Identification of *Leptospira* serovars by RFLP of the RNA polymerase beta subunit gene (*rpoB*)

Lenice Roteia Cardoso Jung\(^1\), Maria Rosa Quaresma Bomfim\(^2\),\(^3\), Erna Geessien Kroon\(^2\), Álvaro Cantini Nunes\(^1\)

\(^1\)Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.  
\(^2\)Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.  
\(^3\)Universidade Ceuma, Departamento de Biologia Parasitária, São Luis, MA, Brazil.

Submitted: September 03, 2012; Approved: October 29, 2014.

**Abstract**

Leptospires are usually classified by methods based on DNA-DNA hybridization and the conventional cross-agglutination absorption test, which uses polyclonal antibodies against lipopolysaccharides. In this study, the amplification of the *rpoB* gene, which encodes the beta-subunit of RNA polymerase, was used as an alternative tool to identify *Leptospira*. DNA extracts from sixty-eight serovars were obtained, and the hypervariable region located between 1990 and 2500-bp in the *rpoB* gene was amplified by polymerase chain reaction (PCR). The 600-bp amplicons of the *rpoB* gene were digested with the restriction endonucleases *TaqI*, *TruI*, *Sau3AI* and *Msil*, and the restriction fragments were separated by 6% polyacrylamide gel electrophoresis. Thirty-five fragment patterns were obtained from the combined data of restriction fragment length polymorphism (PCR-RFLP) analysis and used to infer the phylogenetic relationships among the *Leptospira* species and serovars. The species assignments obtained were in full agreement with the established taxonomic classifications. Twenty-two serovars were effectively identified based on differences in their molecular profiles. However, the other 46 serovars remained clustered in groups that included more than one serovar of different species. This study demonstrates the value of RFLP analysis of PCR-amplified *rpoB* as an initial method for identifying *Leptospira* species and serovars.

**Key words**: *Leptospira*, rpoB gene, RFLP, serovar, DNA typing.

**Introduction**

Leptospirosis is a zoonotic disease of global importance that has emerged as a major cause of morbidity and mortality among impoverished populations (Ko *et al.*, 2009). Based on global data, more than 500,000 new cases of leptospirosis are reported annually, with mortality rates exceeding 10% (WHO, 1999, 2006). Multiple factors, including environmental, demographic, social, and economic factors, have contributed to the emergence of this disease, which affects a broad range of mammalian hosts, including humans, wildlife, and domestic animals (Bharti and Nally, 2003; Lau *et al.*, 2012).

The precise identification and classification of *Leptospira* spp. is important for epidemiological and public health surveillance (Mohammed *et al.*, 2011). Leptospires are usually classified by methods based on DNA-DNA hybridization, whereas the cross-agglutination absorption test (CAAT), which uses polyclonal antibodies against lipopolysaccharides (LPSs), has led to the definition of serovars that are today considered to be the basic systematic units of *Leptospira* spp. (Cerqueira and Picardeau, 2009; Galloway and Levett, 2010). Serological methods for the characterization of *Leptospira* species are complex and costly, restricting their worldwide distribution and use (Ahmed *et al.*, 2010).
Many molecular DNA techniques have been applied to identify and classify the species and serovars of *Leptospira* (Ahmed et al., 2012). These include restriction endonuclease analysis (REA) of chromosomal DNA (Marshall et al., 1981), random amplified polymorphic DNA (RAPD) fingerprinting (Ramadass et al., 1997), DNA-DNA hybridization (Yasuda et al., 1987; Brenner et al., 1999), arbitrarily primed PCR (Ramadass et al., 2002), pulsed-field gel electrophoresis (PFGE) (Galloway and Levett, 2008) and polymerase chain reaction (PCR) of specific genes followed by restriction fragment length polymorphism analysis (RFLP) (Li et al., 2009). Recently, multilocus sequence typing has been applied as an alternative to immunological methods for the identification and classification of pathogenic leptospires (Ahmed et al., 2006; Pavan et al., 2008; Leon et al., 2010; Ahmed et al., 2011; Boonsilp et al., 2013). All of these techniques mentioned above have contributed significantly to the current taxonomic classification of the *Leptospira* genus (Morey et al., 2006; Slack et al., 2009).

Quantitative DNA-DNA hybridization to measure genetic homology has been used as a reference for the classification of serovars within species (Yasuda et al., 1987; Perolat et al., 1998, Brenner et al., 1999). However, this hybridization method is not routinely used for the identification of *Leptospira* species due its complex and laborious execution, which requires the use of radioactive isotopes and is therefore restricted to reference laboratories (Morey et al., 2006). It has also been observed that some serotypes are more characteristic of a single species, while others contain both pathogenic and nonpathogenic serovars (Morey et al., 2006). Furthermore, little correlation has been shown between serological classification and genotypic systems because a given serogroup can often be found in several species of *Leptospira* (Ahmed et al., 2012).

In addition to DNA-DNA hybridization and the other molecular methods mentioned above, specific PCR amplification of the 16S rRNA gene has contributed to the molecular characterization of some species of *Leptospira* (Ahmed et al., 2012). The advantage of this technique is that the use of a DNA template, particularly one designed based on the region that encodes the bacterially conserved 16S rRNA gene, can clearly reveal phylogenetic relationships among species (Morey et al., 2006).

La Scola et al. (2006a) have designed a universal primer pair for the identification of *Leptospira* species based on the gene encoding the β subunit of RNA polymerase (*rpoB*). These primers have been used to amplify and sequence the partial *rpoB* gene from 16 *Leptospira* species. According to the authors, analysis of the *rpoB* gene “may be useful as an initial screening test for the serovar identification of a new isolate of *Leptospira* and the detection or identification of *Leptospira* in clinical or environmental samples”.

