Research article

Elucidating Sequence and Structural Variations Present in RNase P RNA Ribozyme for the Taxonomy of *Leptospira* spp.

Vigneshwaran Ravishankar, Manasha Karunanidhi, Nivetha Sridhar and Stephen Raj Michael Dason Lourdu*

Department of Biotechnology, Mepco Schlenk Engineering College (Autonomous), Sivakasi 626005, Tamilnadu, India

Curr. Appl. Sci. Technol. 2024, Vol. 24 (No. 5), e0259544; https://doi.org/10.55003/cast.2024.259544

Received: 27 June 2023, Revised: 14 December 2023, Accepted: 15 March 2024 Published: 1 May 2024

Keywords

Leptospira;

phylogeny;

RNase P RNA

Abstract

RNase P RNA (RPR) gene responsible for transcribing tRNA processing ribozyme is an essential single-copy gene present in the genome of all organisms and has been proven to be a reliable genetic marker for the differentiation of species. In the present study, phylogenetic analysis was performed by exploiting sequence and structural variations present in the RPRs to determine the taxa of unidentified leptospires. RPR gene-based phylogeny revealed that serovars of *Leptospira borgpetersenii* could be bifurcated into distinct subgroups A and B. The saprophytic leptospiral strains were classified into three groups namely saprophytic group I, saprophytic group II and saprophytic group III. The presumed taxonomic positions of leptospiral strains Hampton, LT 2116, M4, 18R, Pond 2020, ZV016, ICFT and *L. alexanderi* were investigated for the phylogeny using both RPR and *secY* genes. The strains of *L. alexanderi* branched out as a separate clade between strains of *L. santarosai* and *L. borgpetersenii*. Furthermore, a simple restriction digestion assay was performed for the RPR gene-based differentiation of leptospires. PCR amplification and subsequent restriction digestion of the RPR gene amplified helped to distinguish *Leptospira* sp. The present research contributes to our understanding of leptospiral taxonomy and provides a valuable tool for species identification in diagnostic applications.

*Corresponding author: Tel.: (+91) 9245295589 Fax: (+91) 4562 235111
E-mail: stephenraj@mepcoeng.ac.in

1. Introduction

The members of the genus *Leptospira* are ubiquitous in warmer regions due to their ability to thrive in arid and semiarid environments [1-3]. Generally, wild and domestic animals are known to be the reservoir of leptospires, and transmission to humans can occur by direct or indirect contact with urine excretions of animal vectors causing leptospirosis [1, 4]. Although leptospirosis is endemic in tropical and subtropical regions, recent incidence of the disease has been reported worldwide [5, 6]. The intensity of disease transmission is dictated by hot and humid conditions, density of the leptospiral population, and the level of contact with host [4]. To date, 65 species, comprising both pathogenic and non-pathogenic leptospires are serologically defined and catalogued in repositories around the world [7]. While infections due to certain serovars are often found within specific geographical areas, this is by no means completely limiting as certain serovars are extraordinarily widespread. As antigenically distinct strains evolve, *Leptospira* might acquire a broader host range causing more complicated and confusing epidemiologic analysis.

Serovar-specific approaches are routinely followed for understanding the epidemiological behavior of *Leptospira* sp. Antibodies are used for this purpose in reference laboratories for genus-specific and/or serogroup-specific detection of *Leptospira.* But in many instances, serology failed to resolve taxonomic conflicts as individual serogroups happen to encompass strains of different species resulting in poor correlation between serology and genotypic classification [8]. The enhanced efforts at culture and isolation from additional hosts in diverse environments and new geographic areas as well as the increased use and availability of molecular methods including whole genome sequencing from isolated strains has expanded our understanding and concept of leptospiral genetic relatedness, as opposed to genuinely "recent emergence" of new species or strains.

RNase P, an ancient metabolic enzyme, could be used as an elegant tool to study phylogenetic distinctions that exist amongst organisms, including *Leptospira* [9]. RNase P is a ubiquitous enzyme essential for tRNA maturation in all organisms. In bacteria, RNase P holoenzyme comprises a C5 protein and a RNA subunit, and these subunits are coded by *rnpA* and *rnpB* genes, respectively [10, 11]. Conserved patches interspersed with variable sequences present in the RNase P RNA (*RPR*) gene across different species could be exploited as signature moieties to delineate the phylogenetic relationship existing between strains of any bacterial genus. In the past two decades, rDNAs [12-15] and several housekeeping genes [16-19] were widely employed as a genotyping tool for classifying the members of eubacteria. As the ribosomal and other housekeeping genes are highly conserved and present in multiple copies with their homologous pseudogenes in the genomes, genotyping based on these genes is often skeptical and apparently failed to discriminate pathogenic from non-pathogenic leptospires [20-25]. *RPR* gene is known to be an essential single-copy gene present in the genomes of all living organisms. Being a single copy locus, *RPR* gene is recalcitrant for lateral gene transfer amongst bacterial population, thus qualifies to be a reliable gene marker for strain identification [26]. In this study, we used *RPR* gene as a potential gene marker and described strategies on how *RPR* gene could be employed to detect and differentiate leptospires in our laboratory.

