Research article

Evaluation of the genetic stability of *Leptospira* reference strains maintained under two conservation methods

Evaluación de la estabilidad genética de las cepas de referencia de *Leptospira* mantenidas bajo dos métodos de conservación

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Abstract

Objective. The genetic stability of Strains of *Leptospira* spp., maintained under two conservation systems, was evaluated. **Methodology.** The degree of conservation of the *16S rRNA* and *ompL1* genes of 10 reference serovars from the Leptospira spp. collection, belonging to the Sistema de Bancos de Germoplasma de la Nación para la Alimentación y la Agricultura (SBGNAA), was determined. **Results.** It was corroborated that the genes evaluated these have not undergone considerable changes, since similarities greater than 99.69 % were evidenced for *16S rRNA* and 99.02% for *ompL1*, in the paired alignments. **Conclusion.** The genetic stability and purity of the reference strains of *Leptospira* spp. were verified. spp., kept in cryopreservation in liquid nitrogen at -196 °C and at room temperature for approximately eight years.

Keywords: sequence analysis, DNA; *ompL1* protein, *Leptospira*, RNA, Ribosomal, 16S.

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Resumen

Objetivo. Se evaluó la estabilidad genética de cepas de *Leptospira* spp., mantenidas bajo dos sistemas de conservación. **Metodología.** Se determinó el grado de conservación de los genes *16S rRNA* y *ompL1* de 10 serovares de referencia de la colección de *Leptospira* spp., pertenecientes al Sistema de Bancos de Germoplasma de la nación para la Alimentación y la Agricultura (SBGNAA). **Resultados.** Se corroboró que los genes evaluados estos no han sufrido cambios considerables, puesto que se evidenciaron similitudes superiores al 99,69% para *16S rRNA* y de 99,02 % para *ompL1*, en los alineamientos pareados. **Conclusión.** Se comprobó la estabilidad genética y la pureza de las cepas de referencia de *Leptospira* spp., mantenidas en criopreservación en nitrógeno líquido a -196 °C y a temperatura ambiente durante ocho años aproximadamente.

Palabras clave: secuenciación de ADN; proteína *ompL1*, *Leptospira*, ARN ribosómico, 16S.

Introduction

The *Leptospira* genus belongs to the family *Leptospiraceae* that includes Gram-negative oxidase-producing spirochaetes (1–3), which are divided into three clades: pathogenic, intermediate and saprophytes, based in 16S rRNA sequence and their pathogenicity (4–9).

The advancement of molecular biology has made it possible to reveal the enormous genetic diversity in the genus *Leptospira*, which in recent years has radically modified its taxonomy from 35 to 64 species recognized so far and from three pathogenicity groups to two large groups (pathogen-P and saprophyte S) (10, 11). The group of pathogenic species was subdivided into P1 and P2 where P1 comprises the group of traditional pathogenic species and P2 comprises the group of species previously referred to as intermediates. The saprophytic group was subdivided into two subgroups: S1 that comprises the traditionally saprophytic species and S2, a new subgroup not yet fully described, composed of *L. aldonii* and four new species related phylogenetically with saprophytic species (10, 12–16).

The *Leptospira* genome is composed of approximately 4,768 coding sequences, which varies in each species distributed in two circular chromosomes. The large chromosome (C1) contains approximately 4.33 Megabases with most of the essential genes, the ribosomal RNA and the transfer RNA. The small chromosome (C2) con-

tains approximately 460 kilobases (17–20). However, the genome size varies according to the species and reorganization processes such as single nucleotide polymorphisms (SNP), insertion sequences (IS), inverted sequences, and horizontal gene transfer (HGT) between different *Leptospira* species, as an adaptation mechanism to the environment (17–20).

