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**RESEARCH ARTICLE**

# Development of Simplex-PCR assays for Accurate Identification of Nine Staphylococcal Species at Genus and Species Levels

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## ABSTRACT

**Objective:** Species identification of *Staphylococcus* is the prerequisite for precise assessment of microbial dynamics including their transmission and pathogenic significance in the dairy herd environment. The present study aimed to develop simplex PCR assays for rapid and specific identification of nine different *Staphylococcus* species.

**Methods:** Specific primers targeting *sodA* gene for *S. aureus*, *S. chromogenes*, *S. hominis*, *S. haemolyticus*, *S. hyicus*; *gap* gene for *S. sciuri, S. auricularis, S. simulans*; and *rdr* gene for *S. epidermidis* were designed. The PCR assays were evaluated against 28 ATCC reference strains and 209 composite milk samples. Partial *16S rRNA* sequencing was performed to reconfirm the results.

**Results:** The PCR assays allowed species level identification of 348 staphylococcal field isolates recovered from 209 milk samples. The identification pattern was *S. aureus* (n=101), *S. chromogenes* (n=89), *S. epidermidis* (n=57), *S. sciuri* (n=43), *S. haemolyticus* (n=34), *S. hyicus* (n=13), *S. hominis* (n=5), *S. auricularis* (n=3) and S. simulans (n=3). The PCR based species identification was in 100% concordance with the partial *16S rRNA* gene sequencing approach.

**Conclusion:** The simplex PCR assays can be used as a precise tool for routine identification of *Staphylococcus* species from bovine milk as *Staphylococcus* species including coagulase-negative staphylococci is recognized a major cause of bovine mastitis in different parts of the world including India. *J Microbiol Infect Dis 2018; 8(3):120-127*

**Keywords:** *16S rRNA* gene sequencing, *Staphylococcus*, Species-specific PCR

## INTRODUCTION

Bovine mastitis remains the foremost ailment of dairy cattle, causing economic losses up to 1.3 billion $ to dairy farmers [1]. In spite of the fact, diverse organisms have been isolated from bovine mastitis, the epidemiology of bovine intra-mammary infection (IMI) has revealed a high prevalence of *Staphylococcus* [2,3]. Amongst *Staphylococcus spp.*, *Staphylococcus* aureus is the major pathogen responsible for IMI. As of late, a shift has been seen in the microbial populace of the udder towards an increased occurrence of Coagulase Negative Staphylococci (*CoNS*) both in clinically healthy cows as well in cows influenced by mastitis and are designated as emerging pathogen in bovine mastitis [4,5]. However, the complete knowledge of *CoNS* species involved in bovine mastitis is still limited and benefits can accrue from having more reliable diagnostic methods for species identification [4]. In fact, the data pertaining to clinical significance, therapy and/or management of *CoNS* mastitis that depends on exact species identification is still inadequate [6]. Thus, owing to significant losses caused by mastitis to the dairy industry and the possible influence of the *CoNS* in the progression of this disease, precise and reliable strategies are required to unravel the diverse *Staphylococcus spp.* [5].

Pathogen detection has usually been based on traditional diagnostic methods that are

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immensely tedious, time-*CoNS*uming and often lack reliability. Besides, an intrinsic drawback of phenotypic strategies is the changeability in expression, interpretation and reproducibility of phenotypic characteristics [6,7]. In comparison, genotypic methods have superior resolving power and reproducibility. The advent of molecular techniques, especially, the DNA sequence-based methods have made the species identification of *Staphylococcus* more accurate as it is possible to compare with the reference database that aids in determining homology with the known species [6]. The partial *16S rRNA* gene sequencing approach is well established gold standard method for the recognition and classification of wide-variety of organisms including *Staphylococcus* spp [7,8]. Although useful; however, this technique may not be feasible for routine identification as it is relatively expensive, time-*CoNS*uming and labor intensive. Therefore, a simple and dependable molecular test which provides a promising option for rapid and accurate identification of *Staphylococcus* species remains a necessity.

