Summary
VI. SUMMARY

The present study was carried out with the objective to isolate and identify major CoNS associated with bovine mastitis especially in SCM cases, to standardize molecular methods for precise and rapid identification of predominant CoNS species, to study the antibiogram profile of CoNS isolates and finally to detect major antibiotic resistance genes.

A total of 313 bovine milk samples collected from seven organized dairy farms and seven unorganized sectors in and around southern (Bangalore) and northern (Bidar) districts of Karnataka were subjected for the study. In addition, the clinical samples included 42 milk samples collected from clinical mastitis cases presented in the clinics attached to Veterinary College, Bidar/ Bangalore.

Screening for sub clinical mastitis

All the milk samples (n=313) collected from organized/ unorganized sectors were screened for subclinical mastitis by SCC, EC test, CMT and BTB strip test. By employing conventional cut off criteria of SCC ≥ 5,00,000 cells/ ml and EC ≥ 6.5 milli Siemens/cm, evaluation of SCC of 313 milk samples indicated SCM at 42.8% (134/313) while EC indicated overall SCM at 48.2% (151/313). CMT detected SCM in 67 (21.4 %) of the 313 samples screened. A total of 84 (26.8%) samples were found to be positive by BTB strip test.

In total, 220 out of 313 (70.29 %) milk samples were found positive for SCM when any of the four tests used in the study indicated positivity and the remaining 93
(29.71 %) samples were SCM negative by all the four tests. Further, all the samples were subjected for staphylococcal isolation following standard microbiological techniques.

**Isolation and Identification of CoNS isolates by phenotypic methods**

In all, total of 293 Staphylococcal isolates including 20 isolates from clinical samples were subjected for catalase, thermonuclease and coagulase tests. Based on the results of coagulase tests, the isolates were further categorized as either CoNS or CoPS and in all, a total of 152 CoNS and 141 CoPS isolates were obtained.

CoNS were isolated in 44.09% of the milk samples. All CoNS isolates were further characterized by KB004 HiStaph™ Identification Kit™ which consisted of 12 different tests *viz.* Voges Proskauer test, Phosphatase test, O-nitrophenyl-β-D-galactopyranoside test, Urease test, Arginine Utilisation test and, tests for the fermentation of sugars such as Mannitol, Sucrose, Lactose, Arabinose, Raffinose, Trehalose and Maltose.

All 152 CoNS isolates as well as 7 staphylococcal MTCC strains were subjected to speciation based on biochemical profile. Twenty-four different CoNS species were detected from the 313 bovine milk samples. The predominant CoNS species were *S. arlettae, S. fleurettii, S. equorum, S. epidermidis, S. saprophyticus, S. sciuri, S. xylosus, S. muscae* and *S. sciuri subsp. carnaticus*. The staphylococcal species that were isolated less frequently included *S. warneri, S. homini, S. vitulus* and *S. cohnii subsp cohnii*. Twenty-four isolates could not be identified to species level based on their biochemical properties. There was no difference in the variety of the CoNS species detected in SCM positive and SCM negative samples.
In clinical cases of mastitis, the CoNS species identified were *S. fleurettii*, *S. muscae*, *S. aureus*, *S. chromogenes*, *S. felis* and *S. auricularis*. The remaining four isolates did not conform to any of the species. Interestingly, only four out of seven MTCC reference cultures were identified correctly by the Hi-Media identification system used in the study. One was misidentified and remaining two cultures did not correspond to the species designated by MTCC.

**Identification of CoNS isolates by molecular methods**

DNA was extracted from all the CoNS isolates and they were first confirmed at genus level by the partial amplification of *Staphylococcus* genus specific sequence of the *tuf* gene using primers designed earlier. Of the 152 isolates, 149 isolates yielded 235 bp amplicon of *tuf* gene and the remaining three isolates did not yield the desired amplicon specific for the *Staphylococcus* genus and hence they were considered as non-staphylococcal strains.

For development of PCR for detection of the CoNS species, the five predominant CoNS species (*S. arlettae*, *S. fleurettii*, *S. equorum*, *S. epidermidis* and *S. saprophyticus*) identified based on the outcome of phenotype-based speciation and five CoNS species (*S. chromogens*, *S. simulans*, *S. sciuri*, *S. haemolyticus* and *S. xylosus*) which were reported to be predominant elsewhere by molecular methods were considered to be included. For this, a total of 10 different sets of species specific primers were designed to detect CoNS at species level by PCR.

Initially, the gradient PCRs were set up for each of the species targeted in the study by using known positive reference cultures. Although all the primers amplified at
all temperatures tested (51.8 to 60.1 °C), 60 °C was finally considered to be used in the m-PCR to avoid non-specific amplification at lower temperatures.

The primers to be used for multiplex PCR were first evaluated for their specificity by uniplex PCR using known positive and negative controls. Of the 10 species of CoNS targeted by m-PCR, uniplex PCR was successfully standardized for seven species *viz.*, *S. arlettae*, *S. chromogenes*, *S. sciuri*, *S. epidermidis* and *S. saprophyticus* all of them included in tube-1, and the other two species *viz.*, *S. haemolyticus* and *S. xylosus* were included in tube-2. Uniplex PCR for the remaining species; *S. equorum*, *S. fleurettii* and *S. simulans* could not be standardized as the positive reference strains were not available, yet they were included for m-PCR in the second tube with the aim of detecting them among the isolates.

A two-tube m-PCR was successfully standardized and the desired amplicons were obtained in both the tubes. Positive reference cultures used in tube-1 reaction yielded 216 bp, 303 bp, 354 bp, 466 bp and 630 bp size amplicons specific for *S. arlettae*, *S. chromogenes*, *S. sciuri*, *S. epidermidis* and *S. saprophyticus*, respectively. Similarly, those in tube-2 reaction yielded 292 bp and 433 bp amplicons specific for *S. haemolyticus* and *S. xylosus* respectively. All the bands were well resolved on gel.

