6. MOLECULAR CHARACTERISATION OF LEPTOSPIRAL ISOLATES

6.1. Introduction

Traditionally leptospires are classified and identified in serological techniques such as the cross-absorption tests (Wolff, 1954; Cole et al., 1973; Gerritsen et al., 1995). However, these techniques are laborious and show poor inter-laboratory reproducibility (Kobayashi et al., 1985; Terpstra et al., 1985a), 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 (Yasuda et al., 1987; Zuerner et al., 1990, 1993; Van Eys et al., 1991; Ramadass et al., 1992; Pacciarini et al., 1992; Perolat et al., 1993); Pulsed field gel electrophoresis (PFGE) (Herrmann et al., 1992); Polymerase chain reaction followed by RFLP analysis (Woodward et al., 1991; 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). They called the technique arbitrarily primed PCR or random amplified polymorphic DNA (RAPD). RAPD fingerprinting or arbitrarily primed PCR had been used for the classification of pathogenic groups. Using RAPD fingerprinting Corney et al. (1993) had identified the seven genomospecies of Leptospira viz. L. interrogans, L. weilii, L. borgpetersenii, L. noguchii, L. sanitarosai, L. inadai, and L. kirschnerrii and more than 230 pathogenic serovars are assigned to one of the genomospecies using molecular method of classification. Ralph et al. (1993) categorises Leptospira serovars from various serogroups by arbitrarily primed PCR, and by restriction polymorphism of PCR which amplifies rRNA genes. Corney et al. (1993) discriminated between nine reference strains representing nine serovars of seven
*Leptospira* serogroups and identified 26 isolates from Australian cattle with an RAPD fingerprint method based on non leptospiral primers. 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 enlarge genomospecies number of leptospires. Ramadass *et al.* (2002) stated that the RAPD method have shown some promise for differentiating individual serovars and arbitrarily primer PCR amplification and resultant DNA pattern produced could be well used for the typing of leptospires. Ramadass *et al.* (2002) stated that although RAPD give promise for differentiating individual serovars, it needs purified DNA samples and he recommended RFLP analysis. However, Natarajaseenivasan *et al.* (2004) identified RAPD as a valid technique and they have reported that the RAPD method produced bands that were distinct, reproducible and species specific even within the species and this method showed discrimination between the serovar level. With this background the two isolates collected in the present study were subjected to random amplification of polymorphic DNA fingerprinting analysis to find out whether they are new genomospecies or related to the already existing genomospecies. In the present study, RAPD analysis of the two isolates identified (K₁ and K₂) was compared with the other species. The isolates K₁ and K₂ were subjected to random amplified polymorphic DNA fingerprinting analysis and compared with 5 genomospecies obtained from KIT, Royal Topical Research Centre, The Netherlands. Whether the isolates K₁ and K₂ were pathogenic or nonpathogenic was another problem to be solved. To find out its association with pathogenic
serovar, the isolates were tested with SDS-PAGE analysis. The results of the SDS-PAGE were compared with standard pathogenic serovar and nonpathogenic serovar as recommended by Nicholson et al. (1993) and Nally et al. (2001).

6.2. Material and methods

Isolation of genomic DNA from Leptospiral isolates (Boom et al., 1990)

The isolated leptospiral strains (K1 and K2) were grown at 30°C in EMJH medium for 7 days to a density of 10^8 cells/ml and centrifuged at 10,000 rpm for 30 mins at 4°C. Pellets were collected and resuspended in 400 μl of TE buffer, vortexed and centrifuged again to remove the medium debris. After removing the supernatant, 40 μl of lysozyme (10mg/ml) was added to the pellet, vortexed well and incubated at 37°C for 24 hours. After that, 56 μl of 10% sodium dodecyl sulphate and 5 μl of proteinase K (10 mg/ml) were added and incubated in a water bath at 65°C for 30 mins. Then, 80 μl of 5 M NaCl and 64 μl NaCl/CTAB was added, vortexed and incubated at 65°C in water bath 30 mins. After that, 645 μl of chloroform, isooamylic alcohol (24:1 v/v) was added, mixed well and centrifuged at 10,000 rpm for 20 mins. 300 μl of clear supernatant was taken without disturbing inter phase layer and transferred into another tube. To this tube 180 μl 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 4°C to pellet the DNA. The nucleic acid was precipitated by adding 150 μl of ice cold ethanol and centrifuged at 10,000 rpm for 5 mins. The DNA thus obtained was suspended in 20 μl of TE buffer and stored at -20°C until use.
Agarose gel electrophoresis of isolated Leptospiral DNA (Senthilkumar et al., 2001)

