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Detection of mastitis pathogens by multiplex polymerase chain reaction

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Abstract
Mastitis is a multi-etiologic disease of the mammary gland which affects not only the health of cattle but also, economics of the dairy sector. This study was conducted for rapid identification of the etiological agent for mastitis (*Staphylococcus hyicus*, *Mycoplasma bovis*, *Streptococcus uberis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*) directly from mastitis-infected cows by multiplex polymerase chain reaction (mPCR). A total of 40 mastitic milk samples, confirmed by the California mastitis test (CMT) were processed for identification of bacterial pathogens. The prevalence of above-mentioned pathogens was 35% of *S. hyicus*, 15% of *M. bovis*, 12.5% of *Str. uberis*, 12.5% of *P. aeruginosa* and 5% of *L. monocytogenes*. The most prevalent organism was *S. hyicus* whereas, the least one was *L. monocytogenes*. The sequence of *sodA*, *uvrC*, *pauA*, *inlA*, and *FecR* genes were used as a target for designing the primers for *S. hyicus*, *M. bovis*, *Str. uberis*, *L. monocytogenes* and *P. aeruginosa* respectively. mPCR was proved to be quite efficient in the rapid detection and identification of more than one pathogen at a time.

Keywords: california mastitis test (CMT), cattle, Mastitis, mPCR, *Staphylococcus hyicus*

Introduction
Mastitis remains the most common disease of dairy cattle, causing the biggest economic losses to the dairy industry [23]. Costs due to mastitis include reduced milk production, condemnation of milk due to antibiotic residues, veterinary costs and occasional deaths [22]. Moreover, mastitis has a serious zoonotic potential associated with the shedding of bacteria and their toxins in the milk [8]. Bovine mastitis is caused by a wide spectrum of pathogens such as *Staphylococcus* spp., *Streptococcus* spp., *Mycoplasma* spp., *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Escherichia coli*, *Klebsiella* spp. [3, 16].
Reliable identification of the causal bacteria is important for developing mastitis control strategies for dairy herds. Targeting antimicrobial treatment of animal infections such as mastitis against the causal agent is generally recommended [5]. This is only possible with accurate identification of bacteria in the mastitic milk samples. Bacteria do not grow in conventional culture in a substantial proportion of mastitic milk samples. According to the literature, no bacterial growth is detected in at least 20 to 30% of milk samples taken from udder quarters with mastitis [1]. A negative result for a milk sample is not only frustrating for the farmer and the veterinarian submitting the sample, but also for the laboratory responsible for mastitis diagnostics. Possible reasons for no growth in milk samples can include a low concentration of bacteria in the milk sample, pathogens not growing in standard culture media, or presence of substances in the milk decreasing the viability of bacteria in culture. This study aimed to use a multiplex PCR-based assay to study of mastitic milk samples from mastitis that are not detected easily in conventional culturing containing mastitis pathogens. The assay was performed on milk samples without the need for bacterial culturing and it identified a total of 5 mastitis-causing bacterial species. The analytical accuracy of the assay was validated in an earlier study, which demonstrated 100% analytical specificity and sensitivity, across a large collection of culture isolates originating from bovine clinical mastitis. Because the mPCR assay does not provide false-positive results and its sensitivity is not reliant on the viability of the bacteria to grow in culture [12]. It provided an optimal tool to study the bacteriological etiology of the mastitis milk samples.

Materials and Methods

Collection of samples

A total of 40 mastitis milk samples were collected from cattle. It was confirmed by California mastitis test. Before milk collection, the teat end was scrubbed with a cotton swab soaked in 70% ethanol. The first squirt of milk was discarded and approximately 5 ml of milk was collected into a sterile plastic container. All milk samples were frozen at −20 °C and processed within a few hours after collection.

