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**Original Article**

# New Primers for Detection and Differentiation between *Leishmania viannia* and *L. leishmania* Subgenera by Polymerase Chain Reaction

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***Abstract***

***Background:*** *Leishmania* is the parasitic protozoan responsible for leishmaniases, a disease that can cause a range of cutaneous, mucosal, and visceral infections. Two subgenera *L. Viannia* and *L. Leishmania* are known to infect humans in the tropics and subtropics of the Americas. The aim of the present study was to develop a new pair of primers for the two subgenera and test in clinical samples.

***Methods:*** We designed two new pairs of primers for a PCR method from two conserved genes, cysteine proteinase B (*cpb*) and N-acetylglucosamine-6-phosfate deacetylase-like protein (*nag*A), as specific markers for those two respective subgenera. Primers were tested with 16 microscopical positive clinical samples from the Amazon region of Ecuador obtained in 2010-2020 period.

***Results:*** The *cpb* presented a band of 172 bp and the *nag*A a band of 300 bp, thus clearly differentiating *L. viannia* from *L. leishmania*. Additionally, primers identified and differentiated the clinical samples in the two subgenera.

***Conclusion:*** The new primers targeting different two genes and standardized in a PCR assay could identified and differentiated *Leishmania* parasites at subgenus level. This protocol could be used for *Leishmania* genus identification and diagnosis at the subgenus level and for determining the parasite's geographical distribution where different *Leishmania* subgenera are found in the same area.

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## Introduction

*Leishmania* genus is a protozoa agent for leishmaniases, a complex disease. *Leishmania* are classified into four subgenera: *Leishmania*, *Viannia*, *Mundinia* and *Sauroleishmania*; the first three are capable of infecting humans. In the New World *Leishmania* and *Viannia* subgenera are prevalent, being further subdivided into several species (1). In the tropics of the American continent, the four principal species of the *Viannia* include *L.* (*V.*) *braziliensis*, *L.* (*V.*) *panamensis*, *L.* (*V.*) *peruviana*, and *L.* (*V.*) *guyanensis*; whereas the subgenus *Leishmania* contains three main species *L.* (*L.*) *mexicana*, *L.* (*L.*) *amazonensis*, and *L.* (*L.*) *venezuelensis* (2). Nevertheless, some twenty others, have been reported in the Americas *e. g.*, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *naiffi*, *L.* (*L.*) *major-like*, *L.* (*L.*) *infantum*, *L.* (*L.*) *chagasi*, *L.* *(V.)* *colombiensis*, *L.* (*V.*) *shawi* (3).

The species are indistinguishable morphologically, but can be differentiated by isoenzyme analysis, monoclonal antibodies, or molecular-genetic methods (2). In humans the course of infection largely depends on the host-parasite relationship and primarily on the infecting species (4). The cutaneous manifestation can generally be attributed to all the species, whereas the other clinical features are closely related to a more specific range of the parasites: for example, mucocutaneous and mucosal disease is known to be caused by some of the species classified under the *Viannia* subgenus, while diffuse cutaneous leishmaniasis and visceral disease is attributed to certain of the species within the *Leishmania* subgenus (2).

The laboratory diagnosis of leishmaniasis is made by direct parasitological methods, cultivation, isoenzyme analysis, monoclonal antibodies, or molecular-genetic determinations from a given clinical sample. Despite the inability to differentiate among species and a high number of false positives and/or negatives, microscopy remains the method accepted by general consensus in observing amastigotes from skin, mucosa, and spleen or bone-marrow samples (1).

Molecular-genetic assays based on DNA, such as the PCR, constitute important methods on leishmaniasis, in order to facilitate the diagnosis and identification of the specific etiologic agent with a high level of sensitivity and specificity (1). Examples of approaches in the identification of *Leishmania* at level of subgenus and species and in the determination of their geographical distribution include: multilocus sequences for characterizing the species (5), sequencing of genes such as the loci encoding cytochrome b, the kDNA minicircle, mini-exons, or ribosomal genes along with random-amplified–polymorphic-DNA analysis and PCR with restriction-fragment-length polymorphisms, mannose phosphate isomerase (mpi), cysteine proteinase B (*cpb*) and the hsp70 (6–10). Therefore, the strategy of designing new primers of different genes for the detection of *Leishmania* DNA together with developing a PCR technique constitutes a highly practical means of characterizing, identifying, and differentiating the subgenera of *Leishmania*; which capability would, in turn, prove useful in clinical diagnosis. Developing a method for identifying both *Viannia* and *Leishmania* subgenera by PCR would be useful in the diagnosis of New World leishmaniasis, regardless of the specie of *Leishmania* responsible for the clinical picture.

