Optimization of DNA extraction and development of a Multiplex PCR for detection of sub-clinical mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae*

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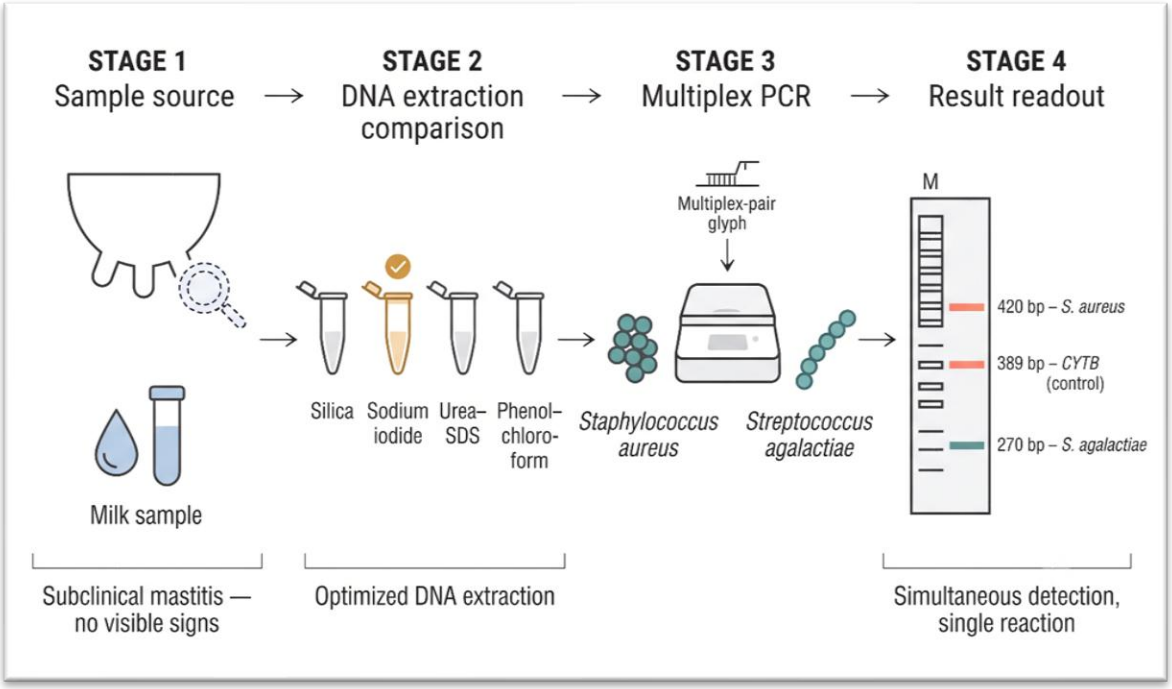

Abstract

The current study was carried out to establish the most suitable DNA extraction protocol to economically obtain pure genomic DNA from cow's milk and to develop a multiplex PCR to detect sub-clinical mastitis caused by *Streptococcus agalactiae* and *Staphylococcus aureus*. Sodium iodide method possessed the least processing time (80 minutes), low cost (0.16$), highest yield (405.0±27.6 ng/ml) and highest purity (1.93±0.30) with up to 10 CFU/ml of minimal detection level. Multiplex PCR was able to detect both bacteria simultaneously up to 10^5 CFU/ml and individually 10 CFU/ml for *S. agalactiae* and 10^3 CFU/ml for *S. aureus* with high specificity of primers indicated by absence of any cross reactivity with each other and with frequent mastitis causing bacteria. Multiplex PCR is more efficient than conventional PCRs and cultures, integrated with the selected DNA extraction method has a high potential to develop as a commercially available kit for routine diagnosis of subclinical mastitis.

