MULTIPLEX PCR TO DETECT *Brucella* AND *Leptospira* SPECIES IN SPIKED MILK SAMPLES

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ABSTRACT

Brucellosis and leptospirosis are zoonotic diseases and cause much economic losses in livestock by causing abortion, stillbirth and infertility. Both organisms are excreted in milk and thus increasing the risk of infecting other animals and human. Early detection of organisms is desirable for effective treatment and control of diseases. In this study, multiplex PCR was attempted to detect Brucella and Leptospira species in spiked milk samples. Brucella genus specific primers were designed and E1E2 primers that target only pathogenic leptospires were used to yield the amplicon of 230bp and 571bp respectively. CTAB and lysis methods were used to extract DNA from spiked milk samples and found that lysis method is more sensitive than CTAB method in detecting both organisms. CTAB method could detect both organisms in 10^4 per ml but lysis method detected both organisms in 10^3 per ml. This multiplex PCR can also be used to detect these bacteria in other clinical samples such as aborted fetus, placenta, uterine discharges, urine and blood.

Keywords: Multiplex PCR, *Brucella, Leptospira,* spiked milk samples

Brucellosis and leptospirosis are contagious, infectious and zoonotic diseases caused by bacteria of genus *Brucella* and *Leptospira* respectively. Both cause economical loss by reducing the quality of meat and milk (cause mastitis, abortion, stillbirth infertility in livestock). Both bacteria are excreted in milk and thus infect other animals and human. Early diagnosis of abortion is crucial in controlling the spread of organisms to other in-contact animals and human. Isolation of organisms is definite but it is less sensitive, laborious, hazardous and time consuming. Amplification of target nucleic acid by polymerase chain reaction (PCR) is more sensitive, specific and advantageous over other techniques for the

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detection of infectious disease. Hence, multiplex PCR was developed to detect *Brucella* and *Leptospira* organisms in the milk samples.

MATERIALS AND METHODS

Leptospira interrogans serovar Autumalis maintained at Leptospirosis Research Laboratory, TANUVAS, Chennai was used for this study. It was subcultured in EMJH enrichment media and incubated at 30°C in BOD incubator for 5-7 days. *Brucella abortus* strain 99 was obtained from Institute of Veterinary Preventive Medicine, Ranipet, Tamil Nadu. It was grown on tryptic soy agar overnight at 37°C. Both organisms were washed twice using PBS and the pellets were suspended in PBS and the concentration was adjusted to 0.5 Mc Farland Standard tube. *Brucella* and *Leptospira* organisms were spiked at the concentration of 10^5 to 10^1 organisms in 1ml of bovine milk samples and 100 µl of the milk samples were processed immediately for DNA extraction by both CTAB and lysis method

DNA extraction by CTAB method :

20 µl of Triton X117 was added to 100 µl of spiked milk samples and diluted with 500 µl of distilled water and then boiled for 10 min. To the supernatant, 100 µl of 10% CTAB solution was added, incubated at 65°C for 15 min and centrifuged at 10000 rpm for 5 min. DNA was extracted from the aqueous solution using routine phenol: chloroform: isoamylalcohol (25:24:1) procedure and finally, DNA was suspended in 50 µl of nuclease free water.

DNA extraction by lysis method

Spiked milk samples of 100 µl were centrifuged and 200 µl of TE buffer and 400 µl of lysis buffer (6M guanidine thiocyanate, 0.1 M Tris pH 6.4, 0.02M EDTA pH 8.0 and 2% Triton X-100) were added to the pellet and were kept at 55°C for 10 min. Aqueous solution was then treated with Phenol: chloroform: isoamylalcohol and DNA was extracted and suspended in 50 µl of nuclease free water.

The entire procedure of spiking and DNA extraction by both CTAB and lysis method were carried out twice for reproducibility of the results.

Polymerase Chain Reaction

To detect pathogenic leptospiral species, E1E2 primers targeting 16S RNA (Vitale *et al*., 2005) were used. To detect *Brucella* organism, genus specific primers targeting IS711 were designed using DNAstar® software and the sequences were as follows: Bru F 5´-AGGAGAACAGCCGTGAGC -3´ and Bru R 5´-GAAAGCGGGCATGAACCG -3´. PCR was performed in 20 µl reaction volume which contained 2X PCR master mix (GeNei, Bangalore), 20 pmole of each primer, 5 µl of DNA. Amplification was carried out in Thermalcycler (Eppendorf, Germany) and conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles comprising 94°C for 30 sec denaturation, 60°C for 30 sec annealing and 72°C for 30 sec extention and final elongation was at 72°C for 10 min. Amplified product was detected by electrophoresis in 1% agarose containing 0.5 µg/ml ethidium bromide and viewed under UV transilluminator.

RESULTS AND DISCUSSION

Brucellosis and leptospirosis are emerging zoonotic diseases affecting human and many animals including marine species. Leptospirosis is endemic in South India and other coastal regions of India. Major sources of infection are urinary excretion, abortive materials and also through milk and milk products. Leptospirosis cause hemorrhagic mastitis and agalactia in cattle whereas, *Brucella* organisms lodge in the supramammary lymph node and are periodically shed in the milk and spread the infection.