The maintenance of *Leptospira* strains, unlike other pathogens, is not such a simple matter. It implies the conservation of virulence and other important features. These issues are of vital importance since it is determinant fact in pathogenesis studies in the design of accurate diagnostic methodologies and the design of vaccines or immunogens. Importantly, in order to maintain the virulence of *Leptospira* strains, it is necessary to perform iterative passages in susceptible animals, since the loss of such virulence may be the consequence of successive passages in culture media for long periods of time (14). Keeping bacterial cultures may lead to the loss of virulence cryopreservation could be considered an optimal Conventional preservation techniques for *Leptospira* strains include successive passes either liquid or semi-solid culture media, using the Ellinghausen-McCullough-Johnson-Harris (EMJH) or the Fletcher media, and conserved at room temperature under aerobic conditions (21, 22). This methodology requires a routine follow-up using dark field microscopy to evaluate motility and morphology, in addition to culturing in enrichment media to assess the purity cultures (21, 22). Moreover, it demands time and increases the risk of cross-contamination among different serovars or with other environmental microorganisms, as well as the appearance of mutant strains and possible loss of virulence (22, 23). On the other hand, conservation at -70 °C or liquid nitrogen using suitable cryopreservants is effective for long-term conservation of *Leptospira* spp. (22, 24). These techniques have achieved successful viability of vaccine serovars preserved at -70 °C for at least seven months (25), meanwhile those preserved in liquid nitrogen have been preserved for at least 19 months (24). However, the viability depends on the age of the culture, the serovar and the initial bacterial concentration used (22, 24, 25). Nonetheless, due to the maintenance procedures that these techniques require, errors might occur handling this type of material (23), therefore, the quality of the collections must be evaluated periodically with DNA sequencing, as this is a viable option given the simplicity of the procedure, the availability of the reagents and the low cost (23).

The 16S rRNA subunit is essential in protein synthesis, for that reason it is a highly conserved nucleotide sequence. Further, its sequencing is standard for the study and identification of almost all prokaryotes, including complex identifiable bacteria

(26–28), and making it useful for diagnosis, contamination detection, verification of bacterial antigen sets, quality control and taxonomical purposes (23, 25–27). The *ompL1* gene (outer membrane protein) synthesizes a transmembrane porine of 320 amino acids unique in pathogenic species (21, 29–31), which shows throughout its sequence, a series of synonymous and non-synonymous variations. These variations are grouped into four regions that code the protein portions exposed on the membrane surface (29, 31, 32).

To improve the identification and characterization achieved by phenotypic methods, molecular tools have been developed to allow a more accurate approach in the differentiation of strains and isolates (27, 33). These apply to the conservation of reference collections since they must be stable and free from contamination. Also, it is necessary to perform periodic quality control to each strain to identify changes or loss of original genetic characteristics caused by the successive passes needed for its conservation (23). Accordingly, this study aimed at assessing the genetic stability of 10 *Leptospira* spp. reference serovars conserved under two conditions: room temperature and cryopreservation in liquid nitrogen, to demonstrate the influence of the conservation conditions on the genetic characteristics of the collection.

Materials and methods

Bacterial strains

Ten *Leptospira* spp. reference serovars belonging to the Sistema de Bancos de Germoplasma de la Nación para la Alimentación y la Agricultura (SBGNAA) [National System of Germplasm Banks for Food and Agriculture] were used in this study. This material is cryopreserved in liquid nitrogen using Dimethyl sulfoxide (DMSO) at 2 % as a cryoprotectant, and its corresponding replicas are preserved at room temperature through periodic subcultures in semi-solid EMJH medium. Around 13 culture passages have been carried out throughout eight years. The *Leptospira* spp. strains included in this work were: *L. interrogans* serovar Autumnalis str. Akiyani A, *L. interrogans* serovar Bataviae str. Van Tiene, *L. interrogans* serovar Australis str. Ballico, *L. santarosai* serovar Shermani str. 1342K, *L. interrogans* serovar Bratislava str. Jez Bratislava, *L. interrogans* serovar Balcanica, *L. interrogans* serovar Butembo str. Butembo, *L. interrogans* serovar Hebdomadis str. Hebdomadis, *L. borgpetersenii* serovar Tarassovi str. Peripecilin, and *L. biflexa* serovar Adamana str. JNS. Additionally, *Leptonema illini* str. 3055 was used as a negative control for the presence of the *ompL1* gene since this gene is absent in this saprophytic *Leptospira* species.

Microbiological cultures

The serovars were cultured in 4 mL of liquid EMJH-DIFCO® medium supplemented with EMJH-DIFCO® medium at 10% (v/v) and rabbit serum at 1 % (v/v) (6). The initial culture of the strain maintained at room temperature was carried out by transferring 1 mL of the culture maintained in semi-solid EMJH medium to 4 mL of liquid EMJH medium. The cryopreserved strains were reactivated by rapid defrosting in a serological bath for 5 min at 37 °C. Subsequently, 1 mL of each cryovial was transferred to 4 mL of liquid EMJH medium (24). These cultures were incubated at 30 °C under aerobic conditions, five weekly passages were performed during a five-week period (five passages in total). After each passage, viability was confirmed by dark field microscopy checking for motility and morphology features compatible with live bacteria, as well as by culturing these in brain heart infusion broth to ensure the absence of contamination. Bacterial concentration in each passage was not conducted since the main interest was to obtain live bacteria along the five passages (24, 33). The liquid media were centrifuged at 17000 gravities for 10 minutes at 4 °C to pellet the bacterial cells, and a final wash with PBS pH 7.2 was carried out. Finally, the bacterial pellets were stored at -20 °C until used (9).

