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

The hospital environment is a major source of nosocomial pathogens. These pathogens could contaminate and colonize surgical wounds leading to infections. These infections otherwise known as the surgical site infections (SSIs) have been shown to increase duration of stay in the hospital by up to 9.7 days, raise cost of treatment due to additional tests and therapeutic antibiotic treatment. It may also lead to additional surgery with accompanied morbidity which can occasionally be permanent and sometimes mortality [1-3].

The exogenous sources of SSI pathogens include surgical personnel (especially members of the surgical team), the operating room environment (including air), tools, instruments, and materials brought to the sterile field during an operation [4-6]. In a health care facility, various items (animate and inanimate) can harbor infectious agents and can serve as a vehicle for transmission of nosocomial infection [7]. This is further exacerbated by the ability of some microorganisms to persist for a long period on surfaces through biofilm formation and the ability of some of the organisms to resist cleaning and disinfection [8]. Frequent contamination of operating theatre air, floor, foot wears, and protective wears by pathogenic bacteria has been reported [8-10]. Dispersal of these pathogens into surgical wards and

immensely tedious, time-consuming 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-consuming 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 amplicons 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 phylogenetically closely related bacterial species.

Reference ATCC cultures used		SAS2F SAS2R	SCHS1F SCHS1R	SHS2F SHS2R	SSCGF SSCGR	SERF SERR	SHYS1F SHYS1R	SHOS1F SHOS1R	SSIGF SSIGR	SAUGF SAUGR
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. sciuri; SER,Staph. epidermidis; SHYS1, Staph. 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	Amplicon size (bp)	Annealing temp. (°C)
Staphylococcus spp.	*16S rRNA*	D83356	SG16P1F[b]	GTG ATC GGC CAC ACT GGA	285 – 302	842	60
			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	974	60
			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	894	56
			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	222	58
			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	531	58
			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	215	58
			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 – 400039	130	56
			SERR	TCG ATA CCA TCA AAA GT TGG	400145 – 400125		
S. sciuri	*Gap*	EU659914	SSCGF	GAT TCC GCG TAA ACG GTA GAG	122 – 142	306	56
			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	278	60
			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	472	60
			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 – 176541	727	60
			SHOS1R	CCA AAT TTA CCA TAT GCA GCA G	177244 – 177223		

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]	Total	GenBank accession no
Species						
S. aureus	8	10	68	15	101	HM452003-2094
S. chromogenes	-	15	36	38	89	HM367743-7826
						HM452095-2099
S. epidermidis	12	9	29	7	57	HM367827-7875
						HM452104-2111
S. sciuri	-	9	25	9	43	HM451958-1991
						HM451994-1999
						HM452101-2103
S. haemolyticus	4	11	12	7	34	HM359218-9243
						HM452114-2121
S. hyicus	-	8	-	5	13	HM451942-1954
S. hominis	-	-	5	-	5	HM451928-1932
S. auricularis	-	3	-	-	3	HM451939-1941
S. simulans	-	3	-	-	3	HM452000
						HM452100
						HM462053

a Number of cows screened are in parentheses.

DISCUSSION

The general learning of the Consortia group and the significance of the causative agents in bovine mastitis stay feeble as most investigations to date were compelled by the constraints 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-consuming, 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 constitute 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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