In previous studies, the utility of the *rpoB* gene for spirochete distinction among various bacterial species has been demonstrated (Renesto et al., 2000; Lee et al., 2000; Khamis et al., 2004; Balamurugan et al., 2013). Thus, the aim of this study was to investigate whether the PCR-amplified fragment of *rpoB* in conjunction with RFLP would allow for the determination of *Leptospira* serovars.

### Material and Methods

#### Bacterial strains, media and growth conditions

For this study, sixty-eight *Leptospira* strains (Table 1) belonging to 11 reference species from the Pan American Institute for Food Protection and Zoonosis (INNPAZ) were used. Leptospires were grown for approximately five days at 30 °C in Ellinghausen-McCullough-

| Species | Serogroup | Serovar | Strain | Number |
|---------|-----------|---------|--------|--------|
| *L. biflexa* | Andamana | Andamana | CH11 | 1 |
| | Semaranga | Patoc | Patoc 1 | 2 |
| *L. borgpetersenii* | Autumnalis | Srebarna | 1409/69 | 3 |
| | Ballum | Ballum | Mus 127 | 4 |
| | Bataviae | Moldaviae | 114-2 | 5 |
| | Celledoni | Withcombi | Withcomb | 6 |
| | Hebdomadis | Nona | Nona | 7 |
| | Hebdomadis | Worsfoldi | Worsfoldi | 8 |
| | Icterohaemorrhagiae | Tonkini | LT 96-68 | 9 |
| | Javanica | Javanica | Veldrat bataviae 46 | 10 |
| | Mini | Mini | Sari | 11 |
| | Pyrogenes | Kwale | Julu | 12 |
| | Sejroe | Sejroe | M 84 | 13 |
| | Tarassovi | Tarassovi | Perepelcin | 14 |
| *L. inadai* | Canicola | Malaya | H6 | 15 |
| | Panama | Mangus | TRVL 137774 | 16 |
| Species          | Serogroup      | Serovar       | Strain       | Number |
|------------------|----------------|---------------|--------------|--------|
| Tarassovi        | Kaup           | LT 64-68      | 17           |
| L. interrogans   | Australis      | Ballico       | 18           |
| Australis        | Muenchen       | Muenchen C90  | 19           |
| Autumnalis       | Autumnalis     | Akiyami A     | 20           |
| Djasimian        | Djasimian      | Djasimian     | 21           |
| Bataviae         | Bataviae       | Van Tienen    | 22           |
| Canicola         | Canicola       | Hond Utrech IV| 23           |
| Djasimian        | Sentot         | Sentot        | 24           |
| Gryppotyphosa    | Valbuzzi       | Valbuzzi      | 25           |
| Hebdomadis       | Hebdomadis     | Hebdomadis    | 26           |
| Icterohaemorrhagiae | Icterohaemorrhagiae | RGA | 27           |
| Louisiania       | Lanka          | LT 25-67      | 28           |
| Mini             | Szwajizak      | Szwajizaki    | 29           |
| Pomonadis        | Pomonadis      | Pomonadis     | 30           |
| Pyrogenes        | Pyrogenes      | Salinem       | 31           |
| Sejiroe          | Hardjo         | Hardjoprjaino | 33           |
| L. kirschneri    | Australis      | Musa          | 34           |
| Bataviae         | Djatzi         | HS 26         | 35           |
| Canicola         | Bafani         | Bafani        | 36           |
| Cynopteris       | Cynopteris     | 3522 C        | 37           |
| Gryppotyphosa    | Gryppotyphosa  | Moskva V      | 38           |
| Hebdomadis       | Kambale        | Kambale       | 39           |
| Icterohaemorrhagiae | Mwogolo       | Mwogolo       | 40           |
| Pomonadis        | Mozdok         | 5621          | 41           |
| L. meyeri        | Mini           | Parameles     | 343          | 42     |
| Ranarum          | Ranarum        | Ranaram ICF   | 43           |
| Semaranga        | Semaranga      | Veldrat Semaranga | 44         |
| L. noguchii      | Autumnalis     | Fort Bragg    | 45           |
| Djasimian        | Huallaga       | M7            | 46           |
| Panama           | Panama         | CZ 214K       | 47           |
| Pyrogenes        | Myocastoris    | LSU 1551      | 48           |
| Shermani         | Carimagua      | 9160          | 49           |
| L. santarosai    | Autumnalis     | Alice         | 50           |
| Bataviae         | Kobbe          | CZ 320K       | 51           |
| Cynopteris       | Tingomariensis | M13           | 52           |
| Gryppotyphosa    | Canalzonae     | CZ188K        | 53           |
| Hebdomadis       | Maru           | CZ 285B       | 54           |
| Javanica         | Vargonicas     | 24            | 55           |
| Mini             | Georgia        | LT 117        | 56           |
| Pomonadis        | Tropica        | CZ 299U       | 57           |
| Pyrogenes        | Alexi          | HS 616        | 58           |
| Sarmin           | Weaveri        | CZ 390U       | 59           |
| Sejiroe          | Trinidad       | TRVL 34056    | 60           |
| Shermani         | Luis           | M6            | 61           |
| Tarassovi        | Bakeri         | LT 79         | 62           |
| L. weilli        | Celledoni      | Celledoni     | 63           |
| Javanica         | Coxi           | Cox           | 64           |
| Sarmin           | Sarmin         | Sarmin        | 65           |
| Tarassovi        | Vughia         | LT 89-68      | 66           |
| L. terpstraiae   | Icterohaemorrhagiae | Hualien | 67           |
| L. yanagirae     | Semaranga      | Sao paulo     | 68           |
Johnson-Harris (EMJH) culture medium (Difco) (Ellinghausen, 1973).

