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Research article

Development of multiplex polymerase chain reaction for the simultaneous detection of bovine mastitis-associated pathogens

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Abstract

A multiplex polymerase chain reaction (mPCR) assay was developed for the simultaneous detection of five bovine mastitis-associated pathogens including *Staphylococcus simulans*, *Staphylococcus hominis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus* genus-specific group. Results showed that the optimal annealing temperature for mPCR was 58 °C. The sensitivity of mPCR to detect the least DNA template concentration was 0.625 ng/µl. The nucleotide sequences of PCR products were identified using the NCBI database. Results showed 99.15-100% homology to the *gap* gene of *Staphylococcus simulans*, 100% homology to the *peptidase* gene of *Staphylococcus hominis*, 98.76-99.69% homology to the *clumping factor A* gene of *Staphylococcus aureus*, 99.09-99.64% homology to the *CAMP factor* gene of *Streptococcus agalactiae*, and 99.13% to the *elongation factor tu* gene of the *Streptococcus* genus-specific group. This developed mPCR technique showed potential as a rapid, specific, highly sensitive, and low-cost diagnostic tool for the simultaneous detection of bovine mastitis-associated pathogens.

Keywords: Bovine mastitis, Pathogen, Multiplex polymerase chain reaction

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INTRODUCTION

Bovine mastitis causes high economic losses in the dairy industry. Direct losses include a decrease in milk production, poor quality milk due to an increase in somatic cells, and rejection of milk in clinical mastitis cases or identified antibiotic residues. Indirect losses negatively impact cow selling prices, with higher veterinary service, pharmaceutical, and diagnostics/laboratory costs (Hogeveen, 2005; Steele, 2015; El-Sayed et al., 2017). Contagious pathogens of bovine mastitis comprise *Escherichia coli* (*E. coli*), *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Streptococcus parauberis* (*S. parauberis*), *Streptococcus uberis* (*S. uberis*), *Staphylococcus aureus* (*S. aureus*), and *Streptococcus agalactiae* (*S. agalactiae*) (Bramley et al., 1996; Smith, 1996; Riffon et al., 2001). The Coagulase-Negative Staphylococci (CoNS) group of *Staphylococcus* spp. comprising *Staphylococcus simulans* (*S. simulans*), *Staphylococcus hominis* (*S. hominis*), *Staphylococcus chromogenes* (*S. chromogenes*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Staphylococcus heamolyticus* (*S. heamolyticus*) (Shome et al., 2011) are the predominant pathogens causing subclinical or clinical mastitis. Milking machines are important keys that transmit contagious pathogens from one animal to another, and the teat cups must be properly disinfected. Toxins from the bacterial pathogens infect the mammary gland as the main cause of clinical manifestations such as endotoxemia, depression, anorexia, fever or hypothermia, muscular weakness, mammary gland inflammation with aberrant mammary secretion, and breast abscess (Menzies et al., 2000; Radostits et al., 2000; Bleul et al., 2006; Kuang et al., 2009; Shome et al., 2011; Zadoks et al., 2011; Ashraf et al., 2017).

Biochemical tests and conventional microbiological methods are the traditional gold standards for determining bovine mastitis-associated pathogens in milk, with phenotypic features such as serotyping and enzymatic profiles used to identify bacteria in most clinical laboratories. However, microbiological approaches are both time-consuming and labor-intensive (González and Wilson, 2003; Gillespie and Oliver, 2005; Kalin et al.,2017).

DNA-based identification methods target specific pathogens to provide rapid screening. Polymerase chain reaction (PCR) is now used for the rapid detection of various mastitis-associated pathogens (Kim et al., 2001; Chotar et al., 2006). The PCR technique requires over an hour to identify mastitis pathogens by detecting one specific gene in each reaction. Multiplex polymerase chain reaction (mPCR) is a PCR variant that detects several bacterial pathogens in one reaction within a short period of time (Henegariu et al., 1997; Kalin et al., 2017). mPCR is preferred in routine diagnostics because it is cost-efficient and less time-consuming than traditional PCR.With the designed primers, the mPCR can specifically amplify target DNA sequences which is practically useful for the simultaneous detection of bovine mastitis-associated pathogens.

