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Research Article

Development of A Multiplex PCR Method for Direct Detection of Common Mastitis Pathogens in Bovine Milk Samples [1] [2]

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Abstract

The aim of this study was to evaluate a simple and rapid DNA extraction method combined with a multiplex polymerase chain reaction (mPCR) for the identification of the major mastitis pathogens *(Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli* and *Mycoplasma bovis)* from milk samples. Of the 200 California Mastitis Test (CMT) positive milk samples, 45 (22.5%), 21 (10.5%) and 11 (5.5%) were detected as positive for the presence of *S. aureus, S. agalactiae* and *E. coli* by culture, respectively. In mPCR by DNA isolation method optimised here, *S. aureus, S. agalactiae* and *E. coli* were detected in 26.5% (53/200), 12% (24/200) and 6% (12/200) of the milk samples, respectively. The abovementioned agents were observed in similar proportions when the samples were analysed by a commercial DNA isolation kit. On the other hand, *M. bovis* was not detected in any of the milk samples by either culture or mPCR methods. A significant difference was determined between the results of culture and mPCRs (P<0.001). Diagnostic sensitivity and specificity of the optimised mPCR were calculated as 100% and 89.2% respectively, when culture results were considered as reference. The results suggest that the mPCR assay employed in this study could be used as an alternative routine diagnostic method for rapid, sensitive, and specific simultaneous detection of major mastitis agents in bovine milk samples.

Keywords: *Mastitis, Major Pathogens, DNA isolation, Multiplex PCR*

Mastitisli İnek Sütlerinde Önemli Patojenlerin Direkt Tespiti İçin Bir Multipleks PCR Yönteminin Geliştirilmesi

Özet

Bu çalışmanın amacı, inek sütlerinde major mastitis patojenlerinin *(Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli* ve *Mycoplasma bovis)* saptanabilmesi için hızlı, basit ve spesifik bir DNA ekstraksiyon ve multipleks PCR yöntemi geliştirmektir. Kaliforniya mastitis testi (CMT) pozitif 200 süt örneğinin kültürü sonrasında *S. aureus, S. agalactiae* ve *E. coli* varlığı sırasıyla 45 (%22.5), 21 (%10.5) ve 11 (%5.5) örnekte saptandı. Çalışmada optimizasyonu yapılan DNA ekstraksiyon metodu ile elde edilen DNA örneklerinin mPCR analizi neticesinde *S. aureus, S. agalactiae* ve *E. coli* varlığı sırasıyla %26.5 (53/200), %12 (24/200) ve %6 (12/200) olarak belirlendi. Ticari DNA izolasyon kitiyle elde edilen DNA örneklerinde de yukarıdaki etkenlerin varlığı benzer oranda bulundu. Öte yandan süt örneklerinin hiçbirinde kültür veya mPCR ile *M. bovis* tespit edilmedi. Kültür ve mPCR sonuçları arasındaki fark istatistiki olarak önemli bulundu (P<0.001). Kültür sonuçları referens olarak kabul edildiğinde, optimize edilen mPCR'ın sensitivitesi ve spesifisitesi sırasıyla %100 ve %89.2 olarak hesaplandı. Bu sonuçlara göre; çalışmada kullanılan mPCR yöntemi sığır süt örneklerinde majör mastitis etkenlerinin hızlı, duyarlı, spesifik ve eş zamanlı tespit edilmesinde alternatif rutin teşhis metodu olarak kullanılabilir.

Anahtar sözcükler: *Mastitis, Majör patojenler, DNA izolasyonu, Multipleks PCR*

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INTRODUCTION

Mastitis is a frequent intramamary infection seen in dairy herds and leads to significant economic losses (estimated at $150-300/cow, per year) in dairy industry all over the world [1-4]. It constitutes the 26% of cattle diseases and losses due to mastitis in the USA have been recorded as two billion dollars annually [3]. Studies conducted in dairy herds in many countries represented the prevalence of disease in cattle as 50% and the ratio of infection in udder quarters as 25% [5].

