High-Resolution Melting Curve Analysis of the 16S Ribosomal Gene to Detect and Identify Pathogenic and Saprophytic *Leptospira* species in Colombian Isolates

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Abstract.   It is important to identify the circulating *Leptospira* agent to enhance the performance of serodiagnostic tests by incorporating specific antigens of native species, develop vaccines that take into account the species/serovars circulating in different regions, and optimize prevention and control strategies. The objectives of this study were to develop a polymerase chain reaction (PCR)–high-resolution melting (HRM) assay for differentiating between species of the genus *Leptospira* and to verify its usefulness in identifying unknown samples to species level. A set of primers from the initial region of the 16S ribosomal gene was designed to detect and differentiate the 22 species of *Leptospira*. Eleven reference strains were used as controls to establish the reference species and differential melting curves. Twenty-five Colombian *Leptospira* isolates were studied to evaluate the usefulness of the PCR–HRM assay in identifying unknown samples to species level. This identification was confirmed by sequencing and phylogenetic analysis of the 16S ribosomal gene. Eleven *Leptospira* species were successfully identified, except for *Leptospira meyeri*/ *Leptospira yanagawae* because the sequences were 100% identical. The 25 isolates from humans, animals, and environmental water sources were identified as *Leptospira santarosai* (twelve), *Leptospira interrogans* (nine), and *L. meyeri*/*L. yanagawae* (four). The species verification was 100% concordant between PCR–HRM and phylogenetic analysis of the 16S ribosomal gene. The PCR–HRM assay designed in this study is a useful tool for identifying *Leptospira* species from isolates.

INTRODUCTION

Leptospirosis is a globally distributed zoonotic disease caused by pathogenic bacteria of the genus *Leptospira*.[1] Previous studies have estimated that annually 1.03 million cases and 58,900 deaths occur due to leptospirosis worldwide.[2] Leptospirosis is considered a neglected disease mainly in tropical regions of developing countries.[3] Leptospirosis is now recognized as an emerging infectious disease due to large outbreaks in different regions of the world, which are associated with environmental disasters and extreme climate changes. In addition, severe forms of the disease such as Weil's disease and pulmonary hemorrhage syndrome have emerged as the leading cause of death in many regions where the disease is endemic.[4] Currently, serological and molecular classifications of *Leptospira* coexist, but there is no direct correlation between them because various serovars are found in more than one species and some species contain both pathogenic and nonpathogenic serovars.[5] The genus *Leptospira* consists of 22 genomic species, according to studies based on DNA/DNA hybridization.[6] This technique is considered the gold standard for identifying *Leptospira* species, but it is complex and time consuming, and it is usually performed in just a few international reference laboratories.[7] As a result, other techniques are gaining importance as molecular typing tools. Currently, the most commonly used methods are ribotyping, sequencing, and phylogenetic analysis of rRNA encoding genes, pulsed-field gel electrophoresis, multilocus variable number of tandem repeats analysis, and multilocus sequence typing (MLST).[8–12] The MLST technique is the most robust and useful tool for assessing *Leptospira* strain diversity. However, these techniques have the disadvantages of requiring bacterial isolation, which is not often routinely performed, and requiring post–polymerase chain reaction (PCR) analysis.

Molecular methods have been used as an alternative to existing serological diagnosis methods. Such methods include the isothermal amplification methods of nucleic acid sequence–based amplification and loop-mediated isothermal amplification.[13–15] Additionally, many primers have been designed from a variety of genes and used in conventional PCR (*rrs*, *rrl*, *flab*, *gyrB*, *ompL1*, *lig*, *lipL32*, *lipL21*, *lipL41*, and *secY*),[16–24] but only a few have been validated and subjected to clinical evaluation. Real-time PCR has been introduced into the field of leptospirosis as a rapid and sensitive alternative to conventional PCR methods.[24–28] High-resolution melting (HRM) analysis is a relatively new technique that enables direct characterization of PCR amplicons in a closed system. HRM is potentially a simpler, faster, and more inexpensive, accurate, and sensitive technique than current *Leptospira* typing methods.[29,30] It technically measures changes in the fluorescence intensity of a DNA intercalating dye during dissociation from double-stranded DNA to single-stranded DNA and it can differentiate single nucleotide polymorphisms. The melting temperature (Tm) and specific shape of the curve depend on the DNA sequence, GC content, and amplicon length. Hence, Tm variations might reflect species-specific sequence variation in PCR products.[31,32] HRM methodology has been used in two studies to identify serovars belonging to the genus *Leptospira*. In the first study, 10 serovars were correctly identified with the random amplified polymorphic DNA (RAPD)–HRM method. Contradictorily, in the second study, the results indicated that intra-serovar heterogeneity and interserovar homogeneity may limit the application of the RAPD–HRM method in typing.[33,34] A third study was conducted in 2015, where four pathogenic *Leptospira* species were identified successfully using primers