This research developed an mPCR protocol for the simultaneous detection of five bovine mastitis-associated pathogens including three *Staphylococcus* species (*S. simulans*, *S. hominis*, and *S. aureus*) and two *Streptococcus* species (*S. agalactiae* and *Streptococcus* genus-specific group).

MATERIALS AND METHODS

Bacterial strains

S. aureus and *S. agalactiae* were obtained from the Department of Biology, Faculty of Science, Chiang Mai University, while *S. simulans*, *S. hominis*, *S. uberis*, *S. chromogenes*, *S. heamolyticus*, and *E. coli* were isolated from cows with mastitis at the Faculty of Animal Science and Technology, Maejo University. *S. uberis* was used as a representative of *Streptococcus* genus-specific positive.

Bacterial growth and selective enrichment

Each bacterial isolate was cultured at 37 °C on sheep blood agar plates and incubated overnight under anaerobic or aerobic conditions. A single colony was transferred from the agar plate to the brain heart infusion broth (Hardy Diagnostics, USA). Universal primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-CTACGGCTACCTTGTTACGA-3'), which target 16s rRNA, were used to identify the mastitis-associated pathogens (Lane, 1991).

DNA extraction

A bacterial culture suspension was pipetted into a 1.5 ml microcentrifuge tube and centrifuged with a centrifuge machine (Universal 320R, Hettich, Germany) at 9,500 g for 1 min at 4 °C. The supernatant was discarded and bacterial DNA was isolated from the cell pellet following the protocol of TIANamp bacteria DNA kit (TIANamp, China).

Oligonucleotide primer design

The five genes *gap*, *peptidase*, *clumping factor A* (*clfA*), *CAMP factor* (*cfb*), and *elongation factor Tu* (*tuf*) were candidates for *S. simulans*, *S. hominis*, *S. aureus*, *S. agalactiae*, and the *Streptococcus* genus-specific group, respectively. Consensus sequences were identified from multiple alignments in ClustalX2 software and used for gene-specific primer design in NCBI via primer blast (data not shown), with primer sequences shown in Table 1.

Table 1 Primers for bovine mastitis-associated pathogens.

Organism	Gene	Primer name	Sequence (5'-3')	Location within gene	Amplicon size (bp)	Ta (°C)	Reference
S. simulans	*gap*	SSMF	AGCTTCGTTTACTTCTTCGATTGT	171-194	472	58	Shome *et al.*, 2011
		SSMR	AAAAGCACAAGCTCACATTGAC	642-621			
S. hominis	*peptidase*	PEP109F	ATTTCAAGCGACGATCCCAAT	95-115	109	58	In this study
		PEP109R	GAACAACATCTCGAGCGTCC	203-184			
S. aureus	*clfA*	CLFA320F	AGCTCCACAGAGTACAGATGC	143-163	320	58	In this study
		CLFA320R	TGGTGGCACTTTAGCAGTTG	463-444			
S. agalactiae	*cfb*	CFB548F	TAATCAAGCCCAGCAAATGGC	132-152	548	58	In this study
		CFB548R	CCAACAGCATGTGTGATTGC	680-661			
Streptococcus genus-specific group	*tuf*	TUF229F	GCTTCAGGACGTATCGACCG	307-326	229	58	In this study
		TUF229R	GTGTGTGGGTTGATTGAACCTG	536-515			

DNA sequencing

The PCR products were purified using PCR clean-up & gel extraction (Bio-Helix, Taiwan) and sequenced by a commercial sequencing service (1st base DNA sequencing services, Malaysia). Nucleotide sequences were compared with the GenBank database.

