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Multiplex PCR for the detection of five important *Staphylococcus* sp. in bovine subclinical mastitis milk

BIBEK RANJAN SHOME[1], SUSWETA DAS MITRA[2], MANI BHUVANA[3], NATESAN KRITHIGA[4], RAJESWARI SHOME[5], DHANIKACHALAM VELU[6] and KRISHNAMSHETTY PRABHUDAS[7]

Project Directorate on Animal Disease Monitoring and Surveillance, Bengaluru, Karnataka 560 024 India

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ABSTRACT

The multiplex PCR (mPCR) was standardized for the direct detection of 5 most significant *Staphylococcus* sp., viz. *Staphylococcus aureus*, *Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus sciuri* and *Staphylococcus haemolyticus* from milk. Early detection and identification of predominantly *Staphylococcus aureus* and recent emergence of coagulase-negative staphylococci (CNS) in causing bovine mastitis is important to improve the udder health by effective treatment and control measures. The mPCR assay successfully achieved bacterial identification up to species level based on specific amplification of conserved regions of genes, viz. 23S rRNA (*S. aureus*), *sod*A (*S. chromogenes* and *S. haemolyticus*), *rdr* (*S. epidermidis*) and *gap* (*S. sciuri)* genes. The evaluation of mPCR assay with 36 ATCC reference strains and validation with 115 milk samples from subclinically infected herd and 36 bulk milk samples rendered the assay 100% specific and highly efficacious than culture method. The detection limit was found to be from 10^3 to 10 cfu/ml for the 5 target *Staphylococcus* species. The results suggest the suitability of mPCR assay to rapidly detect and differentiate 5 important *Staphylococcus* sp. in about 5 h. The method can be adopted for herd surveillance as a part of health management programme.

Key words: Bovine mastitis, Coagulase negative staphylococcus, Diagnosis, Multiplex PCR, *Staphylococcus aureus*

In India, mastitis leads to economical losses to the tune of ₹ 7165.51 crore, of which ₹ 4151.16 crore (57.93%) occurs due to subclinical mastitis (Bansal and Gupta 2009). Identification of mastitis bacterial pathogens in the milk is regarded as the definitive diagnosis of mastitis. *S. aureus* which is frequently isolated from milk is the leading cause of intramammary infections in cows (Singh and Kataria *et al.* 2009). Pyörälä and Taponen (2009) affirmed coagulase-negative *Staphylococcal* (CNS) as emerging pathogens associated with bovine mastitis and emphasized the need for reconsidering the significance of CNS. The involvement of different species CNS in bovine mastitis and their identification using a genotypic identification system (Ruegg *et al.* 2009) is a prequisite.

The conventional methods are relatively insensitive and cannot identify the etiological agents, an essential information for prevention of disease, treatment and control (Kuang *et al.* 2009, Viguier *et al.* 2009). Molecular methods are potential, but require separate PCR assays for different pathogens, which is labour intensive and expensive (Phuektes 2003). Therefore, mPCR can be a technique of choice for quick diagnosis. Thus, in view of the necessity, with the increasing clinical significance of *Staphylococcus* sp. the present study was undertaken to develop a rapid, sensitive and cost-effective mPCR for simultaneous detection of the coagulase-positive *S. aureus* and the most common coagulase-negative *Staphylococcus,* viz. *S. chromogenes, S. epidermidis, S. sciuri* and *S. haemolyticus* directly from milk.

Present address: [1]Consortium Principal Investigator (brshome@gmail.com), [5]Senior Scientist, [2,6]Research Associate, [3,4]Senior Research Fellow, [7]Formerly Project Director.

MATERIALS AND METHODS

Bacterial strains: Bacterial strains representing different species under *Staphylococcus* sp., and other phylogenetically related genera were used in the study. It included 36 American type culture collection (ATCC) reference strains (Manassas VA, USA) (Table 1) and 522 different staphylococcal species identified up to species level by 16S rRNA recovered from subclinical mastitis cases.