Hence, the present study targeted the *Staphylococcus spp.* commonly isolated from bovine milk namely *S. aureus*, *S. chromogenes*, *S. epidermidis*, *S. sciuri*, *S. haemolyticus*, S. simulans, *S. hyicus*, *S. hominis*, and *S. auricularis* for the development of simplex PCR targeting different genes.

## METHODS

### *Standard bacterial strains*

The type strains included 13 *Staphylococcus spp.* comprising of *S. aureus* and *CoNS*, and 15 strains of non-staphylococci that are phylogenetically related to genus *Staphylococcus* procured from American Type Culture Collection (Manassas, VA, USA) (Table 1).

### *Sample collection*

The udder was cleaned thoroughly with an antiseptic solution and the teat ends were sanitized with swabs containing 70% alcohol. The foremilk was discarded and a total of 209 composite milk samples (20 mL pooled, at the rate of 5 mL/quarter) were collected aseptically from all the lactating cows showing no apparent signs of mastitis from three dairy herds maintained in intensive, semi-intensive and/or extensive system in three villages in and around Bangalore, India. The milk samples were transported in a cold chain for laboratory processing.

### *Isolation of Staphylococcus spp.*

Enrichment of each milk sample (0.5 mL) was carried out in brain heart infusion (BHI) broth for 6 h at 37 $^0$C. An inoculum of 10 µL of each enriched sample was streaked on Gelatin Mannitol Salt Agar (*Staphylococcus* 110 medium; Himedia Laboratories, Mumbai, India) followed by incubation at 37 $^0$C for 24 h. pure cultures were obtained by subculturing on Brain Heart Infusion agar (Himedia Laboratories, Mumbai, India). Organisms suspected to be *Staphylococcus* on the basis of colony morphology, pigmentation, Gram staining, catalase and oxidase tests were stored frozen at -40 $^0$C in nutrient broth containing 15% glycerol for further confirmation.

### *Genomic DNA extraction*

Genomic DNA from the overnight grown pure colonies were extracted using QIAamp DNA minikit (Qiagen, Düsseldorf, Germany) according to the manufacturer's recommendations. The concentration of the extracted DNA was determined spectrophotometrically using Nanodrop 2000C (Thermofischer Scientific Inc., Waltham, MA, USA) and stored at -20 $^0$C until use.

### *Simplex PCR assays*

A *Staphylococcus* genus-specific PCR was devised. The genus-specific primer pair, flanking five hypervariable sequences (V3, V4, V5, V6, and V7) was designed to amplify 842 bp fragment of *16S rRNA* for the direct detection of the genus.

Simultaneously, based on the predominance of species distribution observed from partial gene sequencing analysis, species-specific PCR was developed for the major 9 species. Nine different species-specific primer sets were designed for *S. aureus*, *S. chromogenes*, *S. epidermidis*, *S. sciuri*, *S. haemolyticus*, *S. hyicus*, *S. hominis*, *S. auricularis* and S. simulans targeting different gene sequences available in the GenBank (Table 2). The specificity of each primer pair was checked with a panel of 28 reference ATCC strains comprising of *Staphylococcus spp.* and

other bacteria phylogenetically close to *Staphylococcus* (Table 1). Simplex PCR assay for amplification of each organism was performed in 15 µL reaction volume containing 1x PCR master mix (0.025 U Taq polymerase in reaction buffer, 2 mM MgCl2, 200 µM deoxynucleotide triphosphates; Fermentas, Glen Burnie, MD, USA) and 50 ng extracted DNA. The cycling conditions involved an initial denaturation at 94 $^0$C for 5 min, followed by 30 cycles of denaturation at 94 $^0$C for 30 s, annealing (temperature as in Table 2) for 30 s and extension at 72 $^0$C for 45 s followed by final extension at 72 $^0$C for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel with 10 µg mL-1 ethidium bromide and the results were visualized using a gel documentation system (Gbox, Syngene, UK).