2. Materials and Methods

2.1 PCR amplification of *RPR* and *secY* genes.

The genomic DNAs of taxonomically uncertain 11 reference strains of *Leptospira* sp. were obtained from Dr. Rudy Hartskeerl, KIT The Netherlands. The amplification of *RPR* gene sequence of leptospiral strains was carried out using appropriate genomic DNA and gene-specific primers designed based on the conserved sequences present in the P4 domain of RPR. The PCR reaction

mixture of 20 µL volume contained 10 ng of leptospiral genomic DNA, 250 µM of dNTPs, 5 U of Taq polymerase (NEB, Ipswich, MA, USA), 2 µL of 10X Taq buffer and 10 picomoles each of RPR gene-specific sense (5'GAGGAAAGTCCGGGC3') and antisense (5'TAAGCCRKRTTC TGTC3') primers. To amplify the *RPR* gene, the PCR reaction mixture was subjected to initial denaturation at 95ºC for 5 min followed by 1 min denaturation at 95ºC, primer annealing at 48ºC for 1 min and subsequent extension at 72ºC for 1 min performed for 35 cycles on a PCR machine (Bio-Rad, Hercules, CA, USA). To avoid staggered ends, a final extension was allowed at 72ºC for 30 min, after which the reaction was terminated at 4ºC. To corroborate *RPR* based phylogeny with another gene marker, amplification of *secY* gene for the strains of *Leptospira* was carried out using genomic DNA, *secY* gene-specific sense (5'GCATGCCTGTTGTTYCGYATGGG3') and antisense (5'GAAAGCTTTTAGAYTTYTTCATRAAGCC3') primers along with other PCR components as described earlier. The reaction mixture was subjected to initial denaturation at 95ºC for 5 min followed by denaturation at 95ºC for 1.5 min, annealing at 50ºC for 1 min, and extension at 72ºC for 3 min was performed for 35 cycles. A final extension was allowed at 72ºC for 30 min and subsequent termination of reaction at 4ºC. Finally, aliquots of amplified PCR products of *RPR* and *secY* genes were electrophoresed on 1.2% agarose-EtBr gel.

2.2 Cloning of *RPR* and *secY* genes

Fresh PCR products of *RPR* and *secY* genes were used for ligating into pCR™ 4-TOPO® vector and transformed into one shot Top 10 *E. coli* cells (Invitrogen, CA, USA) according to conditions prescribed by the manufacturer. Recombinant plasmids were extracted from the transformants using QIAGEN mini kit (QIAGEN GmbH, Germany) and subjected to restriction with *Eco*R I enzyme (NEB, Ipswich, MA, USA), the RPR gene and *secY* gene release from the vector backbone was confirmed by loading DNA markers in 1.2% agarose-EtBr gel. The recombinant clones were sequenced using M13 reverse (AgriGenom, Cochin) and forward primers, and 100% identity was confirmed for three clones for each serovar.

2.3 RNA structure prediction and phylogenetic relationship among *Leptospira* sp.

The partial *RPR* gene sequences of 11 leptospiral reference strains with uncertain taxonomic positions were determined in this study. To achieve better resolution of phylogram, *RPR* gene sequences of 11 leptospiral strains along with 298 sequences retrieved from NCBI database were included and aligned using CLC Sequence Viewer 8.0. The aligned sequences were subjected to phylogenetic analysis. HKY85+G substitution matrix was used for the analysis; evolutionary distances were estimated using Maximum Composite Likelihood method and clade support was validated with MrBayes algorithm. RPR secondary structure prediction and phylogenetic analyses were performed as described previously [9]. The phylogenetic analysis utilized the Neighbor-Joining algorithm on the MEGA 11 server and the resulting tree underwent validation through the generation of 1000 bootstrap replicates. The deduced DNA sequences for which the taxonomic positions of leptospiral strains determined in this study were deposited in GenBank, and the accession numbers are listed in Table 1. Information pertaining to each strain is available in GenBank with reference to their respective accession numbers.

2.4 Restriction digestion-based diagnosis of *Leptospira* sp.

The *RPR* sequences of various *Leptospira* sp. were fed into NEB cutter 2.0 software and the *in silico* restriction profile of *RPR* genes was obtained. Unique *Eco*R I and *Sma* I sites were chosen for further

Table 1. List of leptospiral strains used for deducing *RPR* and *secY* genes in the present study

Species	Serovar	Strain	Accession Numbers	
			RPR	*secY*
L. alexanderi	Banna	A31	MT233018	MT233030
	Manzhuang	A23	MT233019	MT233031
	Mengla	A85	MT233020	MT233032
L. borgpetersenii	Arborea	Arborea	MT233021	MT233033
	Guangdong	1853	MT233022	MT233034
	Hamptoni	Hampton	MT233023	MT233035
	Kwale	Julu	MT233024	MT233036
	Mini	Sari	MT233025	MT233037
	Nigeria	Vom	MT233026	MT233038
Leptospira sp.	Huanuco	M4	MT233028	MT233040
	Hongchong	18R	MT233029	MT233041

analysis. For wet-lab experiments, the amplified *RPR* gene products of various strains of *Leptospira* sp. were used as templates for performing restriction analysis. Ten microlitre reaction mixture comprising appropriate restriction digestion buffers, 1 µg of the amplified PCR product, and 5 U of each of restriction enzymes *Eco*R I and *Sma* I were added into the reaction mixture and incubated at 37ºC in a water bath for 1 h. The restricted products were electrophoresed using 1.6% agarose-EtBr gel and documented.