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G1 and G2, which amplify the gene *secY*.[35] These previous studies do not differentiate between pathogenic and saprophytic species of the genus *Leptospira*, which is important to monitor the presence of pathogenic *Leptospira* in environmental water sources. Thus, the main goals of this study were to develop a PCR–HRM assay to differentiate the species subgroups of the genus *Leptospira* and verify its usefulness in identifying unknown samples to species level.

METHODS

Source of isolates of *Leptospira*. Twenty-five *Leptospira* isolates were collected during the study. Nine of these isolates were obtained from blood sampling of patients diagnosed with leptospirosis from the municipalities of Apartadó (eight) and Puente Iglesias (one). Four isolates were obtained from the kidneys and blood of capuchin monkeys (*Cebus capucinus*), which had died as a result of jaundiced leptospirosis in the municipality of Barbosa. Three isolates were obtained from urine samples from dogs in the city of Medellín, which were sent to the laboratory for suspected leptospirosis, and five isolates were obtained from samples of kidneys from *Rattus norvegicus* captured in the city of Medellín and the municipality of Turbo. Finally, four isolates were obtained from environmental water sources collected in the municipalities of Triganá and Necoclí; these water sources are used by the people as a source of water for human consumption. All isolates were grown in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium, supplemented with 10% EMJH enrichment medium (Becton-Dickinson Biosciences, San Jose, CA), at a temperature of 26–30°C. They were examined weekly by dark field microscopy over the course of 3 months to detect *Leptospira* growth.

DNA extraction. DNA was extracted from 1 mL of culture. The turbidity of the bacterial suspensions was adjusted to 0.5 McFarland standards. DNA extraction was performed using kit Wizard (Promega®, Madison, WI), according to the manufacturer's instructions for gram-negative bacteria. All experiments were performed at a concentration of 20 ng/μL DNA for reference species and isolates.

Amplification of the 16S ribosomal gene using real-time PCR. A 317–base pair fragment from the 16S ribosomal gene was amplified using the primers F16S (GGCGCGTCTTAA ACATGCAAG) and R16S (GAGCAAGATTCTTAACTGCTGCC). These oligonucleotides were previously reported by Merien and others.[26] Real-time PCR was performed at a final volume of 25 μL, containing 0.8 μM of each primer, 1× of HRM PCR Master Mix 1X (*Type*-it HRM™® Qiagen, Hilden, Germany: this kit used EvaGreen for detection) and 20 ng of DNA. Each sample was analyzed in triplicate. Real-time PCR was performed in a thermocycler Rotor Gene Q System 6000 (Qiagen). The thermal cycling profile was one initial cycle of denaturation at 94°C for 5 minutes, followed by 40 cycles at 95°C for 45 seconds, 60°C for 30 seconds, 72°C for 10 seconds, and finally an extension cycle at 72°C for 2 minutes. After real-time PCR, a melting-curve analysis was performed using a gradual temperature increase of 65°C to 95°C, rising by 0.1°C each step, waiting for 90 seconds of premelt conditioning on first step and waiting for 5 seconds for each step afterward. Fluorescence data were recorded every 2 seconds.