### *Species identification by partial 16S rRNA gene sequence analysis*

*16S rRNA* gene sequence analysis was performed for definitive species identification. Based on the multiple sequence alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html) variable regions of *16S rRNA* were located and a primer pair flanking five hypervariable regions (V2, V3, V4, V5 and V6) was designed to amplify a 974 bp fragment for the partial *16S rRNA* gene sequence analysis, using Primer 3 software (Table 1) [8]. All the isolated Gram-positive bacteria were subjected to PCR for amplification of the 974 bp fragment of the *16S rRNA* gene.

The PCR reaction setup and cycling conditions for amplification of each organism were similar as described in section 2.5 with 60 $^0$C as the specific annealing temperature (Table 2). The amplified products were purified with QIAquick PCR purification kit (Qiagen, Duüsseldorf, Germany) and sequenced commercially (Eurofins Genomics, Bangalore, India) at both strands using the ABI 3730 sequencer. The sequences were analyzed by comparing with those accessible in the GenBank using Blast search and edited with Chromas Lite 2.01 software (http://www.technelysium.com.au/chromas_lite.html). Match score of >99% on nucleotide blast was acknowledged for the species identity. The sequences were submitted to the GenBank.

### *Ethics*

This study was conducted as per the applicable rules and regulations of good clinical practice. The study was approved by the independent institutional ethics committee of ICAR-NIVEDI.

## RESULTS

### *Isolation and identification of Staphylococcus spp.*

Out of 209 composite milk samples processed, a total of 400 catalase-positive Gram-positive cocci with smooth, pigmented colonies were isolated. Out of the 400 Gram-positive cocci, 348 isolates (87%) were confirmed as *Staphylococcus spp.* by genus-specific PCR (Figure 1).

### *Species-specific PCR*

The 9 different species-specific primers successfully amplified their respective species viz., *S. aureus*, *S. chromogenes*, *S. epidermidis*, *S. sciuri*, *S. haemolyticus*, *S. hyicus*, *S. hominis*, *S. auricularis* and S. simulans (Figure 2). The sequence analysis of selected ampli*CoNS* confirmed the amplification of the desired products. None of the oligonucleotide primers amplified with the other closely related *Staphylococcus spp.* or with other bacteria phylogenetically close to *Staphylococcus*. The distribution of 348 field isolates of *Staphylococcus spp.* were *S. aureus* (n=101), *S. chromogenes* (n=89), *S. epidermidis* (n=57), *S. sciuri* (n=43), *S. haemolyticus* (n=34), *S. hyicus* (n=13), *S. hominis* (n=5), *S. auricularis* (n=3) and S. simulans (n=3) (Table 3).

### *Partial 16S rRNA gene sequence analysis*

The Partial *16S rRNA* gene sequences obtained from the 348 *Staphylococcus* isolates indicated high sequence similarity (>99%) with those sequences available in GenBank resulting in definitive identification of *Staphylococcus spp.* The speciation achieved by the partial *16S rRNA* gene sequence analysis was concordant with the PCR based identification results. All the staphylococcal partial *16S rRNA* gene sequences are available in GenBank (Table 3).

Table 1. Evaluation of the specificity of the designed oligonucleotide primers using different *Staphylococcus spp.* and other p species.