PCR amplification

DNA extraction was performed using the phenol-chloroform-isoamyl alcohol method (34), the extraction products were quantified in the NanoDropTM equipment and adjusted to 85 ng/µL.

Amplification of 16S rRNA were performed in 50 µL at final concentrations of the 1X PCR reaction buffer, 1.5 mM of $MgCl_2$, 1.25 IU of Taq polymerase, 0.13 mM of each dNTP, 85 ng of DNA, 1.6 µM of each *EubA* and *EubB* primers (Table 1). The amplification conditions were as follows: initial denaturation at 96 °C for 3 minutes, followed by 35 cycles with denaturation at 95 °C for 90 seconds, annealing at 54 °C for 90 seconds, extension at 72 °C for 2 minutes, final extension at 72 °C for 15 minutes, and final storage at 4 °C.

Amplification of the *ompL1* gene was carried out in 50 µL at final concentrations of 1X PCR reaction buffer, 1.5 mM of $MgCl_2$, 1.25 IU of polymerase, 0.2 mM of dNTP each, 85 ng of DNA, 0.8 µM of *OmpL1F*, and 0.8. µM of *OmpL1R* (Table 1). The amplification conditions were as follows: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles with denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 2 minutes, final extension at 72 °C for 5 minutes, and final storage at 4 °C.

Table 1. Primers used to evaluate the genetic conservation of Leptospira strains.

Target	Primer	Sequence [5'-3']	Reference
16S rRNA	*EubB*	GAGTTTGATCMTGGCTCAG	(35)
	EubA	AAGGAGGTGATCCANCCRCA	
ompL1	*OmpL1F*	ATGATCCGTAACATAAGTAAGGCATTG	(29)
	OmpL1R	TTAGAGTTCGTGTTTATAACCGAATCT	

The PCR products were observed on a 1.5 % agarose gel [m/v] stained with Syber-Safe® at 1 % in an electric field of 90 V for 45 minutes. The approximate size for the 16S rRNA and *ompL1* genes amplicons were 1,500 pb (34) and 960 bp respectively (36). The sequencing of amplified products in both directions was performed using the Sanger method. The sequencing was conducted in an ABI PRISM® 3700 DNA Analyzer sequencer (Applied Biosystems, Foster City, CA, United States), in an external laboratory. These results were then analyzed with BLASTn (37) aligned with ClustalW (38), and the phylogenetic trees were constructed with the Neighbor-Joining method with a Bootstrap of 1,000 replicates using MEGA 7.0 (39).

Results

Cultures

The cultures showed adequate growth using microscopic observations of thin spiral structures typical of *Leptospira* spp., abundant cell mass and free of contamination (25, 33, 40).

PCR products

The PCR amplification products, both for 16S rRNA and for *ompL1*, showed the expected band sizes (1,500 and 960 pb, respectively) for the strains conserved under the two conditions. Either *Leptonema illini* or *L. biflexa* serovar Andamana did not amplify the *ompL1* gene because these are saprophytes and lack of it (29, 31, 41, 42) (Figures 1 and 2); these results are similar to the ones found by Rosario *et al.* (36) and Dezhbord *et al.* (42). However, *L. interrogans serovar* Tarassovi, which is pathogenic, did not show amplification for this gene; this can be attributed to the inability of the primers to amplify all strains as described by Reitstetter *et al.* 2006. This may require the design of another set of primers specifically for this serovar (43).