Keywords: *DNA extraction, Mastitis, Multiplex PCR, S. agalactiae, S. aureus*

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1. Introduction

Mastitis in dairy cattle is an inflammation of the udder which is characterized by an increased number of somatic cells in milk and pathological changes in mammary tissue. The resulting reduction in milk yield, changes in milk composition, discarded milk and loss of genetic potential causes serious economic losses for the farmers and the dairy industry (McDougall *et al*., 2009). In subclinical mastitis, the causative organisms spread in the herd undetected for a long period of time. The reduction in milk production may account for 70%-80% of the total loss for farmers and silently influence the national income of a country (Gianneechini *et al*., 2002). Thus, early diagnosis of causative agents of mastitis is important for reducing production loss and enhancing the prospects of recovery (Ghebremedhin *et al*., 2008). Bovine mastitis remains one of the most economically damaging diseases of the dairy industry worldwide, and subclinical mastitis in particular accounts for the majority of these losses (Kour *et al*., 2023; Morales-Ubaldo *et al*., 2023).

The development of PCR-based methods provides a promising option for rapid identification of bacteria even in the presence of preservatives or residual therapeutic antibiotics in milk, thus avoiding the false negative results and improving the sensitivity. Further, PCR makes it possible to detect early stages of infection in the carrier animals even when the number of bacteria is at an undetectable level by conventional microbiological testing. PCR also has the ability to be highly specific and to discriminate between closely related microorganisms (Ghorbanpoor *et al*., 2007). Finally, multiplex-PCR (M-PCR) allows identification of several different bacterial species simultaneously (Henegariu et al., 1997), which is cost effective and requires less preparation and analysis time than conventional PCRs (Corne *et al*., 1999). Multiplex and real-time PCR assays have since been developed for the simultaneous detection of the principal contagious mastitis pathogens directly from milk (Gillespie and Oliver, 2005; Shome *et al*., 2011). Newer multiplex PCR assays continue to be developed for panels of mastitis-associated pathogens (Takam *et al*., 2025).

Staphylococcus aureus and *Streptococcus agalactiae* are of particular importance because they are the major cause of bovine mastitis and cause subclinical forms of mastitis and are the most frequently isolated pathogens in the dairy farms all over the world (Kuang *et al*., 2009).

Direct isolation of high-quality DNA from the target bacteria found in milk is an essential task for PCR detection of Mastitis pathogens, but it is often problematic and may require overnight selective-enrichment procedures due to small concentrations of the pathogenic DNA present in a typical sample and various factors such as the degree of cellular lysis, binding of DNA to particulate material, and degradation or shearing of DNA (Phuektes *et al*., 2001). Direct detection is hampered by the presence of PCR-inhibitory substances (Ramesh *et al*., 2002) and many current methods typically require multiple steps or specialized equipment, rendering them impractical for use with large sample numbers (Boom *et al*., 1990) and commercial kits are highly expensive. Isothermal and long-read sequencing-based approaches have more recently been explored as complementary or alternative diagnostic routes for mastitis pathogens (Chapagain *et al*., 2025; Jember *et al*., 2026).

In such a background this study is designed with two major objectives; to establish an efficient and effective genomic DNA isolation protocol and to develop M-PCR to detect subclinical mastitis caused by *Streptococcus agalactiae* and *Staphylococcus aureus.*

2. Methodology

2.1 Bacteria employed

The type strains of *S. aureus* (ATCC - 25923) and *S. agalactiae* (ATCC - 27956) were used to inoculate the samples. Bacterial quantification was done using McFarland turbidity standards (McFarland, 1907) and genomic DNA of *S. aureus*, *S. agalactiae*, were isolated based on guanidinium thiocyanate method described by Boom *et al*. (1990).

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2.2 Oligonucleotide primers

All PCR reactions carried out using species specific primers for *S. agalactiae* and *S. aureus* with target amplification at 16S-23S rRNA intergenic spacer region (Table 1). Bovine cytochrome b gene was also amplified as a positive control in the detection of bacteria in milk.

Table 1 Sequences and lengths of the amplification regions of the primers with reference.