Optimization of monoplex PCR

Primer specificity was determined by a monoplex PCR reaction consisting of 1X PCR buffer for KOD-multi & epi-™ (Toyobo, Japan), 500 nM of each of the five gene-specific primers, 0.3U of KOD-multi & epi-™, and 50 ng/µl of DNA

template. Various final concentrations of primer (100, 200, 300, and 400 nM) were used to optimize monoplex PCR assays. The PCR reaction mixtures consisted of 1X PCR buffer for KOD-multi & epi-™, each primer concentration, 0.3U of KOD-multi & epi-™, and 3 ng/µl of DNA template. To ensure cross-reactivity of the primers, a monoplex PCR reaction was carried out with 1X PCR buffer for KOD-multi & epi-™, 300 nM of five gene-specific primers, 0.3U of KOD-multi & epi-™, and DNA template containing 10 ng/µl from 5 isolates. The PCR reaction was performed in a 100A PCR thermocycler (LongGene, China) as follows: 94 °C for 2 min; 35 cycles of 98 °C for 10 s, 58 °C for 30 s, 68 °C for 30 s, and a final step of 68 °C for 10 min. The PCR products were analyzed by 2% agarose gel electrophoresis (Bio Basic, Canada) and visualized with a GelMax® UV Gel Imager (Labortechnik, Germany) after ethidium bromide staining (Biobasic, Canada).

Optimization of mPCR

Different combinations of individual mPCRs with varying primer concentrations of *peptidase*-specific gene primers were used with final concentrations of 200, 300, and 400 nM. Finally, the mPCR reaction mixture consisted of KOD-multi & epi-™ 1X PCR buffer, 400 nM of *peptidase*-specific gene primers, 200 nM of other gene primers, 0.3U of KOD-multi & epi-™, and DNA template (10 ng/µl from 5 isolates).

The mPCR condition was used to evaluate the sensitivity of the least DNA template, specificity, and annealing temperature of the mPCR. The sensitivity of mPCR was measured on the DNA template using a Nanodrop 2000/2000C Spectrophotometer V1.0 (Thermo Fisher Scientific, USA) and serially diluted from 10 to 0.625 ng/µl for each isolate to provide the least DNA concentration that can be detected by the assay developed. The mPCR consisted of KOD-multi & epi-™ 1X PCR buffer, 400 nM of *peptidase*-specific gene primers, 200 nM of other primers, 0.3U of KOD-multi & epi-™, and 10 to 0.625 ng/µl of DNA template. The specificity of mPCR consisted of KOD-multi & epi-™ 1X PCR buffer, 400 nM of *peptidase*-specific gene primers, 200 nM of other gene primers, 0.3U of KOD-multi & epi-™, and DNA template (10 ng/µl from 5 isolates). The mPCR reaction was performed in a 100A PCR thermocycler as follows: 94 °C for 2 min; 25 cycles of 98 °C for 10 s, 58 °C for 30 s, 68 °C for 30 s, and a final step of 68 °C for 10 min. The PCR products were analyzed by 2% agarose gel electrophoresis at 100 V for 1 h. and visualized using a GelMax® UV Gel Imager with ethidium bromide staining.

For optimal annealing, the mPCR contained 1X PCR buffer for KOD-multi & epi-™, 400 nM of *peptidase*-specific gene primers, 200 nM of other gene primers, 0.3U of KOD-multi & epi-™, and DNA template (10 ng/µl from 5 isolates). The reaction was carried out in a 100A PCR thermocycler as follows: 94 °C for 2 min; 25 cycles of 98 °C for 10 s, 58 °C to 64 °C for 30 s, 68 °C for 30 s, and a final step of 68 °C for 10 min. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized using a GelMax® UV Gel Imager with ethidium bromide staining.

RESULTS

DNA sequencing

PCR products of the *gap, peptidase*, *clfA*, *cfb*, and *tuf* genes were specific to *S. simulans*, *S. hominis*, *S. aureus*, *S. agalactiae*, and the *Streptococcus* genus-specific group, respectively. Nucleotide sequence homology of the PCR products compared to the NCBI database was shown in Table 2.

Table 2. Nucleotide sequence homology of the PCR products.

