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Multiplex PCR for rapid detection of *Streptococcus agalactiae, Streptococcus uberis* and *Streptococcus dysgalactiae* in subclinical mastitis milk

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ABSTRACT

To improve mastitis diagnosis and achieve rapid, specific, reliable and cost effective test, a multiplex PCR for simultaneous detection and differentiation of major streptococcal species, viz. *Streptococcus agalactiae, Streptococcus uberis* and *Streptococcus dysgalactiae* was developed. Evaluation with 24 ATCC strains and 606 strains comprising streptococci (84) and staphylococci (522) showed the assay to be highly accurate. The threshold of detection of the mPCR assay was 10fg of genomic DNA and < 10^2 CFU ml^{-1}. Assessment of 115 milk samples collected from subclinically infected herd, showed mPCR assay to be more efficacious than culture method. Identification of *Streptococcus* species using this assay will be crucial to determine prevalence of *Streptococcus* in a herd. This will facilitate early diagnosis, treatment and control of the rate of infection at farm level. The assay can be implemented for routine monitoring of herd health and can be of great value for promoting prevention of streptococcal mastitis.

Key words: Bovine mastitis, Diagnosis, Mastitis, Multiplex PCR, Streptococci

Bovine mastitis remains the most common disease of dairy cattle, causing huge economic losses to the dairy industry (Halasa *et al*. 2007, Taponen *et al*. 2009). Amongst the causative agents, *Streptococcus agalactiae, Streptococcus uberis* and *Streptococcus dysgalactiae* are the major streptococcal species associated with subclinical and clinical mastitis (Dmitriev *et al.* 2006, Pullinger *et al*. 2006, Luther *et al*. 2008, Ebrahimi *et al*. 2008). Identification of the pathogen is crucial to determine an effective antimicrobial treatment as well as to monitor and control the rate of infection at the farm level. Accordingly for a number of mastitis pathogens, PCR based techniques were described (Hassan *et al*. 2001, Daly *et al*. 2002, Lam *et al*. 2009, Sindhu *et al*. 2010, Shome *et al*. 2011). Although useful, but single PCR for each species again becomes labour intensive and expensive in mastitis diagnosis. The objective of the study was to identify unique target sequence corresponding to the streptococcal species, and develop a multiplex PCR for the simultaneous detection and differentiation of 3 major streptococcal species *Streptococcus agalactiae, Streptococcus uberis* and *Streptococcus dysgalactiae.*

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MATERIALS AND METHODS

Bacterial strains: Bacterial strains (630) representing different species or species groups of related genera of *Streptococcus* and *Staphylococcus* were used. It included 24 American type culture collection (ATCC) reference strains, *Staphylococcus* spp. (522) and *Streptococcus* spp. (84) (Table 1a, 1b). *Staphylococcus* and *Streptococcus* spp. were taken from the strain collection of our laboratory which were isolated from 294 milk samples from subclinical mastitis and identified to species level by partial 16S rRNA sequencing. The partial 16S rRNA gene sequencing was carried out using the following pairs of primers: Sseq-F 5'-GCGGACGGGTGAGTAACAC-3', Sseq-R 5'-GACGACAACCATGCACCAC-3'; Stseq-F 5'-AGCGGGGGATAACTATTGGA-3', Stseq-R 5'-ACGCATTTCACCGCTACAC-3'for *Staphylococcus* spp. (894 bp) and *Streptococcus* spp. (569 bp) respectively.

Primer designing: For the identification of the genus *Streptococcus*, a primer was designed targeting the *tuf* gene. Species-specific primers were designed targeting 16S rRNA gene for the specific identification of *Strep. agalactiae* and *Strep. dysgalactiae*. A common reverse primer sequence (ST-DG R) was employed for identification of both *Strep. agalactiae* and *Strep. dysgalactiae*. While for *S. uberis*, a published primer pair based on chaperonin-60 (Dmitriev *et*

al. 2006) was used. 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)).

Extraction of bacterial genomic DNA: DNA was extracted from overnight cultures grown on BHI agar at 37°C using mini kit as per manufacturer's instructions. The concentration of genomic DNA was determined using Nanodrop 2000C and stored at –20 °C until use. Bacterial DNA extraction directly from milk was done as per Cremonesi *et al*. (2006).

