PHYLOGENETIC ANALYSIS OF BOVINE *Leptospira* ISOLATES

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Received 20-09-2014 Accepted 22-12-2014

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ABSTRACT

Leptospira isolates obtained from bovines were subjected to phylogenetic analysis by sequencing 16s rRNA gene of *Leptospira*. The phylogenetic analysis of the 16s rRNA of 9 isolates were grouped into three clusters. Partial sequence analysis of 16s rRNA of the isolates revealed that they were having 99.30 to 100.00 per cent identity among the *australis* isolates with reference strain, Australis *australis* Ballico. The *Leptospira ballum* isolate showed 99.70 per cent identity with reference strain and also encompassed with *Leptospira hebdomadis* isolates and some of the *Leptospira australis* isolates.

KEY WORDS: *Leptospira* - PCR - Phylogenetic analysis - Bovines

INTRODUCTION

Phenotypic tests are unable to distinguish between species of *Leptospira* and often do not reflect the genetic relatedness (Levett, 2001 and Bharti *et al.,* 2003). Hence there is a need to employ a simplified molecular approach for the identification of leptospires. Molecular approaches based on DNA – DNA hybridization have been described for identification of leptospires (Hookey *et al*., 1993; Perolat *et al.,* 1994; Brenner *et al.,* 1999). The application of phylogenetic analysis to the classification of *Leptospira* showed that 16s rRNA analysis was consistent with results obtained by DNA – DNA hybridization and distinguished strains at the species level (Hookey *et al.,* 1993; Postic *et al.,* 2000). 16s rRNA gene sequences have been universally used for species identification of *Leptospira* (Morey *et al.,* 2006). Hence phylogenetic analysis was used to identify bovine *Leptospira* isolates in the present study.

MATERIALS AND METHODS

A total 115 Sera samples collected (from 33 cattle and 82 buffaloes) suffering with abortion (10), repeat breeder problems (9), jaundice (25) and haemorrhagic mastitis (21) as well as from apparently healthy animals (50) in and around Chennai, Tamil Nadu were examined under dark field microscope and were inoculated into Ellinghausen McCullough Johnson Harris medium for the isolation and identified by Microscopic Agglutination test (Balakrishnan and Roy, 2014) using polyclonal antisera procured from Pasteur Institute, Paris, France.

Polymerase chain reaction

A total of nine *Leptospira* isolates obtained from 5 cattle and 4 buffaloes (Table 1) were subjected to PCR to amplify 16s rRNA using E1 and E2 primers (Vitale *et al*., 2005 and Balakrishnan *et al.,* 2014). Similarly 12 reference *Leptospira* strains were subjected to PCR for 16s rRNA for a comparative study (Table 2).

DNA isolation

DNA isolation was done as per the method of Boom *et al.* (1990) and Balakrishnan *et al.* (2014). To 125 µl of serum samples, 900 µl of lysis buffer and 40 µl of diatom suspension were added

and incubated at room temperature for 20 minutes and then centrifuged at 14000 rpm for 10 minutes. Supernatant was discarded and the pellet washed twice with wash buffer by centrifugation at 14000 rpm for 2 min. Then after one ml of 70 per cent cold ethanol was added and washed twice as above. Then one ml of acetone was added and centrifuged at 14000 rpm for 2 min. The pellet was incubated at 56^{0}C for 15 min. To the dried pellet, 5 µl of proteinase K and 125 µl of triple distilled water were added and incubated at 56^{0}C for 15 min and kept in water bath at 100 ^{0}C for 15 min and centrifuged at 10000 rpm for 5 min after resuspending in TE buffer. The supernatant was collected with TE buffer and used as template DNA for PCR.