Isolation of DNA

An one-mL aliquot of each Leptospira serovar was cultured in 5 mL EMJH medium for 7 to 10 days at 30 °C. The culture was then centrifuged at 3000 x g for 30 min, and DNA was extracted from the bacterial pellet by adding 1 mL lysozyme solution (10 mg/mL in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and Wizard Genomic DNA Purification System reagents according to the manufacturer’s instructions (Promega Co.).

PCR assays

PCR amplification of a 600-bp region of the rpoB gene was performed with the primers 1900F (5'-CCTCATGGTTCCAATGCA-3') and 2500R (5'-CGCATCCTCRAAGTTGTATTWCC-3') as described by La Scola et al. (2006a). Each PCR reaction contained 1.5 mM MgCl2, 200 μM dNTPs, 25-50 ng of DNA template, 1.5 units of Taq DNA polymerase, and 50 pmol of each primer. The PCR amplification reactions were carried out in a Veriti 96-well Thermal Cycler (Applied Biosystems) under the following conditions: an initial denaturation step of 2 min at 95 °C, 33 cycles of denaturation for 30 s at 94 °C, annealing at 51 °C for 30 s and extension at 72 °C for 2 min, with a final primer extension step for 10 min at 72 °C.

Restriction fragment length polymorphism (RFLP) analysis

To select enzymes for RFLP analysis, the results from in silico restriction digestion of twenty five rpoB sequences in GenBank® were analyzed with Webcutter 2.0 program (http://bio.lundberg.gu.se/cutter2/) to distinguish the generated fragments following separation by 6% polyacrylamide gel electrophoresis. The genomic sequences used were as follows: AE016823.1, L. interrogans serovar Copenhageni str. Fiocruz L1-130; AE010300.2, L. interrogans serovar Lai str. S6601; CP000350.1, L. borgpetersenii serovar Hardjo-bovis strain JB197; and CP000777.1, and L. biflexa serovar Patoc strain Patoc 1 (Ames). DNA sequences of the rpoB gene reported in La Scola et al. (2006a) and sequences obtained by us in this study were also used. These sequences were deposited in GenBank® under the accession numbers EU747300.1, EU747301.1, EU747302.1, EU747303.1, EU747304.1, EU747305.1, EU747306.1, EU747307.1, EU747308.1, EU747309.1, EU747310.1, EU747311.1, EU747312.1, EU747313.1, EU747314.1, EU747317.1, and EU747299.1, corresponding to L. interrogans serovar Bratislava, L. kirschneri serovar Grippotypphosa, L. borgpetersenii serovar Ballum, L. interrogans serovar Hardjo-prajitno, L. interrogans serovar Hebdomadis, L. borgpetersenii serovar Hardjo-bovis, L. interrogans serovar Pomona, L. borgpetersenii serovar Tarassovi, L. interrogans serovar Wolfii, L. biflexa serovar Andamanara, L. borgpetersenii serovar Castellonis, L. borgpetersenii serovar Sejroe, L. interrogans serovar Djasiman, L. interrogans serovar Schueffneri, L. borgpetersenii serovar Whittcombi, L. interrogans serovar Sentot, and L. interrogans serovar Canicola, respectively.

PCR products were subjected to restriction digestion with TaqI, TruI1, Sau3AI and MslI endonucleases (Promega Co.) for 3 h at the recommended temperatures. To calculate the relative molecular masses of the digested fragments, a 100-bp DNA Ladder was used (Promega Co.). The digestion and separation of the DNA fragments by 6% polyacrylamide gel electrophoresis were repeated at least three times for all serovars to establish the final restriction patterns.

Dendrogram construction

LabImage version 2.7.0 software (Kapelan GMBH) was used for constructing a binary matrix scored on the presence (1) or absence (2) of each fragment generated by PCR-RFLP with the rpoB primers. Cluster analysis based on similarity (Nei, 1972) was performed by the unweighted pair group method (UPGMA) with the arithmetic averages clustering algorithm (Sneath and Sokal, 1973), and the randomization procedure implemented in Tools for Population Genetic Analysis (TFPGA) software package according to Miller (1998) was used to construct the dendrogram.

Results

In silico analysis of rpoB sequences deposited in GenBank indicated that a combination of four possible restriction enzymes was necessary to distinguish the Leptospira serovars as follows: TaqI, TruI1, Sau3AI and MslI. Alone, each enzyme was able to identify only one or two different serovars.

Digestion with TaqI resulted in ten different patterns (A to J), which are schematically represented in Table 2 and had the following frequencies: A, 29.4% (20); B, 10.3% (7); C, 7.35% (5); D, 13.2% (9); E, 11.8% (8); F, 4.41% (3); G, 16.2% (11); H, 7.35% (3); I, 1.47% (1); and J, 1.47% (1). Thus, TaqI identified two serovars, Huallaga of L. noguchii (profile I) and Alice of L. santarosai (profile J), as shown in Figure 1. The G profile pattern was observed in almost all L. santarosai serovars, with the exception of the serovar Alexi (profile D), and it was only identified in the Muenchen serovar L. interrogans.

