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Optimization of a Multiplex PCR Protocol for Detection of *Escherichia coli* and *Staphylococcus aureus* in Bovine Subclinical Mastitis in Sri Lanka

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ABSTRACT

Bovine mastitis is an inflammatory infectious disease of the mammary gland of dairy cows. The most common mastitis pathogens are *Staphylococcus aureus* and *Escherichia coli.* Current diagnostic methods are based on microbial culture of milk. However, these methods have limitations since they are time consuming and labour intensive. The aim of this study was to optimize a rapid Polymerase Chain Reaction (PCR) based multiplex protocol to detect *Escherichia coli* and *Staphylococcus aureus* in bovine subclinical Mastitis in Sri Lankan conditions. Pure DNA was extracted from *Escherichia coli* and *Staphylococcus aureus* using DNA extraction kits. These samples were used as positive controls. Genomic DNA was extracted from California Mastitis Test (CMT) positive milk samples according to the rapid DNA extraction protocol. Species specific primers were synthesized as previously described in the literature to amplify 884 bp and 229 bp PCR fragments for *Escherichia coli* and *Staphylococcus aureus* respectively. For the positive multiplex control, DNA concentration, primer concentration and annealing temperature were optimized separately. The optimized conditions for the multiplex of positive controls were, 50 ng DNA of *Escherichia coli* and 25 ng of *Staphylococcus aureus* DNA, 55 °C of annealing temperature and 200 nmol of each primer concentration from both species. Seventy five percent *Staphylococcus aureus* and 20% dual contaminations can be directly diagnosed by the optimized condition. Optimized multiplex conditions could be used directly to detect these two pathogens by PCR method to diagnose subclinical mastitis however contaminated sample analysis was not 100% successful.

KEYWORDS: Bovine subclinical mastitis, *Escherichia coli*, Multiplex PCR, *Staphylococcus aureus*

INTRODUCTION

Mastitis is the most common infectious disease among dairy cows that lower the milk yield and reduced the quality of milk in affected cows. It is the most important cause that leads to an economic loss in dairy industry of Sri Lanka (Hettiarachchi *et al.,* 2012) and throughout the world (Miller *et al.,* 1993). Mainly mastitis can be seen in two forms, sub clinical and clinical. Subclinical infection is difficult to identify as it does not have any visible change in udder or in milk, except reducing the milk yield and altered milk composition (Pradhan *et al.,* 2011).

In clinical mastitis, the cow displays definitive symptoms of the disease with hot, swollen udders and watery milk sometimes with clots. The most common mastitis pathogens are found either in the udder (Contagious pathogens: *Staphylococcus aureus* and *Streptococcus agalactiae*) or the cow's surroundings (Environmental pathogens: *Escherichia coli, Streptococcus dysgalactiae, Streptococcus parauberis;* Mahammed *et al.,* 2013).

Subclinical mastitis is caused by two main bacterial pathogens, *Staphylococcus aureus* and *Escherichia coli.* These pathogens needed to be identified at subclinical level before progression in to clinical stage to reduce the economic loss due to infection hence, and rapid accurate detection method is necessary to control the infection. The most commonly used methods for identification of bacteria are California Mastitis Test (CMT), somatic cell count (SCC) and culturing of bacteria (Pradhan *et al.,* 2011). Although conventional bacterial culture and biochemical test could identify the microbial pathogens responsible for mastitis, they are time consuming and labour intensive. The application of molecular diagnostic technique such as Polymerase Chain Reaction assay (PCR) is the most preferable, rapid, specific (Riffon *et al.,* 2000) and sensitive identification method of bacteria.

The primary objective of this study was to develop and optimize a multiplex PCR to detect

multiple pathogens that are responsible for subclinical mastitis in a single PCR reaction.

MATERIALS AND METHODS

Experimental Location

The study was conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila and Veterinary Investigation Center (VIC) Pannala, from December 2015 to May 2016.

Sample Collection

California Mastitis Test (CMT) positive milk sample with a score of two or above were collected from small scale dairy farms (Pannala, Kuliyapitiya and Alawwa).

Culturing of Bacteria

California Mastitis Test (CMT) positive milk samples were cultured in Nutrient Agar (NA) plates by streak plate method. Plates were incubated at 37 °C for 18 hours. Gram staining was done to distinguish the Gram positive and negative bacteria. Sub culturing was done in the Macconky Agar plates to confirm the *E. coli* bacteria. Catalase test was done to confirm the *Staphylococcus aureus*.

