured parameters except the duration of the increase phase. It was also observed that the quarters with a longer duration of the decline phase had greater SCC and peak flow rate but had lower milk yield compared with quarters with a shorter duration of the decline phase. It was concluded that for good udder health, the duration of the decline phase at the quarter level should be considered for milking parameters and udder preparation before milking.

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 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 per cent; of *S. aureus*, 30 per cent; of *S. uberis*, 37.5 per cent and of CoNS, 38.3 per cent. The geometric mean of the SCC was 4,65,000 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 subclinical mastitis 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 per cent), Streptococci (26.7 per cent) and Gram-negative bacteria (11 per cent). The most frequent microorganisms related to cured IMI were CoNS (33 per cent). The study revealed that the quarters with SCC > or =2,00,000 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 2,00,000 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 analysed 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.

2.4 Polymerase chain reaction and sequencing

Since the initial development of polymerase chain reaction (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).

2.4.1 DNA extraction

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.

Phuektes 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, S. agalactiae, S. dysgalactiae and S. 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.
Riffón et al. (2001) extracted DNA with and without a pre-PCR enzymatic lysis step of the bacterial cells and observed that latter version eliminated the need of expensive reagents while standardizing a rapid and sensitive PCR assay for identification of major pathogens in bovine mastitis like *E. coli*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis*. The results indicated that PCR could avoid cumbersome cultivation steps, which could be performed within hours, and was sensitive and specific.

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 ‘Tu’ elongation factor (*tuf*) gene

Ke et al. (1999) analyzed the *tuf* sequences from a variety of Streptococci and Lactococci and observed the presence of only one copy of *tuf* gene in their genome. They concluded that the nonspecific amplification of *Enterococcus durans* (*E. durans*) by the *Streptococcus* specific PCR might be due to the presence of two divergent *tuf* genes, one of which sharing a common ancestor with the *tuf* gene of Streptococci.

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.

Picard *et al.* (2004) used *tuf* gene sequences to select *Streptococcus* specific PCR primers and to perform phylogenetic analysis. The specificity of the PCR assay was verified using 102 different bacterial species, including the 28 Streptococcal species. Genomic DNA purified from all streptococcal species was efficiently detected, whereas, there was no amplification with DNA from 72 of the 74 non Streptococcal bacterial species tested. There was cross amplification with DNAs from *E. durans* and *Lactococcus lactis* (*L. lactis*). However, the *tuf* gene provided a better discrimination at the Streptococcal species level that should be particularly useful for the identification of very closely related species. They concluded that, *tuf* gene was more suitable than the 16S ribosomal RNA gene for the development of diagnostic assays for the detection and identification of Streptococcal species because of its higher level of species specific genetic divergence.

Cupakova *et al.* (2005) applied a duplex PCR for identification of Enterococci at genus level targeting *tuf* gene and 16S rRNA gene as internal control. They concluded that PCR based on *tuf* gene has been found specific and suitable for genus confirmation of Enterococci from foods and environmental samples.

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 *S. aureus*
and several strains of other species. It was shown that strains within a species migrated the same distance in the denature 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.

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 per cent sensitive and 100 per cent specific for the detection of the *Staphylococcus* genus and of *S. aureus*.

### 2.4.3 16S rRNA gene based PCR

16S rRNA gene has hyper variable regions which facilitate the design of highly specific oligonucleotide probes. Lee *et al.* (1989) opined that 16S rRNA genes are found in all bacteria and has served as an important tool for determining phylogenetic relationships between bacteria. Sequence analysis of the 16S rRNA gene is a powerful mechanism for identifying new pathogens in patients with suspected bacterial disease. Further, these studies have shown that sequence identification is useful for slow growing, unusual and fastidious bacteria as well as for bacteria that are poorly differentiated by conventional methods.
William et al. (1991) opined that the amplification by PCR of a taxonomically diverse collection of eubacterial 16S rRNA genes is possible with a small number of primers. These products can readily be cloned for sequencing or they can be sequenced directly. The ability to determine rRNA sequences from ATCC lyophilized cultures, without culturing, enables the study of fastidious or pathogenic species without employing tricky or expensive microbiological methods.