The most common pathogens that cause mastitis are known as contagious agents including *S. aureus*, *S. agalactiae*, *Mycoplasma* sp. and, environmental agents including *E. coli*, *S. dysgalactiae*, *S. uberis* [6-10]. It is stated that eleven different *Mycoplasma* species induce mastitis [11]. Losses due to *M. bovis* mastitis in the USA have been estimated as 108 million/year dollars and the proportion of disease in infected herds has been reported over the 70% [12]. Recent studies indicated that the prevalence of mycoplasmal mastitis has increased in most regions of the world in last years [13,14].

Although the culture method has been regarded as a gold standard for identification of mastitis pathogens, it is labor-intensive and time-consuming [15]. The more sensitive and specific PCR assays allow examining a wide range of samples in a short time. Direct DNA extraction from samples, without the need for cultivation of agents, provides to PCR to accomplish a reliable direct identification [16]. mPCR facilitates the simultaneous identification of different bacterial pathogens in a few hour time, although conventional culture methods require several days. So far, in order to determine the causes of mastitis in milk samples directly, either single bacterium (by PCR) or most common species such as *S. aureus* and *S. agalactiae* (together by mPCR) have been studied in researches carried out in several regions of the world [17-20]. However, mycoplasmas have been neglected in mastitis cases in recent years, due to the labor-intensive and time-consuming characteristics and owing to presence of other major agents such as contagious and environmental agents at high proportions in milk.

The purpose of this study was to develop rapid, simple, sensitive and specific DNA extraction and mPCR method for the detection of major mastitis pathogens (*S. aureus*, *S. agalactiae*, *M. bovis* and *E. coli*) in bovine milk samples, without the need for culture and biochemical identification.

MATERIAL and METHODS

Milk Samples

CMT positive milk samples were collected from 200 dairy cattle in 25 different farms located in Elazig province, in eastern Turkey. Approximately 10 mL of milk sample was taken into sterile tubes from udders aseptically and transferred to labortory in cold chain conditions. In addition, 19 *M. bovis* positive milk samples provided from a previous study [14] were included for use in mPCR tests.

Culture and Isolation

Milk samples were inoculated on blood agar base (Merck) supplemented with 5% defibrinated sheep blood and incubated aerobically for 48 h at 37°C for the isolation of *S. aureus*, *S. agalactiae* and *E. coli*. Also, milk samples were inoculated onto Edwards Medium Modified Agar (Oxoid) for *S. agalactiae* and Eosine Methylene Blue Agar (Oxoid) for *E. coli*. Additionally, samples were plated onto supplement G including Mycoplasma Agar (Oxoid) and incubated at 37°C in 5% CO_2 for 7 to 10 days for *M. bovis*. After biochemical identification, suspected isolates were subjected to DNA extraction and PCR. *S. aureus* (ATCC 25923), *S. agalactiae* (ATCC 13813), *E. coli* (ATCC 25922) and *M. bovis* (provided from Department of Microbiology, Faculty of Veterinary Medicine, Firat University) reference strains were used as positive controls in all assays.

Direct DNA Extraction from Milk Samples

Two different extraction procedures were employed for direct DNA extraction from milk samples. In the first, a commercial product named as Milk Bacterial DNA Isolation Kit (Norgen Biotek, Canada) was used to extract the bacterial DNA from milk samples. The extraction was carried out as described by the manufacturer.