Pathogens	Genes	% Homology	Accession
S. simulans	*gap*	99.15-100.00	MF620370.1, JN609231.1, KP325404.1, AF495498.1, DQ321698.1, EU659919.1, JQ728515.1
S. hominis	*peptidase*	100.00	CP033732.1
S. aureus	*clfA*	98.76-99.69	HQ424276.1, HQ424257.1, EF207782.1, AB245457.1, HQ424278.1, JQ278700.1, AB245456.1
S. agalactiae	*cfb*	99.09-99.64	JQ289563.1, JQ289564.1, JQ289567.1, JQ289578.1, HQ148672.1, JQ289562.1, EF694027.1
Streptococcus genus-specific group	*tuf*	99.13%	MK322647.1, GU392968.1, GU392962.1, GU392946.1, GU392929.1

Specificity of monoplex PCR

Bacterial mastitis-associated pathogen DNA was utilized for testing the primers previously identified to species level by partial *16S rRNA* (27F and 1492R) gene sequencing. The *gap*, *peptidase*, *clfA*, *cfb*, and *tuf* gene primers were found to be specific to *S. simulans*, *S. hominis*, *S. aureus*, *S. agalactiae*, and *Streptococcus* genus-specific groups, respectively (Table 3). The positive PCR product showed a single band at the expected size. For example, *S. simulans* showed a single band at 472 bp, specific to the *gap* gene primer.

Table 3 Cross-reactivity of bovine mastitis-associated pathogens.

Primer Name	*S. simulans*	*S. hominis*	*S. aureus*	*S. agalactiae*	*S. uberis*	*S. chromogenes*	*S. haemolyticus*	*E. coli*
SSMF SSMR	+	-	-	-	-	-	-	-
PEP109F PEP109R	-	+	-	-	-	-	-	-
CLFA320F CLFA320R	-	-	+	-	-	-	-	-
CFB548F CFB548R	-	-	-	+	-	-	-	-
TUF229F TUF229R	-	-	-	+	+	-	-	-

+; PCR positive and -; PCR negative

Optimization of primer concentration of monoplex PCR assays

The final primer concentrations for optimization were 100, 200, 300, and 400 nM. Primers that were specific to the targeted gene produced a single, unambiguous band in the experiment. The lowest primer concentration revealed the expected band for bovine mastitis-associated bacteria as 100 nM for *S. aureus* and 200 nM for all other pathogens. We used 400 nM of *peptidase*-specific gene primers and 200 nM of all other gene primers to develop the mPCR method as they generated similar band intensities (Figure 1).