Milk and bacterial DNA extraction: DNA extraction from spiked and field milk samples was based on the lysis and nuclease-inactivating properties of the chaotropic agent, guanidine thiocyanate, together with the nucleic acid binding properties of the silica particles (Cremonesi *et al.* 2006). DNA

Table 1. Reference strains used for evaluation of primers

ATCC no.	Reference strains/species	SAS2	SCHS1	SER	SHS1	SSCG
A 25904	*S. aureus*	+	-	-	--	
A 43764	*S. chromogenes*	-	+	-	--	
A 12228	*S. epidermidis*	-	-	+	--	
A 29970	*S. haemolyticus*	-	-	-	+	-
A 29062	*S. sciuri*	-	-	-	-+	
A 27848	*S. simulans*	-	-	-	--	
A 11249	*S. hyicus*	-	-	-	--	
A 15305	*S. saprophyticus*	-	-	-	--	
A 33753	*S. auricularis*	-	-	-	--	
A 29974	*S. cohnii*	-	-	-	--	
A 27844	*S. hominis*	-	-	-	--	
A 12162	*S. xylosus*	-	-	-	--	
A 27836	*S. warneri*	-	-	-	--	
A 13548	*Macrococcus caseolyticus*	-	-	-	--	
A 11454	*Lactococcus lactis*	-	-	-	--	
A 11731	*Micrococcus aurantiacus*	-	-	-	--	
A 4356	*Lactobacillus acidophilus*	-	-	-	--	
A 14404	*Planococcus citreus*	-	-	-	--	
A 25740	*Pediococcus acidilactici*	-	-	-	--	
A 11563	*Aerococcus viridans*	-	-	-	--	
A 8293	*Leuconostoc mesenteroides*	-	-	-	--	
A 43125	*Saccharococcus thermophilus*	-	-	-	--	
A 10379	*Gemella haemolysans*	-	-	-	--	
A 49258	*Salinicoccus roseus*	-	-	-	--	
A 13813	*Streptococcus agalactiae*	-	-	-	--	
A 43078	*Streptococcus dysgalactiae*	-	-	-	--	
A 9927	*Streptococcus uberis*	-	-	-	--	
A 33317	*Streptococcus bovis*	-	-	-	--	
A 43765	*Streptococcus suis*	-	-	-	--	
A 43138	*Streptococcus porcinus*	-	-	-	--	
A 33398	*Streptococcus equi*	-	-	-	--	
A 19615	*Streptococcus pyogenes*	-	-	-	--	
A 6303	*Streptococcus pneumoniae*	-	-	-	--	
A 25175	*Streptococcus mutans*	-	-	-	--	
A 19258	*Streptococcus thermophilus*	-	-	-	--	
A 19433	*Enterococcus fecalis*	-	-	-	--	

Bacterial strains isolated from bovine subclinical mastitis milk samples.

Strain	Accession no.	Bacterial species	SAS2	SCHS1	SER	SHS2	SSCG
VC443	HM452003	*S. aureus*	+	-	-	-	-
AK19Y	HM452004	*S. aureus*	+	-	-	-	-
AK6W	HM367743	*S. chromogenes*	-	+	-	-	-
AK10Y	HM367744	*S. chromogenes*	-	+	-	-	-
VC206	HM367827	*S. epidermidis*	-	-	+	-	-
VC233	HM367828	*S. epidermidis*	-	-	+	-	-
VC334S2	HM359218	*S. haemolyticus*	-	-	-	+	-
VC401	HM359219	*S. haemolyticus*	-	-	-	+	-
AK11DY	HM451958	*S. sciuri*	-	-	-	-	+
AK11LY	HM451959	*S. sciuri*	-	-	-	-	+

SAS2, *S. aureus*; SCHS1, *S. chromogenes*; SER, *S. epidermidis*; SHS2, *S. haemolyticus*; SSCG, *S. sciuri*.

from purified bacterial cultures was extracted using QIAmp DNA mini kit. The concentration of genomic DNA was determined using Nanodrop 2000 c and stored at –20°C until use.

