VI. SUMMARY

The present study was carried out with an objective of isolation and identification of major bacterial pathogens from the cases of subclinical bovine mastitis and to standardize simplex and multiplex PCR for rapid identification of *S. aureus*, *E. coli* and predominant Streptococcal species and finally apply the standardised multiplex PCR for direct, rapid and simultaneous detection of *S. aureus*, *E. coli* and predominant Streptococcal species in milk samples of subclinical cases of bovine mastitis.

The milk samples were collected from four organized farms and three unorganized sectors in and around Bangalore. A total of 246 milk samples were collected from all the lactating animals in the farm or the unorganized sector with sterile precautions.

All the 246 milk samples were screened for sub clinical mastitis status by employing conventional cut off criteria of SCC $\geq 5,00,000$ cells / mL and EC $\geq 6.5$ milli Siemens / cm. A preliminary evaluation of SCC of 246 milk samples indicated SCM at 45 per cent (111/246) by SCC and by EC indicated overall SCM at 62 per cent (150/246).

Totally, 186 out of 246 milk samples were selected which were positive by either of the two criteria and some randomly picked samples from SCM negative samples. Further, these samples were subjected for isolation of mastitis causing pathogens following conventional microbiological assays.

In all, a total of 85 Streptococci, 95 *S. aureus*, 95 CoNS and 48 *E. coli* isolates were obtained from 186 milk samples subjected for isolation.
Primers were designed targeting *tuf* gene to identify *Streptococcus* at genus level with an amplicons size of 110 bp. Species specific primers targeting 16S rRNA were designed to identify *S. agalactiae*, *S. dysgalactiae* and *S. uberis* with an amplicons size of 329 bp, 549 bp and 854 bp respectively. Primers were also designed targeting *sip* gene of *S. agalactiae* (amplicons size of 266 bp) and *pauA* gene of *S. uberis* (amplicons size of 439 bp). Uniplex PCR was standardized using reference strains and all the streptococcal isolates were screened by the uniplex-PCR. Seven isolates of Streptococci were confirmed as *S. agalactiae* both by species specific 16S rRNA and *sip* gene amplification. None of the isolates were either confirmed as *S. dysgalactiae* or *S. uberis*. The specificity of the designed primers was confirmed by sequence BLAST analysis of PCR amplified products using reference strains.

Primers were designed targeting *tuf* gene to identify *Staphylococcus* at genus level with an amplicons size of 235 bp. To identify *S. aureus*, primers were designed targeting *nuc* gene (amplicons size of 181bp) and *sodA* gene (amplicons size of 159 bp). Uniplex PCR was standardized using reference strains and the screening of all 95 isolates of *S. aureus* revealed the presence of *nuc* gene in all the *S. aureus* isolates while, *sodA* gene was present in 89 *S. aureus* isolates. The specificity of the designed primers was confirmed by sequence BLAST analysis of PCR amplified products using reference strains.

For identifying *E. coli* isolates, *alr* gene based primer was used (Yokogoiva et al., 1999). The *E. coli* isolates were further screened by a multiplex-PCR using the primers designed at Lead centre, NAIP Subproject on Bovine mastitis: Unraveling molecular details of host-pathogen interaction and development of molecular diagnostic methods.
PD_ADMAS, Bangalore. Both alr gene based PCR and multiplex-PCR specifically identified 48 isolates as *E. coli*.

*E. coli* isolates were also screened for the presence of virulent gene *TraT*. The primers designed at NAIP Subproject, Molecular Virology Laboratory, Regional campus, IVRI, Bangalore-24 was used. The screening of 48 *E. coli* isolates revealed the presence of *TraT* gene in 40 isolates.

Simplex PCR could detect 7 pg of DNA for *S. aureus*, 77 pg for *S. dysgalactiae*, 22 pg for *S. agalactiae*, 25.5 pg for *S. uberis* while it was 140 pg DNA for *E. coli*.

Sensitivity of PCR was 1cfu/mL for detection of *S. agalactiae*, *S. dysgalactiae* and *S. aureus*, while it was 1000cfu/mL for *S. uberis* when spiked to both normal saline and milk. The sensitivity of PCR for detection of *E. coli* was 1000 cfu/mL when spiked in normal saline, while it was 10000 cfu/mL in milk.

A two tube multiplex-PCR was standardized to simultaneously detect five major mastitis pathogens viz., *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, *S. aureus* and *E. coli*. The two tube multiplex PCR developed had allowed the simultaneous detection of major mastitis pathogens using smaller amounts of reagents and less time to set up and analyze than uniplex PCR, thus making it more applicable to routine diagnostic use. In one tube, amplification of *sip* and *pauA* gene to detect *S. agalactiae* and *S. uberis* respectively, and in other tube amplification of 16s rRNA *S. dysgalactiae*, alr and nuc gene to detect *S. dysgalactiae*, *E. coli* and *S. aureus* was carried out. The two tube multiplex-PCR was initially standardized using reference strains.
The two tube multiplex-PCR was carried out on the subclinical mastitis milk samples processed for isolation. The DNA was extracted from the frozen milk samples after thawing following the procedure of Cremonesi et al. (2006). The results indicated that *S. agalactiae* and *S. aureus* could be detected and was in accordance with the species isolated from culturing. However, only 44 isolates of *E. coli* could be detected.

A total of 147 bulk milk samples were collected from four milk chilling centres and screened for SCC and EC. The results of SCC indicated prevalence of SCM at 78 per cent since, 115 out of 147 samples were positive for SCM, if the conventional criteria of SCC ≥5 lakh cells / mL is considered to declare positivity. Whereas, EC categorized these samples into two broad groups, 55 (37 per cent) samples revealed a EC value of < 6.5 mS/cm and remaining 92 (63 per cent) revealed EC ≥ 6.5mS/cm.

The screening of the bulk milk samples for detection of five major mastitis pathogens (*S. agalactiae, S. dysgalactiae, S. uberis, S. aureus* and *E. coli*) by two tube mPCR revealed the presence of pathogens in 81 bulk milk samples. *S. aureus* was the predominant pathogen detected (43 per cent), followed by *E. coli* (42 per cent), *S. dysgalactiae* (9 per cent) and *S. uberis* (6 per cent).

The multiplex PCR assay developed in the present study was an easy and rapid method to simultaneously detect the five major mastitis pathogens, *S. aureus, S. agalactiae, S. dysgalactiae, S. uberis* and *E. coli* in bulk milk. PCR required less time to process and results were easier to interpret. Hence, the regular analysis of bulk milk by m-PCR may be a useful tool for determining the herd status with regard to the detection of contagious and environmental mastitis pathogens.