The two-tube m-PCR developed as above was used to screen all the CoNS (n=149) isolates for identification of species. Based on PCR results, *S. epidermidis* (n=34), *S. chromogenes* (n=33), and *S. sciuri* (n=22) were the major CoNS species detected in this study; followed by *S. xylosus* (n=14), *S. saprophyticus* (n=7), *S. haemolyticus* (n=6) and *S. arlettae* (n=3).
Current study revealed the existence of coagulase negative variants of *S. aureus* of bovine origin. CoNS isolates that were negative by two-tube m-PCR including phenotypically thermonuclease positive isolates were subjected for triplex PCR specific for *S. aureus* developed and standardized earlier. Interestingly, 19 of the 20 thermonuclease positive isolates yielded 210 bp, 461 bp and 1250 bp amplicons of *fib, nuc* and *23S rRNA* genes specific for *S. aureus*. The remaining isolates (n=11) did not yield any amplicon. Finally, 11 CoNS isolates were negative by both two-tube m-PCR specific for 10 different CoNS species and triplex PCR specific for *S. aureus*.

The genotyping (m-PCR) of CoNS was compared with phenotyping (using HiStaph™ Identification Kit and supplementary tests) with regard to the speciation. Only 17 (11%) of the 152 strains were identified correctly by the phenotypic method and 107 (71%) strains were misjudged. The remaining 28 (18%) strains had no match in the list of CoNS species provided by the kit manufacturer.

The PCR products/ species specific amplicons of the CoNS isolates (n=30) were purified and commercially sequenced. The sequence data obtained was subjected for BLAST with NCBI nucleotide sequence data library and the alignment studies were carried out and, they were confirmed to be the same species as detected by PCR.

The phylogenetic trees were constructed and the evolutionary history/ genetic relatedness was inferred using the Neighbour-Joining method for all the CoNS species detected in the study using corresponding sequences from both published reference strains as well as strains isolated in this study. Phylogenetic analysis revealed wide genetic diversity among the CoNS with respect to *gap/sodA/rpoB* gene sequences.
signifying their higher discriminating power *vis-à-vis* species detection by molecular methods targeting these genes.

**Antimicrobial resistance of CoNS isolates**

*In-vitro* antimicrobial sensitivity of CoNS isolates by disc diffusion method using 12 different antibiotics that are commonly used for the treatment of mastitis revealed that most of the CoNS isolates (127 of 149, 85.23 %) were found to be multi-drug resistant *i.e.*, resistant to more than 3 class of antibacterials used in the study. Four isolates were found to be resistant to all the antibacterials used in the study.

Highest resistance was detected to oxacillin (95.97%) and methicillin (85.91%) in CoNS as a whole, followed by ceftriaxone with sulbactum/ tazobactum (72.48%; 71.14%), penicillin G (68.46%), ceftriaxone (62.42%), amoxycillin with sulbactum (51.68%), ampicillin (51.01%) and streptomycin (44.3%). Highest sensitivity was recorded for chloramphenicol (81.88%) followed by enrofloxacin (70.47%) and gentamicin (59.73%).

The statistical analysis of the data by the Pearson chisquare as well as Liklihood ratio chisquare and Fisher’s exact test revealed that the association of CoNS species with antimicrobial resistance was significant (p< 0.001).

Based on the phenotypic resistance pattern, the antibiotic resistance genes *mecA* as the indicator of methicillin resistance and, *aacA*-D and *aph3'-IIIa* as the indicators of aminoglycoside resistance were targeted for detection by m-PCR. Upon detection of phenotypically coagulase negative *S. aureus* in the present study, the *coa* gene encoding the enzyme coagulase, was also targeted by m-PCR. The m-PCR was initially developed
and standardized using published primers and used to screen CoNS isolates for the presence of these genes.

Of the 149 strains tested for genotypic methicillin resistance, 10 strains yielded 304bp amplicon of \textit{mec}A and none of the strains yielded 450bp amplicon of novel \textit{mec}A gene. When the genotypic aminoglycoside resistance was tested, 11 of the 149 strains were positive for \textit{aac}A-D gene, whereas, 4 strains were positive to both \textit{aac}A-D and \textit{aph}(3')-\textit{III}a, and only one strain was positive to \textit{aph}(3')-\textit{III}a alone. Of the 15 strains tested positive to either of the aminoglycoside resistance genes, 9 (50\%) strains belonged to \textit{S. epidermidis} alone.

Of 19 coagulase negative \textit{S. aureus} isolates identified in this study; 14 isolates yielded 850 bp amplicon specific for \textit{coa} gene, one isolate yielded an amplicon of higher size (~1100 bp) and the remaining four did not yield any amplicon.

MIC levels of oxacillin and methicillin were determined using commercial MIC paper strips for the \textit{mec}A positive as well as few negative isolates. Likewise, \textit{aac}A-D/\textit{aph}(3')-\textit{III}a positive and negative isolates were used for determination of MIC levels of gentamicin. The MIC values of oxacillin in the \textit{mec}A positive strains ranged from 0.256 to >256 and in \textit{mec}A negative strains, it ranged from 0.064 to >256. Similarly, the MIC values of methicillin in the \textit{mec}A positive and negative strains ranged from 0.5 to 30 and <0.001 to 2, respectively.

The MIC values of gentamicin in \textit{aac}A-D/\textit{aph}(3')–\textit{III}a positive strains were in the range of 0.064 to 8, whereas, in genotypically negative strains the least values (<0.064 to 0.128) were recorded.