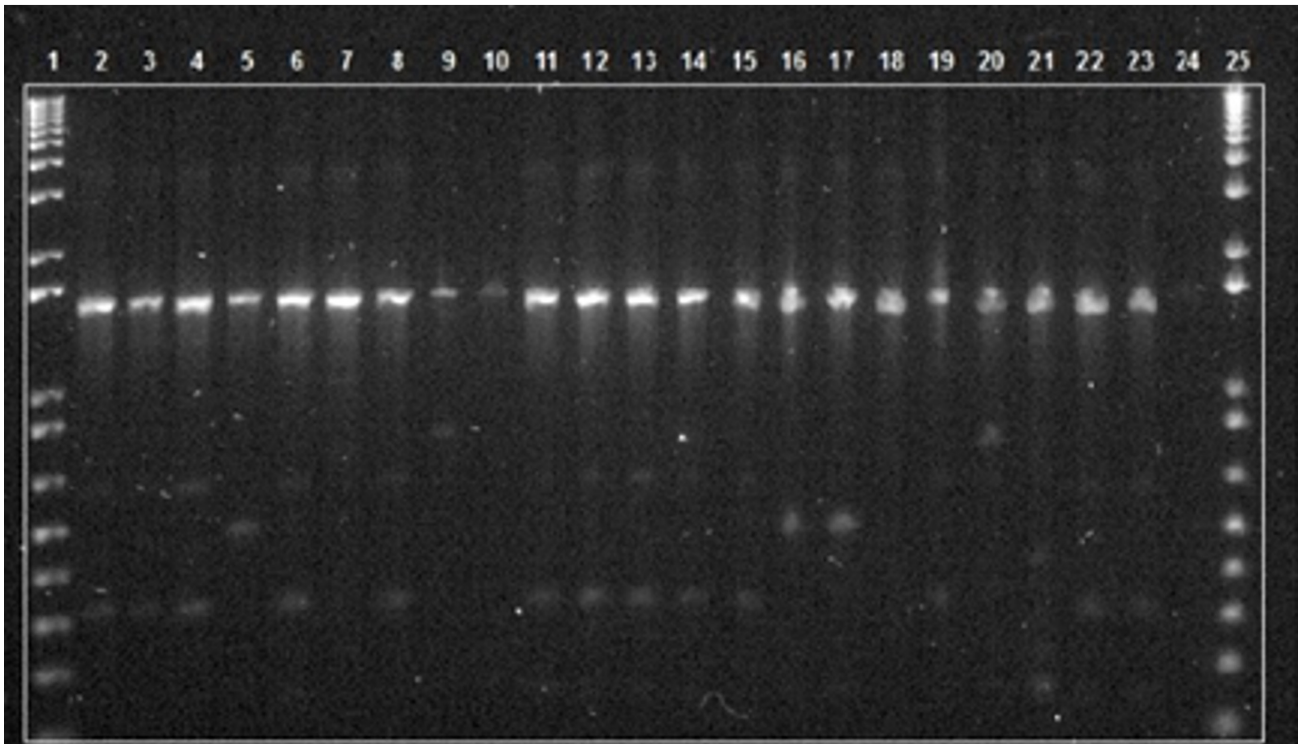

Figure 1. PCR amplification of the 16S *rRNA* gene of the *Leptospira* serovars strains. 1 and 25. Ladder 1Kb plus DNA Ladder-Invitrogen®, (2-12 room temperature), 2. *L. Autumnalis,* 3. *L. Bataviae,* 4. *L. Australis,* 5. *L. Shermani, 6.* L. *Bratislava,* 7. *L. Andamana,* 8. *L. Balcanica,* 9. *L. Tarassovi,* 10. *L. Illini,* 11. *L. Butembo,* 12. *L. Hebdomadis,* (13-23 criopreserved), 13. *L. Autumnalis,* 14. *L. Bataviae,* 15. *L. Australis,* 16. *L. Shermani,* 17. *L. Bratislava,* 18. *L.Andamana,* 19. *L. Balcanica,* 20. *L. Tarassovi,* 21. *L. Illini,* 22. *L. Butembo,* 23. *L. Hebdomadis,* 24. Negative control (Water).