Oligonucleotide primers: Advanced genetic information provided in the database, revealed very close phylogenetic relationship within the *Staphylococcus* sp. hence designing of novel primers targeting different regions of the bacterial genome was done, viz. *sod*A: superoxide dismutase (*S. chromogenes* and *S. haemolyticus*), *rdr*: ribonucleoside diphosphate reductase (*S. epidermidis*), *gap*: glyceraldehyde-3-phosphate dehydrogenase (*S. sciuri*) and 23S rRNA (*S. aureus*). The primer combinations, accession numbers, annealing temperatures and length of the amplified products are summarized in Table 2. All the primers were designed using Primer-3 software (http: //frodo.wi.mit.edu/primer3/ (Rozen and Skaletsky 2000).

Optimization of simplex PCR assays: PCR reactions for *S. aureus* (23S rRNA), *S. chromogenes* (*sod*A), *S. haemolyticus* (*sod*A), *S. epidermidis* (*rdr*) and *S. sciuri* (*gap*) were optimized separately. The PCR in a reaction volume of 15 µl containing 0.025 U/µl *Taq*-DNA polymerase in reaction buffer, 2.0 mM $MgCl_2$, 200 µM each of dATP, dTTP, dCTP and dGTP and 100ng of extracted DNA. PCR amplification was performed in an automated thermocycler, with an initial denaturation of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing temperature for 30 s, and 72°C for 45 s, then a final extension at 72°C for 5 min.

Optimization of mPCR assay

It was performed in reaction volume of 25 µl containing 200 ng of DNA in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 200 µM each of dATP, dTTP, dCTP and dGTP, different concentrations of $MgCl_2$ (2, 2.5 and 3 mM), *Taq* DNA polymerase (1, 1.5, 2 and 2.5 U per reaction) and different combination of primers (0.1, 0.2, 0.3, 0.4 and 0.5µM) were tested. Template DNA was prepared by mixing each of the extracted bacterial DNA from *S. aureus*, *S. chromogenes, S. epidermidis, S. sciuri* and *S. haemolyticus.*

Evaluation of mPCR specificity and sensitivity: The specificity of the primers were tested in mPCR using 36 different ATCC strains relating to *Staphylococcus* sp. and other bacterial species under related genera and staphylococcal field isolates (Table 1). Sensitivity in terms of DNA concentration (pg) for which 10-fold serial dilutions of DNA from each target bacteria separately and from 5 target bacteria together were analyzed by mPCR. Similarly, sensitivity in terms of cfu/ml, 10-fold serial dilutions of spiked milk samples with *S. aureus, S. chromogenes, S. epidermidis, S. sciuri* and *S. haemolyticus* were done.

Validation of the mPCR assay: A total of 115 fourquarter pooled milk samples were collected as per National Mastitis Council (NMC) guidelines from cows from subclinically infected herds. Similarly, 36 bulk milk samples (representing 36 co-operative units) collected from chilling centres were evaluated for conventional *in vitro* culture and DNA extraction for mPCR. The species level identification of the bacterial isolates was based on biochemical tests and further confirmed by partial 16S rRNA gene sequence analysis.

RESULTS AND DISCUSSION

In simplex PCR assays, amplicons of 130bp, 222bp, 306bp, 531bp and 894bp corresponding to *S. epidermidis*, *S chromogenes, S. sciuri, S. haemolyticus* and *S. aureus,* respectively, were observed. Similarly, in mPCR, all the 5 species-specific primers showed expected amplicons (Fig. 1). The 5 PCR amplicons were clearly separated and distinguished after agarose gel electrophoresis due to appreciable difference in their lengths (Table 2). Non-specific amplification with 36 ATCC cultures were not observed

Table 2. Primer sequences, Tm values and predicted sizes of PCR products for the amplification of *Staphylococcus* species-specific target regions.

Organisms	GenBank Accession no.	Gene	Primer designation	Oligonucleotide primer (52 - 32)	Location within gene	Amplicon size (bp)	Annealing temperature (°C)
S. chromogenes	AJ343945	*sod*A	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	EU652775	*sod*A	SHS2F	CAA ATT AAA TTC TGC AGT TGA GG	42–64	531	58
			SHS2R	GGCCTCTTATAGAGACCACATGTTA	572–548		
S. epidermidis	CP000029	*rdr*	SERF	AAG AGC GTG GAG AAA AGT ATC AAG	400016–400039	130	56
			SERR	TCG ATA CCA TCA AAA AGT TGG	400145–400125		
S. sciuri	EU659914	*gap*	SSCGF	GAT TCC GCG TAA ACG GTA GAG	122–142	306	56
			SSCGR	CAT CAT TTA ATA CTT TAG CCA TTG	427–404		
S. aureus	X68425	23S r RNA	SAS2F	AGCGAGTCTGAATAGGGCGTTT	678–699	894	56
			SAS2R	CCCATCACAGCTCAGCCTTAAC	1571–1550		

