Molecular Characterisation Of Leptospiral Isolates Identified In Agricultural Labours Of Salem District Of Tamilnadu

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Citation

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Abstract

In the present study, RAPD analysis of the two Leptospire isolates identified (K_1 and K_2) were subjected to random amplified polymorphic DNA fingerprinting analysis and compared with 5 genomospecies. Genotype characters of isolated K_1 and K_2 have poor resemblance with reference strain Panama CZ 14M, LSU 1945 and Sarmin CZ 390. The genomic profile of K_1 and K_2 were very similar and resemble the genomic profile of Rachmat belongs to the genomospecies Leptospira interrogans. The present study reports the prevalence of the leptospire belongs to Rachmat strain of the genomospecies Leptospira interrogans.

INTRODUCTION

Traditionally leptospires are classified and identified in serological techniques such as the cross-absorption tests (Gerritsen et al., 1995). However, these techniques are laborious and show poor inter-laboratory reproducibility (Kobayashi et al., 1985), but still cannot distinguish all serovars. The DNA based methods for the identification of Leptospira holds good results and these method include restriction fragment length polymorphism (RFLP) analysis (Terpstra et al., 1978; Hookey et al., 1987; Ellis et al., 1991); DNA-DNA hybridisation (Perolat et al., 1993); Pulsed field gel electrophoresis (PFGE) (Herrmann et al., 1992); Polymerase chain reaction followed by RFLP analysis (Ralph et al., 1993) and random amplified polymorphic DNA (RAPD) fingerprinting (Corney et al., 1993; Natarajaseenivasan et al., 2004). RFLP analysis is generally more sensitive and discriminatory than serotyping, but some serovars are not readily distinguished. (Thiermann et al., 1986), and the complex banding patterns are difficult to interpret. Hybridization of labelled DNA probes to RFLP blots greatly simplifies the visualised banding patterns facilitating interpretation. (Van Eys et al., 1991) and allows serovars with similar RFLP banding pattern to be differentiated (Zuerner et al., 1993). RFLP analysis, DNA-DNA hybridization and PFGE are slow and require large amounts of purified DNA. In contrast, PCR - based methods are rapid and require only small amounts of DNA. (Van Eys et al., 1989; Gerritsen et al., 1991; Merien et al., 1992; Gravekamp et al., 1993). PCR-based fingerprinting systems have been well developed for a range of bacteria. (Corney et al., 1993; Giesendorf et al., 1993; Ralph et al., 1993; Van Belkum et al., 1993; Woodward et al., 1993). Because of some disadvantage in the application of cross-absorption agglutination (CAA) technique and in restriction endonuclease analysis (REA) alternative methods were searched. Corney et al. (1993) stressed the need to develop a rapid and simple typing method which can distinguish different genotype without the disadvantages of CAA and REA. Rapid identification of the isolates would allow farmers to start appropriate vaccination regimens with minimal delays. Welsh and McClelland (1990) and Williams et al. (1990) developed a DNA fingerprinting technique based on the random amplification of genomic sequences by using a single primer at low stringency in a polymerase chain reaction (PCR). Gerritsen et al. (1995) investigated the use of leptospiral and non leptospiral primers in RAPD fingerprinting of leptospirosis. Perolat et al. (1998), Brenner et al. (1999) and Levett (2001) reported more than 16 genomospecies of leptospires based on genotypic characters. The molecular analysis of new isolates from different regions may increase genomospecies number of leptospires. With this background the leptospires isolates collected in the present study were subjected to random amplification of

polymorphic DNA finger printing analysis to find out whether they are new genomospecies or related to the already existing genomospecies.