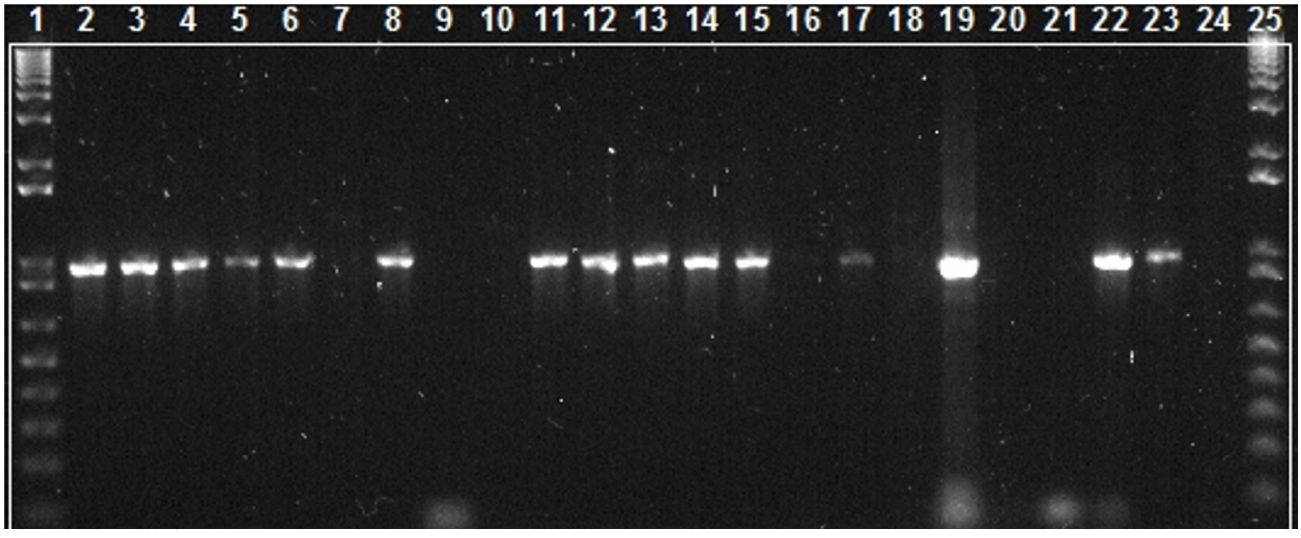

Figure 2. PCR amplification of the *ompL1* genes of the *Leptospira* serovars strains. 1. Ladder 1Kb plus DNA Ladder-Invitrogen®, (2-12 room temperature), 2. *L. Autumnalis,* 3. *L. Bataviae,* 4. *L. Australis,* 5. *L. Shermani, 6.* L. *Bratislava,* 7. *L. Andamana,* 8. *L. Balcanica,* 9. *L. Tarassovi,* 10. *L. Illini,* 11. *L. Butembo,* 12. *L. Hebdomadis,* (13-23 criopreserved), 13. *L. Autumnalis,* 14. *L. Bataviae,* 15. *L. Australis,* 16. *L. Shermani,* 17. *L. Bratislava,* 18. *L.Andamana,* 19. *L. Balcanica,* 20. *L. Tarassovi,* 21. *L. Illini,* 22. *L. Butembo,* 23. *L. Hebdomadis,* 24. Negative control (Water).

Analysis of sequences

The BLASTn results confirmed the identity of the sequences with those reported in GenBank for *Leptospira* spp., i.e., 98 % for 16S rRNA; these results are similar to those from Picardeau *et al*. (19), Fearnley *et al*. (34), and Boonsilp *et al*. (9). Furthermore, it also confirms the absence of culture contamination as described by Cerqueira *et al*. (23). Similarly, for *ompL1*, 96 % of the sequences were confirmed against pathogenic serovars, results that are similar to those published by Haake *et al*. (29), Chang *et al*. (7), Fernandes *et al*. (44), and Dezhbord *et al*. (42). Besides, these genetic distances were expected considering the variations shown by the gene (29, 45).

The paired alignments between the same serovar under two conditions using ClustalW for the two genes showed similarities higher than 99.69 % for 16S rRNA and 99.02 % for *ompL1*. On the other hand, the phylogenetic trees (Figure 3 and 4) showed a low divergence of the sequences due to the stability of the genes and the uniformity in the conservation conditions employed (8, 9, 20, 26).