3. Results and Discussion

3.1 RPR structural differences among leptospiral strains

The leptospiral RPR secondary structures were predicted based on the eubacterial consensus sequences present in the RPRs, and the loops and bulges were manually constructed using the predicted structures available in the RNase P database. The sequence and structural differences analysed in the RPR configuration of strains of *L. borgpetersenii* brought forth two significant observations: (i) Presence of P18 and P19 domains in the RPRs of strains 20091122 and 20091116 was similar to the RPRs of other pathogenic leptospires; (ii) The SNPs present in P1, P3, P8, P9, P12, P13 and P17 domains of *L. borgpetersenii* RPRs bifurcated them into two distinct subgroups viz. A and B (Figure 1). RPRs of *L. stimsonii*, *L. ainlahdjerensis*, *L. tipperaryensis*, *L. ainazelensis*, *L. adleri* and *L. gomenensis* contained GAAA tetraloop in L12 and SNPs present in P1, P8, P9, and P12 brought about a separate clade named pathogenic group IV. All saprophytic leptospiral RPRs were devoid of both P18 and P19 domains. Interestingly, SNPs present in P1, P3, P8, P9, P12, P14 distinguished saprophytic strains and classified them into three sub clades namely saprophytic groups I, II and III.

In our previous study, we reported that RNase P, an ancient metabolic enzyme, could be used as an elegant tool to study phylogenetic distinctions that existed amongst leptospires. Sequence and structural investigations performed on RPRs from members of eubacteria revealed that maximal sequence conservation falls within the region that constitutes P4 helix [11, 27] (Figure 1). Primers

complementary to conserved sequences identified in the P4 helix of known *RPR* sequences of *Leptospira* sp. were used in the present study. Among the strains of *Leptospira* sp., the presence or absence of P18 and P19 domains are distinct features found in the RPRs to distinguish pathogenic from non-pathogenic strains. Sequence and structural diversity present in the leptospiral RPRs help delineating the genus *Leptospira* into pathogenic groups I, II, III and IV, intermediate groups I and II, and saprophytic groups I, II and III.

3.2 Phylogenetic analysis among *Leptospira* sp.

Phylogenetic analysis was performed with deduced RPR sequences of 11 leptospiral strains in addition to the 298 leptospiral sequences available in the public database (Figure 2). Sequences assembled with MEGA 11 software revealed that strains 20091122 and 20091116 were placed amongst pathogenic strains, and they emerged as a distinct clade contiguous to the strains of *L. borgpetersenii* subgroup A (Figure 2). Based on RPR gene-based classification, the strains of *L. alexanderi* emerged as a separate clade and positioned between the strains of *L. santarosai* and *L. borgpetersenii*. RPR gene analysis confirmed that *L. borgpetersenii* serovar Hamptoni strain Hampton was clustered along with serovars of *L. weilii*. Moreover, *secY* gene analysis also confirmed all of the above observations (data not shown). Similarly, *L. weilii* serovar Topaz strain LT 2116 and *L. weilli* serovar Ranarum strain ICFT were also abnormally clustered among *L. alexanderi* and *L. alstonii* clade, respectively. RPR sequence and structural similarities indicate that the strains M4 and ZV016 were most likely to be *L. santarosai* and *L. kirschneri*, respectively (Table 2). Similarly, RPR analysis confirmed the genomospecies of strains 18R and Pond 2020 as most likely to be *L. interrogans*.

The topology of phylogenetic trees showed the species of *Leptospira* segregated into separate clades according to their presumed pathogenic, saprophytic, and intermediate status (Figure 2). RPR based phylogenetic analysis revealed that the strains of *L. borgpetersenii* segregated into two distinct subgroups, and this was in agreement with earlier report [28]. Serovar Balcanica strain 1627 Burgas occupied a separate branch above that of serovar Hardjo-bovis strain L550 and serovar Hardjo-bovis strain Sponselee CDC. The delineation of the two proposed subgroups A and B at the Burgas branch, placed serovar Tarassovi strain Perepelitsin, serovar Pomona strain 200901968 and serovar Hardjo bovis strain JB 197 all on a separate branch from the two other Hardjo bovis strains present (L550 and Sponselee CDC). This analysis suggests the need for further evaluation to ensure the current presentation accurately captures the relationship of these two closely related strains [29].

As a result of RPR sequence homology, *L. borgpetersenii* serovar Hamptoni strain Hampton was grouped under the *L. weilii* cluster in both RPR and *secY* trees which confirmed that strain Hampton belonged to *L. weilii*. Furthermore, *L. weilii* strain LT 2116 and strain ICFT were grouped within *L. alexanderi* clade and *L. alstonii*, respectively. The strains of *L. alexanderi* branch formed a separate clade and were placed between the strains of *L. santarosai* and *L. borgpetersenii*. The conclusions derived from RPR and *secY* based phylogeny indicated that strain M4 could be *L. santarosai* and strain ZV016 could be *L. kirschneri* whereas strains 18R and Pond 2020 could be *L. interrogans*.