Bentley and Leigh (1992) developed oligonucleotide probes specific for 16S rRNA, capable of differentiating *S. uberis* and *S. parauberis* from each other and other aesculin hydrolyzing Streptococci. Use of a mini-RNA extraction technique for gram-positive cocci associated with bovine mastitis has allowed the probes to be used for identification of aesculin hydrolyzing streptococci from two dairy herds. A total of 179 out of 206 isolates were identified as *S. uberis*, three were identified as *S. parauberis*, and 24 were not identified.

George and Bramley (1992) emphasized the fact that effective identity of 16S rRNA sequences was not necessarily a sufficient criterion to guarantee species identity. Thus, although 16S rRNA sequences could be used routinely to distinguish and establish relationships between genera and well resolved species, very recently diverged species may not be recognizable.

Hassan et al. (2001) investigated *S. uberis* and *S. parauberis* reference strains and isolates obtained from routine diagnosis by PCR with oligonucleotide primers designed according to species specific parts of the 16S rRNA gene, the 23S rRNA gene, and the
16S-23S rRNA intergenic spacer region of both species. All three primer pairs allowed an identification of 67 isolates as *S. uberis* and four isolates as *S. parauberis*.

Martinez *et al.* (2001) developed PCR using species specific and universal primers derived from the 16S rRNA gene for direct detection of *S. agalactiae* from cow's milk. The amplification products were verified by restriction endonuclease digest and sequencing. Specific identification was proven on a collection of 147 *S. agalactiae* isolates of bovine and human origin.

Meiri-Bendek *et al.* (2002) detected *S. agalactiae* in raw milk by PCR. The specificity of the PCR reaction was based on unique sequences of *S. agalactiae* DNA sequences within the 16S subunit of the rRNA genes. The method of detection included selective enrichment of *S. agalactiae* in the milk samples, followed by using rapid and simple procedure for preparation of template DNA for use in PCR.

Kathryn *et al.* (2003) have shown that the broad range 16S rRNA gene PCR could detect and identify DNA from a wide range of organisms from culture or in clinical specimens. They concluded that use of broad range 16S rRNA gene PCR in the routine clinical microbiology service improves the diagnosis of bacterial infection from clinical specimens when used alongside routine culture techniques. However, the authors opined that PCR did not replace culture technique and when performed by a single skilled operator, was rapid and reproducible.

Gillespie and Oliver (2005) developed a real-time PCR assay for simultaneous detection of *S. aureus*, *S. agalactiae* and *S. uberis* directly from milk sample. A genetic
marker specific for *S. aureus* was used for primers and dual labeled probe design. The target for *S. agalactiae* primers and dual labeled probe was selected from the *cfb* gene encoding the CAMP factor. The plasminogen activator (*pauA*) gene was the target for primers and dual labeled probe design for *S. uberis*. They analyzed 192 milk samples and concluded that the multiplex real time PCR procedure had the potential to be a valuable diagnostic technique for simultaneous detection of these pathogens directly from milk samples.

Dmitriev *et al.* (2006) sequenced the *cpn60* genes of *S. agalactiae, S. dysgalactiae* and *S. uberis* and observed a certain polymorphism of *cpn60* genes. Further, they standardized *cpn60* gene based multiplex-PCR for simultaneous identification of *S. agalactiae, S. dysgalactiae* and *S. uberis* strains.

Chakrabarti *et al.* (2009) concluded that broad range 16S rRNA PCR was able to detect organism in five cases where conventional techniques were negative. Overall performance of broad range 16S rRNA PCR was found to be promising as a screening test for diagnosis. Using the semi-nested strategy identification up to species level could be done within 4 hr provided the appropriate species specific primers were applied according to the epidemiological pattern in etiology.