Standardization of mPCR: A simplex PCR with genus specific primer (STTUF) was optimized for the identification of the genus *Streptococcus*. To optimize the PCR reaction conditions, DNA from all the 3 species was used in separate reactions. Once the best reaction conditions were found for the single species, a mix of DNA of the 3 species was used. After several attempts, the optimal composition of mPCR consisted in 1X Taq buffer, an optimized primer concentration of STAG: 0.5 µM , STUB 0.45 µM, STDG 0.35 µM, dNTPs 0.2 mM, *Taq* polymerase 1.5 U and 100ng of template DNA. Reaction mixtures were thermally cycled once at 94°C for 5 min, followed by 30 times at 94°C for 30 s; 60°C for 30 s; 72°C for 45 s and then once at 72°C for 10 min. The amplified products were analyzed on 1.5% agarose gel containing ethidium bromide (10 µg ml^{-1}).

Accuracy of the mPCR assay: To evaluate the accuracy of mPCR for the species identification, 24 reference strains and 606 strains derived from subclinical mastitis cases were employed in this study. To determine the threshold of detection, initially pasteurised milk was spiked individually with ATCC strains 13813- *Strep agalactiae*, 43078- *Strep. dysgalactiae*, 9927- *Strep. uberis*. Further, bacterial strains previously isolated and species identified from milk, viz. *Strep. agalactiae* (HM355957), *Strep. dysgalactiae* (HM 359247) and *Strep. uberis* (HM355971) were also similarly used for spiking pasteurised milk. Subsequently, the 3 target bacteria in combinations were spiked in milk. Ten-fold serial dilutions were made from the inoculated milk samples using uninoculated pasteurised milk as a diluent. Parallel dilutions of each organism were made in distilled water and cell count was determined by standard plate count (cfu ml^{-1}). DNA was extracted from the dilutions of spiked milk and threshold of detection of PCR was then compared on dilutions containing organisms at 10^4cfu ml^{-1} to 1 cfu ml^{-1}. Similarly, to determine the detection limit of DNA, the concentration of purified DNA extracted from each target bacterial species were determined using nanodrop and 10-fold serial dilutions of purified DNA were made and subjected to mPCR.

Detection of Streptococcus *spp. in milk samples:* Composite milk samples (115) having SCC >3,00,000 cells/ ml were collected as per National Mastitis Council (NMC) guidelines for milk bacteriology. To determine the comparative efficiency, all these milk samples were processed simultaneously for isolation and DNA extraction from milk. In culture method, the species level identification of selected colonies was done by the partial 16S rRNA gene sequence analysis for *Streptococcus* spp. In mPCR method, detection of the 3 target pathogens was done using the DNA extracted from milk.

RESULTS AND DISCUSSION

The genus-specific primer amplified 96 bp amplicon for all the strains under the genera *Streptococcus,* successfully categorizing the genus (Fig. 1). Subsequently, for species level identification, the primer pairs designed targeting 16Sr RNA were specific, when evaluated against the 24 reference strains (Table 1a, Fig. 2).

Multiplex PCR amplified DNA fragments of specific amplicons of size 871bp, 572 bp and 400 bp corresponding to *Strep. agalactiae* (16S rRNA), *Strep. dysgalactiae* (16S rRNA) and *Strep. uberis* (*cpn60*) with the primer pairs specific for each species (Fig. 3).

The mPCR could detect the target streptococcal species accurately amongst the bacterial strains (606) tested in the study. The threshold for detection of mPCR for DNA isolated from milk samples was found to vary within the 3 target bacteria. When DNA was extracted from dilutions of spiked milk having combination of 3 bacteria, the mPCR had a