Species	Primer	Sequence (5′ – 3′)	Length of target region	Reference
S. aureus	STAA-F	TCTTCAGAAGATGCGGAATA	420 bp	Phuektes *et al* (2001)
	STAA-R	TAAGTCAAACGTTAACATACG		
S. agalactiae	STRA-F	AAGGAAACCTGCCATTTG	270 bp	Phuektes *et al* (2001)
	STRA-R	TTAACCTAGTTTCTTTAAAACT AGAA		
Bovine cytochrome b	CYTB-F	CGATACATACACGCAAACG	389 bp	Meiri *et al* (2002)
	CYTB-R	TGTTGGGTTGTTGGAGCC		

2.3 DNA extraction

Four published methods for DNA extraction from milk were used for the comparison, including Silica method (Cremonesi *et al*., 2006), Sodium iodide method (Burbano *et al*., 2006), Urea - SDS method (Ramesh *et al*., 2002) and Phenol Chloroform method (Phuektes *et al*., 2001). Fresh Bovine milk samples were artificially inoculated with *S. agalactiae* having bacterial cell densities from 10 CFU/ml to 10^8 CFU/ml and DNA extraction was performed by employing the methods stated above. A pure bacterial solution was used as the positive control, and guanidium thiocynate method (Boom *et al*., 1990) was applied to extract DNA having same bacterial densities. Then the Sodium iodide method was applied for extraction of DNA from *S. aureus* using the same methodology.

2.4 DNA extraction procedure 1 – Silica method

500 µl aliquot of milk sample was diluted with 500 µl of sterile saline solution (NaCl 0.9%) and centrifuged for 15 min at 600x g at 4°C.The supernatant was discarded and repeated the step once more and resuspended the pellet in 50 µL of saline solution. A portion of 300 µL of lysis buffer and 200 µL of binding solution was added to the suspension and it was mixed and incubated for 5 min at room temperature. Then centrifuged for 30 s at 450 × g and the supernatant was discarded. A portion of 200 µl of lysis buffer was added to the pellet and mixed well. It was centrifuged for 30 s at 450 × g and the supernatant was discarded. This step was repeated once. Then 200 µl of washing solution was added to the pellet and mixed well. It was centrifuged for 30 s at 450 × g and the supernatant was discarded. This step was repeated once. A portion of 200 µl of absolute ethanol solution was added to the pellet and mixed it well. It was centrifuged for 30 s at 450 × g and the supernatant was discarded. The pellet was vacuum-dried in a thin wall micro tube heat block for 10 min. A portion of 100 µl elution buffer was added and the pellet was resuspended. Following the incubation of 15 min at 65°C, suspension was centrifuged for 30 s at 450 × g. Then the supernatant was transferred in to a clean tube and stored at -20 °C until further use.

2.5 DNA extraction procedure 2 – Sodium iodide method

A 200 µl aliquot of raw milk sample was homogenized by vortexing and mixed with 400 µl of lysis buffer. After vortexing for 1 min, the mixture was centrifuged at 15,000 rpm for 5 min and the supernatant was removed. Pellet was suspended in 200 µl of lysis buffer containing 0.03 µg/µl of glycogen and 4 µl of proteinase K (2 mg/ml). After incubation for 1 hour at 37ºC, 300 µl of NaI

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solution and 500 µl of isopropyl alcohol were added to the suspension and then centrifuged at 10000 x g for 5 min. The pellet was washed with 35% (v/v) of isopropyl alcohol, dried for 5 min at 37 ºC, and then suspended in 20 µl of sterilized water.