### 2.4.4 Surface immunogenic protein (*sip*) gene

The *sip* is an antigenic protein localized on the surface of *S. agalactiae* which is capable of raising an antibody response. It is also known that *sip* is highly conserved at the gene level. The PCR was developed to rapidly identify the *S. agalactiae* by targeting the *sip* specific sequence along with the 16S and 23S sequences (Tettelin *et al.*, 2000).
Brodeur and Sneath (2000) evaluated the level of molecular conservation in the sip gene of S. agalactiae; sip genes were amplified by PCR from five additional Group B Streptococcal (GBS) strains, three of serotype III, one of serotype II, and one of serotype V. The nucleotide and deduced amino acid sequences of these six sip genes were found to be highly conserved. Indeed, at the nucleotide level, those six sip genes showed differences in only 19 positions out of the 1,305 bp, which made them 98 per cent identical. Furthermore, the sip gene was found conserved for S. agalactiae and might be used as the specific marker to detect S. agalactiae.

The method based on PCR using specific sequence primers only for group B Streptococci (GBS) and species specific primers derived from 16S rRNA for S. agalactiae, S. aureus and E. coli was developed. The sequence coding for Surface immunogenic protein from GBS human isolates named as sip specific sequence (SSS) was selected for specific primer design. This sequence unique for GBS was constructed as a consensus of all known sip gene sequences. The specific identification was proven on a collection of 75 GBS bovine isolates from different localities in Slovakia. 16S and 23S rRNA PCR detection was also performed with S. aureus and E. coli. The detection limit for GBS and E. coli was 6000 cfu/µL and 16cfu/ µL for S. aureus. Seventy-four isolates were positive to SSS and 16S rRNA sequence (Chotar et al., 2006).

A multiplex PCR method was developed to characterize the potential variability within the sip gene from bovine isolates of S. agalactiae. An incomplete sip gene (ncosip) was found in four isolates of S. agalactiae. According to the in silico analysis of the missing region at amino acid level, the N-terminal of surface immunogenic protein
(sip) was found to contain a LysM domain motif, whilst the incomplete sip (ncosip) lacked a part of this motif. Immune response against sip was confirmed by immunization of mice (Vidová et al., 2006)

### 2.4.5 Plasminogen activator (pauA) gene

Bacteria that have a limited capacity to synthesize amino acids exhibit a reduced ability to grow in milk (Leigh and Lincoln, 1997). To overcome this deficiency, some bacteria such as *S. uberis* utilize host proteolytic enzymes e.g. plasmin to digest the casein in milk. In the animal host, plasmin is generated from plasminogen following cleavage by plasminogen activators that are present in blood plasma and tissues (Leigh and Lincoln, 1997). Certain Streptococci are capable of producing a plasminogen activator which can also cleave plasminogen to plasmin (Leigh and Lincoln, 1997). The *S. uberis* streptokinase, known as pauA, has been shown to specifically activate both bovine (Rosey et al., 1999) and ovine plasminogen (Kliem and Hillerton, 2002).

The *S. uberis* streptokinase pauA is a putative virulence factor and encodes the plasminogen activator which converts plasminogen in blood plasma and tissues in cattle to plasmin (Leigh, 1999; Leigh, 2000), facilitating early colonization of the mammary gland prior to inflammation (Leigh, 1993; Smith et al., 2002 and Rambeaud et al., 2004). According to the authors pauA gene specific PCR performed in their study provided useful supplemental data to differentiate *S. uberis* from closely related species.

Ward and Leigh (2004) demonstrated that pauA appeared to be restricted solely to *S. uberis* and was absent in other *Streptococcus* species or other bacteria commonly associated with bovine mastitis.
A pauA gene specific PCR was used to investigate the presence of pauA in the Australian isolates using a modification of the method described by Zadoks et al. (2005). Tomita et al. (2008) characterized 53 isolates conventionally as *Streptococcus* species. Of the 53 isolates, seven were negative for pauA gene and were identified as non *S. uberis* species by 16S rRNA gene sequence identification. The remaining 46 isolates were all positive for pauA gene and were confirmed as *S. uberis* following 16S rRNA gene sequencing.

2.4.6 nuc gene based PCR for *S. aureus*

Wilson et al. (1991) used PCR to amplify the staphylococcal enterotoxin B and C genes (*entB* and *entC1*) and the staphylococcal nuclease gene (*nuc*). They inferred that two sets of primers ("nested primers") were necessary for the detection of low copy numbers of purified DNA in diluents.