| Reference ATCC cultures used | | SAS2F SAS2R | SCHS1F SCHS1R | SHS2F SHS2R | SSCGF SSCGR | SERF SERR | SH SH |
|---|---|---|---|---|---|---|---|
| *S. aureus* subsp. *Aureus* | ATCC12598 | + | - | - | - | - | |
| *S. chromogenes* | ATCC 43764 | - | + | - | - | - | |
| *S. haemolyticus* | ATCC 29970 | - | - | + | - | - | |
| *S. sciuri* subsp. *Sciuri* | ATCC 29062 | - | - | - | + | - | |
| *S. epidermidis* | ATCC 12228 | - | - | - | - | + | |
| *S. hyicus* | ATCC11249 | - | - | - | - | - | |
| *S. hominis* subsp. *Hominis* | ATCC 27844 | - | - | - | - | - | |
| *S. simulans* | ATCC 27848 | - | - | - | - | - | |
| *S. cohnii* subsp. *Cohnii* | ATCC 29974 | - | - | - | - | - | |
| *S. xylosus* | ATCC 12162 | - | - | - | - | - | |
| *S. auricularis* | ATCC 33753 | - | - | - | - | - | |
| *S. saprophyticus* | ATCC 15305 | - | - | - | - | - | |
| *S. warneri* | ATCC 27836 | - | - | - | - | - | |
| *Streptococcus agalactiae* | ATCC13813 | - | - | - | - | - | |
| *Streptococcus dysgalactiae* subsp. *dysgalactiae* | ATCC43078 | - | - | - | - | - | |
| *Streptococcus uberis* | ATCC19436 | - | - | - | - | - | |
| *E. coli* | ATCC 25922 | - | - | - | - | - | |
| *Lactococcus lactis* subsp. *Lactis* | ATCC11454 | - | - | - | - | - | |
| *Salinicoccus roseus* | ATCC 49258 | - | - | - | - | - | |
| *Leuconostoc mesenteroides* subsp. *mesenteroides* | ATCC 8293 | - | - | - | - | - | |
| *Aerococcus viridians* | ATCC11563 | - | - | - | - | - | |
| *Micrococcus aurantiacus* | ATCC 11731 | - | - | - | - | - | |
| *Lactobacillus acidophilus* | ATCC 4356 | - | - | - | - | - | |
| *Pediococcus acidilactici* | ATCC 25740 | - | - | - | - | - | |
| *Planococcus citreus* | ATCC 14404 | - | - | - | - | - | |
| *Saccharococcus thermophilus* | ATCC 43125 | - | - | - | - | - | |
| *Gemella haemolysans* | ATCC 10379 | - | - | - | - | - | |
| *Macrococcus caseolyticus* | ATCC 13548 | - | - | - | - | - | |

Positive (+): PCR amplification for the primers, Negative (-): No PCR amplification for the primers.
ATCC: American type culture collection. SAS2, Staph. aureus; SCHS1, Staph. chromogenes; SHS2, Staph. haemolyticus; SSCG, Staph. sci hyicus; SHOS1, Staph. hominis; SSIG, Staph. simulans; SAUG, Staph. auricularis.

Table 2. The details of oligonucleotide primers designed for genus- and species -specific identifications of *Staphylococcus spp.*