PCR to amplify 16s RNA

The PCR reaction was carried out as per the method described by Vitale *et al.* (2005) and Balakrishnan *et al.* (2014). Amplification of DNA was performed in 25 µl reaction mixture containing 12.5 µl of super mix (Genei, Bangalore), 100 pmol of each primer, E1 and E2 (Sigma - Aldrich, Bangalore) and 11.5 µl of extracted DNA. For positive control, 5 µl of DNA template (culture) and 6.5 µl of nuclease free water were used. The reaction components were mixed and subjected to amplification in a thermal cycler (Eppendorf, Germany). The temperature profile consisted of initial denaturation at 94^{0}C for 5 minutes, followed by 40 cycles of denaturation at 94^{0}C for 1 minute, primer annealing at 60^{0}C for 1 minute and extension at 72^{0}C for 1 minute. The final extension was carried out at 72^{0}C for 5 minutes.

Detection of PCR products

The PCR products were analysed by electrophoresis in 1.5 per cent agarose gel in 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 0.5 µg per ml of molten gel. The PCR products (10 µl) were loaded into different wells along with the molecular marker (100bp ladder DNA) for 16s rRNA. The electrophoresis was carried out at 100 V for 20 to 30 minutes. The gel was placed under the gel documentation unit (Vilber Lourmet, France)

Phylogenetic analysis

The PCR products were further confirmed by sequencing using the automated ABI Prism BioEdit module. Nucleotide sequence analysis and homology study were carried out using Lasergene Version. 7.0. using the softwares namely Editseq and Megalign.

RESULTS AND DISCUSSION

Out of 115 animals, 30 animals were found positive by dark field microscopical examination. Out of 30 serum samples positive by dark field microscopical examination subjected to isolation study, only nine *Leptospira* isolates were obtained, of which five were from cattle and four from buffaloes (Table 1).

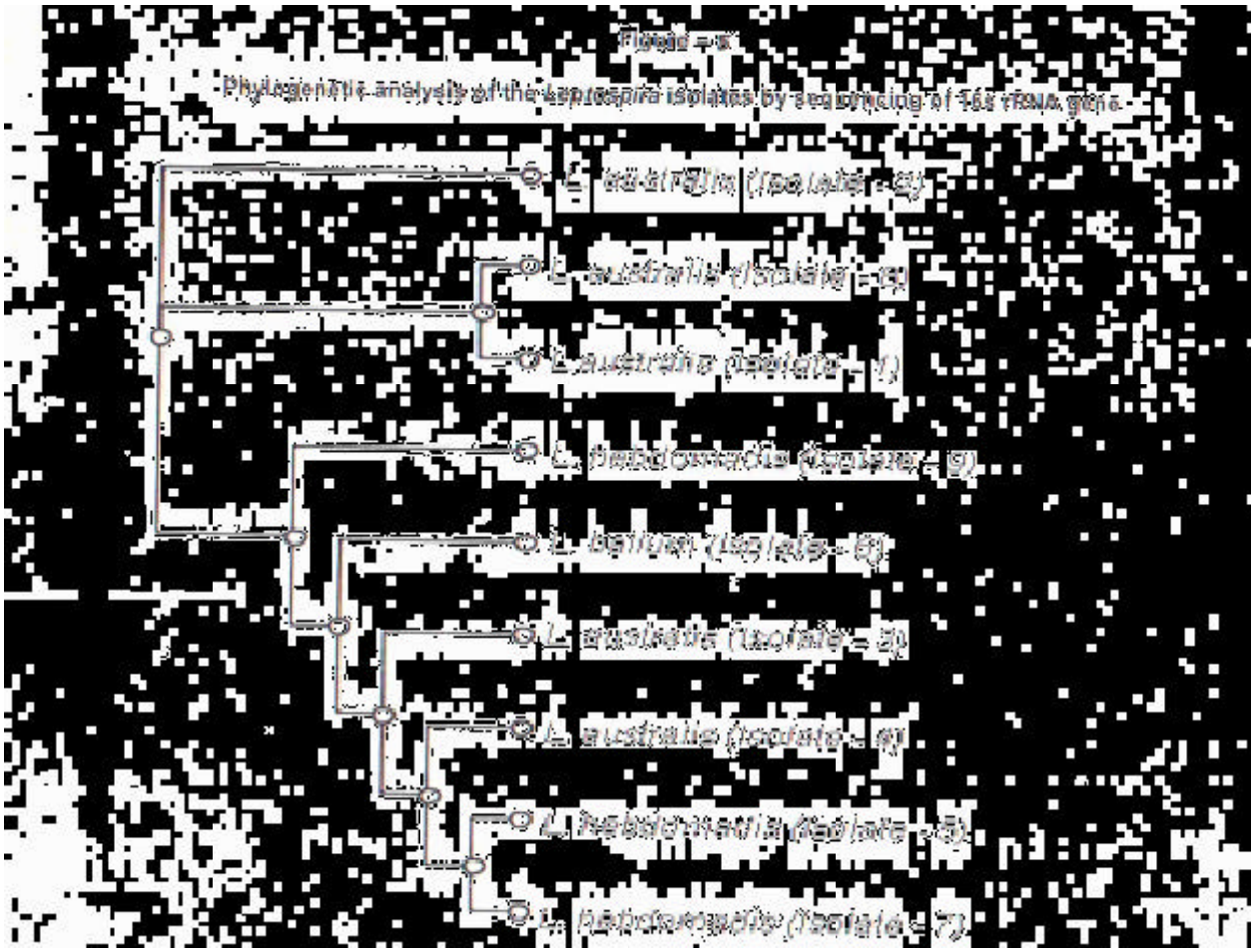
All the isolates were identified by microscopic agglutination test using polyclonal antisera as *australis* (5 No's), *ballum* (1 No's) and *hebdomadis* (3 No's) (Table 1). All the 9 *Leptospira* isolates obtained from bovines were subjected to phylogenetic analysis by sequencing of 16s rRNA gene of *Leptospira*. The details of *Leptospira* field isolates and phylogenetic analysis of *Leptospira* field isolates by sequencing of 16 s rRNA gene are presented in Table 1 and Figure 1 respectively. All the 9 isolates could be grouped into three clusters. The *Leptospira* serovar *australis* isolates 1, 2 and 8 were found to be in a single clad. Other *australis* isolates (Isolate – 3 and 4) belong to cluster – 3 encompassed other isolates also namely *ballum* (Isolate – 6 in) and *hebdomadis* (Isolates – 5, 7 and 9). The 16s rRNA Blast N search revealed comparative blast hit between 457th to 813th position of the 16s rRNA gene showing homology of all isolates.