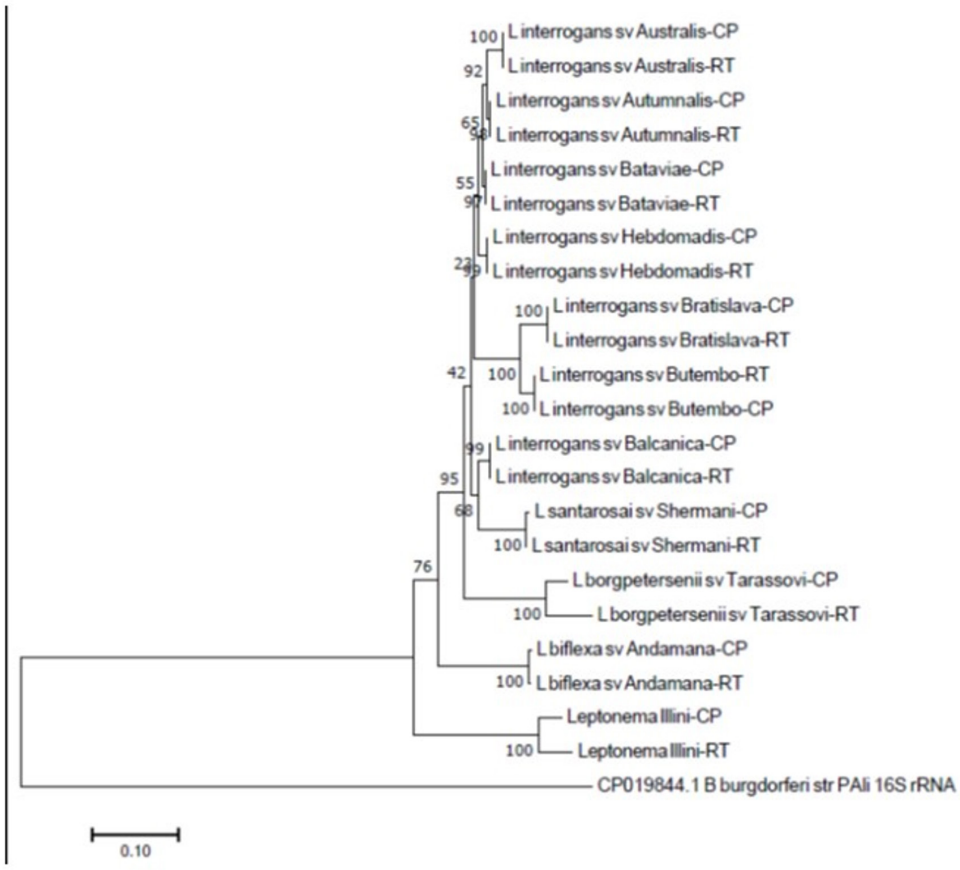

Figure 3. Phylogenetic tree obtained for 16S rRNA. Numbers represent bootstrap support generated from 1000 replications. CP: cryopreserved; TA: room temperature NR_074481.1: reference sequence for 16S rRNA.

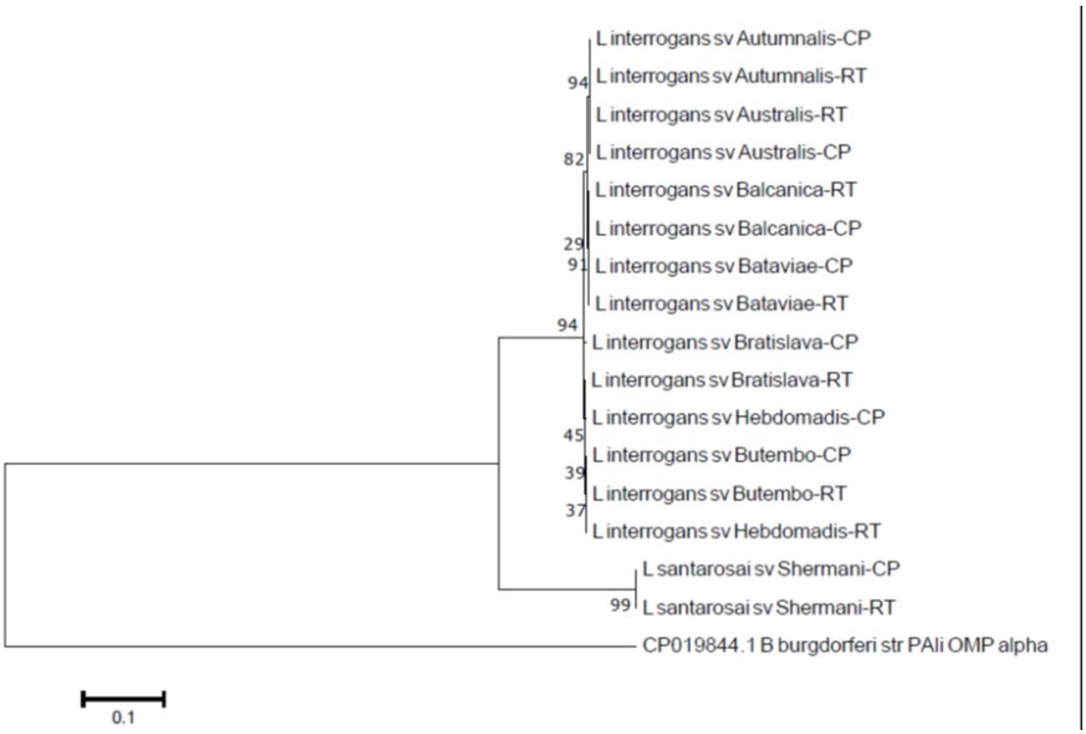

Figure 4. Phylogenetic tree obtained for *ompL1*. Numbers represent bootstrap support generated from 1000 replications. CP: cryopreserved; TA: room temperature; AY461984.1: reference sequence for *ompL1*.