HRM curve analysis. HRM analysis was performed using Rotor Gene Q software v2.2, with a normalization region between 80°C and 85°C in the first active melting area. Eleven reference strains of *Leptospira* were used to define the reference species. The Rotor-Gene ScreenClust HRM software® auto-called the reference species, melting temperatures of each amplicon, and provided a confidence percentage based on the square root of the correlation coefficient between samples and the reference species. To make a correct identification of the unknown species, a confidence percentage between 80% and 100% for the reference species and the unknown species was established.

Sequencing. To confirm species identification by HRM analysis, a 317-bp fragment of the 16S ribosomal gene from each isolate and reference strain was purified using the Gel Extraction Kit (Qiagen®). Concentration and purity were determined by Nanodrop, whereas integrity was assessed by agarose gel electrophoresis at 1%. All amplification products were sent to the Macrogen® company (Seoul, Korea) for sequencing. For each specimen, both forward and reverse sequences were used to generate a consensus sequence using Bioedit v. 7.0.5.[36]

Species identification by phylogenetic analysis. The 16S ribosomal genes from 22 currently known species of *Leptospira* were used as reference sequences. These sequences and those obtained from isolates by sequencing were aligned using the ClustalX program[37] (see GenBank accession numbers in Table 1). The phylogenetic analysis was performed with the MEGA5 phylogenetic program,[38] using the neighbor-joining method with 1,000 replicates of bootstrap. Evolutionary distances were computed using the parametric method Kimura-2.

TABLE 1
Sequences used for species identification by phylogenetic analysis

Reference strains		Colombian isolates of *Leptospira*	
Number of access	Species	Code isolates	Number of access GenBank
AY631891.1	*Leptospira inadai*	JET	KP031552
Y19243.1	*Leptospira broomii*	AIM	KP031553
AY996789.1	*Leptospira fainei*	JEC	KP031554
NR_044042.1	*Leptospira wolffii*	MCO	KP031555
NR_044310.1	*Leptospira licerasiae*	OV	KP031556
AY631876.1	*Leptospira biflexa*	OLP	KP031557
NR_043046.1	*Leptospira wolbachii*	JCM	KP031558
NR_041544.1	*Leptospira kmetyi*	LG	KP031559
NR_043045.1	*Leptospira meyeri*	VR	KP031560
EF536990.1	*Leptospira interrogans*	C35	KP031561
EF536995.1	*Leptospira kirschneri*	C45	KP031562
EF537006.1	*Leptospira borgpetersenii*	C8011	KP031563
DQ991495.1	*Leptospira santarosai*	C51849	KP031564
NR_043050.1	*Leptospira noguchii*	P828	KP031565
DQ483058.1	*Leptospira weilii*	P046	KP031566
AY996803.1	*Leptospira alexanderi*	P153	KP031567
DQ991480.1	*Leptospira alstoni*	RN040	KP031568
AY631897.1	*Leptospira vanthielii*	RN046	KP031569
AY631888.1	*Leptospira terpstrae*	RN111	KP031570
AY631882.1	*Leptospira yanagawae*	RN235	KP031571
AB721966.1	*Leptospira idonni*	MIN187	KP031572
NR_134067	*Leptospira mayottensis*	NECO007	KP031573
		TRIGA30	KP031574
		TRIGA32	KP031575
		TRIGA37	KP031576

FIGURE 1. Polymorphisms found along a fragment of 317 base pairs from the 16S ribosomal gene used in this study. (**A**) Pathogenic species, (**B**) intermediate species, and (**C**) saprophytic species.

Assessing the detection limit of PCR–HRM assay. This assay was performed to determine the minimum amount of *Leptospira interrogans* DNA that the technique PCR–HRM can detect. This assay is performed in triplicate using 100 ng/µL of DNA as starting genetic material and performing serial dilutions in base ten, until 10 pg, which is the minimum amount of DNA detected by PCR–HRM assay using the 16S ribosomal gene as target amplification.