sod A: superoxide dismutase, *rdr*, ribonucleoside-di-phosphate reductase; *gap*, glyceraldehyde-3- phosphate dehydrogenase.

A: American type culture collection (ATCC). SAS2, *S. aureus*, SCHS1, *S. chromogenes*; SER, *S. epidermidis*; SHS2, *S. haemolyticus*; SSCG, *S. sciuri*.

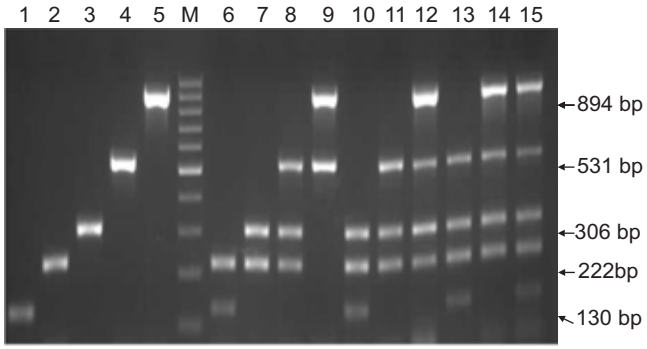

Fig 1. mPCR for detection of *Staphylococcus* sp.

Products amplified from DNA from lanes 1-5 represents: *S. epidermidis* (130bp); *S. chromogenes* (222bp); *S. sciuri* (306bp); *S. haemolyticus* (531bp); *S. aureus* (894bp) respectively; lane M: 100bp DNA marker; lane 6: combination of *S. epidermidis* and *S. chromogenes*; lane 7: combination of *S. chromogenes* and *S. sciuri*; lane 8: combination of *S. chromogenes, S. haemolyticus* and *S. sciuri;* lane 9: *S. haemolyticus and S. aureus*; lane 10: combination of *S. epidermidis, S. sciuri* and *S. chromogenes*; lane 11: combination of *S. chromogenes, S. haemolyticus* and *S. sciuri*; lane 12*:* combination of *S. chromogenes, S. aureus, S. haemolyticus* and *S. sciuri*; lane 13: combination of *S. chromogenes, S. epidermidis, S. haemolyticus* and *S. sciuri*; lane 14: combination of *S. chromogenes, S. aureus, S. haemolyticus* and *S. sciuri*; lane 15: combination of *S. chromogenes, S. aureus, S. epidermidis, S. haemolyticus* and *S. sciuri.*

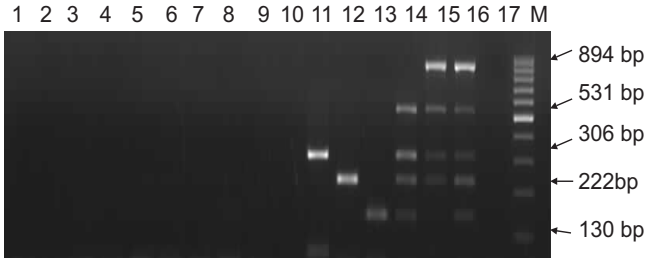

Fig 2. Specificity of mPCR.

Products amplified from DNA from lanes 1-10 represents: *E. coli*; *S. agalactiae, S. auricularis*, *S. hyicus*, *S. hominis*, *S. saprophyticus*, *S. Simulans*, *S. warneri*, *S. cohini* and *S. xylosus* strains respectively; lane 11: *S. sciuri* (306 bp); lane 12: *S.chromogenes* (222 bp); lane 13: *S. epidermidis* (130 bp); lane 14: combination of *S. chromogenes, S. epidermidis, S. haemolyticus* and *S. sciuri*; lane 15: *S. aureus , S. chromogenes, S. haemolyticus* and *S. sciuri*; lane 16: *S. chromogenes, S. aureus, S. epidermidis, S. haemolyticus* and *S. sciuri*; lane 17 and M: NTC (No Template Control) and 100bp DNA ladder respectively.