2.6 DNA extraction procedure 3 – Urea – SDS method

A 250 µl aliquot from each of diethyl ether and chloroform were added to a 500 µl milk sample and vortexed briefly. The samples were centrifuged at 8000 x g for 20 min at 25 ^{0}C. The aqueous phase was transferred to a fresh tube and 500 µl of 6 M urea and 100 µl of 10% SDS were added. The samples were incubated at 37 ^{0}C for 20 min and then centrifuged at 8000 x g for 20 min at 25 ^{0}C. The supernatant was discarded and 100 µl of 0.2 N NaOH was added to the samples and incubated at 37 ^{0}C for 10 min. DNA was precipitated by adding 1.0 ml of chilled absolute ethanol and one-tenth volume of 3 M sodium acetate (pH 4.8) and holding the samples at -20 ^{0}C for 2 hours. Samples were then centrifuged at 8000 x g for 20 minutes at 4 ^{0}C. Excess salt in the DNA preparation was removed by 70% ethanol wash. The DNA pellet was air-dried and resuspended in 15 µl of sterile deionized water.

2.7 DNA extraction procedure 4 – Phenol chloroform method

A 300 µl aliquot of milk sample was thoroughly mixed with 300 µl of NTE (0.1 M NaCl, 20 mM Tris-HCl [pH7.4], 1mM EDTA [pH7.5]) buffer containing 0.5% SDS, and 100 µg/ml of proteinase K. The solution was then incubated at 37^{0}C for 4 h. An equal volume of Phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the solution was gently mixed for 3 min. The solution was centrifuged at 10,000 x g for 3 min, and the upper phase was collected without disturbing the interface. This process was repeated twice. The upper phase was collected and 60 µl of 3 M sodium acetate (pH 4.8) and 1.2 ml of cold 100% ethanol were added. The solution was mixed and held at −20 ^{0}C for 30 min to precipitate the DNA. The DNA was recovered by centrifugation at 10,000 x g for 15 min at 4 ^{0}C. The supernatant was discarded and the pelleted DNA was washed with 70% ethanol and centrifuged at 10,000 x g for 5 min at room temperature. The DNA pellet was then air-dried and 50 µl of TE (10 mM Tris HCl − 5mM EDTA, pH 7.8) buffer was added to dissolve the DNA.

2.8 Comparison of four DNA extraction procedures

The DNA concentration was measured by using UV/Visible spectrophotometer at 260 nm and the amount of DNA was calculated (1.0 A_{260} unit = 50 µg/ml) (Yeates *et al* 1998). The purity of extracted DNA was determined by ratio of A_{260}/A_{280} (Sambrook *et al*., 2001). The chemical cost and the total time per single extraction was calculated for each method. Approximately 10 ng of genomic DNA from *S. agalactiae* was amplified in a final volume of 50 µL with the optimized reaction mixture containing 0.4 µM of each primer, 2.5 mM $MgCl_2$, 0.5 U of Taq polymerase (Fermentas, USA), 200 µM of each dNTPs, 10 mM Tris HCl (pH 8.3.) The amplification reaction was performed in a Veriti® 96 well thermo cycler, with an initial denaturation at 95°C for 5 min, followed by 36 cycles of 95°C for 1 min, 59°C for 30 S, and 72°C for 30 S, then a final incubation at 72°C for 7 minutes. PCR amplified products were analyzed by gel electrophoresis in 2% agarose containing 0.5 µg/mL ethidium bromide in TBE buffer (Henegariu *et al*., 1997). Gel band intensities were analyzed using Image J software and minimum detection limit for each extraction method was determined by choosing the band having minimal bacterial density with optical density above 100. Subsequently, a DNA extract from *S. aureus* obtained earlier was subjected to PCR reaction to analyze the sensitivity with same conditions except annealing temperature at 61°C.

2.9 Optimization of M-PCR assay

M-PCR was performed in a final volume of 25 µL reaction mixture with approximately 10 ng of each *S. agalactiae* and *S. aureus* DNA along with milk somatic cell DNA, 2 mM $MgCl_2$, 1 µM of

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each STAA (F+R), STRA (F+R) and CYTB (F+R), 2.5µl of 10X PCR buffer, 1 unit of Taq DNA polymerase (Fermentas, USA), and 200 µM dNTP mixture. The amplification reactions were performed in a Veriti® 96 well thermo cycler, with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 30 S, and 72°C for 30 S, then a final incubation at 72°C for 7 min. The amplified DNA was visualized in 2% agarose gel.

