ISOLATION AND CONFIRMATION OF *LEPTOSPIRA CANICOLA* FROM THE ABORTED BOVINE FOETUS

Shivaraj, D.Rathnamma, S. Isloor, K. Sanganagouda, V.Balmurugan, M.D. Venkatesha and C.Renukaprasad .

Department of Veterinary Microbiology, Veterinary College Bangalore, KVAFSU, Hebbal, Bangalore-24

Received 5-11-2013 Accepted 30-11-2013

Corresponding Author: shivaraj.murag@gmail.com

ABSTRACT

A study was conducted on isolation and confirmation of *leptospira canicola* from the aborted bovine foetus. Out of the 24 aborted bovine samples subjected for isolation, one sample has yielded the growth after two weeks of inoculation. The conventional polymerase chain reaction (PCR) using G1G2 and LipL32 primers have shown the amplicon size of 285 bp and 756 bp respectively. The positive PCR sample/isolate was confirmed as serogroup *canicola* by subjecting it to “O” gene specific cluster multiplex PCR with an amplicon size of 341bp.

KEYWORDS: *Leptospira canicola*, Leptospirosis, Lip L32, Multiplex PCR.

INTRODUCTION

Leptospirosis is a zoonosis of worldwide distribution and it causes major economic losses in the cattle industry due to abortion and decreased milk production and fertility (de Castro *et al*. 2011; Subharat *et al*. 2011). In prevention of leptospirosis, detection of infecting serovar, serogroup of leptospires involved is very important. Basicallly Microscopic Agglutination Test (MAT) is widely used to detect the type of serovar prevalent in given area. But it requires maintenance of several serovars and handling live culture repeatedly leading to the chance of spread of infection to the handlers. Keeping in view of the above facts an attempt was made to standardize the”O” gene cluster multiplex PCR to identify the type of sero group involved in aborted bovine foetus.

MATERIALS AND METHODS

An attempt for isolation of *L.interrogans* from bovines was carried out from December 2012 to April 2013 in and around Bangalore. A total of 24 aborted bovine foetus samples (above six months pregnancy) were collected. The uterine discharge, cotyledons, foetal materials like heart, kidney and intestinal contents were inoculated into the Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (DIFCO-USA) using 0.22 µ syringe filter. The above medium was enriched with 1% rabbit serum for better leptospira growth. The cultures were incubated at 30^0C with weekly assessment using dark field microscope (DFM) for eight weeks, before discarding them as negative. Reference serovars/strains were procured from the National reference laboratory for Leptospirosis, Port Blair, Andaman and Nicobar.

Primers and PCR assay

DNA extraction: DNA extraction was done using the Qiagen kit and the procedure was followed as per the specifications of the kit using 200 ul each of the isolate and reference strain. The purified DNA extract was used for the PCR assay.

Setting up of PCR

The conventional PCR reaction was carried out as per the method described by Gravekemp *et al.* (1993) using G1 G2 primers designed from the 5’ end (nucleotides 1 to 20 G1) and 3’ end

(nucleotides 264-285 G2), representing the sequence of recombinant plasmid pLIPs60 selected from the genomic library of *L.interrogans*. The PCR was performed with the total volume of 25 µl reaction mixture using the Primers (Forward) G1 5'- CTG AAT CGC TGT ATA AAA GT- 3', (Reverse) G2 5'- GGA AAA CAA ATG GTC GGA AG- 3'. An amplicon size of 285 bp was observed for positive reactions. Subsequently they were subjected to the LipL32 gene primers as per the method described by Meenambigai *et al*. (2011), using the forward primer 5'- CAT ATG GGT CTG CCA AGC CTA AA-3', and reverse primer 5'-CTC GAG TTA CTT AGT CGC GTC AGA A-3'. An amplicon size of 756 bp was observed for positive reactions.

After the confirmation of pathogenic *leptospira* from above primers the sample was subjected to Multiplex "O" gene cluster serogroup specific primers as per the method described by Cheng-song *et al*. (2010) for serogroups *canicola, hebdomadis, icterohaemorrhagiae* and *sejroe* with an amplicon size of 341bp, 656bp, 590bp and 319bp respectively.