The isolated Leptospiral DNA was analysed by agarose gel electrophoresis in 1.0% agarose with 5 µl 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 20°C 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 4°C until use.
**Quantitation of DNA using UV Spectrophotometer**

The eluted DNA (50 µl) 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**

(Gerritsen et al., 1995; Natarajaseenivasan et al., 2004)

DNA prepared from the isolated strain (Boom et al., 1990), the RAPD fingerprinting technique was carried out using B₁₁ and B₁₂ primer as per the method described by Gerritsen et al. (1995). The amplification of isolated leptosiral DNA was carried out in a total 50 µl volume which contains 50 ng of isolated chloromosomal DNA, 10 mM Tris HCl (pH 9.0), 50 mM KCl, 4 mM MgCl₂, 0.1 mM of each of the four dNTP mixture (dATP, dTTP, dCTP and dGTP), 300pm of each of primer (B₁₁ and B₁₂) and 0.5 U of Taq DNA polymerase.

Primer B₁₁ : 5'CCGGAAGAAGGGCGCCCAT 3'

B₁₂ : 5'CGATTAAGAAGGACTTGAACAC 3'

**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 95°C for 1 min.
annealing of the primers at 60°C for 1 min and extension for 3 mins at 72°C with a final extension for 10 mins in last cycles.

**Agarose gel electrophoresis of amplified DNA**

The amplified DNA products were analysed by agarose gel electrophoresis (1.2% Agarose with 5 μl EtBr (10 mg/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 camera 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 B11 and B12 primers.

**Protein profile study of Leptospiral isolates using SDS-PAGE electrophoresis**

**Preparation of SDS-PAGE gel (Maniatis, et al., 1989)**

The SDS-PAGE gel was prepared by a mixture of two component (1) a separating gel in the bottom and (2) a stacking gel on top. The 10% of separating gel that contains distilled water (19.80 ml), 1.5 M Tris, pH 8.8 (12.50 ml) 30% bis acrylamide monomer (16.70 ml) and 10% SDS (0.50 ml) along with 10% APS (0.50 ml) and TEMED (0.02 ml) was added just before loading into glass plate. Distilled
water was added over the surface of acrylamide layer, and allowed it for polymerization for 20-40 minutes. When the separating gel has fully polymerized, water was carefully removed by absorbing it with a piece of filter paper and then 5.0% of stacking gel that contains distilled water (6.8 ml) 1 M Tris, pH 6.8 (1.25 ml), 10% SDS (0.1 ml) 30% Bisacrylamide monomer (1.70 ml) along with 10% ammonium per sulphate (0.1 ml) and TEMED (0.01 ml) was added, mixed and poured over polymerized separating gel along with comb and allowed it for polymerisation for 20-40 mins. After polymerization, the comb was removed from the gel and fixed to the gel in a appropriate SDS-PAGE apparatus vertically with tank buffer in a bottom reservoir and top reservoir.

**Preparation of Leptospiral antigen**

Leptospiral strain isolated (K1 and K2), Leptospiral Reference strain Australis (Pathogenic) and strain Patoc I (Nonpathogenic) were grown separately in EMJH medium for 7 days at 30°C in a shaking incubator. After checking the growth and the purity, the leptospires were killed with formalin. After half an hour, the killed *Leptospira* culture was heated in a boiling water bath and centrifuged at 10,000 rpm for 30 mins. The pellet was collected and used as antigen. 25 μl of standard protein molecular weight marker was mixed with 15 μl of sample loading buffer and heated at 100°C for 3 mins to denature proteins were used to compare the protein profile of reference strain and isolates K1 and K2.
The samples (15 μl) was loaded into the wells using micropipette that was washed with buffer from the bottom reservoir after each sample was loaded. An equal volume of 1X SDS gel loading buffer was loaded into any wells that were unused. The electrophoresis apparatus was attached to an electric power supply. A voltage of 8 V/cm was applied to the gel electrophoresis. After the dye front had moved into the resolving or separating gel, the voltage was increased to 15 V/cm and the gel was allowed to run until the bromophenol blue reaching the bottom of the gel (about 4h). Then the power supply was turned off and the glass plate were removed from the electrophoresis apparatus and placed on a paper towel. Using a spatula, the plates were separated. The orientation of the gel was marked by cutting a corner from the bottom of the gel that was closest to the left most well. The gel was immersed in at least 5 volume of staining solution (20 ml Coomassie brilliant blue stain) and placed on a slowly rotating platform for 4 hrs at room temperature. After the staining of the gel, it was destained by soaking it in the glacial acetic acid solution on a slowly rocking platform for 1-4 hrs and changed the destaining solution for three or four times. After destaining, the gel was stored in water containing 20% glycerol. The molecular weight of the protein of the both pathogenic, non pathogenic and leptospiral isolated (K1 and K2) strain were measured by comparing with the standard molecular weight protein marker. The stained gel with protein band was photographed.
6.3. Results and Discussion