(Fig. 2). The 200ng of DNA, 1.5 U of *Taq* DNA polymerase; primer concentrations of 0.5µM for *S. chromogenes*; 0.45µM for *S. haemolyticus*; 0.4µM each of *S. epidermidis* and *S. sciuri*, and 0.35µM of *S. aureus* primer, 2 mM of $MgCl_2$ were found optimum concentrations for mPCR with 60°C annealing temperature.

In simplex PCR, 2 pg of each DNA whereas in mPCR 2 pg of DNA of *S. epidermidis* and *S. chromogenes,* 20 pg

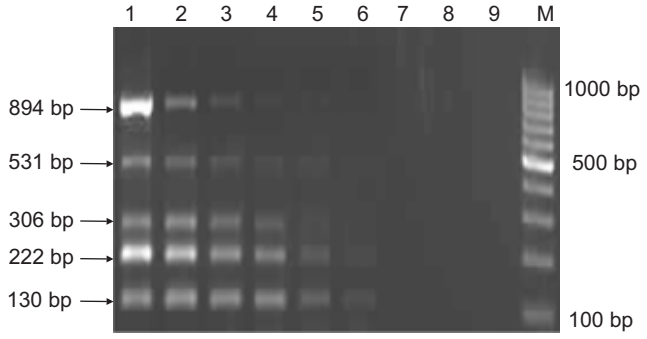

Fig 3. Sensitivity of mPCR for DNA concentration.

DNA extracted from cultures of *S. aureus*, *S. chromogenes*, *S. epidermidis, S. sciuri*, and *S. haemolyticus*. Lanes 1-8 represents concentration of DNA 200 ng, 20 ng, 2 ng, 200 pg, 20 pg, 2 pg, 200 pg and 20 pg respectively; lane 9: NTC and lane M: 100 bp DNA ladder.

from *S. haemolyticus* and 200 pg from *S. sciuri* and *S. aureus* could be detected (Fig. 3). The mPCR had a DNA detection limit of 10 cfu /ml for *S. aureus* and *S. chromogenes*; 10^2cfu /ml for *S. haemolyticus* and 10^3cfu /ml for *S. sciuri* and *S. epidermidis* in spiked milk samples. In PCR, the sensitivity detection level varied from 2 to 20 pg of DNA whereas in mPCR it ranged from 10 to 10^3 cfu/ml of milk indicating that the assay can even detect and differentiate as low as 10 bacterial counts/ml making it sensitive enough to be used as a diagnostic tool for bovine mastitis.

Isolation and partial 16S rRNA gene sequence based identification showed that, out of 115 milk samples cultured, 110 samples yielded at-least one of the 5 target pathogens (total 168), which include *S. aureus* (68), *S. chromogenes* (44), *S. epidermidis* (37), *S. haemolyticus* (12), and *S. sciuri* (7). Five samples did not show any growth.

The cumulative number of detection by mPCR were *S. aureus, S. chromogenes*, *S. epidermidis*, *S. sciuri* and *S. haemolyticus* 73, 62, 41, 8 and 13, respectively (total 197). In 36 bulk milk samples, mPCR detected *S. aureus* in 16, *S. chromogenes* in 3 and *S. epidermidis* in 2 samples. In

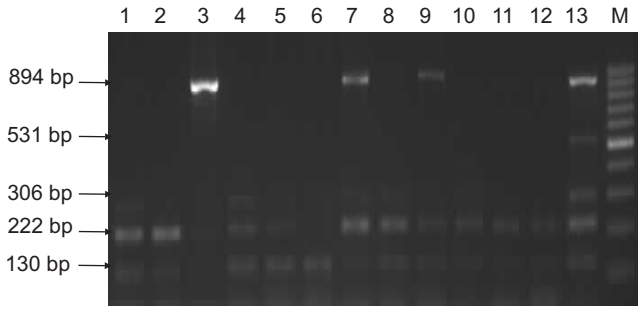

Fig 4. Representative picture showing direct detection of pathogens in field milk samples from subclinical mastitis.