Brakstad et al. (1992) used PCR to amplify a sequence of the *nuc* gene, for the detection of *S. aureus*. A DNA fragment of approximately 270 bp was amplified from lysed *S. aureus* cells or isolated DNA. With *S. aureus* cells, the lower detection limit was less than ten colony forming unit, and with the isolated target the lower detection limit was 0.69 picogram (pg) of DNA. They opined that the PCR for amplification of the *nuc* gene has potential for the rapid diagnosis of *S. aureus* infections by direct testing of clinical specimens, including specimens from patients with ongoing antimicrobial therapy.

Khan et al. (1998) evaluated PCR analysis targeting *nuc* gene for detection of *S. aureus* in fresh and formalin-preserved milk. Samples from 80 lactating sheep and 100
lactating dairy cows were screened by bacteriologic culture and PCR. The PCR assay was 100 per cent specific and sensitive. They opined that the assay was particularly suitable for analysis of samples shipped or stored without refrigeration.

Kim et al. (2001) optimized the PCR for detection of *S. aureus nuc* gene in bovine milk. In the study, a set of oligonucleotide primers of 21 and 24 bases was used in Taq-PCR to amplify DNA from *S. aureus* isolates from bovine mastitis with a specific amplicon of 270 bp. They observed that replacing Taq DNA polymerase with *Thermus thermophilus* (Tth) DNA polymerase alone (Tth-PCR) raised the sensitivity of *S. aureus* detection in milk from experimentally infected cows from 65 to 80 per cent. In a random survey involving 100 milk samples from cattle not infected with *S. aureus*, the test was 100 per cent specific. With milk samples from clinical cases of bovine mastitis, 100 per cent sensitivity and specificity were also observed. It was concluded that Tth-PCR on milk samples with the purification of crude DNA extracts was as sensitive as, but faster than conventional milk bacteriological culture.

Kuzma et al. (2003) standardized PCR for detection of *S. aureus* targeting *nuc* gene from intramammary infections. The *nuc* gene based PCR amplified all the 29 isolates of *S. aureus* from mastitis cow milk with an amplicons size of 270 bp.

Pinto et al. (2005) explored the possibility of using PCR for rapid identification of food-borne *S. aureus* isolates as an alternative to the API-Staph system. The study involved a total of 158 strains, 15 *S. aureus*, 12 other staphylococcal species and 131 isolates recovered from 164 food samples. They were phenotypically characterized by API-Staph profiles and tested for PCR amplification with specific primers directed to
thermonuclease (nuc) and enterotoxin (sea to see) genes. The high concordance between
*S. aureus* and nuc PCR positive strains (99 per cent) corroborated the specificity of the
primers used and the suitability of nuc PCR for rapid identification of *S. aureus* in routine
food analysis.

Alarcón *et al.* (2006) evaluated the specificity of nuc targeted primers for PCR
detection of *S. aureus* in different food matrices. They tested the specificity of nuc
targeted primers on a total of 157 strains of genetically confirmed identity, including
reference and food isolates. They opined that PCR approaches, using nuc targeted
primers proved specific and combined with growth techniques may improve detection of
*S. aureus* in different types of food.

Kalorey *et al.* (2007) studied the detection of genes encoding virulence
determinants in *S. aureus* from bovine subclinical mastitis cases by PCR. A total of 37
strains of *S. aureus* isolated during processing of 552 milk samples from 140 cows were
characterized phenotypically, and further genotypically by PCR using oligonucleotide
primers that amplified genes encoding coagulase (coa), clumping factor (clfA),
thermonuclease (nuc), enterotoxin A (entA), and the gene segments encoding the
immunoglobulin G binding region and the X region of protein A gene spa. The
amplification of the thermonuclease gene, *nuc*, was observed in 36 out of 37 isolates.

Yamagishi *et al.* (2007) developed and evaluated screening of milk samples from
cases of bovine mastitis for *S. aureus* by overnight cultivation followed by PCR to
amplify the nuc gene. The assay could detect concentrations of *S. aureus* as low as one
cfu/mL milk. Among 106 milk samples collected from individual quarters of lactating
cows in one dairy herd and from a bulk tank, *S. aureus* was detected in nine samples by PCR but in only three samples by conventional microbiological culture.