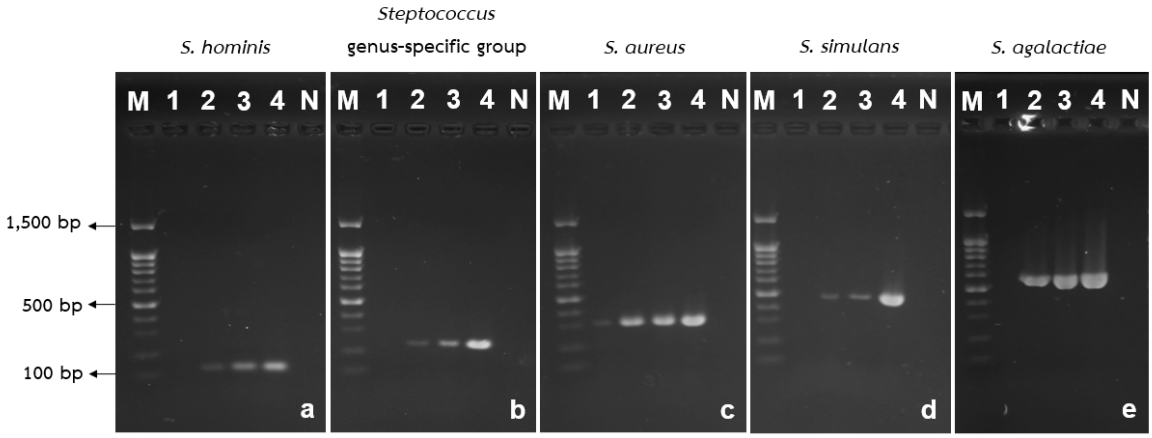

Figure 1 Optimization of primer concentration; lane M = 100 bp plus DNA ladder, lane 1 = 100 nM, lane 2 = 200 nM, lane 3 = 300 nM, lane 4 = 400 nM, and lane N = negative control (a,b,c,d,e = *the peptidase*, *tuf*, *clfA*, *gap*, *cfb* genes, respectively).

Cross-reactivity

A DNA template containing 10 ng/µl of each isolated pathogen was used to verify the PCR reaction. Various types of DNA templates were placed in the same tube, with results showing individual bands of each pathogen (Figure 2) because the primers used in this experiment were specific to their targets.

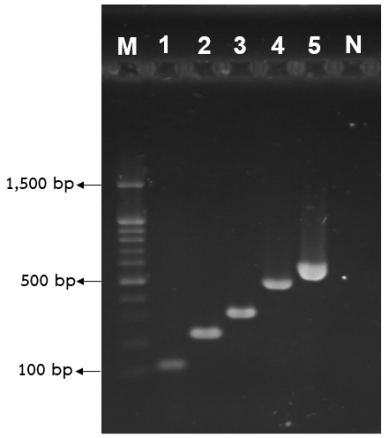

Figure 2 Cross-reactivity of mastitis-associated bacteria; lane M = 100 bp plus DNA ladder, lane 1 = *S. hominis*, lane 2 = *Streptococcus* genus-specific group, lane 3 = *S. aureus*, lane 4 = *S. simulans*, lane 5 = *S. agalactiae*, and lane N = negative control.

mPCR development

The mPCR was composed of individual PCR products with different combinations and various primer concentrations. The mPCR using 200 nM primers for the *gap*, *clfA*, *cfb*, and *tuf* and 400 nM for the *peptidase* genes yielded similar band intensities (Figure 3a).

The sensitivity of mPCR was examined. Similar band intensities were obtained for DNA templates using 10, 5, and 2.5 ng/µl. The band intensity of *S. hominis*

became faint at 1.25 and 0.625 ng/µl. The lowest amount of detectable bacterial DNA was 0.625 ng/µl (Figure 3b).

To optimize mPCR annealing temperature, a DNA template concentration of 5 ng/µl for each pathogen was utilized. The mPCR product of *S. hominis* faded out when the temperature increased to 60, 62, or 64 °C (Figure 3c). Therefore, the optimal annealing temperature for the mPCR assay was 58 °C.

The mPCR reaction consisted of 5 primers in one DNA template. The developed mPCR in this study was specific to the *peptidase*, *tuf*, *clfA*, *gap*, and *cfb* genes of *S. hominis*, the *Streptococcus* genus-specific group, *S. aureus*, *S. simulans*, and *S. agalactiae*, respectively. The mPCR product was highly accurate, as shown on lane 5, which used *S. agalactiae* as a DNA template. Results indicated that the PCR product of *S. agalactiae* was specific to the *tuf* and *cfb* gene primers, while the mPCR product showed two bands in one reaction (Figure 4).