2.10 Sensitivity of the M-PCR for simultaneous detection.

DNA extracted from milk inoculated with bacterial densities from 10 to 10^8 CFU/ml of *S. agalactiae* and *S. aureus* using Sodium Iodide method were subjected to M-PCR. Sensitivity was analyzed based on the band intensities.

2.11 Sensitivity of the M-PCR for individual detection.

DNA extracts from 10 to 10^8 CFU/ml of *S. agalactiae* and *S. aureus* were separately subjected to M-PCR. Sensitivity was analyzed based on the band intensities.

2.12 Specificity of the M-PCR

Three separate DNA extracts of *S. aureus*, *S. agalactia* (from 10^8 CFU/ml bacterial density solutions) and fresh milk was mixed with DNA from 4 frequent mastitis pathogens *Streptococcus disgalactiae, Streptococcus pyogenes, Streptococcus uberis* and *Escherichia coli* having 10^8 CFU/ml concentrations. Then M-PCR was carried out.

3. Results and Discussion

Results of the comparison of DNA extractions are given in Table 2 and further details are shown in Figure 1. The selection of the published procedures was based on target species, cost, specialized instruments or chemicals and pre-enrichment steps. All 4 methods are targeting gram positive organisms, because most of the mastitis pathogens are gram positive and they require specific extractions due to their resistant cell walls (Boom *et al*., 1990). On the other hand, these methods can be easily applicable to gram negative organisms as well. Cost per single extraction is minimal and did not require specialized instruments like columns and specific chemicals which are not easily found in normal laboratories. This method did not demand time consuming selective pre-enrichment procedures for the detection of target species. Subsequent analysis of sodium iodide method for *S. aureus* revealed minimum detection limit as 10 CFU/ml.

Sodium iodide method gave the highest yield correlated with the least number of centrifugation steps. The highest sensitivity shown by this method may be due to its efficient removal of impurities along with proper lysis and use of molecules such as Glycogen as a carrier for DNA. Silica method resulted in a relatively low yield, most probably due to highest number of centrifugation steps. However, it showed the second highest detection limit indicating that removal of PCR inhibitory substances effectively. Sensitivity results of sodium iodide and phenol chloroform methods have agreed with its initial publications but neither Silica nor Urea-SDS methods, initially reported as 10 CFU/ ml.

Phenol chloroform method resulted in the second highest DNA yield but lowest sensitivity, which could be due to contamination of the phenol, a known PCR inhibitor and the inhibitory compounds that accumulated in the DNA extract of this method, especially EDTA, which is not removed by phenol chloroform extraction and ethanol precipitation, as it co-precipitates with nucleic acid (Sambrook *et al*., 2001). The lowest purity of Urea-SDS method might due to lower number of centrifugation steps & inefficient lysis that may have been responsible for the lowest DNA yield. When NaOH was used in the lysis buffer amplifications became weaker and it has been reported that NaOH solutions can affect the sensitivity of the PCR (Ramesh *et al*., 2002). This might be the reason for lower sensitivity in this method.