Extraction of DNA

All the samples were exposed to the extraction of DNA directly from mastitis milk. It was carried out by using the Phenol-chloroform extraction method which was described by Phueketes *et al*. [16] with certain alterations. The gel electrophoresis (containing 1.5% agarose) was used to check the integrity of DNA whereas, quantification was carried out by spectrophotometric measurements.

Designing and grouping of oligonucleotide primers

The sequence of genes i.e., *sodA* for *S. hyicus* [26], *uvrC* for *M. bovis* [24], *pauA* for *S. uberis* [11], *inlA* for *L. monocytogenes* [10] and *FecR* for *P. aeruginosa* [2] were used as a target for designing of the oligonucleotide primers, by using National Centre for Biotechnology Information (NCBI) website. To establish a combination of these two sets of primers for mPCR, they were adjusted to had 100bp differences between two primers and similar Tm values, as predicted by the computer program (Oligodt Analyzer tool). The primers are listed in Table 1.

Table 1: Oligonucleotide primers used for amplification of target gene along with annealing temperature

Set no.	Bacteria	Target gene	Primers	Primer sequence (5'-3')	Annealing temp.
1.	*Staphylococcus hyicus*	*sodA*,	SH-F	TAACAATGGTGGCGGTCACT	60^0C
			SH-R	AAGCCCAGCCAGATCCAAAT	
	Mycoplasma bovis	*uvrC*	MB-F	GCGCAGTGCTGATGTTGAAT	
			MB-R	ACAAAAATCAATAGGAAAGCACCCT	
	Streptococcus uberis	*pauA*	StrU-F	AACTAGTCGACTTTGCGCCT	
			StrU-R	GTCAGGGTAGCGTTGCAAAA	
2.	*Pseudomonas aeruginosa*	*FecR*	PA-F	TGACCACGAAGAACACCTCG	56^0C
			PA-R	TTCGCAGACGAAACCGAAGA	
	Listeria monocytogenes	*inlA*	LM-F	TTCGCAGACGAAACCGAAGA	
			LM-R	CGTGAATTGAGCGTACAGCG	

Amplification by multiplex PCR

The reaction for multiplex PCR was carried out for set 1 and set 2 in a final volume of 25 µl consisted of 5.0 µl 5X Go Taq® Flexi buffer, 3.0 µl MgCl2 (25mM), 1µl of each forward primer (10 pM/µl), 1µl of each reverse primer (10 pM/µl), 1 µl dNTP (25mM each), 0.25 µl Taq DNA polymerase (5 U/µl), 3 µl template DNA (30ng/µl) and remaining added nucleus free water. The mPCR conditions for the target gene consisted of an initial denaturing step at 96 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 60 °C (set no.1), 56 °C (set no.2) for 1 min, and 72 °C for 1min, and a final elongation step at 72 °C for 7 min.

Results and Discussion

Multiplex PCR based analysis of samples

The current study reported that the mPCR assay was used in diagnosis for rapid, sensitive, and specific simultaneous detection of the organism directly from milk samples which showed agreement with the previous finding of Gangwal & Kashyap [7] and Taponen *et al*., [25]. It can be applied for the assessment of bulk milk samples and concluded in less than 6 hours.

The study indicated the target pathogens which were responsible for mastitis and diagnosed by multiplexing, has been shown in Table 2 and Fig.1. Out of all the samples, few showed single followed by two or more co-infections. This proved that such organisms were involved in causing mastitis. It also determined that *S. hyicus* was a major cause of mastitis followed by *M. bovis, Str. uberis, P. aeruginosa* while *L. monocytogenes* was the least one among target bacteria. Our result was in agreement with Schoder *et al*. [21]; Winter *et al*. [27] who reported that *L. monocytogenes* was less commonly reported in bovine mastitis whereas, Coimbra *et al.* [4] informed that *S. hyicus* (22%) also observed to be major cause of bovine mastitis.