| Organism | Target/ gene[a] | GenBank Nucleotide/Contig Accession no. | Primer designation | Oligonucleotide primer (5`-3`) | Location |
|---|---|---|---|---|---|
| *Staphylococcus spp.* | *16S rRNA* | D83356 | SG16P1F[b] | GTG ATC GGC CAC ACT GGA | 285 – 302 |
| | | | SG16P1R[b] | CAA CTT AAT GAT GGC AAC TAA GC | 1126 – 1104 |
| *Staphylococcus spp.* | *16S rRNA* | D83356 | SSEQIF[c] | GCG GAC GGG TGA GTA ACA C | 78 – 96 |
| | | | SSEQIR[c] | GAC GAC AAC CAT GCA CCA C | 1051 – 1033 |
| *S. aureus* | 23S r RNA | X68425 | SAS2F | AGC GAG TCT GAA TAG GGC GTT T | 678 – 699 |
| | | | SAS2R | CCC ATC ACA GCT CAG CCT TAA C | 1571 - 1550 |
| *S. chromogenes* | *SodA* | AJ343945 | SCHS1F | GCG TAC CAG AAG ATA AAC AAA CTC | 134 -157 |
| | | | SCHS1R | CAT TAT TTA CAA CGA GCC ATG C | 355 – 334 |
| *S. haemolyticus* | *SodA* | EU652775 | SHS2F | CAA ATT AAA TTC TGC AGT TGA GG | 42 – 64 |
| | | | SHS2R | GGC CTC TTA TAG AGA CCA CAT GTT A | 572 – 548 |
| *S. hyicus* | *SodA* | AJ343913 | SHYS1F | TAT TGA AGA GCT TAT CGC GAA TGT | 105 – 128 |
| | | | SHYS1R | ATC GTG CTG CTG CTT TAT CTG AG | 319 – 297 |
| *S. epidermidis* | *Rdr* | CP000029 | SERF | AAG AGC GTG GAG AAA AGT ATC AAG | 400016 – 40003 |
| | | | SERR | TCG ATA CCA TCA AAA AGT TGG | 400145 – 40011 |
| *S. sciuri* | *Gap* | EU659914 | SSCGF | GAT TCC GCG TAA ACG GTA GAG | 122 – 142 |
| | | | SSCGR | CAT CAT TTA ATA CTT TAG CCA TTG GA | 427 – 402 |
| *S. auricularis* | *Gap* | AF495476 | SAUGF | TGC AAG GTC GTT TCA CAA GT | 127 – 146 |
| | | | SAUGR | TGT ACC ATC AAG TGT ATC GTG GT | 404 – 382 |
| *S. simulans* | *Gap* | DQ321698 | SSIGF | AGC TTC GTT TAC TTC TTC GAT TGT | 171 - 194 |
| | | | SSIGR | AAA AGC ACA AGC TCA CAT TGA C | 642 – 621 |
| *S. hominis* | *SodA* | NZ_ACLP01000030 | SHOS1F | TTT TAA GCA AGA CAA TCG ACC TCA | 176518 – 17654 |
| | | | SHOS1R | CCA AAT TTA CCA TAT GCA GCA G | 177244 – 17722 |

a *sodA*: superoxide dismutase A; *rdr*: ribonucleoside diphosphate reductase gene; *gap*: glyceraldehyde-3-Phosphate Dehydrogenase gene.
b Genus-specific.
c Primer for amplification of partial *16S rRNA* gene for sequence based identification.

Table 3. Distribution of identified *Staphylococcus spp.* from bovine milk samples from organised and unorganised dairy sectors.

| | Organised Farms | | | | |
|---|---|---|---|---|---|
| | **farmA (30) [a]** | **farmB (42) [a]** | **farmC (61) [a]** | **unorganised sector (76) [a]** | **T** |
| Species | | | | | |
| *S. aureus* | 8 | 10 | 68 | 15 | 1 |
| *S. chromogenes* | - | 15 | 36 | 38 | |
| *S. epidermidis* | 12 | 9 | 29 | 7 | |
| *S. sciuri* | - | 9 | 25 | 9 | |
| *S. haemolyticus* | 4 | 11 | 12 | 7 | |
| *S. hyicus* | - | 8 | - | 5 | |
| *S. hominis* | - | - | 5 | - | |
| *S. auricularis* | - | 3 | - | - | |
| *S. simulans* | - | 3 | - | - | |

a Number of cows screened are in parentheses.

## DISCUSSION

The general learning of the *CoNS*ortia group and the significance of the causative agents in bovine mastitis stay feeble as most investigations to date were compelled by the *CoNS*traints of traditional microbiological methods [9,10]. Despite the high prevalence of *Staphylococcus spp.* especially the *CoNS* as the main pathogen causing bovine intramammary infection (IMI) in numerous nations, still a precise, quick and advantageous method that can differentiate between the bacterial species of this family is not available [11]. Though phenotypic identification of *CoNS* is widely used, molecular assays have been appeared to be more discriminatory between the *CoNS* species [12].