Assessing the ability of PCR–HRM to detect genomic DNA of *Leptospira* in blood. A blind study was made to assess the ability of PCR–HRM to detect genomic DNA of *Leptospira* in human blood. For the test, a peripheral blood sample of a patient without clinical symptoms of leptospirosis was taken and divided into 50 aliquots containing 1 mL of peripheral blood. Twenty-five of these blood aliquots were spiked with 20 ng/µL DNA of *L. interrogans* and 25 blood aliquots were used as negative controls (blood aliquots without *Leptospira* DNA). The process of DNA extraction, 16S ribosomal gene PCR amplification, and HRM analysis were performed on 50 samples as was previously described in the methodology section.

Ethics statement. This project was approved by the ethics committee of the Colombian Institute of Tropical Medicine (ICMT).

RESULTS

Polymorphism analysis. The alignment of the 16S ribosomal gene reference sequences showed 78 polymorphisms in the amplified fragment (Figure 1). These polymorphisms represent about 50% of the total polymorphisms of the complete gene. A comparison of polymorphism patterns for each species showed that 17 of 22 species had unique polymorphism patterns, thus providing a basis for differentiating among species. The patterns for the species *Leptospira meyeri*/*Leptospira yanagawae* and *Leptospira biflexa*/*Leptospira wolbachii* were 100% identical in this fragment, making it impossible to distinguish between them with this method (Figure 1). The sequences were more conserved among the saprophytic species than among the pathogenic or intermediate species. Furthermore, there was a much closer phylogenetic relationship among the pathogenic and intermediate species than among the saprophytic species (Figure 1).

Establishing melting curves for 11 reference species. Eleven reference strains representing groups of pathogenic (*L. interrogans*, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira borgpetersenii*, *Leptospira weilii*, *Leptospira santarosai*), intermediate (*Leptospira inadai*, *Leptospira fainei*), and saprophytic (*L. meyeri*, *L. biflexa*, *L. yanagawae*) species were used to standardize the PCR–HRM assay (the strains were supplied by the World Health Organization Collaborating Center for Leptospirosis, Oswaldo Cruz Institute/FIOCRUZ, Rio de Janeiro, Brazil). The real-time PCR detected all of the reference species and classified them according to their pathogenicity status, using exclusively the average value of their melting temperature (Figure 2). HRM analysis also established nine different species-specific profiles (Figure 3). The species belonging to the three groups displayed differential behavior in the HRM analysis difference graph.

Identification to species level of Colombian isolates. We used 25 isolates provided by the Colombian Institute of Tropical Medicine (ICMT-CES) to evaluate the usefulness of PCR–HRM in identifying the different *Leptospira* species. These isolates were obtained from seven Colombian