Genomic DNA Extraction

The genomic DNA from pure cultures was extracted by Column purification method using QIAGEN blood tissue kit (Catalogue no 69506).

The DNA was extracted from CMT positive milk samples according to rapid DNA extraction method developed by Iranpur and Esmailizadesh (2010). To 500 µL of milk sample 1000 µL of red cell lysis buffer (0.01M Tris-HCl pH 7.6, 320 mM sucrose, 5 mM $MgCl_2$, 1% Triton X 100) was added and mixed gently by inverting. Homogenate was spun for 2 minutes at 7 000 rpm. Supernatant was discarded, and to the pellet 400 µL of nucleic lysis buffer (0.01 M Tris-HCl, 11.4 mM sodium citrate, 1% sodium dodecyl sulphate (SDS), 1 mM EDTA), 100 µL of saturated sodium chloride (5 M) and 600 µL of pre chilled chloroform were added and mixed on a rotating blood mixture. Then it was spun for 2 minutes at 7 000 rpm. Four hundred microliters of supernatant was transferred to a new tube and 800 µL of ice cold absolute ethanol was added, contents were gently mixed and then vortexed. The tube was spun for 10 min at 7 000 rpm to precipitate DNA. The supernatant was discarded, the pellet was air dried and dissolved in 20 µL of TE buffer (1 M tris-HCl, 0.5 M EDTA, pH 8).

The extracted genomic DNA was quantified by running with a 100 bp ladder before PCR amplification.

PCR Amplification

The PCR conditions were initially optimized for *E. coli* and *Staphylococcus aureus* separately with species specific primers. Forward and reverse primers of *E. coli* were (884 bp) 5' CCG ATA CGC TGC CAA TCA GT 3', and 5' ACG CAG ACC GTA GGC CAG AT 3' respectively. The forward and reverse primers of *Staphylococcus aureus* were (229 bp) 5'TCT TCA GCG CAT CAC CAA TGC C 3' and 5' TTC GTA CCA GCC AGA GGT GGA 3' respectively (Pradhan *et al.*, 2011). For a 25 µL PCR reaction volume, added 1 µL of template DNA (100 ng/µL DNA of *Escherichia coli* and 25 ng/µL of *Staphylococcus aureus)*, 2.5 µL of 10X PCR buffer with 20 mM $MgCl_2$, 0.2 µL of 5 U/µL Taq polymerase, 2.5 µL of 2.5 mM dNTPs and 1 µL of 20 µM of each forward and reverse primers.

Multiplex PCR

Multiplex PCR was initially performed using the same concentrations and conditions that were used to amplify the bands of each of the species separately. Then it was optimized for DNA concentration (0.5, 0.75, and 1 µL) from 100 ng/µL DNA of *Escherichia coli* and 25 ng/µL of *Staphylococcus aureus*, primer concentration (200 nmol, 300 nmol 400 nmol) for each of the species, and annealing temperature (47.9, 52.9 and 55 °C).

Initial PCR was performed with following parameters, initial denaturation at 95 °C for 2 min, followed by 35 cycles, of denaturation at 95 °C for 45 sec, annealing at 45 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Polymerase Chain Reaction products were run in 1% Agarose with 0.5 mg/mL ethidium bromide.

RESULTS AND DISCUSSION

DNA Extraction

The agarose gel image of the DNA extracted from positive controls is given in Figure 1.

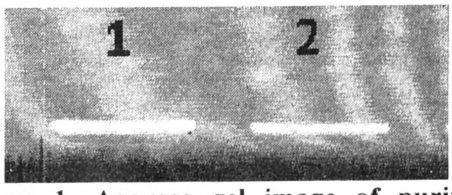

Figure 1. Agarose gel image of purified genomic DNA from positive controls. *Lane 1- Escherichia coli, Lane 2- Staphylococcus aureus*

Quantification of DNA

Extracted DNA was quantified approximately by agarose gel electrophoresis method, comparing the band intensity of 100 bp ladder which was run in parallel with the sample. It was quantified approximately as 100 ng/μL and 25 ng/μL for DNA of *Escherichia coli* and *Staphylococcus aureus* respectively (Figure 2).