The gene sequencing approach, as a last resort for unambiguous identification, provides an exact molecular identification of *CoNS* species, specifically through sequencing of hsp60, *tuf*, rpoB, *16S rRNA*, *sodA*, dnaJ, and/or *gap* gene [5, 13, 14]. However, this strategy may not be feasible for routine identification on daily basis in clinical diagnostic set-up, since it is time-*CoNS*uming, labor-intensive, and economically unviable for analysis of a large number of isolates. In order to overcome these limitations, a PCR based identification protocol was standardized in the present study for 9 different *Staphylococcus spp.*

For genus-level identification of *Staphylococcus*, analysis of partial *16S rRNA* gene sequence was carried out targeting hypervariable regions V2 and V3 which allows differentiation between organisms across all major phyla of bacteria [15]. Accordingly, the genus *Staphylococcus* was identified using a novel set of the *16S rRNA* gene specific primer designed in the present study. For bacterial phylogeny and taxonomy, *16S rRNA* is the most commonly targeted gene. However, it does not possess adequate discriminatory ability to differentiate all the species within the genera *Staphylococcus* [6, 13,16]. Hence, several studies have been reported on species identification system based on other target regions mainly housekeeping genes like *cpn*60 (chaperonin or heat shock protein 60), *sodA* [16,17], *gap* [16], *tuf* [18] for differentiation of *Staphylococcus spp.* Besides, the 16S-23S rRNA intergenic spacer region is also useful for identification of strains as it suffers lesser evolutionary pressure [19]. Similarly, the *sodA* gene was found to *CoNS*titute a highly discriminative target sequence for differentiating closely related bacterial species [3,20]. Ghebremedhin et al., suggested *gap* gene to be an ideal target even for taxonomic analysis of *Staphylococcus spp.* [16]. Accordingly, in the present study, 9 different species-specific PCR primers were targeted for the important staphylococcal species. Species-specific PCR assay targeting the DNA sequences showing potential discriminatory power namely, *sodA* gene for *S. aureus*, *S. chromogenes*, *S. hominis, S. haemolyticus, S. hyicus*; *gap* gene for *S. sciuri, S. auricularis, S. simulans*; and *rdr* gene for *S. epidermidis* was developed. The PCR was verified on 348 field isolates and evaluation with the 28 standard ATCC strains confirmed the specificity of the primers, thereby, eliminating the likelihood of any kind of misidentification.

Subsequently, to validate the accuracy and reliability of the described assays, the partial *16S rRNA* gene sequence analysis, a methodology broadly acknowledged as a standard technique for identification of *Staphylococcus spp.* as well as other micro-organisms was utilized [21]. The observations achieved using partial *16S rRNA* gene sequence analyses were concordant with the species-specific PCR results confirming the accuracy of the primers and the PCR protocol developed.

In conclusion, the PCR assays described in this study were demonstrated to be a valuable and efficient tool for swift and specific identification of 9 *Staphylococcus spp.* In view of the fact that appropriate species identification is imperative for curbing the mastitis burden and monitor epidemiological profile, this species-specific PCR will prove to be a satisfactory tool for the identification of the common staphylococcal species, independent of their phenotypic traits. In turn, identification of bacteria will help to determine an effective antimicrobial treatment as well to monitor and control the rate of infection at the farm level. Besides, the assay may likewise be utilized for the quality control of milk and other edibles in addition to the detection of this pathogen in clinical and ecological settings [22].