Lane 1, 4 & 8: *S. epidermidis*, *S. sciuri*, *S. chromogenes*; lane 2, 5, 10, 11 & 12: *S. chromogenes* and *S. epidermidis*; lane 7 & 9: *S. chromogenes* and *S. aureus*; lane 13: Positive control (DNA from milk spiked with five organism); lane M: 100bp DNA ladder.

isolation, 12 and 1 isolates of *S. aureus* and *S. epidermidis*, respectively, were recovered. Thus collectively in 115 field and 36 bulk milk samples, 218 organisms were detected by mPCR, of which, *S. aureus* (89) was predominant followed by *S. chromogenes* (65), *S. epidermidis* (43), *S. haemolyticus* (13), and *S. sciuri* (8) (Fig. 4).

Sindhu *et al.* (2010) analysed 770 milk samples, of which 50.74 and 52.21% were diagnosed positive for *Staphylococcus sp.* by cultural examination and gap gene based genus specific PCR. Similarly, as per the isolation data, staphylococcal group was found to be predominant in mastitis in India (Sasidhar *et al.* 2002, Kumar 2009,NAIP Annual Report 2010). This appears to be the first report of species level comparison of CNS isolates from mastitis in the country.

Currently, the increase of genetic information in database revealed a better view of the close phylogenetic relationship within the *Staphylococcus* sp. Indeed for the candidate bacteria in the study, it was difficult to design primers that would specifically yield corresponding species-specific amplification of 16S rRNA or 16S–23S rRNA due to lack of considerable sequence diversity. Consequently, housekeeping genes which are considered to be useful for species identification (Ghebremedhin *et al.* 2008,Capurro *et al.* 2009), was targeted and after extensive comparative study of the molecular targets, novel species-specific mPCR primers were finally derived from 23S rRNA (*S. aureus*), *sod*A (*S. chromogenes* and *S. haemolyticus*), *rdr* (*S. epidermidis*) and *gap* (*S. sciuri*) genes.

A usual hindrance encountered with mastitis diagnosis, is PCR-inhibiting substances such as lipopolysaccharide, proteinases and high concentration of Ca^{2+} found in milk (Wilson *et al.* 1997). Accordingly DNA extraction protocol described by Cremonesi *et al.* (2006) preferred in the study.

The mPCR with 36 ATCC reference strains showed 100% specificity and all negative controls from the closest phylogenetic bacteria confirmed the specificity. The sensitivity of mPCR for detection of five different species of *Staphylococcus* showed variation with the minimum detection level of 2 pg in *S. epidermidis* and *S. chromogenes* to as high as 200 pg in *S. aureus*. The sensitivity of mPCR for 5 targets in milk varied from 10^3 to 10 cfu^2/ml of milk and these levels of detection limit are sensitive to be used as a diagnosis tool for bovine mastitis. A probable reason for variation in sensitivity between the species may be due to a difference in copy numbers of the coding regions used as prime targets.

The mPCR assay was found more efficacious than microbiological methods for direct detection of subclinical mastitis and bulk milk samples. The ability of mPCR to detect pathogens directly in milk samples was found suitable, which otherwise did not show any isolation on cultural examination. Using conventional bacteriological techniques it has been observed that on certain occasions no growth is obtained from milk samples leading to false negatives (Sharma *et al*. 2009, Taponen *et al.* 2009).

In such situations, the mPCR could be promising besides techniques like DNA microarray and Real Time PCR (RT PCR) that provide high level of sensitivity (Koskinen *et al.* 2008). However, these assays are difficult to perform in the absence of facilities and expertise (Lee *et al.* 2008). The method developed in this study is not only cost-effective but also reduces pre-enrichment step.

In conclusion, besides being economically feasible, this method can be adopted for rapid detection of staphylococcus species in milk up to species level. The test is sensitive, specific, rapid and can be completed in about 5 h. This method requires minimum affordable infrastructure and relatively little hands-on training and can thus be easily performed at a farm, district level diagnostic laboratory or at centralized laboratories. Thus, it should prove useful to the dairy industry for better health management practices.

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