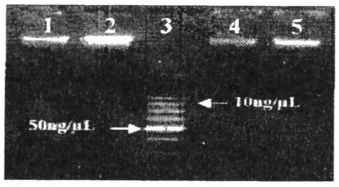

Figure 2. Agarose gel image: genomic DNA quantification of positive controls. *Lane 1- E coli (2 μL), Lane 2- E coli (4 μL), Lane 3- 100 bp ladder, Lane 4- Staphylococcus aureus, (2 μL), Lane 5- Staphylococcus aureus (4 μL)*

PCR Amplification of Positive Control

Polymerase Chain Reaction amplification of positive controls by species specific primers yielded 229 bp and 884 bp bands for *Staphylococcus aureus* and *E. coli* respectively (Figure 3).

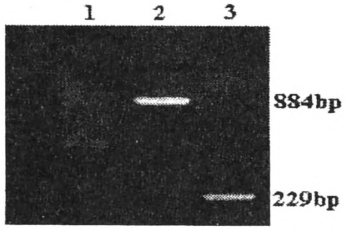

Figure 3. Agarose gel electrophoresis of PCR products for positive controls. *Lane 1- ladder (100 bp), Lane 2 - PCR of Escherichia coli. Lane 3- PCR of Staphylococcus aureus*

Multiplex PCR Amplification of Positive Control

Multiplex PCR assay was standardized for DNA concentration of each of the species, primer concentrations, annealing temperature and PCR master mixture volume.

The annealing temperature optimization was done for different temperatures (47.9 °C 52.9 °C and 55 °C Figure 4A). According to results, sharp bands were observed for *Staphylococcus aureus* at all three temperatures with less nonspecific bands; however *E. coli* bands were less apparent at this temperature. With increasing temperature the nonspecific bands for *Staphylococcus aureus* were increased but *E. coli* bands appeared much sharper. Therefore, 55 °C was selected as the optimized temperature as to avoid non specificity for *Staphylococcus aureus* and to obtain better band for *E. coli.*

During primer optimization, primer concentrations were reduced from 800 nmol to 200 nmol, 300 nmol and 400 nmol for both organisms. Different primer concentrations were optimized here to avoid the primer dimer formation and nonspecific bindings which were observed in the previous amplifications. According to the results, 200 nmol primers (forward and reverse) from both organisms were finalized as the standard primer concentration for multiplex (Figure 4B).

Results indicated in (Figure 4C) shows that with 0.5 μL of 100 ng/μL DNA of *E coli* and 0.5 μL of 25 ng/μL DNA of *Staphylococcus aureus* amplify both bands in a one reaction mixture without any nonspecific bands. The results indicate that the given quantities of DNA is adequate enough to amplify both bands, hence, can be used as a positive control to run in parallel with the contaminated samples to diagnose the causative organisms.

Finally, the PCR mixture was assembled in a 12.5 μL reaction volume. Similar to the results observed for 25 μL of reaction volume which showed positive results. This indicates that by reducing reaction volume it is possible to cut down the cost of amplification of positive control each time when it is run in parallel with contaminated samples to diagnose contaminating species.

DNA Extraction of CMT Positive Milk Sample

Thirty five CMT positive milk samples were collected from dairy farms. Bacteriological culture studies confirmed that 32 samples were positive for contamination.

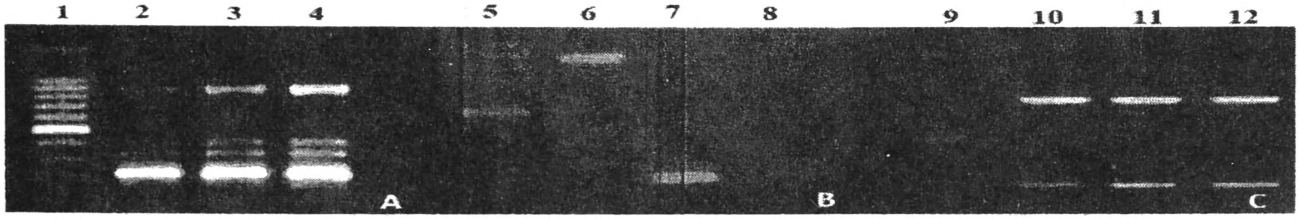

Figure 4. PCR optimization for (positive sample). A: PCR optimization for Annealing temperature B: PCR optimization for primer concentration C: PCR optimization for DNA concentrations. *Lane 1, Lane 5, Lane 9 - Ladder (100 bp), Lane 2 - 47.9 °C, Lane 3-52.9 °C, Lane4 - 55 °C. Lane 6- 200 nmol, Lane 7- 300 nmol, Lane 8 - 400 nmol. Lane 10- 0.5 μL, Lane 11- 0.75 μL, Lane 12- 1 μL*

Twenty of the 32 (62.5%) were contaminated only with *Staphylococcus aureus,* 7 of 32 (21.88%) were contaminated only with *E. coli* and 5/32 (15.63%) were contaminated with both organisms.