In the second, one mL of milk sample was transferred into an eppendorf tube and was mixed with the same volume of Tris-EDTA buffer for washing. Then, the mixture was centrifuged at 11.600 g for one min. The washing process was repeated until a clear suspension was seen. Clear suspension was centrifuged at 11.600 g for 1 min, the pellet was resuspended in 300 µL distilled water and was used for DNA extraction. The spiked milk suspension was treated with 300 µL TNES buffer (20mM Tris, pH 8.0, 150mM sodium chloride, 10mM EDTA, 0.2% sodium dodecyl sulphate) and 200 µg/mL proteinase K, and was incubated at 56°C for one hour. Then, 5 µL Lysostaphin (Sigma, 100 µg/mL) and 10 µL Mutanolysin (Sigma, 5U/µL) were added onto this suspension that was re-incubated at 37°C for 1 h. The suspension was then heated at 95°C for 10 min to inactivate the enzymes, followed by centrifugation for 10 min at 11.600 g. Finally, the suspension was removed; the pellet was dried and resuspended in 100 µL sterile distilled water.

Limit of Detection

S. aureus ATCC 25923 was used for evaluating the second DNA extraction method which was developed in this study. Several *S. aureus* colonies were suspended in sterile phosphate-buffered saline (PBS) and tenfold dilution series containing $6x10^7$ to $6x10^0$ CFU per mL were prepared. All the tubes were centrifuged and the suspension was

removed. One ml sterile milk was added to each tube and tubes were gently vortexed until cells were resuspended. Finally, DNA extraction procedure described above was applied and the detection limit was calculated.

Multiplex PCR Method

DNA samples of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis* reference strains were subjected to amplification alone and in combinations in order to optimize mPCR. Appropriate PCR mix was arranged for mPCR conditions. The mPCRs were performed in a total reaction volume of 50 µL, containing 5 µL 10x PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 8 µL 25 mM $MgCl_2$ 200µM of each deoxynucleotide triphosphate, 2 U Taq DNA Polymerase (MBI Fermentas), 20 pmol of each species specific primer *(Table 1)* and 5 µL of template DNA. Techne 512 Gradient PCR (Techne, England) was used to optimize the best annealing temperature for all primer pairs. The optimal annealing temperature (58°C) was achieved by using an automated program ranging from 50°C to 62°C. The conditions for the mPCR were an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min (denaturation), 58°C for 1 min (primer annealing), 72°C for 1 min (extension) and a final extension step at 72°C for 5 min. The amplified products were detected by ethidium bromide (10 mg/mL) staining after electrophoresis at 80 V for two hours in 2% agarose gels.

Statistical Analyses

Comparison of the methods (culture and mPCRs) with regard to their rate of positivity in milk samples was carried out by the Cochran Q test, followed by pairwise comparisons applying McNemar tests. In all statistical tests, $P<0.05$ value was considered statistically significant. Analyses were performed using the commercial software SPSS 14.0.

RESULTS

Bacteriological Findings

After the incubation period, suspected colonies grown on specific media were examined and identified by primary identification tests. Of the 200 milk samples 114 (57%) were found to be positive by culture. When the culture results were assessed at bacterial species level, 45 (22.5%), 21 (10.5%) and 11 (5.5%) of the milk samples were detected as positive for the presence of *S. aureus, S. agalactiae* and *E. coli,* respectively. The isolation percentages of mixed infections of *S. aureus* + *S. agalactiae* and *S. aureus* + *E. coli* were calculated as 3.5% (7/200) and 2.5% (5/200), respectively. Other bacteria which were detected in 25 samples were beyond the scope of this study. On the other hand, none of the milk samples were positive for *M. bovis*. The overall isolation percentage of the major pathogens examined in this study was determined as 44.5% (89/200) *(Table 2)*.

mPCR Findings

DNAs obtained from milk samples by commercial DNA isolation kit were subjected to optimized mPCR combined with species specific primer pairs of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis.* Amplification products at the molecular sizes of approximately 232, 447, 586 and 1318 bp which were considered indicative for *E. coli*, *M. bovis*, *S. agalactiae* and *S. aureus,* respectively, were detected *(Fig. 1)*. This indicated that the mPCR assay optimised in this study was working well.