RPR and *secY* gene phylogeny depicted that the strains of *L. alexanderi* emerged as a separate clade, which was positioned between strains of *L. santarosai* and *L. borgpetersenii*, and thus became an integral part of pathogenic group III. *Leptospira borgpetersenii* serovar Hamptoni strain Hampton was clustered along with the strains of *L. weilii*. This strain was originally classified as *L. borgpetersenii* in a previous study [30] using DNA hybridization. But RPR sequence and structural differences clearly showed that the strain Hampton belongs to *L. weilii* rather than *L. borgpetersenii*. Furthermore, speciation of the strains 18R, Pond 2020, ZV016 and M4 are resolved

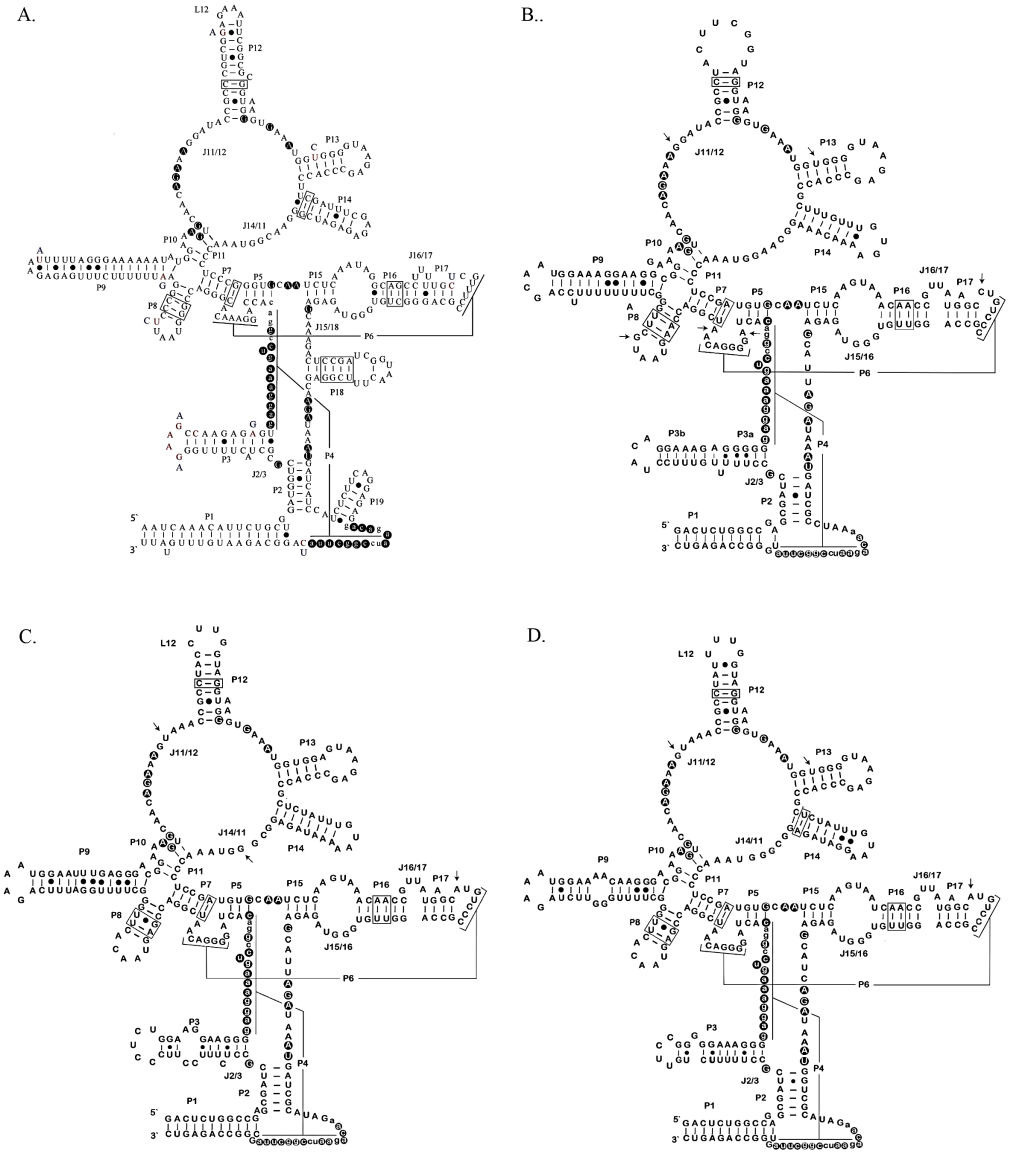

Figure 1. RPR secondary structures of *L. borgpetersenii* serovar Undetermined strain 20091122 (A), *L. bouyouniensis* serovar Undetermined strain 201800295 (B), *L. kanakyensis* serovar Undertermined strain 201800293 (C) and *L. bourretii* serovar Undetermined strain 201800281(D), showing paired and unpaired helices. Non-canonical base pairings (G•U and C•U) are denoted as a dot (•). Nucleotides conserved across all bacterial RPRs are embossed within dark circles. Nucleotides shown in blue indicate conserved positions for *L. borgpetersenii* subgroup A strains, whereas nucleotides in red are conserved for *L. borgpetersenii* subgroup B strains. The RPR structures are constructed using RNAfold server (http://rna.tbi.univie.ac.at/).