Table 2: Number of positive samples for target pathogens by mPCR

S. No.	Isolates	Number of positive samples	Prevalence (%)
1.	*Staphylococcus hyicus*	14	35
2.	*Mycoplasma bovis*	6	15
3.	*Streptococcus uberis*	5	12.5
4.	*Pseudomonas aeruginosa*	5	12.5
5.	*Listeria monocytogenus*	2	5

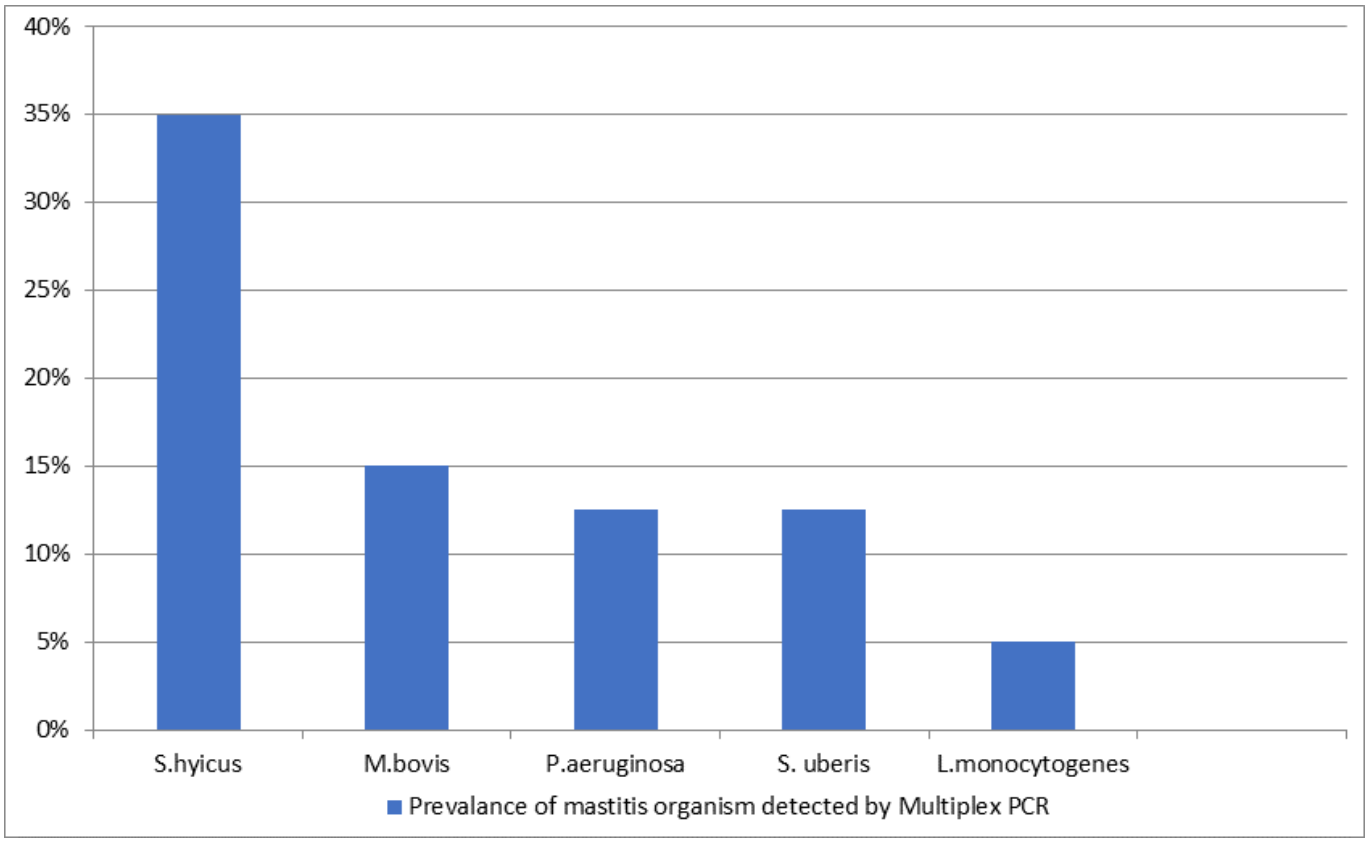
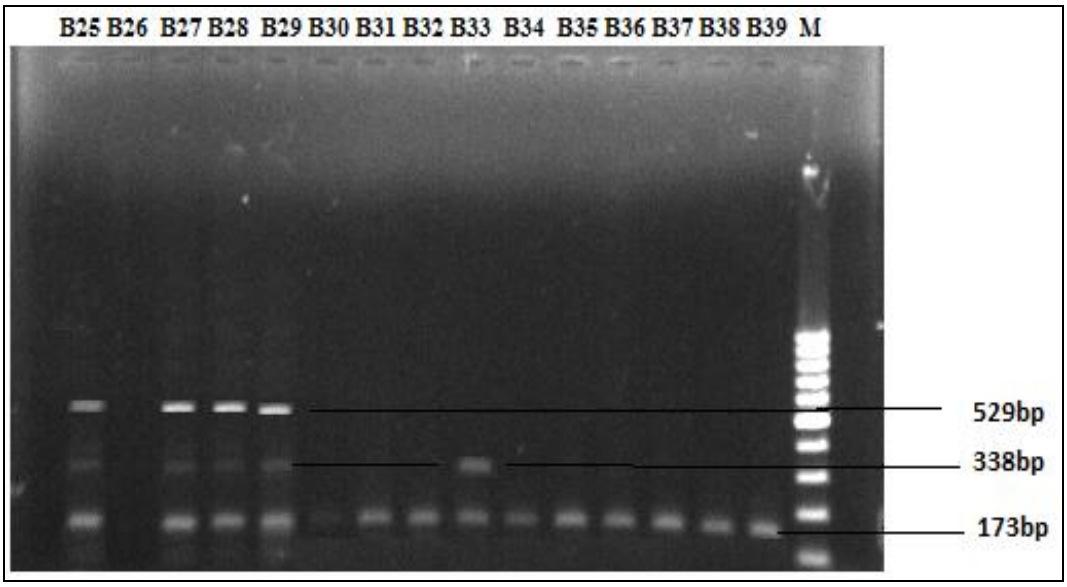