Similar results were observed in the mPCR analysis of DNAs extracted from CMT positive milk samples by both commercial DNA isolation kit (mPCR-1) and optimised direct isolation method (mPCR-2). In total, 104 (52%) and 101 (50.5%) of milk samples were found positive for either of the mastitis agents examined here by the commercial kit and optimised method, respectively. In mPCR analysis of milk samples by optimised DNA isolation method (mPCR-2), specific amplification products that were considered indicative for *S. aureus*, *S. agalactiae* and *E. coli* were detected in 26.5% (53/200), 12% (24/200) and 6% (12/200) of the milk samples respectively. In addition, mixed infections of *S. aureus* + *S. agalactiae* (7/200) and *S. aureus* + *E. coli* (5/200) were identified by mPCR examination. *M. bovis* was not detected in mPCR analysis, either. On the other hand, detection rates of *S. aureus*, *S. agalactiae* and *E. coli* were estimated as 27% (54/200), 12.5% (25/200) and 6% (12/200) respectively in mPCR analysis of milk samples

Table 1. *Primer sequences and lengths of mPCR amplification products*

Primer	Specificity	Sequences (5'-3')	Fragment Size (bp)	References
Sau327	*S. aureus* (f)	GGACGACATTAGACGAATCA	1318	[16]
Sau1645	*S. aureus* (r)	CGGGCACCTATTTTCTATCT		
Sag432	*S. agalactiae* (f)	CGTTGGTAGGAGTGGAAAAT	586	[16]
Sag1018	*S. agalactiae* (r)	CTGCTCCGAAGAGAAAGCCT		
Eco223	*E. coli* (f)	ATCAACCGAGATTCCCCCAGT	232	[16]
Eco455	*E. coli* (r)	TCACTATCGGTCAGTCAGGAG		
Mb-Mp1	*M. bovis* (f)	TATTGGATCAACTGCTGGAT	447	[21]
Mb-Mp2	*M. bovis* (r)	AGATGCTCCACTTATCTTAG		

Table 2. *Bacteriological and mPCR findings obtained from bovine milk samples*

Bacterial strains	**Culture**	**mPCR-1***	**mPCR-2****
S. aureus	45 (22.5%)	54 (27%)	53 (26.5%)
S. agalactiae	21 (10.5%)	25 (12.5%)	24 (12%)
E. coli	11(5.5%)	12 (6%)	12 (6%)
S. aureus + *E. coli*	5 (2.5%)	5 (2.5%)	5 (2.5%)
S. aureus + *S. agalactiae*	7 (3.5%)	8 (4%)	7 (3.5%)
M. bovis	-	-	-
Other bacteria	25 (12.5%)	ND	ND
Total	**114 (57%)*****[a]**	**104 (52%)[b]**	**101 (50.5%)[b]**

** Commercial Milk Bacterial DNA Isolation Kit (Norgen Biotek, Canada); ** Optimized Direct DNA Isolation Method; *** Statistical tests applied for S. aureus, S. agalactiae and E. coli isolation ratio (44.5%); [a,b] different letters represents significancy of difference between groups;* ***ND:*** *Not determined*