Discussion

The reference strains, such as those used in the current study, are used for research work aimed at classifying autochthonous isolates and epidemiological studies of human and animal diseases. For this reason, conserving the genetic features of microorganisms is a priority in the preservation of biological reference material (23, 24). The growth results of the cultures in liquid EMJH medium for all strains showed that this medium, with periodic agitation, provides the necessary growth conditions for this demanding microorganism (7, 25, 33). In addition, the excellent recovery of the strains that were cryopreserved reaffirms the advantage of the use of freezing conservation and defrosting methods, as it has been described by Borrero *et al*. (25), who recovered three vaccine serovars that were maintained at -70 °C using DMSO at 2.5 % and at 5 % as cryoprotectant. Further, Torres *et al*. (46) recovered six serovars cryopreserved for one year in liquid nitrogen at -196 °C using 2 % Dimethyl sulfoxide (DMSO) as cryoprotectant and Borrero *et al*. (24) recovered six serovars kept in 2.5 % and 5 % DMSO and 2.5 % glycerol cryopreservants in liquid nitrogen for 19 months.

The amplification of the 16S rRNA gene corroborates the usefulness of the *EubA* and *EubB* primers (34) for the amplification of

this gene in *Leptospira* spp. strains. Whereas primers *OmpL1F* and *OmpL1R* (29) used for the amplification of the *ompL1* gene were able to amplify the pathogenic reference strains, with the exception of *L. interrogans* serovar Tarassovi. In spite of the confirmation of the primer design by multiple alignments with several *Leptospira* serovars, including *L. interrogans* serovar Tarassovi, non-specificity of the primers for this serovar of this study might have been attributed to polymorphisms resulting from genetic recombination phenomena (29, 36), as described by Reitstetter (43) and not for the absence of the gene in this serovar. These results are compatible with those reported by Dong *et al*. (41), Mgode *et al*. (6), Fernandes *et al*. (44), and Dezhbord *et al*. (42). An alternative approach to solve this issue may include protein analysis by western-blot with specific monoclonal antibodies against *ompL1* protein or designing a new set of specific primers for this serovar. On the other hand, this method confirms that the saprophyte *L. biflexa* serovar Andamana does not hold this gene (29, 31, 36, 42).

Commonly, the 16S rRNA sequencing has been used to identify *Leptospira* spp., molecular characterization of isolates, taxonomic purposes, and panel certification. However, although it is not adequate for the identification of the serovar, it is useful for multiple purposes like monitoring quality and contamination absence (4, 7, 23, 29). Sequencing of this gene has been used to differentiate *Leptospira* species (23, 47). Some authors suggest the use of this method for the verification and quality control of *Leptospira* spp. Collections (23). Moreover, the sequence of the 16S rRNA gene has been complementary for a MLST scheme in (8, 28, 48–50). Additionally, the 16S rRNA analysis method for bacterial characterization has shown similar performance when compared with Multilocus sequence typing (MLST) and Matrix assisted laser desorption/ionization-time of flight mass spectrometry MALDI-TOF MS (51). The 16S rRNA sequencing has also been used to confirmed genomic DNA integrity (44) and to diagnose human patients with negative blood cultures (9).

The presence of *ompL1* is restricted to pathogenic serovars (29, 31, 32, 36, 42), as it was demonstrated by Rosario *et al*. (36) who affirm that its protein product participates in tissue invasion. This argument is supported by Dezhbord *et al*. (42), Fernandes *et al*. (44), Ferreira *et al*. (3), Haake *et al* (21, 29), Lin *et al*. (32), and Reitstetter (43). In this case, the *ompL1* gene was useful to corroborate the presence of pathogenic serovars, in addition to establishing the genetic conservation status of the strains that can be affected by continuous subculturing (19, 24, 25). Though, the presence of this gene does not imply virulence, as it is dependent on its expression level, which can be low or inexistent when maintained in an artificial culture medium (7, 29, 36).