FIGURE 2. Melting temperature analysis of (**A**) pathogenic species, (**B**) intermediate species, and (**C**) saprophytic species.
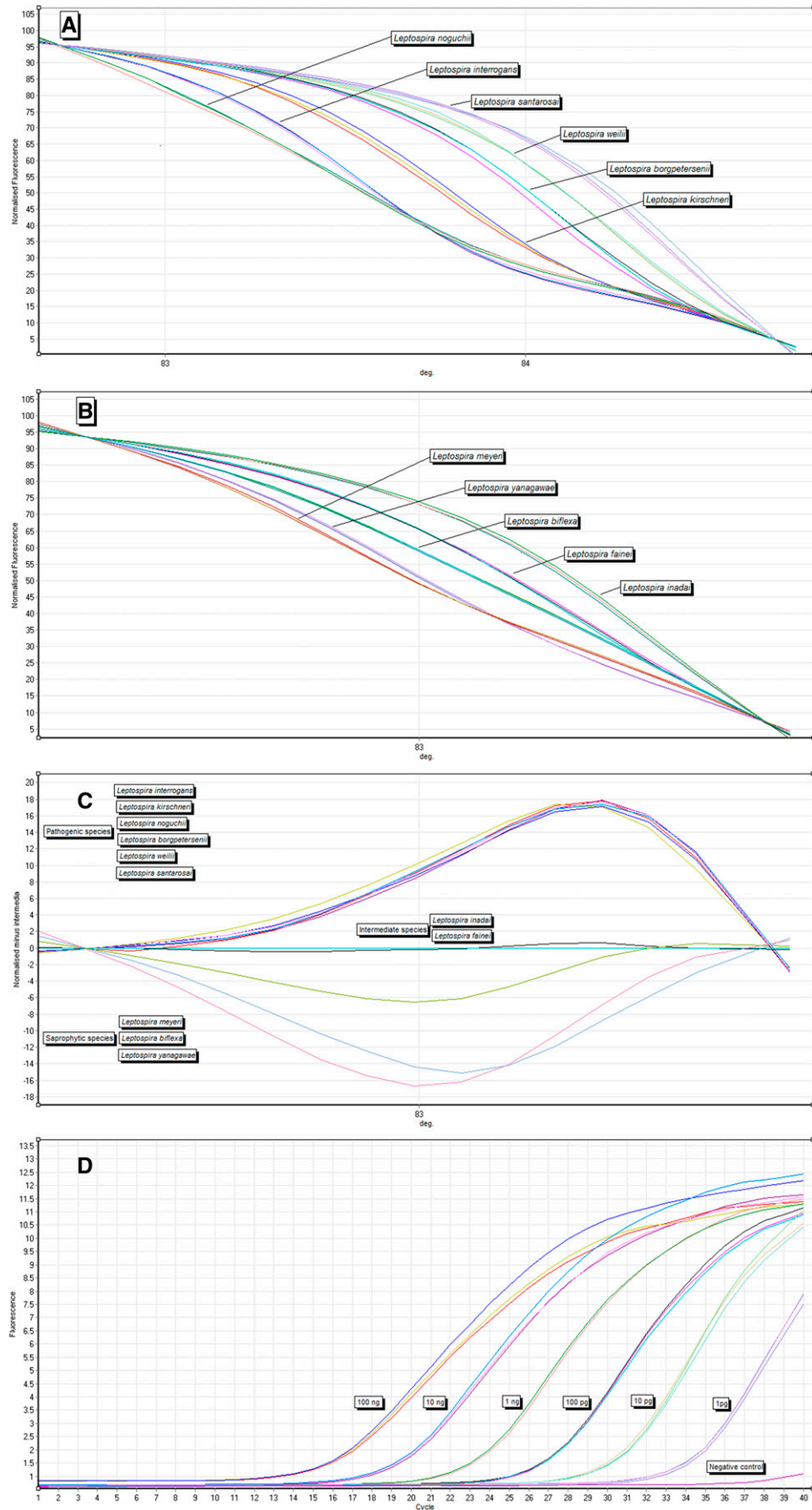
FIGURE 3. Differential melting curves for (**A**) pathogenic, (**B**) intermediate and saprophytic *Leptospira* species, (**C**) graph of differences among the melting curves of pathogenic, saprophytic, and intermediate species, and (**D**) level detection test.

TABLE 2
Species identification of Colombian isolates by phylogenetic analysis and PCR–HRM