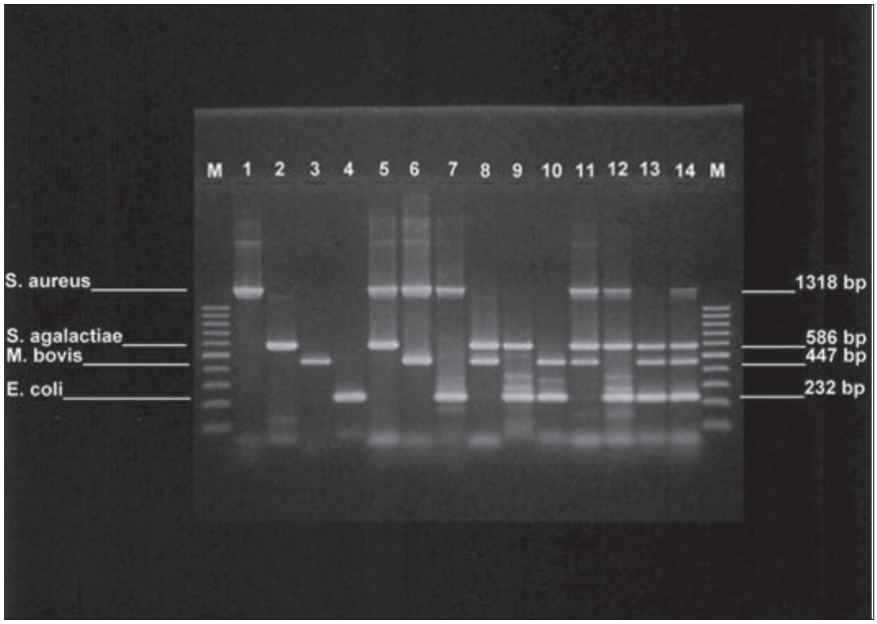
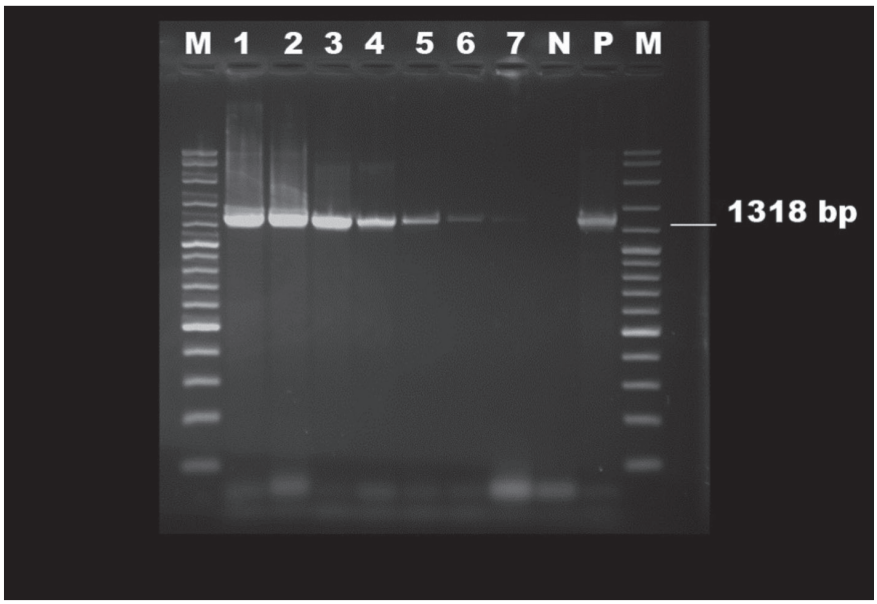


Fig 1. Agarose gel electrophoresis of products of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis* isolates determined by optimized multiplex polymerase chain reaction M: 100 bp DNA Ladder (Fermentas, SM 0241). Lane 1: *S. aureus*, Lane 2: *S. agalactiae*, Lane 3: *M. bovis*, Lane 4: *E. coli*, Lane 5: *S. aureus* + *S. agalactiae*, Lane 6: *S. aureus* + *M. bovis*, Lane 7: *S. aureus* + *E. coli*, Lane 8: *S. agalactiae* + *M. bovis*, Lane 9: *S. agalactiae* + *E. coli*, Lane 10: *M. bovis* + *E. coli*, Lane 11: *S. aureus* + *S. agalactiae* + *M. bovis*, Lane 12: *S. aureus* + *S. agalactiae* + *E. coli*, Lane 13: *S. agalactiae* + *M. bovis* + *E. coli*, Lane 14: *S. aureus* + *S. agalactiae* + *M. bovis* + *E. coli*