To establish a rapid identification system based on RAPD fingerprinting, RAPD profiles of 5 genospecies were prepared. The molecular weight of the isolated leptospiral strains (K₁ and K₂) were 23,000 bp and it has the A₂₆₀/₂₈₀ ratio of 1.70. This 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 genospecies (*L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. borgpetersenii*, and *L. noguchii*) were obtained from KIT, Royal Tropical Research Centre, The Netherlands. 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 (K₁ and K₂) had a number of common bands, they were very close to strain Rachmat (80-90%) (Fig 6.1 a and b). Dendogram analysis revealed that the genotype characters of the isolates K₁ and K₂ has poor resemblance with Panama CZ 214K, LSU 1945 and Sarmin CZ 390 (Table 6.1, Fig. 6.1a, b). The genomic profile of K₁ and K₂ were very similar and resemble the genomic profile of Rachmat. Dendogram analysis also reveals that the strain Rachmat belongs to the genospecies *Leptospira interrogans*. Hence the isolates K₁ and K₂ undoubtedly belong to the genospecies *Leptospira interrogans*. 
(a) RAPD fingerprinting of Leptospiral isolates in comparison with strains from five genomospecies

(b). Phylogenetic tree for the isolates (K₁ & K₂) in comparison with different genomospecies 
(Dendogram analysis)
Table 6.1. Table showing the percentage of similarities as per Dendogram Analysis

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Genomospecies</th>
<th>% of similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rachmat</td>
<td>L. interrogans</td>
<td>80-90</td>
</tr>
<tr>
<td>Ballico</td>
<td>L. interrogans</td>
<td>70-75</td>
</tr>
<tr>
<td>RGA</td>
<td>L. interrrogans</td>
<td>65-70</td>
</tr>
<tr>
<td>Perepelitsin</td>
<td>L. borgpetersenii</td>
<td>60-65</td>
</tr>
<tr>
<td>Mini Sari</td>
<td>L. borgpetersenii</td>
<td>60-50</td>
</tr>
<tr>
<td>Wumalasena</td>
<td>L. kirschnei</td>
<td>40-59</td>
</tr>
<tr>
<td>Cynopteri 3522 C</td>
<td>L. borgpetersenii</td>
<td>40-50</td>
</tr>
<tr>
<td>Moskva V</td>
<td>L. kirschneri</td>
<td>40-50</td>
</tr>
<tr>
<td>CZ 188</td>
<td>L. santarasai</td>
<td>40-50</td>
</tr>
<tr>
<td>Sarmin CZ 390</td>
<td>L. santarasai</td>
<td>40-50</td>
</tr>
<tr>
<td>Panama CZ 214K</td>
<td>L. noguchii</td>
<td>30-40</td>
</tr>
<tr>
<td>LSU 1945</td>
<td>L. noguchii</td>
<td>30-40</td>
</tr>
</tbody>
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![Fig 6.2 SDS - PAGE Protein profile of *Leptospira* isolates](image)

Fig 6.2 SDS - PAGE Protein profile of *Leptospira* isolates
Lane 1 Protein standard markers (phosphorylase b 97.4 KDa, bovine serum albumin 66 KDa, ovalbumin 45 KDa, carbonic anhydrase 29KDa, soyabean trypsin inhibitor 20.1KDa)
Lane 2 *Leptospira interrogans* serovar Australis (Reference strain)
Lane 3 *Leptospira interrogans* serovar Patoc 1(Reference strain)
Lane 4 *Leptospira* isolates K₁
Lane 5 *Leptospira* isolates K₂
SDS-PAGE protein analysis of the isolates K₁ and K₂ showed 3 major bands (45 kDa, 60 kDa and 110 kDa) and many minor bands. The bands 60 kDa and 110 kDa are common in both pathogenic and nonpathogenic groups but 45 kDa is a characteristic band observed in most of the pathogenic serovars (Fig. 6.2). Hence the isolates K₁ and K₂ studied in the present investigation were pathogenic serovars.