Genotype HRM	Genotype Tm	Isolate	Source	Origin	Tm value	% Confidence in the identification
Leptospira santarosai	(84.35–84.58)	JET	Human	Apartadó	84.35	99.47%
L. santarosai	(84.35–84.58)	AIM	Human	P. Iglesias	84.39	95.43%
L. santarosai	(84.35–84.58)	JEC	Human	Apartadó	84.41	97.38%
L. santarosai	(84.35–84.58)	MCO	Human	Apartadó	84.41	99.42%
L. santarosai	(84.35–84.58)	OV	Human	Apartadó	84.50	99.25%
L. santarosai	(84.35–84.58)	OLP	Human	Apartadó	84.54	99.02%
L. santarosai	(84.35–84.58)	JCM	Human	Apartadó	84.53	99.65%
L. santarosai	(84.35–84.58)	LG	Human	Apartadó	84.58	99.23%
L. santarosai	(84.35–84.58)	VR	Human	Apartadó	84.51	99.17%
Leptospira interrogans	(84.11–84.18)	C35	*Cebus capucinus*	Barbosa	84.12	85.49%
L. interrogans	(84.11–84.18)	C45	*C. capucinus*	Barbosa	84.15	90.32%
L. interrogans	(84.11–84.18)	C8011	*C. capucinus*	Barbosa	84.13	89.96%
L. interrogans	(84.11–84.18)	C51849	*C. capucinus*	Barbosa	84.17	87.72%
L. santarosai	(84.35–84.58)	P828	Canine	Medellín	84.38	99.47%
L. santarosai	(84.35–84.58)	P046	Canine	Medellín	84.52	99.25%
L. santarosai	(84.35–84.58)	P153	Canine	Medellín	84.45	99.38%
L. interrogans	(84.11–84.18)	RN040	*Rattus norvegicus*	Turbo	84.16	92.25%
L. interrogans	(84.11–84.18)	RN046	*R. norvegicus*	Turbo	84.17	86.45%
L. interrogans	(84.11–84.18)	RN111	*R. norvegicus*	Turbo	84.13	93.22%
L. interrogans	(84.11–84.18)	RN235	*R. norvegicus*	Turbo	84.18	84.26%
L. interrogans	(84.11–84.18)	MIN 187	*R. norvegicus*	Medellín	84.12	89.32%
Leptospira meyeri/ Leptospira yanagawae	(82.89–82.97)	NECO 007	Environmental water	Necoclí	82.91	87.32%
L. meyeri/L. yanagawae	(82.89–82.97)	TRIGA 30	Environmental water	Triganá	82.89	87.57%
L. meyeri/L. yanagawae	(82.89–82.97)	TRIGA 32	Environmental water	Triganá	82.96	88.23%
L. meyeri/L. yanagawae	(82.89–82.97)	TRIGA 37	Environmental water	Triganá	82.95	88.48%

PCR–HRM = polymerase chain reaction–high-resolution melting; Tm = melting temperature.

municipalities during different research projects, as shown in (Table 2). The isolates from humans and canines were identified as *L. santarosai*. The isolates obtained from cebids and rodents were identified as *L. interrogans* and the isolates obtained from environmental water sources were identified as *L. meyeri/L. yanagawae*, because these two species were indistinguishable with this method. The pathogenicity status of new clinical, environmental, and animal isolates can be identified based on these results (Figure 3). The final detection level of the technique was one picogram (Figure 3). The HRM amplicon curve and Tm showed low intraspecies variability and a specific profile was observed for each species analyzed, with confidence percentages (%C) ranging from 84.26% to 99.65%, indicating that the samples were correctly identified (Table 2).

Verification of results by sequencing and phylogenetic analysis. Sequencing and phylogenetic analysis of amplified 16S rRNA fragments were used to verify that the PCR–HRM assay had correctly identified the species. The species were divided into three main branches according to their pathogenicity status (pathogenic, intermediate, and saprophytic). Branch support values ranged between 45% and 100%, permitting the identification of 17 *Leptospira* species. However, the species *L. meyeri/L. yanagawae* and *L. biflexa/L. wolbachii* were indistinguishable by this method (Figure 4). Isolates from humans, dogs, rodents, and cebidae clustered with the pathogenic species. Isolates from humans and canines were identified as *L. santarosai* (branch support values of 99%); whereas isolates from cebids and rodents were identified as *L. interrogans* (branch support values of 65%). Finally, isolates from environmental water sources were grouped with saprophytic species (branch support values of 77%), but could not be identified to species level because the species *L. meyeri/L. yanagawae* were indistin-

guishable using this method (Figure 4). There was a 100% correlation between the results obtained by the PCR–HRM assay and the phylogenetic identification method.

Assessing the ability of PCR–HRM to detect genomic DNA of *Leptospira* in blood. The results of the study were that 19/25 samples were detected as positive and 25/25 samples were detected as negative. These values correspond to sensitivity (76%), specificity (100%), positive predictive value (100%) and negative predictive value (80%). Additionally, all positive samples were correctly identified as *L. interrogans*.

DISCUSSION

PCR–HRM is an attractive molecular technique in the field of leptospirosis due to its ability to diagnose the acute phase of the disease and to identify the infecting bacteria to species level without prior isolation or sequencing. This technique can avoid the complicated isolation of the bacteria and the post-PCR analysis as electrophoresis, using restriction enzymes, hybridization, and sequencing methods. For this purpose, we designed a PCR–HRM assay that uses a set of primers from the 16S ribosomal gene and has the ability to detect 22 *Leptospira* species (Figure 1), with a detection level of approximately one picogram per sample (Figure 3D). This could also be useful in environmental monitoring of pathogenic, intermediate, and saprophytic species present in environmental water sources, which in turn could help build eco-epidemiological knowledge of the disease in Colombia and other regions where leptospirosis transmission is endemic.