Fig 2. Agarose gel electrophoresis representing detection limit of optimized DNA extraction method. M: 100 bp DNA Ladder (Fermentas, SM 0321) Lane 1: 6 x 10^7 bacteria/mL milk, Lane 2: 6 x 10^6 bacteria/mL milk, Lane 3: 6 x 10^5 bacteria/mL milk, Lane 4: 6 x 10^4 bacteria/mL milk, Lane 5: 6 x 10^3 bacteria/mL milk, Lane 6: 6 x 10^2 bacteria/mL milk, Lane 7: 6 x 10^1 bacteria/mL milk, N: 6 x 10^0 bacteria/mL milk, P: *S. aureus* ATCC 25923



by commercial DNA isolation kit (mPCR-1). Also, mixed infections of *S. aureus* + *S. agalactiae* (8/200) and *S. aureus* + *E. coli* (5/200) were identified by mPCR examination *(Table 2)*.

When the culture was considered as gold standard, the sensitivity and specificity of the mPCR-2 were calculated as 100% (89/89) and 89.2% (99/111), respectively. On the other hand, when mPCR assay combined with the commercial isolation kit was considered as gold standard, the sensitivity and specificity of the mPCR-2 were evaluated as 97% and 100%, respectively. The isolation percentages of the major mastitis pathogens by culture, mPCR-1 and mPCR-2 were compared with Cochran's Q statistical test and the difference was significant ($P<0.001$). The McNemar test indicated a significant difference between the culture and two mPCR methods ($P<0.001$). However, the difference between the mPCR-1 and mPCR-2 was not significant ($P>0.05$).

Although no positive results were obtained for *M. bovis* in the analysis of 200 milk samples by both culture and molecular tests, mPCR analysis of DNA samples extracted by both procedures from 19 *M. bovis* positive cultures which were provided from a previous study yielded amplification products at the molecular size of approximately 447 bp, which was indicative for the presence of *M. bovis*.

The Limit of Detection

DNA samples obtained from tenfold dilution series of spiked milk samples containing *S. aureus* ATCC 25923 ($6x10^7$ to $6x10^0$ CFU/mL) were tested in order to compare detection limit of the optimized DNA extraction method with the commercial DNA isolation kit. The minimum detection limit of the optimized method was estimated as $6x10^1$ CFU/mL with the specific band at the molecular size of approximately 1318 bp *(Fig. 2)*. On the other hand, the commercial DNA isolation kit has been declared to detect as few as 10 CFU/mL in milk samples by the manufacturer.

DISCUSSION

Mastitis is a disease of dairy cattle and cause significant economic losses in milk industries [22]. In order to develop control strategies in dairy farms, rapid and reliable identification of pathogens that cause mastitis is very important [23]. Identification of bacteria by culture methods requires 2 to 10 days and mix infections or closer species cannot be distinguished by biochemical assays. Although the culture method has been regarded as a gold standard for identification of pathogens of mammary gland, it is arduous, time-consuming and sometimes incapable [15,24]. In order to overcome identification problems such as growth failure of bacteria, a mPCR in combination with direct bacterial DNA isolation from milk samples was employed to detect the most common mastitis pathogens *(S. aureus*, *S. agalactiae*, *M. bovis* and *E. coli)*. This is the first study conducted in Turkey for direct identification of four mastitis agents in bovine milk simultaneously.

Bacterial growth was observed in 44.5% of 200 CMT positive milk samples in the present study. This rate was lower than those obtained in mPCR assays (mPCR-1: 52% and mPCR-2: 50.5%) combined with both extraction procedures indicating that mPCR assays were superior to culture in terms of sensitivity. Multiple factors may influence the performance of cultivation results. The absence of bacterial growth has been reported as 20-30% of milk samples in clinical mastitis [25-28]. Besides, in Canada 43.9% of milk samples have been declared as culture negative [29]. In a study conducted in subclinical-clinical mastitis (not separated), no growth was observed in almost half of the samples [24]. It was mentioned that failure of bacterial growth in milk samples may be due to low bacterial concentration, pathogens not growing on standard medium or existence of substances that suppress the observation [30,31]. Milk samples may contain external inhibitor substances such as antimicrobial or disinfectant residues due to treatment of udders before sampling and this situation may suppress the bacterial growth [32].