Figure 2. Phylogenetic tree was constructed with partial RPR gene sequences of 309 leptospiral strains (65 species) using Neighbor-Joining algorithm and validated using 1000 bootstrap replicates. The analysis was performed in MEGA 11. The saprophytic leptospires were classified into three groups namely saprophytic group I, saprophytic group II and saprophytic group III. Discrepant strains (RPR sequences derived from the present study) with debatable taxonomic positions are marked in black. Strains of *L. alexanderi* are positioned between *L. santarosai* and *L. borgpetersenii*.

in the present study using RPR and *secY* gene-based phylogeny. The RPRs of strains 18 R and Pond 2020 comprised the signature P18 and P19 domains and were devoid of GAAA tetraloop (L12) which showed that the strain 18R and Pond 2020 belonged to *L. interrogans*. The RPR of strain M4 contained P18, P19 domains and GAAA tetraloop in L12 demonstrateing that strain M4 belonged to *L. santarosai*. RPR sequence and structural similarities confirmed that strain ZV016 belonged to *L. kirschneri*. Although *secY* and *RPR* gene-based phylogeny confirmed the taxonomic positions of

Table 2. Typing of *Leptospira* spp. based on the specific structural features of RPRs

Species	Serovar	Strain	RPR Configuration		
			P18	P19	GAAA in L12
L. alexanderi	Banna	A31	+	+	+
	Manzhuang	A23			
	Mengla	A85			
L. borgpetersenii	Arborea	Arborea	+	+	+
	Guangdong	1853			
	Hamptoni	Hampton			
	Kwale	Julu			
	Mini	Sari			
	Nigeria	Vom			
Leptospira sp.	Huanuco	M4	+	+	+
Leptospira sp.	Hongchong	18R	+	+	-

+/- denotes the presence or absence of specific component.

most of the leptospiral strains investigated, it failed to corroborate with each other while classifying *L. weilii* serovar Topaz strain LT 2116. RPR sequence similarity (>98%) with the strains of *L. alexanderi* and the presence of SNPs in P8, P9, P12, P13 and P17 domains indicated that *L. weilii* serovar Topaz strain LT 2116 RPR belonged to *L. alexanderi*. However, this observation requires further investigation as *secY* gene-based phylogeny retained *L. weilii* serovar Topaz strain LT 2116 within *L. weilii* cluster. Similarly, our studies on *LipL21*, *fliD* and *dnaK* gene marker-based phylogeny failed to corroborate with *RPR* gene. We presume that the resolution of phylogeny appears to be superior in the case of single copy *RPR* gene. In the case of other gene markers, inconsistent phylogenetic maps were observed due to presence of multiple copies of those genes in the genomes of *Leptospira* sp. (data not shown).

3.3 Restriction based typing of *Leptospira* sp.

In silico analysis of leptospiral *RPR* gene sequences revealed the presence of unique *Eco*R I and *Sma* I restriction sites. The *Eco*R I and *Sma* I cleavage products of *RPR* gene amplicons can help distinguish pathogenic, intermediate and saprophytic strain (Figure 3). Restriction digestion of leptospiral *RPR* gene with *Eco*R I and *Sma* I enzymes produced three digestion products with molecular masses of 145 bp, 134 bp and 47 bp for the strains of pathogenic group I (Figure 3, Lane 2-4) and two restriction cleavage products of molecular masses of 201 bp and 135 bp for the strains of pathogenic group III (Figure 3, Lane 6-9). Similarly, restriction digestion on RPR gene of the pathogenic group II leptospiral strain produced two digests of sizes 153 bp and 136 bp (Figure 3, Lane 5) and among the intermediate leptospires, strains of subgroup I (Figure 3, Lane 10, 11) produced two distinct products of molecular sizes 181 bp and 127 bp while strains of subgroup II (Figure 3, Lane 12, 13) produced two cleavage products of sizes 232 bp and 62 bp. Saprophytic leptospires produced an intact 285 bp fragment (Figure 3, Lane 14) even after performing *Eco*R I and *Sma* I digestion as they were devoid of the above restriction sites in the sequences of *RPR* gene amplicons.

These observations are in agreement with the *in silico Eco*R I and *Sma* I restricted profiles of *RPR* amplicon.