### 2.4.7 *alr* gene based PCR for *E. coli*

Specific oligonucleotide primers for detecting *E. coli* in various foods were designed based upon the conserved sequences of the *E. coli alr* gene. The oligonucleotide primers were specific to *E. coli*, except for *Shigella* species, when tested with 67 strains of *E. coli*, including such serotypes as O157:H7 and O111 and 32 strains of non-*E. coli* species. The oligonucleotide primers proved useful for detecting *E. coli* in beef, chicken, pork, tomato, soybean, potato, cow’s milk, and egg (Yokoigawa *et al*., 1999).

Daly *et al.* (2002) developed PCR-ELISA for the detection of *E. coli* in milk. They used primers specific for *E. coli alr* gene for amplification and further evaluated by ELISA to improve the sensitivity of detection. It was found that five *E. coli* cfu could be detected per PCR reaction using the PCR-ELISA system, equating to a sensitivity of detection of 100 *E. coli* cfu/mL pasteurized milk. They opined that the approach should facilitate evaluation of milk contamination and enable rapid detection of *E. coli* mastitis, leading to correct deployment of relevant antibiotic therapy and improved animal welfare.

### 2.4.8 *TraT* gene PCR for *E. coli*

Acik *et al.* (2004) investigated the presence of *TraT* and cytotoxic necrotising factor-2 (*CNF2*) virulence genes by multiplex–PCR from the milk collected from healthy cows and sheep. *E. coli* was isolated from 61 samples of the 1028 milk samples collected
from apparently healthy ruminants (737 cows and 291 sheep) in eastern Turkey. Multiplex PCR showed that TraT and CNF2 genes were present in 62.3 per cent and 6.6 per cent of all isolates, respectively. Both genes were present in 16.4 per cent of the isolates, with only 14.7 per cent having neither gene.

Altalhi and Hassan (2009) analyzed the bacterial quality and safety of raw milk sources for the natural contamination of fecal coliform and E. coli by standard most probable number method. Thirty-three E. coli strains were recovered from raw milk sample sources, which were contaminated by fecal coliform. The isolates were screened for virulent genes and the most frequent virulence markers were TraT (17 strains), IutA (11 strains) and FyuA (8 strains). Results suggested a possibility of potential public health threat of E. coli originating from raw milk sources.

### 2.4.9 Multiplex polymerase chain reaction

Phuektes et al. (2001) developed a Multiplex polymerase chain reaction (m-PCR) assay for simultaneous detection of four major bacterial causes of bovine mastitis, S. aureus, S. agalactiae, S. dysgalactiae, and S. uberis. The target sequence was the 16S to 23S rRNA spacer regions. The performance of the assay was examined with 117 milk samples collected from a subclinically infected herd, and the diagnostic specificities and sensitivities of the multiplex PCR were compared with conventional culture. PCR was significantly more sensitive than culture for detection of S. aureus and S. uberis, but there were no significant differences in sensitivities between PCR and culture for the detection of S. agalactiae and S. dysgalactiae. The results suggested that this multiplex PCR assay could be used as an alternative method in routine diagnosis for rapid, sensitive, and
specific simultaneous detection of *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, and *S. uberis* in milk samples.

Phuektes *et al.* (2003) used a m-PCR assay as a mastitis screening test for *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* in bulk milk samples. PCR was used to detect the presence of these organisms in bulk milk samples. Correlations with bulk milk somatic cell counts (BMSCC), total bacteria counts and thermoduric bacteria counts were evaluated. A total of 176 bulk milk samples were collected from 42 herds on five consecutive occasions at approximately ten days intervals. *S. uberis* was the most common organism in these bulk milk samples. There was no relationship between presence of either *S. aureus*, *S. dysgalactiae* or *S. uberis* and BMSCC, total bacteria counts or thermoduric bacteria counts. However, presence of *S. agalactiae* was associated with high BMSCC and total bacteria counts. The results of this study showed that regular analysis of bulk milk using multiplex PCR assay might be a useful tool for monitoring herd status with respect to *S. agalactiae*, but is of less value for monitoring occurrence of *S. aureus*, *S. dysgalactiae* and *S. uberis*. They opined the necessity of further investigations needed to clarify the relationship between positive PCR results and the prevalence of infected cows in the herd.