Table 1a. Reference strains used for evaluation of primers

ATCC no.	Reference strains/species	STAG	STDG	STUB
A 25904	*Staph. aureus*	-	-	-
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 49258	*Salinicoccus roseus*	-	-	-
A 13813	*Strep. agalactiae*	+	-	-
A 43078	*Strep. dysgalactiae*	-	+	-
A 9927	*Strep. uberis*	-	-	+
A 33317	*Strep. bovis*	-	-	-
A 43765	*Strep. suis*	-	-	-
A 43138	*Strep. porcinus*	-	-	-
A 33398	*Strep. equi*	-	-	-
A 19615	*Strep. pyogenes*	-	-	-
A 6303	*Strep. pneumoniae*	-	-	-
A 25175	*Strep. mutans*	-	-	-
A 19258	*Strep. thermophilus*	-	-	-
A 19433	*Enterococcus fecalis*	-	-	-
A25922	*E. coli*	-	-	-

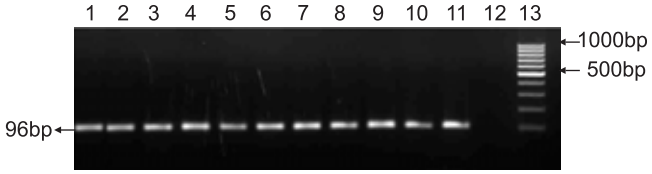

Fig. 1. Genus-specific PCR amplification of DNA from different Streptococcal species amplifying the partial *tuf* gene (96bp). Lane 1, *S. uberis* (ATCC 19436); lane 2, *S. agalactiae* (ATCC 13813); lane 3, *S. dysgalactiae* (ATCC 43078); lane 4, *S. bovis* (ATCC 33317); lane 5, *S. equi* (ATCC 33398); lane 6, *S. porcinus* (ATCC 43138); lane 7, *S. suis* (ATCC 43765); lane 8, *S. pyogenes* (ATCC 19615); lane 9, *S. pneumoniae* (ATCC 6303); lane 10, *S. mutans* (ATCC 25175); lane 11, *S. thermophilus* (ATCC 19258); lane 12, no template control; and lane 13, 100 bp ladder.

Fig. **2.** Specificity of mPCR. Products amplified from lane 1, *S. agalactiae* (ATCC 13813) 871 bp; lane 2, *S. dysgalactiae* (ATCC 43078) 572 bp; lane 3: *S. uberis* (ATCC 19436) 400 bp; lane 4, *S. bovis* (ATCC 33317); lane 5, *S. equi* (ATCC 33398); lane 6, *S. porcinus* (ATCC 43138); lane 7, *S. suis* (ATCC 43765); lane 8, *S. pyogenes* (ATCC 19615); lane 9, *S. pneumoniae* (ATCC 6303); lane 10, *S. mutans* (ATCC 25175); lane 11, *S. thermophilus* (ATCC 19258); lane 12, no template control; and lane 13, 100 bp ladder.

detection limit of 10^2 cfu ml^{-1} for *Strep. agalactiae* and *Strep. uberis*; 10 cfu ml^{-1} for *Strep. dysgalactiae*. The detection limit for genomic DNA was approximately 10 fg (Fig. 4).

For detection of pathogens in milk samples, the isolation studies showed that out of 115 milk samples cultured, 24 samples yielded at least 1 of the 3 target pathogens. A total of 24 strains were isolated. No growth was observed in 5 samples and among target species, *Strep. dysgalactiae* could not be recovered from any of the 110 samples.

All culture positive samples were detected positive by mPCR for the corresponding bacteria. The comparative detection of *Strep. agalactiae, Strep. dysgalactiae,* and *Strep. uberis* by culture and mPCR are depicted in Table 3. In comparison of mPCR with isolation of the pathogens, the mPCR could detect more strains (30) from the DNA isolated from milk samples than could be detected using conventional bacteriological culture.