In order to evolve *RPR* based strain identification into a routine diagnostic procedure, a simple two-step PCR and subsequent restriction analysis of amplicons were demonstrated for the possible diagnosis of leptospires present in the clinical and environmental samples. For restriction analysis, amplicons of RPR genes from representative leptospires were digested with *EcoR* I and *Sma* I enzymes, and the restricted products were resolved on agarose gel. The nucleotide sequences of the RPR gene were analysed for the presence of specific restriction sites that could produce restriction digests thus revealing banding patterns unique to pathogenic, intermediate, and saprophytic groups of *Leptospira* sp. The double digestion of *RPR* gene by *Eco*R I and *Sma* I enzymes produced restriction profiles that were unique to different *Leptospira* sp. (Figure 3). This analysis can help distinguishing pathogenic from non-pathogenic leptospires quickly without resorting to cumbersome DNA sequencing procedures. By using the above technique, clinicians can diagnose the pathogenic infection early during outbreaks and can eliminating potential false positives.

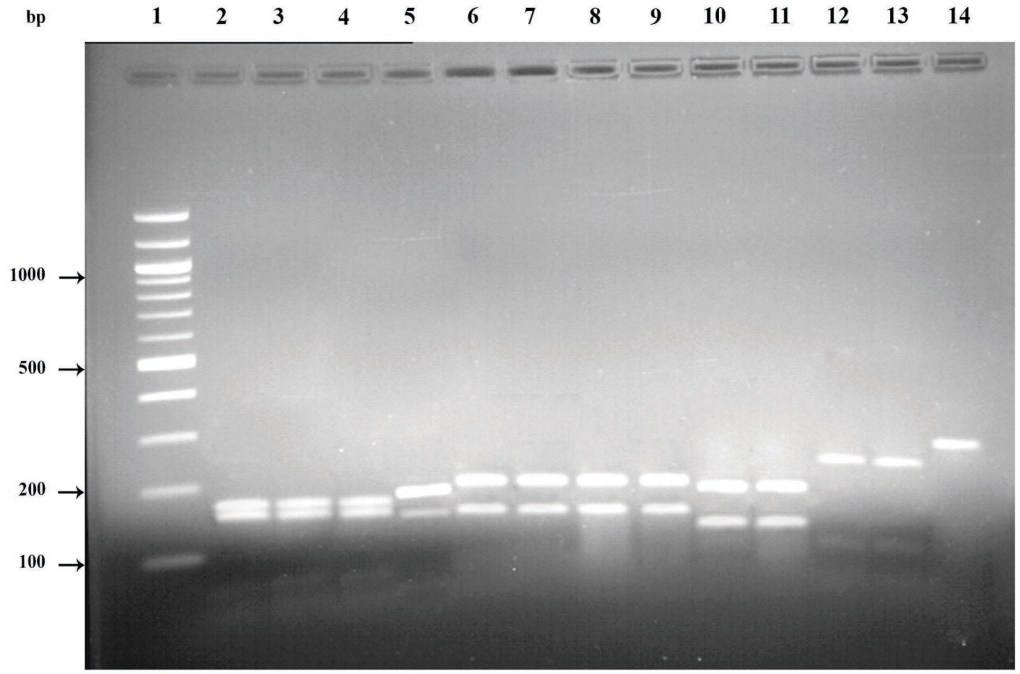

Figure 3. RPR gene amplicon restricted with *Eco*R I and *Sma* I and resolved in 1.6% agarose-EtBr gel. Lane 1 corresponds to 100 bp DNA marker, Lanes 2–14 correspond to RPR gene products of strains *L. interrogans* strain Fiocruz L (1-130), *L. kirschneri* strain 1051, *L. noguchii* strain CZ 214, *L. kmetyi* strain Bejo-Iso 9, *L. borgpetersenii* strain Mus 127, *L. alexanderi* strain A 31, *L. weilii* strain Ecochallenge, *L. santarosai* strain 1342 KT, *L. wolffii* strain Khorat-H2, *L. licerasiae* strain VAR 010, *L. fainei* strain BUT 6, *L. inadai* strain 10, *L. biflexa* strain Patoc1 (Ames), respectively.

4. Conclusions

Among the taxonomically uncertain 11 reference strains included in this study, the phylogenetic positions of at least seven strains using *RPR* based phylogeny suggested that these strains were erroneously classified previously as a result of either sample swapping or serological misrepresentation in the repository. The sequence and structure-based differences observed between RPRs of different leptospires indicated that analysis using *RPR* gene was more discriminatory than analysis with many other gene markers already described in the literature. The presence or absence of P18 and/or P19 in the secondary structure of RPR clearly distinguishes pathogenic, saprophytic, and intermediate leptospiral strains. If RPR based leptospiral typing were to be extended for diagnostic application, the simplicity of the technique would allow a large number of clinical and environmental samples to be screened within a short span of time in the event of an epidemic.

5. Acknowledgements

The authors sincerely thank Dr. Rudy A. Hartskeerl and Dr. Ahmed Ahmed for providing leptospiral genomic DNAs. Dr. Eugene Raj, UCL, London, UK is gratefully acknowledged for his critical comments.

References

[1] Hartskeerl, R.A., Collares-Pereira, M. and Ellis, W.A., 2011. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology and Infectious Diseases*, 17(4), 494-501, https://doi.org/10.1111/j.1469-0691.2011.03474.x.

[2] Andre-Fontaine, G., Aviat, F. and Thorin, C., 2015. Waterborne Leptospirosis: Survival and preservation of the virulence of pathogenic *Leptospira* spp. in fresh water. *Current Microbiology*, 71(1), 136-142, https://doi.org/10.1007/s00284-015-0836-4.