Fig 1: Prevalence of target pathogens in mastitis milk samples

The prevalence of *S. uberis* (between 2.7 to 8%) had been reported by several researchers in India [3 ,6]. Sayed *et al*. [20] revealed that *P. aeruginosa* (4.2%) was also reported to be cause of bovine mastitis. In another, according to Punyapornwithaya *et al*. [17] incidence rates of *M. bovis* mastitis cases in the milking and hospital pens were found to be 0.01 and 1.7 cases per 100 cow-days at risk. The isolation and subsequent sub culturing for mycoplasma identification is a time-consuming process that may take up to 15 days before a sample is considered negative or positive for *Mycoplasma* [19, 9] so, PCR is an efficient technique to overcome these problems.

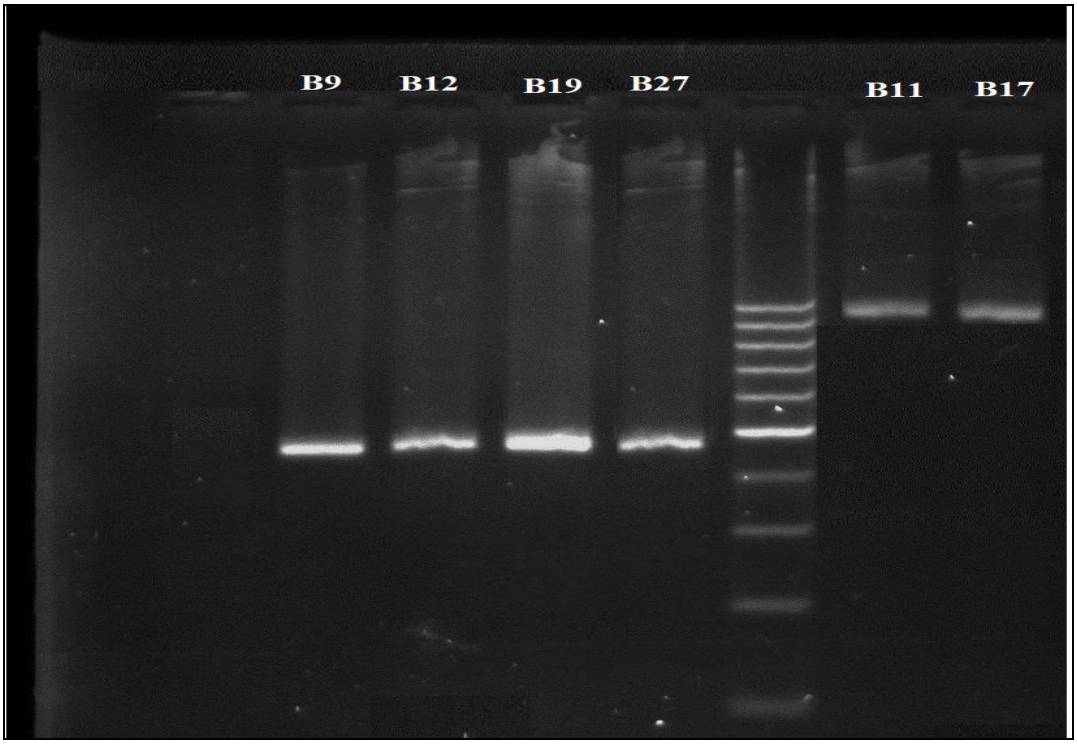
A clear appearance of the different amplicons of respective pathogens was also visible under agarose gel electrophoresis where in set no.1 of the primers *S. hyicus, Strep. uberis, M. bovis* having amplicon size of 173bp, 338bp, 529bp, respectively (Fig. 2) appeared. Similarly, *L. monocytogenes and P. aeruginosa* with amplicons of 954bp and 472bp (Fig. 3) were appeaered in set no. 2 of primers.

The designed primers for the target gene (Table 1) were allowed rapid and reliable identification of target bacteria. *inlA* and *FecR* gene has been exploited as a marker for highly specific confirmatory identification of pathogenic *L. monocytogenes* and *P. aeruginosa*, respectively [10, 18].



M- marker 100bp, C- control, B- sample no.
529bp - *M. bovis*; 338bp – *Strep. uberis*; 173bp – *S. hyicus*

Fig 2: Sensitivity of multiplex PCR in direct detection of pathogens directly from milk samples



M- marker 100bp, C- control, B- sample no.
954bp - *L. monocytogenes*; 472bp - *P. aeruginosa*

Fig 3: Sensitivity of multiplex PCR in direct detection of pathogens directly from milk samples

Perrig *et al.* [15] demonstrated that the *pauA* gene was prevalent and highly conserved in *S. uberis*, showing their importance to be included in future vaccine studies to prevent bovine mastitis. *sodA* genes encoding "manganese-dependent superoxide dismutase A" was found to be present in *S. hyicus* [26]. uvrC gene, highly conserved housekeeping gene that encodes for a DNA repair enzyme "Deoxyribodipyrimidine photolyase" was reported to be very sensitive and specific for *M. bovis* [13, 14].

Conclusion

Mastitis is a heavy economic burden in the dairy sector so, the corrective identification of causative agents is important. As, correct species identification is significant for mastitis treatment, prevention, control and in epidemiological investigations, as well as for the understanding of the significance of infections caused by different bacterial species. The use of multiplex PCR is saving the time used during conventional determination which was found to be more laborious and required more expertise for detection such as *M. bovis* and *L. monocytogenes*. This assay could prove to be an adequate tool for the rapid identification of the mastitis pathogens directly from milk, independent of their phenotypic characteristics which would ultimately contribute to economic development.

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