Zhao and Liu (2004) developed a m-PCR assay for detection of *E. coli* O157:H7 in foodstuffs using 4 pairs primers targeting *fliC*, *rfbE*, *SLT I* and *SLT II* genes. The m-PCR had very high specificity to match all the 71 tested strains. The study also revealed that, with the enrichment procedure, the strain of *E. coli* O157:H7 were recovered at the detection limit of 0.1 cfu/mL from artificial inoculated pasteurized milk.
Gillespie and Oliver (2005) developed multiplex real-time PCR for simultaneous detection of mastitis pathogens, *S. aureus*, *S. uberis*, and *S. agalactiae* directly from milk. A genetic marker specific for *S. aureus* was used for primers and dual-labeled probe design. The target for *S. agalactiae* primers and dual-labeled probe was selected from the *cfb* gene encoding the Christie-Atkins-Munch-Petersen factor. The plasminogen activator gene was the target for primers and dual-labeled probe design for *S. uberis*. In all, 192 quarter milk samples were analyzed by the multiplex real-time PCR assay and conventional microbiological methods. An additional 57 quarter milk samples were analyzed in a separate real-time PCR assay for *S. agalactiae* only. Using an overnight enrichment step, the real-time PCR technique correctly identified 96.4 per cent of all quarter milk samples; 91.7 per cent of *S. aureus*, 98.2 per cent of *S. agalactiae*, and 100 per cent of *S. uberis*. Results of conventional microbiological methods were used to determine the sensitivity and specificity of the multiplex real-time PCR procedure. The sensitivity of the procedure to correctly identify *S. aureus*, *S. agalactiae*, and *S. uberis* directly from milk was 95.5 per cent, and the specificity was 99.6 per cent. Results of the study indicated that the multiplex real-time PCR procedure has the potential to be a valuable diagnostic technique for simultaneous identification of *S. aureus*, *S. agalactiae*, and *S. uberis* directly from quarter milk samples.

Dmitrive *et al.* (2006) standardized *cpn60* gene based m-PCR assay for simultaneous identification of *S. agalactiae*, *S. dysgalactiae*, *S. uberis* and concluded that the technique can be employed for analysis of the milk products and for monitoring mastitis.
Lee et al. (2008) developed a novel biochip for rapid multiplex detection of seven mastitis-causing pathogens in bovine milk samples. The technique was based on DNA amplification of genes specific to the target pathogens and involved DNA extraction of bacteria, PCR, DNA hybridization, and colorimetric reaction procedures. The test could detect 7 common species of mastitis-causing pathogens, including *Corynebacterium bovis*, *Mycoplasma bovis*, *S. aureus*, and the *Streptococcus* spp., *S. agalactiae*, *S. bovis*, *S. dysgalactiae*, and *S. uberis*, within 6 hr. To examine the accuracy and specificity of the biochip, a preliminary test was carried out with 82 random quarter milk samples and compared with results from conventional microbiological methods. They opined that the biochip could be a feasible tool for rapidly diagnosing mastitis-causing pathogens in milk and providing information for a more effective treatment to cure mastitis.

Chiang et al. (2008) used primers based on the heat shock protein genes *hsp70*, *hsp40*, and *hsp10*, for the detection of bovine mastitis pathogens *S. agalactiae*, *S. uberis* and *S. bovis* by PCR. The study revealed that using these primers, all the randomly selected target strains could be specifically detected. Bacterial species other than the target organisms, including strains of other *Streptococcus* spp., and strains of non-*Streptococcus* spp., did not generate any false positive results. The detection limit was $N (N=1-9) \times 10^{(3)} \text{ cfu/mL}$ of cell dilutions, and a 10 hr pre-enrichment step had increased the detection limit at $N \times 10^{(0)} \text{cfu/mL}$.