Genomic DNA was extracted from CMT positive milk sample (n=32) according to rapid DNA extraction method. Figure 5 shows the agarose gel image of the DNA extracted from some of the infected samples.

Figure 5. Agarose gel image of genomic DNA from some of the infected milk sample

PCR Amplification of Milk Sample

Twenty DNA samples were PCR amplified under optimized conditions developed for multiplex. According to results, 15/20 (75%) of *Staphylococcus aureus*, 1/5 (20%) of dual contaminated samples were correctly identified. Samples which were contaminated only with *E. coli* were failed to amplify under the given conditions. There could be several reasons for the optimized conditions for not working in the present study. At the subclinical level, the number of bacterial cells present in the milk samples may be very less and also a portion of the extracted DNA comes from somatic cells of the bovine.

There were few studies that used molecular technique as an alternative method to microbiological methods for the detection of pathogens in food (Pozzoben *et al.,* 2015). Most of the molecular techniques used for identification of contaminating species from milk are directly taken from culture mediums, hence not contaminated with other organisms, DNA or milk (Perez *et al.*, 2002; Zocche *et al.*, 2009). There may be some inhibitory substances in milk affecting successful amplification of the pathogen DNA. Some studies also indicated that milk fat could cover bacterial surface and make lysis more difficult, thus lowering sensitivity to PCR (Kim *et al.,* 2001; Aslam *et al.,* 2003).

Therefore, further studies are needed to optimize PCR for samples to detect the pathogen in order to amplify the sample successfully in parallel with multiplex. It is also recommended that attempts should be made to remove fat before DNA extraction and column purification of DNA from samples to obtain maximum recovery of DNA from the contaminating species DNA.

Amplification results of the samples from *Staphylococcus aureus* have shown two different bands in the 229 bp region (Figure 6, Lane 1). This may be due to the presence of another sub species. This is needed to be verified by further analyzing the sequence variation. If the problem merely lies as a result of lower annealing temperature or as a result of secondary structure formation in the PCR product, the PCR can be optimized again with Dimethyl Sulfoxide (DMSO) which inhibits secondary structure formations in the DNA template or the DNA primers.

The present study is successful in optimizing PCR for multiplex; however, successful rate is not 100%. Therefore, sample analysis procedure should be optimized through a sensitivity test and larger number of samples should be analyzed to obtain statistically significant result in order to use this method against the conventional culture based method.

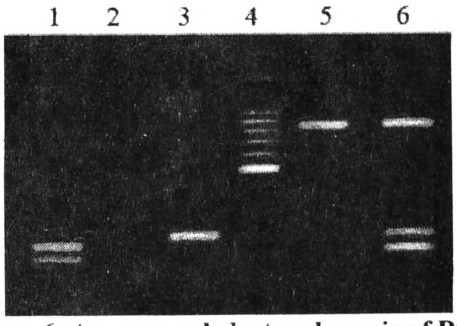

Figure 6. Agarose gel electrophoresis of PCR of California Mastitis Test (CMT) positive milk sample. *Lane 1- Staphylococcus aureus (milk sample), Lane 2 - Negative control, Lane 3- Staphylococcus aureus (positive control), Lane 4 - ladder (100 bp), Lane 5 – E. coli (positive control), Lane 6 - Multiplex bands of milk sample (Staphylococcus aureus and E. coli)*

CONCLUSIONS

Optimization of PCR conditions revealed that the best conditions for the multiplex PCR were, 50 ng DNA of *Escherichia coli* and 25 ng of *Staphylococcus aureus* DNA, 55 °C of annealing temperature and 200 nmol of each primer for both species. Optimized multiplex conditions work for 25 µL and 12.5 µL reaction volume. Seventy five percent *Staphylococcus aureus* and 20% dual contaminations can be directly diagnosed by the optimized condition. However, *E. coli* contaminated do not work under present conditions. Therefore, considering the presence of other DNA in contaminated sample, optimization should be carried out for samples separately for DNA and primer concentrations. The optimized PCR amplification conditions for multiplex PCR were initial denaturation at 95 °C for 2 min, followed 35 cycles, denaturation at 95 °C for 45 sec, annealing at 55 °C for 1 min, extension 72 °C for 1 min, final extension 72 °C for 5 min.

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