Bovine mastitis, the most significant disease of dairy herds, has huge effects on farm economics due to reduction in milk production and treatment costs. The principles of mastitis control have been understood for decades and are based on reducing new infections and limiting the duration of existing infections (Ruegg 2009). 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). Subclinical mastitis apart from

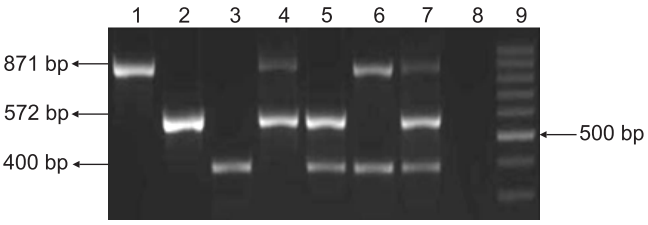

Fig. 3. Multiplex PCR for detection of *Streptococcus* spp. Lane 1, *S. agalactiae* (871bp); lane 2, *S. dysgalactiae* (572bp); lane 3, *S. uberis* (400bp); lane 4, combination *of S. agalactiae, S. dysgalactiae;* lane 5, combination of *S. dysgalactiae, S. uberis;* lane 6, combination *of S. agalactiae, S. uberis;* lane 7, combination *of S. agalactiae, S. dysgalactiae, S. uberis;* lane 8, no template control; lane 9, 100bp DNA ladder.

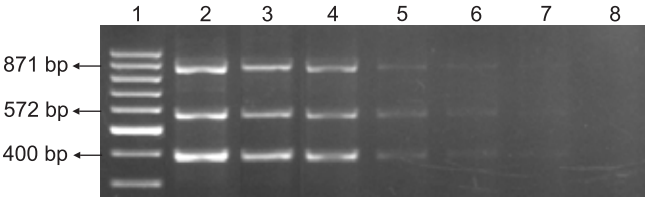

Fig. 4. Sensitivity of mPCR in detection of DNA isolated from *Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis*. Lane 1, 100bp DNA ladder; lane 2-7 represents 100pg, 10pg, 1pg, 100fg, 10fg, 1fg, respectively, and lane 8, no template control.

Table 1b. Bacterial isolates tested by mPCR

Bacterial species	Total no.	GenBank accession no.
Staphylococcus sp.	358	
Staph. aureus	92	HM452003-HM452094
Staph. chromogenes	89	HM367743-HM367826; HM452095- HM452099
Staph. epidermidis	57	HM367827-HM367875; HM452104- HM452111
Staph. sciuri	45	HM451958-HM451999; HM452101- HM452103
Staph. haemolyticus	37	HM359218-HM359246; HM452114-HM452121
Staph. hyicus	13	HM451942-HM451954
Staph. saprophyticus	8	HM451933-HM451938; HM452112-HM452113
Staph. hominis	5	HM451928-HM451932
Staph. auricularis	3	HM451939-HM451941
Staph. pasteuri	3	HM451955-HM451956
Staph. gallinarum	2	HM452122- HM452123
Staph. simulans	2	HM452000; HM452100
Staph. devrisei	1	HM452124
Staph. warneri	1	HM452001
Streptococcus sp.	33	
Strep. agalactiae	17	HM355957-970, HM452002; HM590574-75
Strep. dysgalactiae	3	HM359247–249
Strep. uberis	13	HM355971-HM355983

Table 2. Primer sequences, annealing temperature and predicted sizes of PCR products for the amplification of *Streptococcus* genus and *Strep. agalactiae*, *Strep. dysgalactiae* and *Strep. uberis* species-specific target regions

Organisms	GenBank Acc. No.	Gene	Primer designation*	Oligonucleotide primer —52-32 —	Location within gene	Annealing temperature	Ampliconsize (bp)	Reference
Strep. Genus	AY266996	*Tuf*	STTUF	GGTGTTGAAATGTTCCGTAAAC	445–466	60°C	96	This study
				ACGTTCGATTTCRTCACGTTG	540–520			
Strep. agalactiae	AB297817	16S rRNA	STAGF	GCTAATACCGCATAAGAGTAATTAACA	145–171	60°C	871	This study
			STDGR	AAGGGAAAGCCTATCTCTAGACC	993–1015			
Strep. dysgalactiae	AB002488	16S rRNA	STDGF	GGGAGTGGAAAATCCACCAT	459–478	60°C	572	This study
			STDGR	AAGGGAAAGCCTATCTCTAGACC	1030–1008			
Strep. uberis	AF485804	*cpn60*	STUBF	TCGCGGTATTGAAAAAGCAACAT	57–794	60°C	400	Dmitriev *et al.* 2006
			STUBR	TGCAATAATGAGAAGGGGACGAC	56–434			

* STAG: *S. agalactiae*; STDG: *S. dysgalactiae*; STUB: *S. uberis*.

