Lipl 41 GENE SPECIFIC PCR FOR THE DETECTION OF PATHOGENIC LEPTOSPIRES IN CATTLE

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ABSTRACT

Primers capable of amplifying the gene coding for the outer membrane protein Lipl 41 of Leptospira sp. were used in a PCR for the rapid diagnosis of leptospirosis in cattle. This gene was found to be conserved among the pathogenic serovars of Leptospira interrogans viz., Australis, Autumnalis, Canicola, Grippotyphosa, Hebdomadis, Icterrohemorrhagiae, Javanica, Pomona and Pyrogenes as the amplicon of 1077 bp were obtained. Blood and urine samples from six cows suspected of leptospirosis were subjected to PCR using the above primers. Two blood samples and one urine sample were found to be positive. The specificity of the primers was checked was by using the cultures of Pasteurella, E. coli, Staphylococcus aureus and the saprophytic Leptospira biflexa and it was found that these were not amplified by these primers.

INTRODUCTION

Leptospirosis is an emerging zoonotic disease, with a great impact to bovine herds, causing infertility, abortion, agalactia, and an increase of mortality in infected herds. Leptospiral mastitis is popularly known as flabby udder in cattle. The animal responds to antibiotics, if the infection is diagnosed in early stages. Microscopic Agglutination Test (MAT) is considered as the gold standard test (OIE, 2005). But it is difficult to differentiate acute infections from chronic infections by MAT, without paired serum samples. Polymerase Chain Reaction (PCR) can be useful for a rapid diagnosis of leptospirosis particularly acute infections (Ramadass, *et al*., 1997 and Gravekamp, *et al*., 1993). Many of the previously described primers were amplifying both pathogenic and non pathogenic serovars (Merien *et al*., 1992) whereas several others like G1/G2 failed to amplify some of the most pathogenic serovars like *L. interrogans* serovar Grippotyphosa (Gravekamp, *et al*., 1993). The present study describes the diagnosis of leptospirosis in cattle by PCR using *lipl 41* gene which was reported to be conserved among the pathogenic serovars.

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MATERIALS AND METHODS

Blood and urine samples (5 ml each) were collected from a private dairy farm in Thrissur which was reported to be having incidence of haemorrhagic mastitis. Blood was collected in citrated vials. The plasma was separated and centrifuged at 12000 rpm for 15 minutes to pellet the leptospires. The pellet was washed thrice in PBS by centrifuging at 12000 rpm for 15 minutes and resuspended in 100 μl sterile nuclease free water. This suspension was kept in a boiling water bath for 10 minutes and snap chilled on ice and used as template in PCR. Serum was separated and used in MAT.

Amplification of DNA was performed in 25 μl reaction mixture containing 14.5 μl of nuclease free water, 2.5 μl of 10 X Assay Buffer, 1μl of dNTP mix (Sigma), 1μl (25 pmol) of each primer (Sigma), 1 μl of *Taq* DNA Polymerase (3U/ μl) and 5 μl of extracted DNA. The reaction components were mixed and subjected to amplification in a thermal cycler with an initial denaturation at 94 °C for 5 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at 57 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was carried out at 72 °C for 10 minutes. The primers which amplify 1077bp fragment of *lipl41* gene of pathogenic leptospira were used (Senthilkumar *et al*., 2007). The sequence of the forward primer was 5'-TG TTA CCC ATG GGG AGA AAA TTA TCT TCT CT-3'.and the reverse primer was 5'-AAA GGA CTC GAG TTA CTT TGC GTT GCT TTC-3'. The analysis of PCR products were carried out in one per cent agarose gel stained with ethidium bromide (0.5 μg / ml). A 100 bp DNA ladder (MBI Fermentas) and appropriate controls were incorporated to rule out false positive and false negative results. The gel was viewed under UV transillumination.

Microscopic Agglutination Test was performed as per the OIE (2005). Serum samples were serially diluted from 1:50 to 1:25600. Five day old cultures of leptospires (Australis, Autumnalis, Canicola, Grippotyphosa, Hebdomadis, Icterohemorrhagiae, Javanica, Pomona and Pyrogenes *and L.biflexa serovar Patoc*) grown in Ellinghausen McCullough Johnson Harris (EMJH) medium containing 2 x 10^8 organisms/ml were used as antigens. The serum showing 50 per cent reduction in motility of leptospires with or without agglutination with a titre of 1 in 100 was considered as positive (OIE, 2005).

RESULTS AND DISCUSSION

In the present study, the affected cow was reported to be having pyrexia, anorexia and agalactia. The milk was blood tinged with clots. The udder was flabby without heat and pain and all four quarters were equally affected which was suggestive of leptospirosis (Durfee and Allen, 1980). As the course of this economically important zoonotic disease varies from mild to acute fatal forms, laboratory based techniques are essential for arriving at a definitive diagnosis. Detection of antibodies using MAT has been the most common diagnostic method. However, there are several difficulties that confuse interpretation of MAT titers. Many animals with leptospirosis present with clinical signs of disease prior to the development of antibodies measurable by MAT. On the other hand, there is a high prevalence of subclinical infections that result in the persistence of antibodies.

In recent years, PCR has been proved to be a rapid diagnostic tool for the detection of leptospiral DNA in clinical samples like urine (Van Eys, *et al*., 1989) and serum (Merien, *et al*., 1992 and Gravekamp, *et al*., 1993), the success of which depends on the method of DNA extraction. Boom *et al*. (1997) opined that the addition of Guanidine thiocyanate and Cetyl trimethyl ammonium bromide followed by phenol extraction is an ideal protocol for DNA extraction. Senthilkumar *et al*. (2001) described DNA extraction using lysozyme. In the present study, we used simple boiling method to extract the leptospiral DNA which makes the PCR technique an even more rapid and cost effective technique. The presence of 1077 bp amplicon was detected in the present study which confirms the amplification of *lipl41* gene of pathogenic *Leptospira* (Senthil Kumar *et al*., 2007).

The presence of antibodies against *L. Pomona* in the serum was detected by MAT and the antibody titre was found to be 1: 400 which could be considered as positive as per OIE (2005). In an endemic area like Kerala, most of the animals posses antibodies against leptospira and hence antibody detection from serum samples is of limited significance in diagnosing acute infections. The gold standard test MAT is unable to differentiate the active infection from carrier state although it is the only test by which serovars can be identified. Polymerase chain reaction is found to be a promising tool for the early, rapid and specific diagnosis of leptospirosis.

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