[3] Casanovas-Massana, A., Pedra, G.G., Wunder Jr., E.A., Diggle, P.J., Begon, M. and Ko, A.I., 2018. Quantification of *Leptospira interrogans* survival in soil and water microcosms. *Applied and Environmental Microbiology*, 84(13), https://doi.org/10.1128/AEM.00507-18.

[4] Levett, P.N., 2001. Leptospirosis. *Clinical Microbiology Reviews*, 14(2), 296-326, https://doi.org/10.1128/CMR.14.2.296-326.2001.

[5] Jiménez, J.I.S., Marroquin, J.L.H., Richards, G.A. and Amin, P., 2018. Leptospirosis: Report from the task force on tropical diseases by the World Federation of Societies of Intensive and Critical Care Medicine. *Journal of Critical Care*, 43 361-365, https://doi.org/10.1016/j.jcrc.2017.11.005.

[6] Mendoza, M.V. and Rivera, W.L., 2019. Identification of *Leptospira* spp. from environmental sources in areas with high human leptospirosis incidence in the Philippines. *Pathogens and Global Health*, 113(3), 109-116, https://doi.org/10.1080/20477724.2019.1607460.

[7] Vincent, A.T., Schiettekatte, O., Goarant, C., Neela, V.K., Bernet, E., Thibeaux, R., Ismail, N., Khalid, M.K.N.M., Amran, F., Masuzawa, T., Nakao, R., Korba, A.A., Bourhy, P., Veyrier, F.J. and Picardeau, M., 2019. Revisiting the taxonomy and evolution of pathogenicity of the genus *Leptospira* through the prism of genomics. *PLoS Neglected Tropical Diseases*, 13(5), https://doi.org/10.1371/journal.pntd.0007270.

[8] Bourhy, P., Storck, C.H., Theodose, R., Olive, C., Nicolas, M., Hochedez, P., Lamaury, I., Zinini, F., Bremont, S., Landier, A., Cassadou, S., Rosine, J. and Picardeau, M., 2013. Serovar diversity of pathogenic *Leptospira* circulating in the French West Indies. *PLoS Neglected Tropical Diseases*, 7(3), https://doi.org/10.1371/journal.pntd.0002114.

[9] Ravishankar, V., Ahmed, A., Sivagnanam, U., Muthuraman, K., Karthikaichamy, A., Wilson, H.A., Devendran, A., Hartskeerl, R.A. and Raj, S.M.L., 2014. Evolution of the RNase P RNA structural domain in *Leptospira* spp. *Research in Microbiology*, 165(10), 813-825, https://doi.org/10.1016/j.resmic.2014.10.007.

[10] Guerrier-Takada, C. and Altman, S., 1993. A physical assay for and kinetic analysis of the interactions between M1 RNA and tRNA precursor substrates. *Biochemistry*, 32(28), 7152-7161.

[11] Frank, D.N. and Pace, N.R., 1998. Ribonuclease P: unity and diversity in a tRNA processing ribozyme. *Annual Review of Biochemistry*, 67, 153-180, https://doi.org/10.1146/annurev.biochem.67.1.153.

[12] Innings, A., Krabbe, M., Ullberg, M. and Herrmann, B., 2005. Identification of 43 *Streptococcus* species by pyrosequencing analysis of the *rnpB* gene. *Journal of Clinical Microbiology*, 43(12), 5983-5991, https://doi.org/10.1128/JCM.43.12.5983-5991.2005.

[13] Täpp, J., Thollesson, M. and Herrmann, B., 2003. Phylogenetic relationships and genotyping of the genus *Streptococcus* by sequence determination of the RNase P RNA gene, *rnpB*. *International Journal of Systematic and Evolutionary Microbiology*, 53(6), 1861-1871, https://doi.org/10.1099/ijs.0.02639-0.

[14] Peters, I.R., Helps, C.R., McAuliffe, L., Neimark, H., Lappin, M.R., Gruffydd-Jones, T.J., Day, M.J., Hoelzle, L.E., Willi, B., Meli, M., Hofmann-Lehmann, R. and Tasker, S., 2008. RNase P RNA gene (*rnpB*) phylogeny of *Hemoplasmas* and other *Mycoplasma* species. *Journal of Clinical Microbiology*, 46(5), 1873-1877, https://doi.org/10.1128/JCM.01859-07.

[15] Schön, A., Fingerhut, C. and Hess, W.R., 2002. Conserved and variable domains within divergent RNase P RNA gene sequences of *Prochlorococcus* strains. *International Journal of Systematic and Evolutionary Microbiology*, 52(4), 1383-1389, https://doi.org/10.1099/00207713-52-4-1383.

[16] Guglielmini, J., Bourhy, P., Schiettekatte, O., Zinini, F., Brisse, S. and Picardeau, M., 2019. Genus-wide *Leptospira* core genome multilocus sequence typing for strain taxonomy and global surveillance. *PLoS Neglected Tropical Diseases*, 13(4), https://doi.org/10.1371/journal.pntd.0007374.