The paired alignments show a high conservation status of the sequences among the strains conserved in two different conditions, i.e., cryopreservation in liquid nitrogen and room temperature. Hence, the methodologies used are correct for the conservation of these genes and the strains had not undergone considerable changes as a result of the microorganism biology, conservation technique or bad handling. Additionally, there was an absence of contamination as it was demonstrated by the identity results of 16S rRNA gene. According to the studies conducted by Borrero *et al*. (27) and Torres *et al*. (46), inadequate conservation methodologies can lead to genotypic changes such as insertions, deletions, transposons, pseudogenes, punctual mutations and horizontal transfer (4, 17, 18, 27, 29), which has been also supported by the work published by Haake *et al*. (29). These authors studied the molecular evolution mechanisms of 38 pathogenic *Leptospira* spp. reference strains using polymorphism comparisons in preserved (16S rRNA and *lipL*32) and variable (*lipL*41 and *ompL1*) genes. They concluded that genetic mosaicism in 16S rRNA can also occur on a lesser extent compared to the one described for *ompL1*, as a result of the partial or total horizontal gene transferring. This argument is supported by Morey *et al*. (27) and Tian *et al*. (52) who also confirm the inability to amplify these genes due to variations that may occur in the primers annealing points, as reported by Reitstetter (43), Mgode *et al*. (6) and Fernandes *et al*. (44). Even so, the genome reorganization processes can hinder the construction of phylogenetic trees and the taxonomic classification of material (4, 8, 17, 18, 47). In spite of the low variance in the *ompL1* and 16S rRNA genes found in this study, in conclusion, any of the two conservations methods are recommended for preservation of *Leptospira* spp. strains. Choosing any of these methods will depend on financial sources and appropriated facilities.

The variation rate must be calculated by determining the molecular clock, however this can be calibrated from the MRCA (most recent common ancestor) for closely related species, like in *Leptospira* species, where the model assumes that the members of the lineage share a uniform rate of evolution (53). However, their calibration is limited by the causal differences generated by the mutations and in the evolutionary history of the traits of interest (53, 54).

Taking into account that the molecular markers used in the present study are submitted to different selection pressures, according to their biological functions, the use of the molecular clock would introduce uncertainty to the analysis, which may constrain its implementation.

Specifically, for *Leptospira*, growth rates are different among species (22, 55) and the different passages may lead to the generation of new variants, their evolution rates

may be affected distinctly. In addition, in organisms that hold segmented genomes, like *Leptospira*, differences in evolutionary rates have been demonstrated between each of the passages and a reduction in the negative selection pressure of the secondary chromosome, which allows the continuous variation of the population (56).

On the other hand, due to the absence of replicative plasmids and the slow growth of the bacteria, genetic experimentation in pathogenic leptospires remains a complex topic. In concordance with this, complementary studies of the genetic variation during long preservation of *Leptospira* strains may include analysis of the persistent presence of plasmids.

Since the analyzed strains belong to a strain collection, which is maintained and replicated in isolated groups in order to reduce the risk of cross-contamination and in spite of keeping standardized protocols for replication and conservation, changes in lab personnel as well as the butch of production of the culture media have not been a guarantee for completely homogeneous conditions for their maintenance. This issue obligated us in this study to use genetic distance as a measure to estimate the paired differences among the strains kept under the two conservation methods. Importantly, the paired alignment was parameterized with a Gap Open Penalty of 15 and Gap Extension Penalty of 6,6 and the DNA weight matrix used for the sequence comparison was IUB.

The phylogenetic analyses showed high preservation between the strains conserved under the two different conditions, and a high degree of genetic stability was found among the strains assessed. Regarding the 16S rRNA gene, the distances found are those used in species differentiation (4, 23, 27, 28, 47). Meanwhile, the conservation of *ompL1* is achieved because all the strains were treated with the same protocol before the DNA extraction, which ensures a similar behavior of the microorganism (18, 23, 29).

Conclusion

The conservation methods used in the preservation of *Leptospira* spp. strains belonging to the SBGNAA are adequate to ensure the stability of the *ompL1* and 16S rRNA genes since their nucleotide sequences were highly conserved.

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Ethical approval: The authors declare that:

- This article does not contain any studies with human participants or animals performed by any of the authors.

- This article does not include clinical essays.
- The data presented in the figures and tables shown in the manuscript are included in the results section and the conclusion.

Informed consent: This work did not carry on human or animal interventons, for that reason informed consent was unnecessary.

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