We found a clear difference between the melting temperatures of the pathogenic (84.13–84.47), intermediate (83.36–83.51), and saprophytic (82.92–82.97) species groups.
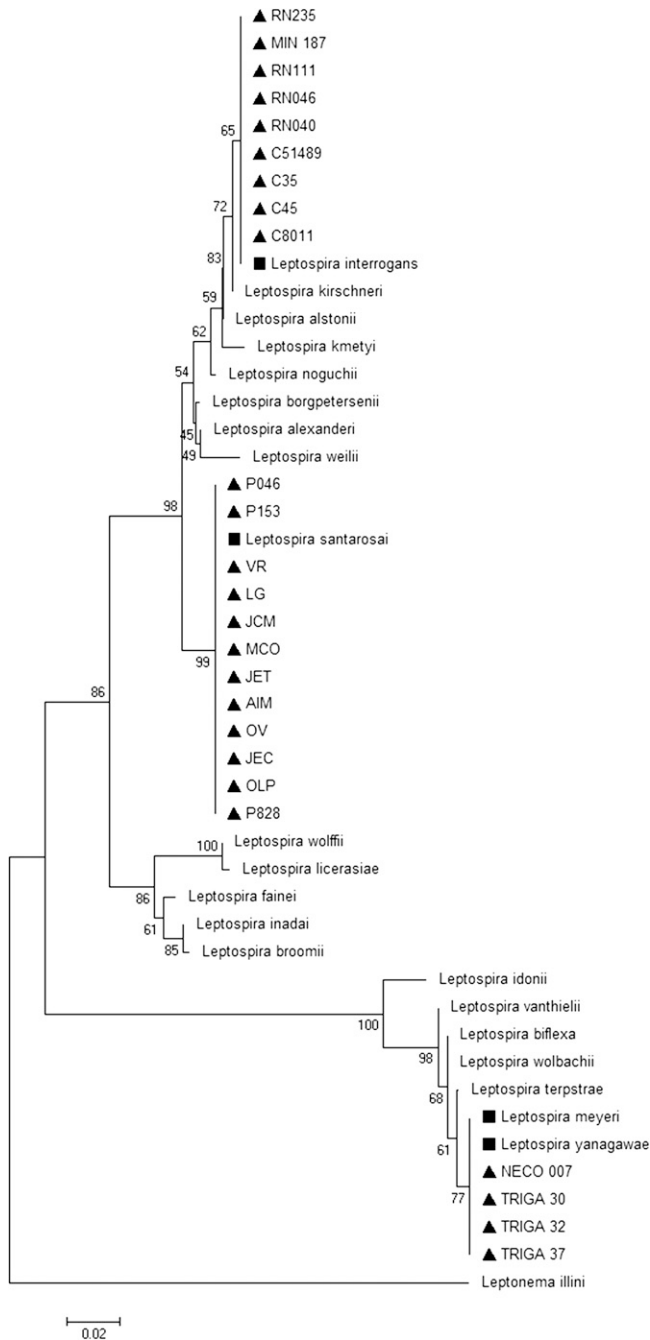
FIGURE 4. Species level identification of 25 Colombian *Leptospira* isolates by phylogenetic analysis of the 16S ribosomal gene. Squares and triangles indicate the reference species and isolates, respectively.