[17] Cosate, M.R.V., Sakamoto, T., Mendes, T.A.D.O., Moreira, É.C., da Silva, C.G.R., Brasil, B.S.A.F., Oliveira, C.S.F., de Azevedo, V.A., Ortega, J.M., Leite, R.C. and Haddad, J.P., 2017. Molecular typing of *Leptospira interrogans* serovar Hardjo isolates from leptospirosis outbreaks in Brazilian livestock. *BMC Veterinary Research*, 13(1), https://doi.org/10.1186/s12917-017-1081-9.

[18] Leon, A., Pronost, S., Fortier, G., Andre-Fontaine, G. and Leclercq, R., 2010. Multilocus sequence analysis for typing *Leptospira interrogans* and *Leptospira kirschneri*. *Journal of Clinical Microbiology*, 48(2), 581-585, https://doi.org/10.1128/JCM.00543-09.

[19] Ahmed, N., Devi, S.M., Valverde, M.D.L., Vijayachari, P., Machang'u, R.S., Ellis, W.A. and Hartskeerl, R.A., 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Annals of Clinical Microbiology and Antimicrobials*, 5, https://doi.org/10.1186/1476-0711-5-28.

[20] Koizumi, N., Muto, M.M., Akachi, S., Okano, S., Yamamoto, S., Horikawa, K., Harada, S., Funatsumaru, S. and Ohnishi, M., 2013. Molecular and serological investigation of *Leptospira* and leptospirosis in dogs in Japan. *Journal of Medical Microbiology*, 62(4), 630-636, https://doi.org/10.1099/jmm.0.050039-0.

[21] Goarant, C., Colot, J., Faelchlin, E., Ponchet, M., Soupé-Gilbert, M.-E., Descloux, E. and Gourinat, A.-C., 2014. An exotic case of leptospirosis imported into an endemic area. *Travel Medicine and Infectious Disease*, 12(2), 198-200, https://doi.org/10.1016/j.tmaid.2013.12.005.

[22] Pagès, F., Kuli, B., Moiton, M.P., Goarant, C. and Jaffar-Bandjee, M.C., 2015. Leptospirosis after a stay in Madagascar. *Journal of Travel Medicine*, 22(2), 136-139, https://doi.org/10.1111/jtm.12163.

[23] Thibeaux, R., Geroult, S., Benezech, C., Chabaud, S., Soupé-Gilbert, M.-E., Girault, D., Bierque, E. and Goarant, C., 2017. Seeking the environmental source of Leptospirosis reveals durable bacterial viability in river soils. *PLoS Neglected Tropical Diseases*, 11(2), https://doi.org/10.1371/journal.pntd.0005414.

[24] Han, H.-J., Wen, H.-L., Liu, J.-W., Qin, X.-R., Zhao, M., Wang, L.-J., Luo, L.-M., Zhou, C.-M., Zhu, Y.-L., Qi, R., Li, W.-Q., Yu, H. and Yu, X.-J., 2018. Pathogenic *Leptospira* species in insectivorous bats, China, 2015. *Emerging Infectious Diseases*, 24(6), 1123-1126, https://doi.org/10.3201/eid2406.171585.

[25] Mason, M.R., Encina, C., Sreevatsan, S. and Muñoz-Zanzi, C., 2016. Distribution and diversity of pathogenic *Leptospira* species in peri-domestic surface waters from South Central Chile. *PLoS Neglected Tropical Diseases*, 10(8), https://doi.org/10.1371/journal.pntd.0004895.

[26] Li, Y. and Altman, S., 2004. In search of RNase P RNA from microbial genomes. *RNA*, 10(10), 1533-1540, https://doi.org/10.1261/rna.7970404.

[27] Torres-Larios, A., Swinger, K.K., Krasilnikov, A.S., Pan, T. and Mondragón, A., 2005. Crystal structure of the RNA component of bacterial ribonuclease P. *Nature*, 437, 584-587, https://doi.org/10.1038/nature04074.

[28] Bourhy, P., Collet, L., Lernout, T., Zinini, F., Hartskeerl, R.A., van der Linden, H., Thiberge, J.M., Diancourt, L., Brisse, S., Giry, C., Pettinelli, F. and Picardeau, M., 2012. Human *leptospira* isolates circulating in Mayotte (Indian Ocean) have unique serological and molecular features. *Journal of Clinical Microbiology*, 50(2), 307-311, https://doi.org/10.1128/JCM.05931-11.

[29] Bulach, D.M., Zuerner, R.L., Wilson, P., Seemann, T., McGrath, A., Cullen, P.A., Davis, J., Johnson, M., Kuczek, E., Alt, D.P., Peterson-Burch, B., Coppel, R.L., Rood, J.I., Davies, J.K. and Adler, B., 2006. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proceedings of the National Academy of Sciences of the United States of America*, 103(39), 14560-14565, https://doi.org/10.1073/pnas.0603979103.

[30] Brenner, D.J., Kaufmann, A.F., Sulzer, K.R., Steigerwalt, A.G., Rogers, F.C. and Weyant, R.S., 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. *International Journal of Systematic Bacteriology*, 49(2), 839-858, https://doi.org/10.1099/00207713-49-2-839.