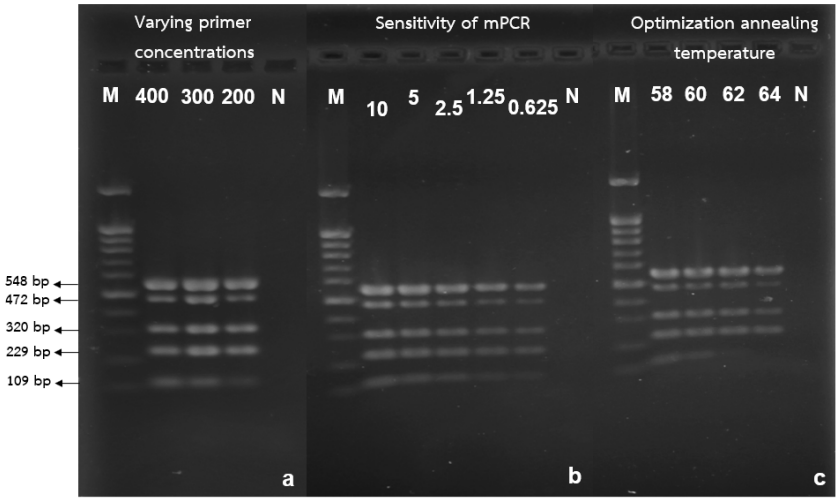

Figure 3 Development of mPCR; (a) varying primer concentrations of *peptidase* specific gene primer for detection of *S. hominis*, (b) sensitivity of mPCR, (c) optimization of annealing temperature. Lane M = 100 bp plus DNA ladder, and lane N = negative control.

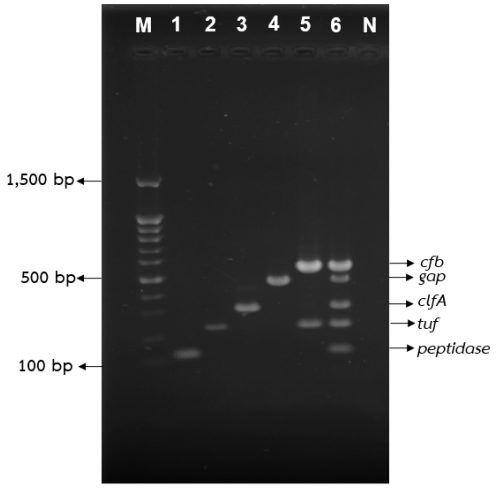

Figure 4 Specificity of mPCR; lane M = 100 bp plus DNA ladder, lane 1 = *S. hominis*, lane 2 = *Streptococcus* genus-specific group, lane 3 = *S. aureus*, lane 4 = *S. simulans*, lane 5 = *S. agalactiae* and lane N = negative control.

DISCUSSION

Rapid diagnostic methods with high sensitivity and specificity are required to control bovine mastitis. Historically, biochemical tests and conventional microbiological methods have been the gold standards to identify pathogenic bacteria in milk but these are both labor-intensive and time-consuming procedures. PCR-based approaches for detecting mastitis have been extensively reported (Riffon et al., 2001; Vieira-da-Motta et al., 2001; Kalorey et al., 2007; Amin et al., 2011).

This study developed an mPCR protocol to simultaneously detect *S. simulans*, *S. hominis*, *S. aureus S. agalactiae*, and *Streptococcus* genus-specific group. The *gap* gene for *S. simulans* and the *peptidase* gene for *S. hominis* (Shome et al., 2011) have been previously used in primer design. The *S. aureus* virulence factor *clfA* contributes to adherence and penetration of phagocytic cells, along with evading the host immune system (El-Behiry et al., 2015). The *cfb* gene, which encodes the Christie-Atkins-Munch-Petersen (CAMP) factor for *S. agalactiae* (group B *Streptococcus*; GBS) has been found in most GBS isolates (Podbielski et al., 1994; Ke et al., 2000; Gosiewski et al., 2012). For *streptococcal* species, the *tuf* gene encodes the elongation factor Tu, with a large amount of species-specific genetic divergence in the *tuf* gene (Picard et al., 2004). Primer selection for mPCR was based on the melting temperature (Tm), specificity, difference in amplification size, concentration of PCR buffer, balance between magnesium chloride and deoxyribonucleotide triphosphate concentrations, and cycling temperature (Henegariu et al., 1997).