MATERIAL AND METHODS

ISOLATION OF GENOMIC DNA FROM LEPTOSPIRAL ISOLATES

Blood samples from Leptospiral infected patients were inoculated into sterile EMJH medium and the isolated leptospiral strains (K_1 and K_2) were grown at 300C in EMJH medium for 7 days to a density of 108 cells/m1 and centrifuged at 10,000 rpm for 30 mins at 4oC. Pellets were collected and resuspended in 400 l of TE buffer, vortexed and centrifuged again to remove the medium debris. After removing the supernatant, 40 l of lysozyme (10mg/ml) was added to the pellet, vortexed well and incubated at 370C for 24 hours. After that, 56 l of 10% sodium dodecyl sulphate and 5 l of proteinase K (10 mg/ml) were added and incubated in a water bath at 650C for 30 mins. Then, 80 l of 5 M NaCl and 64 l NaCl/CTAB was added, vortexed and incubated at 650C in water bath 30mins. After that, 645 l of chloroform, Isoamylalcohol (24: 1 v/v) was added, mixed well and centrifuged at 10,000 rpm for 20mins. 300 l of clear supernatant was taken without disturbing inter phase layer and transferred into another tube. To this tube 180 l of isopropanol was added and kept the tube in a deep freezer for 1 hour to precipitate the DNA and centrifuged at 10,000 rpm for 20 mins at 40C to pellet the DNA. The nucleic acid was precipitated by adding 150 l of ice cold ethanol and centrifuged at 10,000 rpm for 5 mins. The DNA thus obtained was suspended in 20 l of TE buffer and stored at -200C until use.

The isolated Leptospiral DNA was analysed by agarose gel electrophoresis in 1.0% agarose with 5 l of Ethidium bromide (10 mg/ml) and the electrophoresis was carried out in a submarine gel electrophoresis apparatus at 100 V for 4 to 5 hours in TBE buffer (pH 8.0). At the end of the electrophoresis, the EtBr stained band in gel was visualized in UV transilluminator.

ELUTION OF ISOLATED LEPTOSPIRAL DNA FROM THE GEL

The agarose gel containing the DNA was cutout with a sterile scalp. These agarose pieces were then transferred into dialysis membrane and TBE buffer was added so that the agarose pieces were surrounded by buffer. The dialysis bag was placed at the bottom of a gel apparatus filled with buffer in such a way that the pieces of agarose was in the same position with respect to the electrodes as it was in the gel. A current of 100-200 mV was applied for 2 hours to elute the DNA out of gel. The buffer containing the electroeluted DNA was transferred through cotton plugged 1 ml tip into an eppendorf tube and the collected DNA was centrifuged at 10,000 rpm for 2mins. The supernatant was then extracted with phenol and then with chloroform: Isoamylalcohol (24:1 v/v). The extracted DNA solution was mixed with 3 M Tris-HCl (pH 7.5) to give a final concentration of 0.5 M and two volume of isopropanol was added and allowed to stand at 200C for 30mins and centrifuged at 15,000 rpm for 10mins for repeated time to remove EtBr. The DNA pellet was then dried under vacuum and resuspended in a TE buffer and stored at 40C until use.

QUANTIFICATION OF DNA USING UV SPECTROPHOTOMETER

The eluted DNA (50 l) was dissolved in 3.0 ml of 1X SSC buffer. The absorbance of DNA sample was determined at 220nm using 0.1X SSC buffer as blank in a UV spectrophotometer. The wavelength was increased by 10 nm up to 300 nm, the absorbance of the sample was measured. The absorbance ratio of 260 to 280 nm were measured.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) FINGERPRINTING OF ISOLATED STRAIN

DNA prepared from the isolated strain (Boom et al., 1990), the RAPD fingerprinting technique was carried out using B_{11} and B_{12} primer as per the method described by Gerritsen et al. (1995). The amplification of isolated leptospiral DNA was carried out in a total volume of 50 l which contains 50 ng of isolated chloromosomal DNA, 10 mM Tris HCl (pH 9.0), 50 mM KCl, 4 mM $MgCl_2$, 0.1mM of each of the four dNTP mixture (dATP, dTTP, dCTP and dGTP), 300pm of each of primer (B_{11} and B_{12}) and 0.5 U of Taq DNA polymerase.

Primer B11 - CCGGAAGAAGGGGCGCCAT

B12 - CGATTAAGAAGGACTTGAACAC

AMPLIFICATION OF DNA

The amplification of DNA was carried out in a total of 35 cycles in a MJ Thermal cycler. Each cycle consist of denaturation of DNA at 950C for 1 min, annealing of the primers at 60oC for 1 min and extension for 3mins at 72oC with a final extension for 10mins in last cycles.