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## REFERENCES

1. Bansal BK, Gupta DK. Economic analysis of bovine mastitis in India and Punjab – A Review. Ind J Dai Sci 2009; 62:337-344.
2. Sumathi BR, Veeregowda BM, Gomes AR. Prevalence and antibiogram profile of bacterial isolates from clinical bovine mastitis. Vet World 2008; 1:237-238.
3. Shome BR, Mitra SD, Bhuvana M, et al. Multiplex PCR assay for species identification of bovine mastitis pathogens. J Appl Microbiol 2011; 111:1349-1356.
4. Pyorala S, Taponen S. Coagulase-negative staphylococci- emerging mastitis pathogens. Vet Microbiol 2009; 134:3-8.
5. Braem G, De Vliegher S, Supre K, Haesebrouck F, Leroy F, De Vuyst L. (GTG)5-PCR fingerprinting for the classification and identification of coagulase-negative *Staphylococcus* species from bovine milk and teat apices: A comparison of type strains and field isolates. Vet Microbiol 2011; 147:67-74.
6. Zadoks RN, Watts JL. Species identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. Vet Microbiol 2009; 134:20-28.
7. Park JY, Fox LK, Seo KS, et al. Comparison of phenotypic and genotypic methods for the species identification of coagulase-negative staphylococcal isolates from bovine intramammary infections. Vet Microbiol 2011; 147:142-148.
8. Rozen S, Skaletsky HJ. Primer-3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (Eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, USA; 2000.p.365-386.
9. Kuang Y, Tani K, Synnott AJ, et al. Characterization of bacterial population of raw milk from bovine mastitis by culture- independent PCR-DGGE method. J Biochem Engg 2009; 45:76–81.
10. Cremonesi P, Pisoni G, Severgnini M, et al. Pathogen detection in milk samples by ligation detection reaction-mediated universal array method. J Dairy Sci 2009; 92:3027-3039.
11. Supre K, Haesebrouck F, Zadoks RN, Vaneechoutte M, Piepers S, De Vliegher S. Some coagulase-negative *Staphylococcus* species affect udder health more than others. J Dairy Sci 2011; 94:2329-2340.
12. Patteet L, Goossens H, Ieven M. Validation of the MicroScan-96 for the species identification and methicillin susceptibility testing of clinical significant coagulase-negative staphylococci. Eur J Clin Microbiol Infect Dis 2012;31:747–751.
13. Shah MM, Iihara H, Noda M, et al. DnaJ gene sequence-based assay for species identification and phylogenetic grouping in the genus *Staphylococcus*. Int J Syst Evol Microbiol 2007;57:25–30.
14. Supre K, De-Vliegher S, Sampimon OC, et al. Use of transfer RNA-intergenic spacer PCR combined with capillary electrophoresis to identify coagulase-negative *Staphylococcus* species originating from bovine milk and teat apices. J Dairy Sci 2009; 92:3204–3210.
15. Chakravorty S, Helb D, Burday M, Connell C, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 2007; 69:330-339.
16. Ghebremedhin B, Layer F, Konig W, Konig B. Genetic classification and Distinguishing of *Staphylococcus* Species Based on Different Partial *gap*, *16S rRNA*, hsp60, rpoB, *sodA* and *tuf* gene sequences. J Clin Microbiol 2008; 46:1019–1025.
17. Heikens E, Fleer A, Paauw A, Florijn AC, Fluit AC. Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. J Clin Microbiol 2005; 43:2286-2290.
18. Capurro A, Artursson K, Waller KP, Bengtsson B, Ericsson-Unnerstad H, Aspan A. Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and *tuf* gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis. Vet Microbiol 2009; 134:327–333
19. Gurtler V, Stanisich VA. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiol 1996; 142:3-16.
20. Poyart C, Quesne G, Boumaila C, Trieu-cuot P. Rapid and accurate species-level identification of coagulase-negative staphylococci by using the *sodA* gene as a target. J Clin Microbiol 2001; 39:4296-4301.
21. Becker K, Harmsen D, Mellmann A, et al. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. J Clin Microbiol 2004; 42:4988–4995.
22. Schukken YH, Gonzalez RN, Tikofsky LL, et al. CNS mastitis: Nothing to worry about? Vet Microbiol 2009:134:9–14.