The TruI1 enzyme also exhibited ten distinct restriction patterns (A to J) with the following frequencies: A, 2.94% (2); B, 23.5% (16); C, 16.2% (11); D, 11.8% (8); E, 22.1% (15); F, 14.7% (10); G, 2.94% (2); H, 2.94% (2); I, 1.47% (1); and J, 1.47% (1). These patterns are summarized in Table 2 and identified the serovars Huallaga of L. noguchii (profile I) and Trinidad of L. santarosai (profile J).
The combination of both enzymes, TaqI and TruI, generated 23 distinct patterns with some interesting results as follows: profile A of TaqI and TruI (profile AA) was species-specific and was only observed for *L. biflexa*. Pro-

**Figure 1** - Polyacrylamide gel electrophoresis (6%) of the PCR products resulting from the digestion of the *rpoB* gene with the restriction endonuclease *TaqI*. was consistent with the 100-bp molecular weight ladder.
file AC was displayed by all serovars of *L. kirschneri* and by serovar Hualien of *L. terpstrae*; therefore, it is nearly species-specific. Finally, the profiles AG, FG, FE, CE, FD, BE and GE were unique to the serovars Mini, Kaup, Lanka, Szwajizak, Waskurin, Myocastoris and Maru, respectively.

Digestion with the Sau3AI enzyme generated nine distinct restriction patterns, which are summarized in Table 2 with the following frequencies: A, 2.94% (2); B, 22.1% (15); C, 30.9% (21); D, 4.41% (3); E, 7.35% (5); F, 16.2% (11); G, 8.82% (6); H, 5.88% (4); and I, 1.47% (1). Sau3AI digestion identified only the serovar Ranarum to have a serovar-specific profile. However, the combination of all three enzymes generated 30 distinct profiles, including EEE, DFD, ACC, HDD, GBB and AHH, which were specific for the serovars Whitcomb, Icterohaemorrhagiae, Hardjo, Ramisi, Semaranga, Vargonicas and Sarmin, respectively.

Finally, digestion with the enzyme MslI produced only five distinct restriction patterns, which are summarized in Table 2 with the following frequencies: A, 10.3% (7); B, 20.6% (14); C, 57.4% (39); D, 10.3% (7); and E, 1.47% (1). Only the serovar Saopaulo was identified by this enzyme to have a serovar-specific profile.

The combination of the four enzymes TaqI, TruI, Sau3AI and MslI generated 35 distinct profiles and identified the serovars Parameles (EFFD) and Celledoni (HDFA). In addition, this combination helped to distinguish the serovars Valbuzzi and Tropica, which had the profiles DFFC and GBCC, respectively (Table 3).

Out of sixty-eight serovars analyzed for RFLP polymorphisms in the region of the coding sequence containing the \(\beta\)-subunit gene of RNA polymerase, 22 serovars from nine species (32%) were identified by digestion with the enzymes TaqI, TruI, Sau3AI and MslI (Table 3), and the other 46 strains were clustered into 13 groups with two to seven serovars.

A dendrogram obtained from a matrix constructed with the results from the fragments generated by PCR-RFLP with the four restriction endonucleases (Figure 2) showed clustering of the sixty-eight reference serovars. Several of the tested strains appeared to be distant from others of the same species in relation to the current taxonomic classification. The serovar Kaup (*L. inadai*) was grouped with the serovar Waskurin (*L. interrogans*); the serovar Ranarum (*L. meyeri*) was similar to the nonpathogenic *L. biflexa*; the serovar Muenchen (*L. interrogans*) clustered with those of *L. santarosai*; the serovar Nona (*L. borgpetersenii*) was closer to the serovar Mangus (*L. inadai*); the serovar Hualien (*L. terpstrae*) grouped with the those of *L. kirschneri*; the Huallaga and Myocastoris serovars (*L. noguchii*) were located in different branches; the serovar Tonkini (*L. borgpetersenii*) was closer to those of *L. borgpetersenii*; and the serovar Alexi (*L. santarosai*) was grouped with those of Djasiman, Pyrogenes and Sentot (*L. interrogans*), Malaya (*L. inadai*) and Sejroe (*L. borgpetersenii*).
Discussion

The correlation between the serological and genotypic classifications of leptospires is low, and identification is complicated because the same serovar can be distributed among different species (Ahmed et al., 2012; Balamurugan et al., 2013). It is assumed that this lack of correlation between species and serovars is the result of horizontal transference between species of the genes that determine serotypes, but the basis of this transference, which is responsible for exchanging genetic determinants, is still unknown (Cerqueira and Picardeau, 2009). A single base difference differentiated many strains of L. interrogans and L. kirschneri; therefore, phylogenetic representation may be less meaningful than sequence identities at variable positions (Cerqueira and Picardeau, 2009).

The aim of this work was to identify Leptospira strains at the serovar level by performing PCR-RFLP to

Figure 2 - Dendrogram constructed by joint analysis of the bands generated by the restriction endonucleases TaqI, TruI II, Sau3AI, and MslI.
Table 3 - Grouping of the serovars, serogroups and species of the *Leptospira* genus based on the restriction patterns generated with the four endonucleases.