Phenotypic tests currently used are often unable to reflect the genetic relatedness (Levett, 2001 and Bharti *et al.,* 2003). Hence the nucleotide sequences of the *australis* isolates (Isolates – 1,

Table – 1

Details of *Leptospira* field isolates

Isolate	Serovars	Species of animal	Clinical condition
1	*L. australis*	Cattle	Jaundice
2	*L. australis*	Cattle	Jaundice
3	*L. australis*	Cattle	Haemorrhagic Mastitis
4	*L. australis*	Buffaloe	Apparently healthy
5	*L. hebdomadis*	Cattle	Haemorrhagic Mastitis
6	*L. ballum*	Buffaloe	Jaundice
7	*L. hebdomadis*	Cattle	Haemorrhagic Mastitis
8	*L. australis*	Buffaloe	Apparently healthy
9	*L. hebdomadis*	Buffaloe	Haemorrhagic Mastitis



2, 3, 4 and 8) were compared with the sequence of reference strain *australis* Ballico (GenBank FJ154556) and all the isolates were found to show homology with reference strain. The percentages of homology among the isolates varied from 99.30 to 100.00 per cent. *Leptospira ballum* isolate did not show any variation with reference strain. The application of phylogenetic analysis to the classification of *Leptospira* showed that 16s rRNA analysis was consistent with results obtained by DNA – DNA hybridization (Hookey *et al.,* 1993 and Postic *et al.,* 2000). Thus the phylogenetic analysis of 16s rRNA could be used as a simplified approach to the identification of *Leptospira*.

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