Rapid diagnosis is important in control and possible eradication of these diseases to improve health and productivity of the animals. Multiplex PCR for detecting *Brucella* and *Leptospira* species were developed. CTAB and lysis methods were employed to extract DNA from spiked milk samples to assess the sensitivity of the multiplex PCR. The size of the amplified product for *Brucella* species was 230bp and for *Leptospira* species, size of the amplicon was 571bp. CTAB method could detect both species in 10^5 and 10^4/ml concentration (Fig 1A) and could detect *Brucella* species alone in 10^3/ml (10 copies of bacteria in a reaction) concentration. However, lysis method had yielded more DNA and upto 10^3/ml of both bacterial DNA were amplified but it could detect the *Brucella* organisms alone in 10^2/ml (Fig.1B). Hence the sensitivity of this multiplex PCR in milk samples using CTAB and lysis methods was 10^4/ml and 10^3/ml of bacteria respectively.

Lee *et al*. (2011) developed lyophilized reagent-based PCR assay which was able to detect 1 x 10^2 leptospires per ml in the spiked urine and 1 x 10^3 leptospires per ml in the spiked blood sample. Ahmed *et al*. (2012) developed a multiplex (mPCR) for the rapid detection of leptospiral infection based on the amplification of 2 genes: 16S rRNA gene and *LipL*32 gene. It was also shown that the mPCR is a notably sensitive test, detecting up to 1 x 10^3 leptospires per ml in spiked urine.

The success of PCR depends on the DNA yield of the extraction technique used. Milk contains immunoglobulin G (IgG) and lactoferrin which may not have been completely removed during DNA extraction (Al-Soud *et al.*, 2000; Al-Soud & Radstrom, 2001). Despite a perfectly optimized PCR assay, accurate results may not be produced when the extraction of DNA is performed improperly. Therefore, DNA extraction of clinical specimens has to be performed precisely in order to exclude all possible effects of PCR inhibitors that can significantly decrease the sensitivity of PCR.

The sensitivity of this multiplex PCR can be improved by employing simple silica column based kits or magnetic beads to extract DNA from milk samples. In agarose gel electrophoresis, detection of quantity of DNA in the bands of interest can be affected by the detection method, amount of DNA loaded per well and fluorescent dye used to detect nucleic acids. Apart from the standardization of multiplex PCR, all the above techniques also influence the sensitivity of the PCR techniques. However, this multiplex PCR can also be used to detect these bacteria in other clinical samples such as aborted fetus, placenta, uterine discharges, urine and blood.

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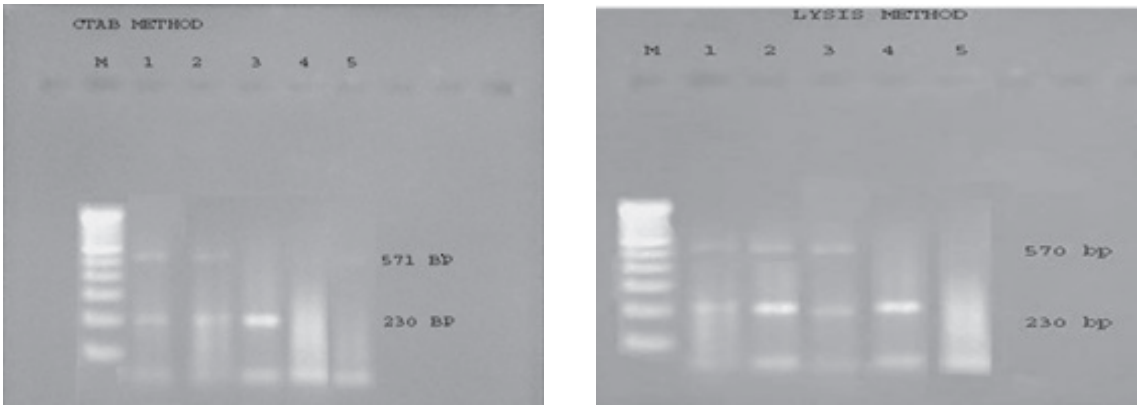
Fig.1 Agarose gel electrophoresis showing the results of PCR using pure cultures of *leptospira* and *Brucella* with DNA extracted by CTAB and Lysis method.



Lane M–100 bp ladder. Lanes 1–5: PCR amplification performed with the DNA extracted from 10^5, 10^4, 10^3, 10^2 and 10^1 per ml of *Leptospira* and *Brucella* respectively. Note using genus specific primers, no amplification with 10 & 100 *Brucella* and leptospires organisms in both methods respectively.

Fig 1 (A): Note: Amplification noticed in 10^5/ml and 10^4/ml concentration and 10^3/ml for leptospires and *Brucella* organisms respectively using CTAB method.

Fig 1 (B): Note: Amplification noticed in 10^3/ml concentration and 10^2/ml for leptospires and *Brucella* organisms respectively using lysis method