| Number | TaqI | TruI | Sau3AI | MslI | Pattern | Species/Serogroup/Serovar |
|--------|------|------|--------|------|---------|--------------------------|
| 1      | A    | A    | A      | A    | 1       | *L. biflexa*/Andamana/Andamana |
| 2      | A    | A    | A      | A    | 1       | *L. biflexa*/Semarang/Patoc |
| 3      | B    | B    | B      | B    | 2       | *L. borgpetersenii*/Autumnalis/Srebarna |
| 5      | B    | B    | B      | B    | 2       | *L. borgpetersenii*/Batavia/Moldavia |
| 12     | B    | B    | B      | B    | 2       | *L. borgpetersenii*/Pyrogenes/Kwale |
| 14     | B    | B    | B      | B    | 2       | *L. borgpetersenii*/Tarassovi/Tarassovi |
| 4      | B    | C    | C      | B    | 3       | *L. borgpetersenii*/Ballum/Ballum |
| 10     | B    | C    | C      | B    | 3       | *L. borgpetersenii*/Javanica/Javanica |
| 6      | C    | D    | D      | B    | 4       | *L. borgpetersenii*/Celledoni/Withcombi |
| 7      | A    | E    | C      | C    | 5       | *L. borgpetersenii*/Hebdomadis/Nona |
| 16     | A    | E    | C      | C    | 5       | *L. inadai*/Panama/Mangus |
| 8      | C    | D    | E      | C    | 6       | *L. borgpetersenii*/Hebdomadis/Worsfoldi |
| 64     | C    | D    | E      | C    | 6       | *L. weilii*/Javanica/Coxi |
| 66     | C    | D    | E      | C    | 6       | *L. weilii*/Tarassovi/Vughia |
| 9      | D    | F    | F      | C    | 7       | *L. borgpetersenii*/Icterohaemorrhagiae/Tonkini |
| 20     | D    | F    | F      | C    | 7       | *L. interrogans*/Autumnalis/Autumnalis |
| 26     | D    | F    | F      | C    | 7       | *L. interrogans*/Hebdomadis/Hebdomadis |
| 30     | D    | F    | F      | C    | 7       | *L. interrogans*/Pomona/Pomona |
| 23     | D    | F    | F      | C    | 7       | *L. interrogans*/Canicola/Canicola |
| 25     | D    | F    | F      | C    | 7       | *L. interrogans*/Gryppotyphosa/Valbuzzi |
| 11     | A    | G    | C      | B    | 8       | *L. borgpetersenii*/Mini/Mini |
| 13     | E    | E    | G      | C    | 9       | *L. borgpetersenii*/Sejroe/Sejroe |
| 15     | E    | E    | G      | C    | 9       | *L. inadai*/Canicola/Malaya |
| 24     | E    | E    | G      | C    | 9       | *L. interrogans*/Djasiman/Sentot |
| 21     | E    | E    | G      | C    | 9       | *L. interrogans*/Djasiman/Djasiman |
| 17     | F    | G    | H      | C    | 10      | *L. inadai*/Tarassovi/Kaup |
| 18     | E    | F    | F      | C    | 11      | *L. interrogans*/Australis/Australis |
| 22     | E    | F    | F      | C    | 11      | *L. interrogans*/Bataviae/Bataviae |
| 19     | G    | B    | C      | C    | 12      | *L. interrogans*/Australis/Muenchen |
| 51     | G    | B    | C      | C    | 12      | *L. santarosai*/Bataviae/Kobbe |
| 53     | G    | B    | C      | C    | 12      | *L. santarosai*/Gryppotyphosa/Canalzonae |
| 56     | G    | B    | C      | C    | 12      | *L. santarosai*/Mini/Georgia |
| 61     | G    | B    | C      | C    | 12      | *L. santarosai*/Shermani/Luis |
| 62     | G    | B    | C      | C    | 12      | *L. santarosai*/Tarassovi/Bakeri |
| 59     | G    | B    | C      | C    | 12      | *L. santarosai*/Sarmin/Weaveri |
| 27     | E    | E    | E      | D    | 13      | *L. interrogans*/Icterohaemorrhagiae/Icterohaemorrhagiae |
| 28     | F    | E    | E      | C    | 14      | *L. interrogans*/Louisiania/Lanka |
| 29     | C    | E    | H      | C    | 15      | *L. interrogans*/Mini/Szwajizak |
| 31     | D    | B    | G      | C    | 16      | *L. interrogans*/Pyrogenes/Pyrogenes |
| 58     | D    | B    | G      | C    | 16      | *L. santarosai*/Pyrogenes/Alexi |
| 32     | F    | D    | H      | C    | 17      | *L. interrogans*/Sarmin/Waskurin |
| 33     | D    | F    | D      | C    | 18      | *L. interrogans*/Sejroe/Hardjo |
| 34     | A    | C    | C      | A    | 19      | *L. kirschneri*/Australis/Ramisi |
| 35     | A    | C    | B      | B    | 20      | *L. kirschneri*/Bataviae/Djatzi |
| 36     | A    | C    | B      | B    | 20      | *L. kirschneri*/Canicola/Bafani |
amplify a 600-bp fragment of the coding sequence of the β subunit of the RNA polymerase gene. The rpoB gene has been widely studied in other organisms and is considered by many researchers to be more useful than the 16S ribosomal RNA gene for the differentiation of bacterial species (La Scola et al., 2006a; Ahmed et al., 2006; Macheras et al., 2011; Ahmed et al., 2012). In addition, twenty-five sequences of the rpoB gene of *Leptospira* are already available in databases, thereby facilitating access and minimizing project costs.

In a previous report, La Scola et al. (2006a) have compared similarities in the *rrs* and *rpoB* genes between different *Leptospira* serovars. Using the *rpoB* gene, they were able to effectively distinguish 11 of 19 serovars tested, differentiating them from other species and showing greater numbers of polymorphisms in both genes, leading to the conclusion that the *rpoB* gene could distinguish species with a higher number of differences between base pairs.

In this study, 68 *Leptospira* serovars were analyzed for polymorphisms in a specific region of the *rpoB* gene using the endonucleases *TaqI*, *TruI*, *Sau3AI* and *MslI*. These enzymes were selected after *in silico* restriction digestion of the *rpoB* sequences deposited in GenBank. We were able to identify 22 strains from nine species at the serovar level (32%). The *rpoB* gene has been widely used as an alternative tool in the phylogeny and identification of different species of bacteria, such as *Coxiella burnetii* (Mollet et al., 1998), *Afipia* (Khamis et al., 2003), *Mycoplasma* (Kim et al., 2003), *Corynebacterium* (Khamis et al., 2004), *Acinetobacter* (La Scola et al., 2006b), *Mycobacterium* (Adekambi et al., 2006; Ben et al., 2008), *Halobacterium* (Minegishi et al., 2010) and *Cyanobacteria* (Gaget et al., 2011).