AGAROSE GEL ELECTROPHORESIS OF AMPLIFIED DNA

The amplified DNA products were analysed by agarose gel electrophoresis (1.2% Agarose with 5 l EtBr (10mg/ml)) and the electrophoresis was carried out in a submarine electrophoresis apparatus at 100 V for 4-5 hours in TBE buffer (pH 8.0). At the end of the electrophoresis, the gel was visualized under UV transilluminator and photograph was taken using polaroid camara with wratten gelatin filter. The RAPD profiles were documented by using a gel documentation system (Bio-Rad, Hercules, Calif., U.S.A) and analysed by using Quantity I-D analysis software, and the dendogram were formed with 4.0% tolerance in UPGAMA. To confirm the specificity of the primers, DNA isolated from other bacteria such as E. coil and S. aureus were subjected to the same procedure with B_{11} and B_{12} primers.

RESULTS AND DISCUSSION

To establish a rapid identification system based on RAPD fingerprinting, RAPD profiles of 5 genomospecies were prepared. The molecular weight of the isolated leptospiral strains (K_1 and K_2) were 23,000 bp and it has the $A_{260-280}$ ratio of 1.70. DNA were purified and subjected to RAPD analysis. Profiles generated from field isolates were compared with this profile to enable identification of the isolates.

Purified DNA from 12 Leptospira reference strains (Ballico, Rachmat, RGA, Wumalasena, Cynopteri 3522C, Moskva V, CZ 188, Sarmin CZ 390, Perepelitsin, Mini Sari, Panama CZ 214K, and LSU 1945) comprising five different genomospecies (L. interrogans, L. kirschneri, L. santarosai, L. borgpetersenii, and L. noguchii) Profiles containing up to eight intense bands and a number of fainter bands were generated when the reaction products were run on agarose gel. Although genomic profile of the isolates (K_1 and K_2) had a number of common bands, they were very close to strain Rachmat (80-90%) (Fig 1 and Table 1].

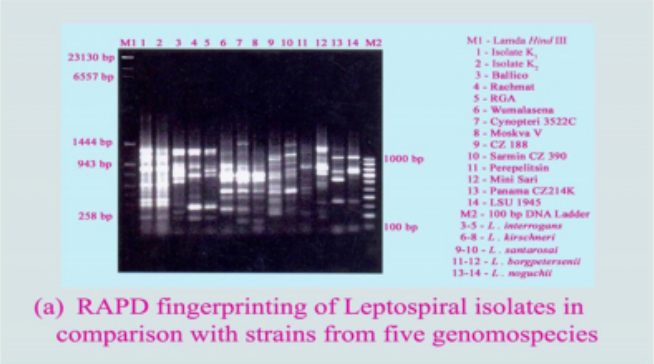
Figure 1

Table 1. Table showing the percentage of similarities as per Dendogram

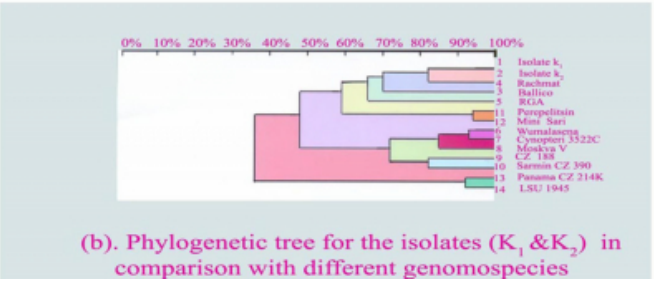
Serovar	Genomospecies	% of similarity
Rachmat	*L. interrogans*	80-90
Ballico	*L. interrogans*	70-75
RGA	*L. interrrogans*	65-70
Perepelitsin	*L. borgpetersenii*	60-65
Mini Sari	*L. borgpetersenii*	60-50
Wumalasena	*L. kirschnei*	40-59
Cynopteri 3522 C	*L. borgpetersenii*	40-50
Moskva V	*L. kirschneri*	40-50
CZ 188	*L. santarasai*	40-50
Sarmin CZ 390	*L. santarasai*	40-50
Panama CZ 214K	*L. noguchii*	30-40
LSU 1945	*L. noguchii*	30-40

Figure 2

Fig. 1



(a) RAPD fingerprinting of Leptospiral isolates in comparison with strains from five genomospecies



(b). Phylogenetic tree for the isolates (K_1 &K_2) in comparison with different genomospecies

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