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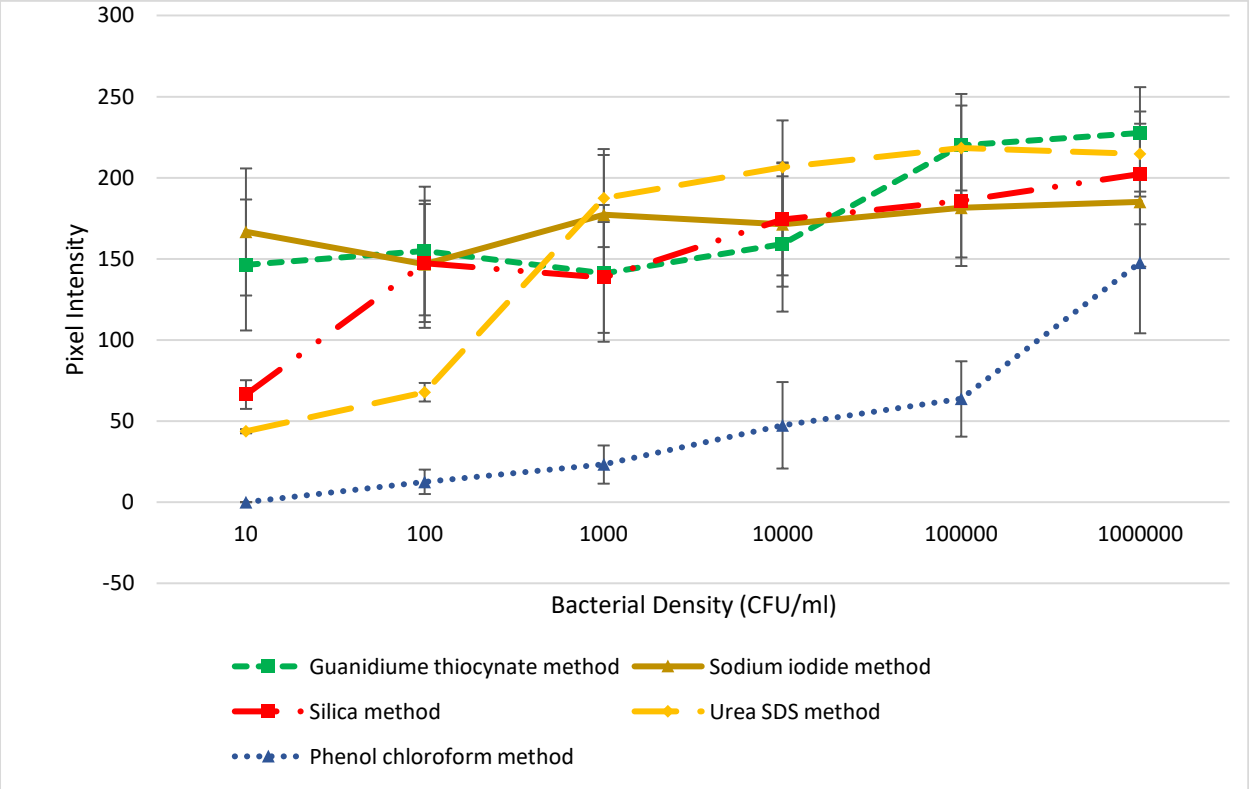

Figure 1. Optical densities of gel bands for different bacterial densities.

Table 2. Comparison of the efficiency of the four DNA extraction methods employed in the study

Method	Number of Centrifuga -tion steps	Sample processing time	cost per single extraction (**USD**)	Total DNA yield (ng/ml)	Purity (absorbance at 260/280 nm)	Minimum detection limit (CFU/ml)
Silica	10	90 minutes	0.30	52.92 (±5.8)	1.4 (±0.083)	10^2
Sodium iodide	2	80 minutes	0.16	405.0 (±27.6)	1.93 (±0.30)	10
Urea-SDS	3	240 minutes	0.10	21.3 (±3.4)	0.83 (±0.33)	10^3
Phenol Chloroform	3	325 minutes	0.09	269.25 (±23.0)	1.21 (±0.15)	10^6

Phenol chloroform method has the least cost for single extraction whereas Silica method has the highest. The almost linear relationship between the cost and the sensitivity indicates that reagents that are needed for the extraction process that yields a higher sensitivity also drives the unit cost. Time is an indicator of the rapidity of the procedure which is a sum of steps and time spent in each step. If the procedure needs lots of manipulation time, the probability of the degradation of the extracted DNA is high (Sambrook *et al*., 2001). On the other hand, decreased processing time improve the ease of sample handling and minimizes the risk of cross-contamination. Silica and Sodium iodide method indicated the minimal time requirement of manipulation.