Table 3. Comparative detection of target pathogens in milk samples (n=115) by culture and mPCR

Organism	Culture	Culture total	mPCR	
			Positive	Negative
S. agalactiae	Positive	22	22	0
	Negative	88	2	86
	Total	110	24	86
S. dysgalactiae	Positive	0	0	0
	Negative	110	2	108
	Total	110	2	108
S. uberis	Positive	2	2	0
	Negative	108	2	106
	Total	110	4	106
No growth	Total	5		

leading to production losses can be a source of new infections too (Lee *et al*. 2008). Amongst causative agents, *Streptococcus* spp. are frequently isolated from subclinical mastitis in dairy cows. Continuous monitoring of mastitis, and its careful management, is essential for the well being of dairy herd. If eradication programs for *Streptococcus* spp. are to be effective, methods for identification of the pathogen at both the herd and individual cow level need to be inexpensive, accurate and non labour intensive. To achieve a sensitive, rapid and specific test for mastitis, a multiplex PCR capable of simultaneous detection of the 3 major *Streptococcus* species causing bovine mastitis was developed.

The traditional and well established tests include SCC and culture based methods. Assays mainly used on-site are only indicative but not conclusive of the infection status of the animal (Viguier *et al*. 2009). The development of PCR based methods provided a promising option for the rapid detection of bacteria, even in cases difficult to detect subclinical mastitis (Cremonesi *et al*. 2009)

Multiplex PCR further enhances the efficiency of PCR technology by reducing both cost and time. However due to close phylogenetic relationship between bacterial species, it sometimes becomes difficult to simultaneously differentiate species under the same genera. The developed mPCR could successfully differentiate the 3 candidate bacteria. The evaluation of primers with 24 ATCC reference strains, unambiguously confirmed the accuracy of the primers. Earlier an mPCR for the identification of the 3 *Streptococcus* species targeting the *cpn*60 gene was devised by Dmitriev *et al.* (2006) but the sensitivity of the assay to detect the pathogens directly in milk was not assessed. In contrast, the mPCR developed in this study has the ability to detect the pathogens directly in milk. Further, the mPCR showed detection limit in the range of 10^2 to 10 CFU ml^{-1} of milk, which is higher in comparison with sensitivity of previously reported assays (Phuektes *et al*. 2001, Gillespie and Oliver 2005). Moreover, the previously developed mPCR for simultaneous detection of *Staphylococcus aureus*, *Streptococcus dysgalactiae, Streptococcus agalactiae* and *Streptococcus uberis* could not

differentiate *Strep. agalactiae* and *Strep. dysgalactiae* due to the size difference of only 6 base pairs, making them indistinguishable in agarose gel (Phuektes *et al*. 2001). However, this was overcome in the present study by use of a common reverse primer targeting 16S rRNA and 2 specific forward primers corresponding to *Strep. agalactiae* and *Strep. dysgalactiae* which yielded 2 distinguishable amplicons of 871 bp and 572 bp respectively, allowing its successful differentiation and detection in agarose gel.

When used for detection of the pathogens in composite subclinical mastitis milk samples, mPCR was found to be more efficacious than microbiological studies. The mPCR successfully detected all bacteria, as isolated in culture method. Besides, mPCR detected organisms in samples found culture negative. These culture negative milk samples represent a large proportion of samples in conventional bacteriology (Taponen *et al*. 2009). Indeed in conventional culturing, 25–50% of all samples from clinical mastitis harbor no bacterial growth (Koivula *et al*. 2007). In such cases, the mPCR can be promising. Thus, the correct identification of streptococcal species amongst the bacterial strains tested (606) and subsequent assessment of 115 milk samples rendered the assay to be highly specific. As a rapid tool, the mPCR assay can be used to resolve bacterial etiology allowing diagnosis of mastitis. In conclusion, owing to specificity, rapidity and ease of use, the mPCR method described in this study can be profitably applied for research, diagnosis and epidemiological studies in streptococcal mastitis.

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