We were able to classify unknown samples according to their pathogenicity status by calculating the average value of the melting temperature. This could be important for laboratories that only have equipment for real-time PCR and do not have access to DNA sequencing (Figure 2, Table 2). The main advantage of the PCR–HRM assay is its ability to identify isolates to species level. To optimize the test, it is necessary to choose a gene with unique polymorphism patterns in all species. There are two copies of the 16S ribosomal gene in the bacterial genome. These have an

approximate size of 1,430 bp and the most polymorphic region is at the start of the gene. For these reasons, we chose the 16S ribosomal gene as a target in our study. Eleven reference strains were used to standardize the PCR–HRM assay. These included a representative sample of pathogenic, intermediate, and saprophytic species groups. Surprisingly, we found that the amplified 16S ribosomal gene presented two areas of active melting (data not shown), suggesting that there are differences between the two copies of the gene. The first region with active melting, which is between 80°C and 85°C, had a greater ability to differentiate *Leptospira* species by melting curves. We defined nine differential melting curves for the 11 *Leptospira* species tested, but we could not differentiate between the species *L. meyeri/L. yanagawae* due to the homology between their sequences (Figure 3). These results support the idea that the 78 polymorphisms found in the amplified region are responsible for species-specific melting curves. In the difference graph constructed from the melting curves, we observed a differential behavior between species groups, as shown in (Figure 3); these data confirmed the results previously obtained by analyzing the melting temperature for each species (Figure 2). With these results, we can verify the usefulness of PCR–HRM assay in identification species according to their pathogenicity status and in identifying the different *Leptospira* species.

To evaluate the PCR–HRM assay, we performed a species-level identification of 25 Colombian *Leptospira* isolates that had been previously identified to species level by sequencing and phylogenetic analysis of the 16S ribosomal gene, as shown in Figure 4. The results obtained by both techniques were 100% similar; therefore, we can conclude that the results obtained by PCR–HRM assay can be reproduced by other molecular techniques as the phylogenetic identification of species in the genus *Leptospira*. Finally, to evaluate the usefulness of the test in early disease diagnosis, the blood samples were contaminated with *L. interrogans* DNA. The results showed a good specificity (100%) and a decrease in sensitivity (76%). These results highlight the need to improve methods for extracting and concentrating genetic material to increase the percentage of positivity and thus improve the test. PCR optimization plays a critical role in HRM analysis; therefore, it is important to use the same extraction method and similar DNA concentrations in samples and controls. The absence of strains representing all 22 *Leptospira* species was a limitation in the study; consequently, it was not possible to confirm the ability of the technique to differentiate the 17 *Leptospira* species. In subsequent research it is important to validate the PCR–HRM assay as a diagnostic and typing tool directly from clinical, animal, or environmental samples and to use a more polymorphic gene (possibly *secY*) with the ability to differentiate between the 22 *Leptospira* species and their different serovars. This would provide more information to improve the diagnosis, vaccines, and strategies for preventing and controlling the disease.

With this assay, human and canine isolates were identified as *L. santarosai*. This species has been reported as a causative agent of human leptospirosis in Costa Rica, the French West Indies, and Peru.[39–41] Brazil and Mexico have not reported cases of human leptospirosis yet, but this species has been found to have infected buffalo and cattle.[42,43]

The isolates from cebids and rodents were identified as *L. interrogans*; this species is the major causative agent of leptospirosis worldwide,[44] and in Colombia, it has been found to infect capuchin monkeys, rodents, and pigs.[45–47] The isolates from environmental water sources were identified as *L. meyeri* or *L. yanagawae*; the *L. meyeri* species is difficult to taxonomically classify because it has clearly established pathogenic (Sofia) and saprophytic (Semaranga) serovars, but some serovars such as Perameles and Ranarum have isolates in both the pathogenic and saprophytic species groups.[48] Therefore, it is important to control the presence of this species in environmental water sources due to its ability to produce disease. *Leptospira yanagawae* is a saprophytic species that was originally isolated from environmental water sources in Sao Paulo, Brazil.[49] This saprophytic species has not been reported in Colombia; therefore, future molecular studies are needed to which species the environmental isolates belong. The diversity of species found in clinics, animals, and environmental samples reflects the importance of *Leptospira* in our eco-epidemiological context and highlights the need to implement public health measures to reduce the impact on human and animal populations in Colombia. Based on the above results, we can conclude that the PCR–HRM assay we designed could be a simple solution for diagnosing and species identification belonging to the genus *Leptospira*. In the future, this assay could be used as a diagnostic tool for leptospirosis.

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