Considering the results of the M-PCR, the optimized reaction contained 0.5 µM, 0.4 µM and 0.1 µM of each of STAA (F+R), STRA (F+R) and CYTB (F+R) respectively, 2 mM $MgCl_2$ and 1.5

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U of Taq DNA polymerase and 200 µM from each dNTPs. The reaction profile was 5 min of denaturation at 95°C and 36 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by final incubation of 7 min at 72°C. M-PCR was able to detect both bacteria simultaneously up to 10^5 CFU/ml and individually 10 CFU/ml for *S. agalactiae* and 10^3 CFU/ml for *S. aureus* with high specificity of primers indicated by absence of any cross reactivity with each other and frequent mastitis causing bacteria. Details about sensitivity and specificity are shown in Figures 2 to 5.

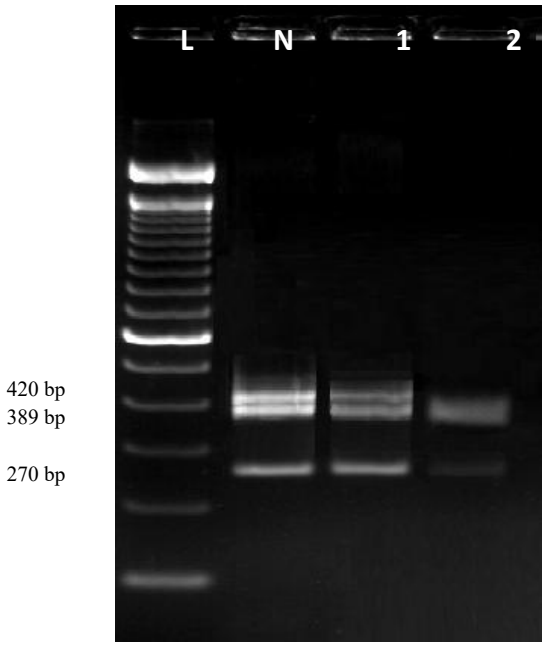

Figure 2. Sensitivity of the M-PCR for simultaneous detection. L – 100 bp ladder, lane 1 to 3 are *S. aureus* 10^6 to 10^4 CFU/ml extracts.

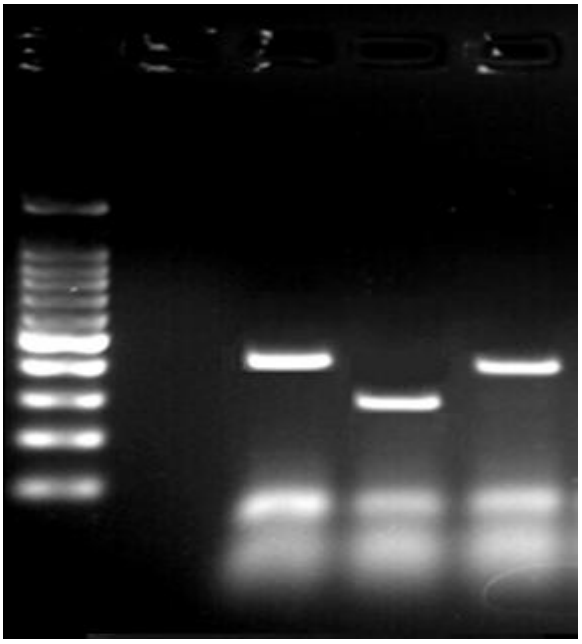

Figure 3. Specificity of the M-PCR. L – 100 bp ladder, lane 1 – DNA of *S. aureus* 10^8 CFU/ml extract, lane 2 – DNA of *S agalctiae* 10^8 CFU/ml extract, Lane 3 –DNA of fresh milk & N is the negative control. All three DNA samples were mixed with *Streptococcus disgalactiae, Streptococcus pyogenes Streptococcus uberis* and *Escherichia coli* DNA with 10^8 CFU/ml extracts.