In a recent study, the *rpoB* gene has been successfully used to identify or detect *Leptospira* species in animals and humans in India (Balamurugan et al., 2013). Because each *Leptospira* serovar is associated with specific host symptoms, their identification is essential for the development of epidemiological studies (Cerqueira and Picardeau, 2009, Li et al., 2009).

Clustering analysis of the results of this study correctly grouped 22 serovars by species. Considerable similarities in the analyzed genomic region were observed among all serovars. Analysis of dendrograms constructed from the results of each restriction enzyme and from the collective results for all of the enzymes showed the formation of clusters, for which serovars of various species had identical profiles. The groups formed by the *rpoB* gene pro-

| Number | TaqI | TruI | Sau3AI | MslI | Pattern | Species/Serogroup/Serovar |
|--------|------|------|--------|------|---------|---------------------------|
| 37     | A    | C    | B      | B    | 20       | *L. kirschneri/Cynopteri/Cynopteri* |
| 38     | A    | C    | B      | B    | 20       | *L. kirschneri/Gryppotyphosa/Gryppotyphosa* |
| 41     | A    | C    | B      | B    | 20       | *L. kirschneri/Pomona/Mozdok* |
| 67     | A    | C    | B      | B    | 20       | *L. terpstrae/Icteroaemorrhagiae/Hualien* |
| 39     | A    | C    | B      | C    | 21       | *L. kirschneri/Hebdomadis/Kambale* |
| 40     | A    | C    | B      | C    | 21       | *L. kirschneri/Icteroaemorrhagiae/Mwogolo* |
| 42     | E    | F    | F      | D    | 22       | *L. meyeri/Mini/Parameles* |
| 43     | A    | H    | I      | C    | 23       | *L. meyeri/Ranarum/Ranarum* |
| 44     | H    | D    | D      | A    | 24       | *L. meyeri/Semaranga/Semaranga* |
| 45     | A    | E    | C      | D    | 25       | *L. noguchii/Autumnalis/Fortbragg* |
| 47     | A    | E    | C      | D    | 25       | *L. noguchii/Panama/Panama* |
| 49     | A    | E    | C      | D    | 25       | *L. noguchii/Shermani/Carinagua* |
| 52     | A    | E    | C      | D    | 25       | *L. santarosai/Cynopteri/Tingomariensis* |
| 46     | I    | I    | B      | A    | 26       | *L. noguchii/Djasiman/Huallaga* |
| 48     | B    | E    | B      | D    | 27       | *L. noguchii/Pyrogenes/Myocastorius* |
| 50     | J    | B    | C      | C    | 28       | *L. santarosai/Autumnalis/Alice* |
| 54     | G    | E    | C      | A    | 29       | *L. santarosai/Hebdomadis/Maru* |
| 55     | G    | B    | B      | C    | 30       | *L. santarosai/Javanica/Vargonicas* |
| 57     | G    | B    | C      | C    | 31       | *L. santarosai/Pomona/Tropica* |
| 60     | G    | J    | C      | C    | 32       | *L. santarosai/Sejroe/Trinidad* |
| 63     | H    | D    | F      | A    | 33       | *L. weilli/Celledoni/Celledoni* |
| 65     | A    | H    | H      | C    | 34       | *L. weilli/Sarmin/Sarmin* |
| 68     | H    | D    | F      | E    | 35       | *L. yanagowae/Semaranga/Saopaulo* |
files showed varying degrees of similarity and clade forma-
tion. Based on this, similar banding patterns were observed
among the serovars Mangus, Nona, Alexi, Pyrogenes, Sen-
tot, Malaya and Sejroe, despite the fact that they belonged
to different species. These findings are in accordance with
similar dendrogram analyses reported previously (Perolat
et al., 1998; Morey et al., 2006; Cerqueira and Picardeau,
2009; Balamurugan et al., 2013), showing similar cluster
formations and variations in serovar-species grouping.

The addition of new enzymes for the production of ad-
ditional profiles should clarify the positions of other
serovars. Still, these results suggest that the use of this
technique to assess gene sequences may reveal a precise sensu
stricto classification of these serovars.

Molecular techniques have been used for the charac-
terization of Leptospira isolates; however, most can only
make identifications to the species level (Galloway and
Levett, 2010), such as 16S RNA sequence analysis (Morey
et al., 2006), RFLP (Li et al., 2009) and MLST (Boonsilp
et al., 2013). PFGE has demonstrated the reliable and repro-
ducible identification of Leptospira at the serovar level
(Galloway and Levett, 2010). These approaches have greatly
tributed to a revolution in both Leptospira detection and
characterization (Ahmed et al., 2012). On the other
hand, the molecular tools described so far for the character-
zation of Leptospira suffer from significant drawbacks.
For example, PFGE, RFLP, and REA require large quanti-
ties of purified DNA, have low levels of discrimination,
produce data that is difficult to interpret, suffer from a lack
of reproducibility and require specialized equipment
(Ahmed et al., 2006).

Notably, the 16S rRNA gene has been considered the
gold standard in molecular surveys of bacterial and archaeal
diversity, but it has several disadvantages as follows: it is often present in multiple copies, has little resolu-
tion below the species level and cannot be readily inter-
preted in an evolutionary framework (Vos et al., 2012).