The mPCR assays were compared with conventional culture and a statistically significant difference between the tests was noted. This is not surprising because milk harbor several organisms that can be difficult to culture, particularly when samples are not plated immediately. The sensitivity and the specifity of the optimised mPCR were calculated as 100% and 89.2% respectively, when culture results were considered as gold standard. Amplification products for at least one of the mastitis agents studied here were obtained in twelve milk samples from which no growth was observed.

The PCR facilitates sensitive, rapid, reliable, objective and user-independent detection of bacterial and antimicrobial resistance genes. mPCR ensures rapid identification of many species simultaneously and reduces false negative results, when used in addition to conventional methods in mastitis control programs [33,34]. In this study, identification of pathogens from milk samples was accomplished in a short time (six hours) by mPCR, without a bacterial culture step. It has been reported that rapid detection of mastitis reduces the treatment time, enhances the cure rates and decreases inappropriate and redundant antimicrobial usage [35,36].

Although the detection of DNA in milk samples is not sufficient to indicate the presence of the disease, identification of pathogens by mPCR could be helpful to get rapid information and to develop control strategies. Despite the fact that molecular methods are regarded as integrant or alternative for conventional methods [16], for direct detection of milk pathogens, milk samples may contain PCR inhibitor substances. In the examination of mastitis, highly qualified DNA extraction protocols and reagents should be implemented to milk samples to obtain decisive results.

Many commercial extraction kits were developed and marketed for this purpose but they are costly. In this study, a DNA extraction method was developed and compared with a commercial kit. The direct DNA extraction method from milk was evaluated to detect as few as 6 x 10^1 CFU/mL, while the minimum detection limit of the commercial DNA isolation kit has been declared as 10 bacteria/mL by the manufacturer. This slight difference might be overlooked when the cost is taken into consideration. Commercial kit requires enzyme (lysostaphin) addition for isolating DNA from Gram positive (especially *S. aureus*) bacteria. Besides, isolation percentages of two methods were compared and the difference between the mPCR1 and mPCR 2 was not significant (P>0.05). The new optimised DNA extraction method can therefore be suggested as an alternative to commercial kits owing to the fact that it could easily be modified and performed in many routine laboratories and the cost of the extraction per sample is cheaper than commercial kits.

S. aureus and *S. agalactiae* are regarded as major mastitis agents. In previous studies, mastitis pathogens have been studied merely by PCR or at most two agents by mPCR. Thus other mastitis pathogens and especially labor-intensive mycoplasmas which require adequate laboratory facilities, experienced personnel were ignored [37]. Four different bacteria including *M. bovis* were successfully identified in spiked milk samples by mPCR method that was developed in the current study. This may lead to clarify mix infections in mastitis. Additionally, both the commercial kit and optimized DNA extraction method were tested with 19 milk samples submitted to our laboratory which were indicated as positive for *M. bovis* by culture and PCR. Direct DNA extraction from these milk samples by both methods followed by mPCR produced positive results for *M. bovis* which requires 7-10 days for cultivation.

It can be concluded that DNA isolation and mPCR methods developed in this study are more sensitive and faster than conventional culture, and can be easily applied for detection of *S. aureus*, *S. agalactiae*, *E. coli* and *M. bovis* in milk samples simultaneously. It can therefore be used as alternative to conventional culture method in the routine diagnosis. Early detection of mastitis by mPCR may contribute to surveillance programs, and to planning prevention and control strategies. Also this study would be basis of further studies that aim to optimize and perform new processes for examining other bacterial agents that cause mastitis.

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