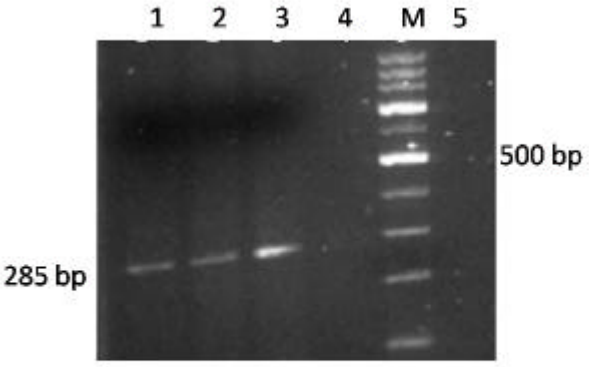
Detection of PCR products

The PCR products were detected by electrophoresis in a 1.5 per cent agarose gel in Tris Borate EDTA (TBE) buffer (1x). Agarose was dissolved in TBE buffer by heating and cooled to $50^{0}C$. To this Ethidium bromide was added to a final concentration of 0.3 µg per ml. The PCR product (10 µl) was loaded into the respective wells along with the molecular marker 100bp for conventional PCR and 50bp for multiplex PCR and positive control. The electrophoresis was carried out at 100 V for 20 to 30 minutes until the tracking dye (Bromophenol blue) migrated more than two third of the length of the gel tray in the buffer. The gel was placed under UV transilluminator and the results were documented in a gel documentation system (BioRad).

RESULTS AND DISCUSSION

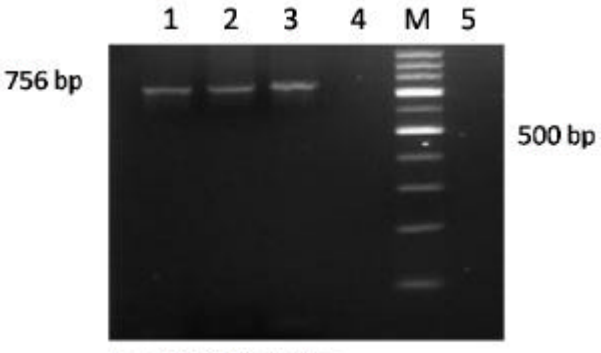
Out of the 24 aborted bovine samples subjected for isolation, one sample has yielded the growth (dingers ring) after two weeks of inoculation. The organisms were observed using dark field microscope with 200X magnification. The conventional PCR using the G1 and G2 primers has shown the amplicon size of 285 bp (Fig.1) and the same was reconfirmed with LipL32 gene primer with an amplicon size of 756 bp (Fig.2). The positive isolate of the above PCR was confirmed as serogroup *canicola* by subjecting it to "O" gene specific cluster multiplex PCR with an amplicon size

Fig 1 : Gel showing PCR positive reactions with G1 and G2 Primers



Lane M: 100 bp ladder
Lane 1: Positive isolate
Lane 2: Positive sample
Lane 3: Positive control
Lane 4: Negative control
Lane 5: No template control

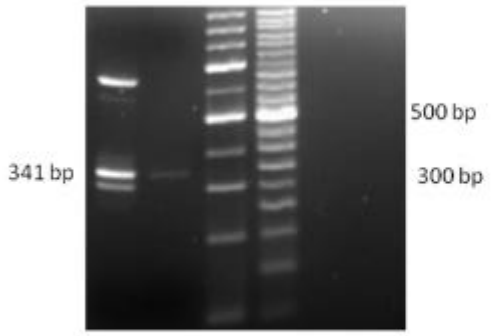
Fig 2 : Gel showing PCR positive reactions with LipL32 Primers



Lane M: 100 bp ladder
Lane 1: Positive isolate
Lane 2: Positive sample
Lane 3: Positive control
Lane 4: Negative control
Lane 5: No template control

of 341bp (Fig.3).

Fig. 3: Gel showing PCR positive reactions with Multiplex Primers



Lane 1: Positive controls
Lane 2: Positive isolate
Lane M1: 100 bp ladder
Lane M2: 50 bp ladder
Lane 3: No template control
Lane 4: Negative control

Leptospirosis is re-emerging throughout the world and assumes importance in both veterinary and medical fields. It is responsible for great economic loss to dairy producers mainly due to abortion and reduction in milk yield. In prevention of leptospirosis, knowledge on the number and frequency of either human or animal cases, infecting serovar, serogroup of leptospires is more important. As the infection being subclinical in bovines the rapid diagnosis is very important to avoid the spread of infection. The MAT has got several major disadvantages hence initially confirmation by Genome specific PCR followed by serogroup specific Multiplex PCR will give an idea and diagnosis of leptopspira and the serogroup prevalent in the given area which will help in developing a specific diagnostic kit and vaccine against these serogroups. The culture requires more period for growth and due to the fastidious nature of the organism, other organisms will take upper hand. Hence the serogroup specific multiplex PCR can be effectively used along with the conventional genome specific PCR for rapid diagnosis of leptospirosis instead of MAT and Cultural isolation studies (Cheng-song *et al*., 2010, Heinemann *et al.,* 1999, Kocabiyik and Cetin, 2003 and Meenambigai *et al.*,2006). Generally leptospira serovar *hardjo* is quite prevalent in the bovine abortion cases (Langoni *et al.,* 1999) but the findings in the present study are in correlation with the Zacarias *et al*. (2008). The infection with the *canicola* serogroup which is commonly seen in dogs might have spread to the bovines mainly due to the close contact and sharing of contaminated water and other sources in the high dog populated places like in and around Bangalore.

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