The mPCR protocol developed in this study showed high sensitivity, specificity, rapidity, and cost-effectiveness for the identification of bovine mastitis-associated pathogens, with minimal concentration of bacterial DNA detection 0.625 ng/µl. Ashraf et al. (2017) developed mPCR to detect nine bacterial pathogens associated with bovine mastitis. They determined detection limits of mPCR assays ranging from 1.0 to 50 pg DNA for all possible random combinations of the target species. Phuektes et al. (2001) developed mPCR for *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, and *S. uberis* based on amplification of the *16S* to *23S rRNA* spacer regions. Monoplex PCR could identify DNA at 5 pg, while mPCR sensitivity was shown to be lower. In the mPCR assay, 500 pg of DNA from *S. agalactiae* and *S. aureus* and 50 pg of DNA from *S. dysgalactiae* and *S. uberis* were detected due to competition for dNTPs and Taq polymerase when numerous primer sets were combined in a single reaction (Madico et al., 2000).

The PCR-based method was proved a suitable tool for detection of bovine mastitis-associated pathogens in milk samples (Vieira-da-Motta et al., 2001; Riffon et al., 2001; Kalorey et al., 2007; Amin et al., 2011), while the mPCR limitation of detection was reported at 10 fg of genomic DNA (Shome *et al.*, 2011), and the detection limit of the biochip method was determined as 10^3-10^5 CFU ml^{-1} (Lee et al., 2008). In another experiment, the limit of detection by real-time mPCR utilizing non-enriched milk samples was 10^3 CFU ml^{-1} for *S. aureus* and 10^2 CFU ml^{-1} for *S. uberis*. The milk samples were positive for *S. aureus* and *S. uberis* when evaluated by conventional methods; however, negative results were recorded for real-time PCR in non-enriched milk containing 400 to 10,000 CFU ml^{-1} of *S. aureus* and *S. uberis* (Gillespie and Oliver, 2005). Limits of detection for the PCR method on *S. agalactiae*, *S. uberis*, and *S. bovis* cultures were found to be N × 10^3 CFU ml^{-1} without pre-enrichment (Chiang et al., 2008), while Graber et al. (2007) detected *S. aureus* with a quantitative sensitivity as low as 460 CFU per assay, equivalent to 1.15 × 10^3 CFU ml^{-1} of milk. Consequently, milk enrichment is necessary to detect low numbers of bacterial pathogens and may be required to dilute inhibitory compounds that are found in milk (Gillespie and Oliver, 2005).

An enrichment step was reported for detecting low numbers of bacteria (< 10^3 CFU ml^{-1}) (Gillespie and Oliver, 2005; Graber et al., 2007; Chiang et al., 2008) using the PCR-based method. The sensitivity of this assay increased from –0^4 - 10^5 CFU ml^{-1} to 1 CFU ml^{-1} after adding milk samples containing *S. agalactiae* as a selective enrichment step (Strep Select Broth) (Meiri-Bendek et al., 2002).

Corresponding to the study of Tran and Nguyen (2023) which shown that multiple strain of bacteria was found in bovine mastitis, with the advantage of mPCR that is able to detect multiple bacterial strains in a PCR reaction, it provides time saving and economical method compared to the traditional PCR method. Therefore, rapid and accurate treatment can be performed with higher efficiency as compared to other detecting methods.

CONCLUSIONS

An mPCR technique was developed for the simultaneous detection of five bovine mastitis-associated pathogens *Staphylococcus simulans*, *Staphylococcus hominis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus* genus-specific group. This developed mPCR technique showed potential as a rapid and cost-effective diagnostic protocol, with optimal annealing temperature of 58 °C and lowest DNA template concentration of 0.625 ng/µl.

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AUTHOR CONTRIBUTIONS

SP : Conceptualization, Methodology, investigation, Validation, data analysis ,Visualization
TC : Conceptualization, Validation, data analysis, Visualization
WC : Conceptualization, Methodology, investigation,Validation, data analysis, data curation, Visualization, Writing—review and editing
PT : Methodology, investigation, Validation, data analysis, Visualization, Writing original draft
PS : Validation, data analysis, Visualization
PL : Writing—review and editing

CONFLICT OF INTEREST

All authors declare no conflicts of interest. This project is financially supported by the National Research Council of Thailand (grant #NRCT-62-006.1).

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