The main advantages of the use of the rpoB gene over
the 16S rRNA gene are as follows: (i) it is universally pres-
ent in all prokaryotes; (ii) it typically occurs in a sin-
gle-copy, essential protein-encoding gene, and sequence
ears can be readily identified and removed if they intro-
duce disruptions in the reading frame; (iii) it possesses both
slowly and quickly evolving regions, enabling the design of
probes and primers of differing specificities; (iv) it has a
housekeeping function, making it less susceptible to some
forms of lateral gene transfer; and (v) it is large enough in
size to contain phylogenetic information, even after the
removal of regions that are difficult to align (Case et al.,
2007; Vos et al., 2012).

Our findings “in vitro” indicate that the PCR-RFLP
 technique is a powerful and reproducible test that may be
used as a complement or alternative tool to assess the dis-
bution of Leptospira strains within species. Additionally,
we recommend the use of PCR-RFLP with in silico diges-
tion of the polymorphic sequences of other conserved
genes already deposited in GenBank as a promising tech-
nique for the genomic classification of the Leptospira ge-
nus.

Conclusion

This study demonstrated that PCR-RFLP is practical
and efficient, enabling the differentiation of species and
serovars with good discriminatory power, reproducibility
and easily interpretable results. In addition, this method is
cost-effective for most research laboratories. This tech-
nique has also been shown to be suitable for phylogenetic
studies and the classifications of species, serovars and
strains. The selected 600-bp polymorphic sequence of the
rpoB gene produced restriction profiles that allowed for the
accurate and timely identification of 32% of the 68 tested
strains. We demonstrated that this approach achieves the
stated purpose and that serological typing is unreliable for
the classification of pathogenic Leptospira. However, addi-
tional studies should be undertaken to reclassify these
serovars within the species with which they have greater
genotypic affinities based on analysis of hypervariable re-
gions of multiple housekeeping genes and especially to
investigate whether the clinical leptospirosis symptoms in-
duced by these serovars are presented according to the spe-
cies with which they are most phylogenetically related.

Acknowledgments

This study was supported by the Fundação de Amparo
à Pesquisa de Minas Gerais (FAPEMIG), the Conselho
Nacional de Desenvolvimento Científico e Tecnológico
(CNPq), the Fundação de Amparo à Pesquisa e Desen-
volvimento Científico do Maranhão (FAPEMA) and the
PRPq/UFMG. We thank Dr. Élvio C. Moreira from the De-
partment of Preventive Medicine of the Veterinary School
of UFMG for providing the Leptospira strains and Dr. Re-
gina M. Nardi Drummond and Dr. Vera Lúcia dos Santos
for their assistance at the laboratory facilities with the
growing and maintenance of the Leptospira strains.

References

Adekambi T, Berger P, Raoult D et al. (2006) rpoB gene se-
quence-based characterization of emerging non-tuberculous
mycobacteria with descriptions of Mycobacterium bolletii
sp. nov., Mycobacterium phocaicum sp. nov. and Mycobac-
terium aubagnense sp. nov. Int J Syst Evol Microbiol
56:133-143.

Ahmed N, Devi SM, Valverde MA et al. (2006) Multilocus se-
dence typing method for identification and genotypic clas-
sification of pathogenic Leptospira species. Ann Clin
Microbiol Antimicrob 5:28.

Ahmed A, Anthony RM, Hartskeerl RA (2010) A simple and
rapid molecular method for Leptospira species identifica-
tion. Infect Genet Evol 7:955-962.
Ahmed A, Thaipadungpanit J, Boonsilp S et al. (2011) Comparison of two multilocus sequence based genotyping schemes for Leptospira species. PLoS Negl Trop Dis 5:e1374.

Ahmed A, Grobusch MP, Klater PR et al. (2012) Molecular Approaches in the Detection and Characterization of Leptospira. J Bacteriol Parasitol 3:2.

Balamurugan V, Gangadhar NL, Mohandoss N et al. (2013) Characterization of Leptospira isolates from animals and humans: phylogenetic analysis identifies the prevalence of intermediate species in India. Springer Plus 2:362.

Ben Salah I, Adekambi T, Raoul D et al. (2008) rpoB sequence-based identification of Mycobacterium avium complex species. Microbiol 154:3715-3723.

Bharti AR, Nally JE (2003) Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3:757-771.

Brenner DJ, Kaufmann AF, Sulzer KR et al. (1999) Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for Leptospira alexanderi sp. nov. and four new Leptospira genomospecies. Int J Syst Bacteriol 49 Pt 2:839-858.

Boonsilp S, Thaipadungpanit J, Amornchai P et al. (2013) A Single Multilocus Sequence Typing (MLST) Scheme for Seven Pathogenic Leptospira Species. PLoS Negl Trop Dis 9:e824.

Case RJ, Bouyer V, Dahllöf I et al. (2007) Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. Appl Environ Microbiol 1:278-288.

Cerqueira G, Picardeau M (2009) A century of Leptospira strain typing. Infect Genet Evol 9:760-768.

Ellingshausen HC (1973) Virulence, nutrition and antigenicity of Leptospira interrogans serotype Pomona in supplemented and nutrient deleted bovine albumin medium. Ann Microbiol (Paris) 124:477-493.

Gaget V, Gribaldo S, Tandeau de Marsac N (2011) A rpoB signature sequence provides unique resolution for the molecular typing of cyanobacteria. Int J Syst Evol Microbiol 61:170-183.

Galloway RL, Levett PN (2008) Evaluation of a modified pulsed-field gel electrophoresis approach for the identification of Leptospira serovars. Am J Trop Med Hyg 78:628-632.