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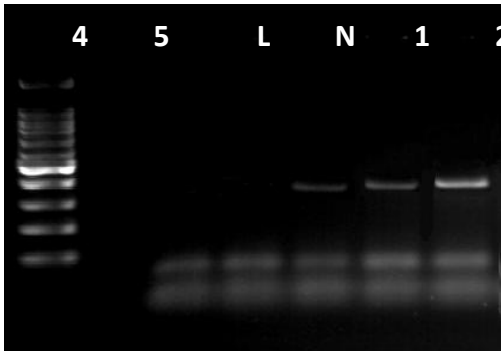

Figure 4. Sensitivity of the M-PCR for *S. aureus*. L – 100 bp ladder, lane 1 to 5 are *S. aureus* 10 to 10^5 CFU/ml extracts and N is the negative control.

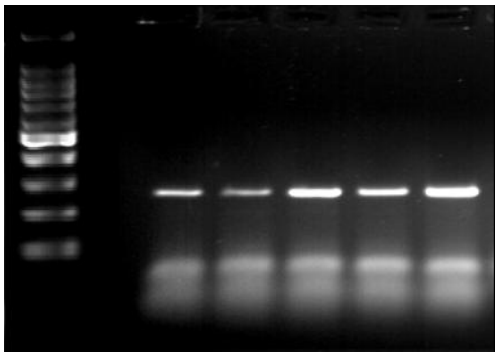

Figure 5. Sensitivity of the M-PCR for *S. agalactiae*. L – 100 bp ladder, lane 1 to 5 are *S. agalactiae* 10 to 10^5 CFU/ml extracts and N is the negative control.

One of the problems often encountered with M-PCR is a reduction of sensitivity, which was practically encountered for *S. aureus*; detection limit 10 CFU/ml in the simplex PCR was increased from 10^2 CFU/ml in M-PCR individual detection and further 10^2 CFU/ml in the simultaneous detection. This result is in total agreement with the results obtained by Phuektes *et al*., 2001), had 10-to-100-fold reduction in sensitivity. This may be because of the competition between individual reactions for dNTPs and Taq polymerase when multiple primer sets are combined in a single reaction (Madico *et al*., 2000). These unequal amplifications indicate that S *aureus* amplification loci is particularly weaker than *S. agalactiae.*

Primers which are specific to the bovine mitochondrial cytochrome B gene are intended to react with bovine somatic cells that are normally present in milk. Thus, in M-PCR, the amplification product of CYTB (F+R) should always appear for milk samples. This was proven by the results with presence of CYTB (F+R), even when the bacterial amplification targets disappeared in 10^4 CFU/ml bacterial density. Complete absence of product indicates a technical problem in the amplification or DNA extraction. Integration of CYTB (F+R) to the M-PCR demanded precise optimization steps with other primers, because bovine DNA always be the high concentrated majority, its amplification would decrease the amplification of the low concentrated bacterial targets by competition for dNTPs and Taq polymerase in the reaction.

Mammary gland is considered infected by subclinical mastitis when having no clinical signs or abnormal milk, in which ≥500 CFU/ml of bacteria were isolated according to the Ribeiro *et al*., 1999). Sodium Iodide method was able to detect less density of bacteria in milk than above limit for both species and developed M-PCR was also same for individual detection of *S. agalactiae* but slightly high detection limit for *S. aureus* than above. With compatible sensitivities, both the extraction method and M-PCR indicate their suitability for subclinical mastitis detection.

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4. Conclusions

Four DNA extraction methods from cow's milk were compared, where sodium iodide method was identified as the most convenient, efficient and cost-effective method for the bacterial DNA extraction from bovine milk. Multiplex-PCR is sensitive, specific and reliable in identification of the major mastitis pathogens from milk, especially in the sub clinically infected animals. Simultaneous identification of *S. agalactiae* and *S. aureus* integrated with the Sodium iodide method make it more efficient and convenient in the detection compared to conventional PCRs and culture methods. Thus, there is a high potential to develop the Multiplex-PCR based detection as a commercially available kit for routine diagnosis of subclinical mastitis.

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