Galloway RL, Levett PN (2010) Application and Validation of PFGE for Serovar Identification of Leptospira Clinical Isolates. PLoS Negl Trop Dis 4:e824.

Khamis A, Colson P, Raoul D et al. (2003) Usefulness of rpoB gene sequencing for identification of Afipia and Bosea species, including a strategy for choosing discriminative partial sequences. Appl Environ Microbiol 69:6740-6749.

Khamis A, Raoul D, La Scola B (2004) rpoB gene sequencing for identification of Corinebacterium species. J Clin Microbiol 42:3925-3931.

Kim KS, Ko KS, Chang MW et al. (2003) Use of rpoB sequences for phylogenetic study of Mycoplasma species. FEMS Microbiol Lett 226:299-305.

Ko AI, Goarant C, Picardeau M (2009) Leptospira: The Dawn of the Molecular Genetics Era for an Emerging Zoonotic Pathogen. Nat Rev Microbiol 10:736-747.

La Scola B, Bui LTM, Baranton G et al. (2006a) Partial rpoB gene sequencing for identification of Leptospira species. FEMS Microbiol Lett 263:142-147.

La Scola B, Gundl VA, Khamis A et al. (2006b) Sequencing of the rpoB gene and flanking spacers for molecular identification of Acinetobacter species. J Clin Microbiol 44:827-832.

Lau C, Clements A, Skelly C et al. (2012) Leptospirosis in American Samoa: Estimating and mapping risk using environmental data. PLoS Negl Trop Dis 6:e1669.

Lee SH, Kim BJ, Kim JH et al. (2000) Differentiation of Borrelia burgdorferi sensu lato on the basis of RNA polymerase gene (rpoB) sequences. J Clin Microbiol 38:2557-2562.

Leon A, Pronost S, Fortier G et al. (2010) Multilocus Sequence Analysis for Typing Leptospira interrogans and Leptospira kirschneri. J Clin Microbiol 48:581-585.

Li W, Raoul D, Fournier PE (2009) Bacterial strain typing in the genomic era. FEMS Microbiol Rev 33:892-916.

Macheras E, Roux AL, Bastian S et al. (2011) Multilocus sequence analysis and rpoB sequencing of Mycobacterium abscessus (sensu lato) strains. J Clin Microbiol 49:491-499.

Marshall RB, Wilton BE, Robinson AJ (1981) Identification of Leptospira serovars by restriction-endonuclease analysis. J Med Microbiol 14:163-166.

Miller MP (1998) TFP GA: Tools for Population Genetic Analyses for Windows. Arizona State University, USA.

Minegishi H, Kamekura M, Itoh T et al. (2010) Further refinement of the phylogeny of the Halobacteriaceae based on the full-length RNA polymerase subunit B9 (rpoB9) gene. Int J Syst Evol Microbiol 60:2398-2408.

Mohammed H, Nozha C, Hakim K et al. (2011) Leptospira: Morphology, Classification and Pathogenesis. Bacteriol Parasitol 2:6.

Mollet C, Drancourt M, Raoul D (1998) Determination of Coxiellaburnetii rpoB sequence and its use for phylogenetic analysis. Gene 207:97-103.

Morey RE, Galloway RL, Bragg SL et al. (2006) Species-specific identification of Leptospiraceae by 16S rRNA gene sequencing. J Clin Microbiol 44:3510-3516.

Nei M (1972) Genetic distance between populations. Am Nat 106:283-292.

Pavan ME, Cairo F, Brihuega B et al. (2008) Multiple-locus variable-number tandem repeat analysis MLVA of Leptospira interrogans serovar Pomona from Argentina reveals four new genotypes. Comp Immunol Microbiol Infec Dis 31:37-45.

Perolat P, Chappel RJ, Adler B et al. (1998) Leptospira fainei sp. nov., isolated from pigs in Australia. Int J Syst Bacteriol 48:851-858.

Ramadass P, Meerarani S, Venkatesha MD et al. (1997) Characterization of Leptospiral Serovars by Randomly Amplified Polymorphic DNA Fingerprinting. Int J Syst Bacteriol 47:575-576.

Ramadass P, Latha D, Senthilkumar A et al. (2002) Arbitrarily primed PCR- a rapid and simple method for typing leptospiral serovars. Indian J Med Microbiol 20:25-28.

Renesto P, Lorraine-Cuillen K, Drancourt M et al. (2000) rpoB gene analysis as a novel strategy for identification of spirochetes from the genera Borrelia, Treponema and Leptospira. J Clin Microbiol 38:2200-2203.

Slack AT, Khairani-Bejo S, Symonds ML et al. (2009) Leptospira kerryi sp. nov., isolated from an environmental source in Malaysia. Int J Syst Evol Microbiol 59:707-708.

Sneath PHA, Sokal RR (1973) Numerical Taxonomy. Freeman, San Francisco, C.A.

Vos M, Quince C, Pijl AS et al. (2012) A comparison of rpoB and 16S rRNA as markers in pyrosequencing studies of bacterial diversity. PLoS One 2:e30600.
World Health Organization. Leptospirosis worldwide. (1999). Weekly Epidemiol Rec 74:237-242.

World Health Organization (2006) Informal Consultation on Global Burden of Leptospirosis: Methods of Assessment. Available at: http://www.who.int/entity/foodsafety/zoonoses/InformalConsultationOnBoDLeptospirosis.pdf.

Yasuda PH, Steigerwalt AG, Sulzer KR et al. (1987) Deoxyribonucleic acid relatedness between serogroups and serovars in the family Leptospiraceae with proposals for seven new Leptospira species. Int J Syst Bacteriol 37:407-415.

Associate Editor: Elizabeth de Andrade Marques

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.