Therefore, we designed a new set of primers based on separate genes specifically conserved in *Viannia* and in *Leishmania* *cpb* and *nag*A, respectively, to differentiate between the two subgenera using PCR. We tested those primers against clinical samples from the Ecuadorian Amazon.

## Materials and Methods

### *Primer design*

We searched *in silico* for marker chromosomes that demonstrated strong DNA conservation among species pertaining to each subgenera, carrying out multiple sequence alignments using *Geneious* software (https://www.geneious.com) based on sequences available from the NCBI database (http://www.ncbi.nlm.nih.gov). The cysteine proteinase B (*cpb*) gene was selected for *L. viannia* identification, in which subgenus a single locus may contain a several number of copies. In that regard, the *cpb* gene had polymorphic properties—in, for example, the C-terminal region (11). The N-acetylglucosamine-6-phosfate deacetylase-like protein (*nagA*) was chosen for *L. leishmania* because that locus present in chromosome 20 of New-World species and chromosome 36 of Old-World species, reported to be highly conserved (12).

At this time findings comprised the following accession numbers: *L. panamensis* (XM_010705915.1) (EU289030.1), *L. guyanensis* (GQ180933.1), *L. peruviana* (LN609243.1), *L. braziliensis* (XM_001562089.1), *L. donovani* (XM_003865074.1), *L. major* (XM_001686521.1), *L. infantum* (XM_001469566.1), *L. mexicana* (XM_003874297.1), *L. amazonensis* (Scaffold367 10914.12212), *L. arabica* (CM004641.1), and *L. aethiopica* (CM007213.1) (Fig. 1). Consensus sequences were extracted from the multiple sequence alignments by means of the *Primer3* algorithm (http://primer3.ut.ee/). In total, two pairs of primers were designed that could produce amplicons of variable sizes suitable for the differentiation of the two subgenera. The level of specificity of the primers was tested in Primer-BLAST with the Mega Blast algorithm (https://www.ncbi.nlm.nih.gov/tools/primer-blast), a bioinformatics software tool. The physicochemical and thermodynamic properties of the primers were calculated using the Oligo Analyzer tool (https://www.idtdna.com/calc/analyzer).

### *Preparation of DNA samples for PCR*

Sixteen skin Giemsa-stained glass slides-smears positive for *Leishmania* spp. from the Amazon-Ecuador were provided from the Instituto Nacional de Investigación en Salud Pública (INSPI) located in the Amazon. These samples had been taken from cutaneous lesions that were subsequently confirmed as positive for *Leishmania* spp. amastigotes by microscopy. These specimens were used as positive controls in the standardization of the PCR process. The smears from glass slides were scraped into a 99% (v/v) aqueous-ethanol solution with a sterile scalpel and centrifuged at 10,000 g for 2 min. All the samples were stored in 1.5 mL Eppendorf tubes at –20 °C.

For DNA extraction, 100 µl of 10% (w/v) solution of the Chelex resin (Sigma-Aldrich, USA) was added to each tube. The mixture was then homogenized by vortexing for 2 min before centrifugation at 10,000 g for 1 min. After the addition of 5 µl of proteinase-K solution, the mixture was heated in a water bath at 56 °C for 60 min, then vortexed a second time for 15 s. The resulting homogenate was incubated in a thermoblock at 95 °C for 30 min, before a second centrifugation under the same conditions for 1 min. The supernatant was then extracted and transferred to a tube for storage at –20 °C.

### *DNA quantification and DNA quality*

It was performed by means of a microplate reader (Biotech Instruments, USA). Out of a 10% (w/v) Chelex solution, 2 µl was added to the first set of microspots to act as the blank and 2 µl of the DNA samples were then added to each of the remaining microspots. The purity of the DNA was

established through the absorbance ratios of A260/A280 and A260/A230 nm as measured with a NanoDrop spectrophotometer (Thermo Fisher, USA).

### *PCR amplification*

The PCR master mix, in a total volume of 10 µl, contained the following reagents: 0.5 µM concentration of *cpb* forward and reverse primers (lCPB-F470 / lCPB-R641) and 0.7 µM concentration of *nagA* forward and reverse primers (lNAGA-F537 / lNAGA-R836) plus 1.5 IU/µl of Platinum® Taq DNA polymerase, 0.7 mM dNTP, 2.5 mM $MgCl_2$, 1X Buffer (all from Invitrogen, Brazil); in ultrapure water; and 10 ng/µl of genomic DNA. The amplification was carried out in a thermal cycler (Eppendorf, Germany). After an initial denaturation phase, where the temperature was raised to 94 °C for 5 min, the sample underwent the following 3-phase cycle for a total of 40 times: denaturation at 94 °C for 30 s; annealing at 64 °C for 30 s, and an extension phase at 72 °C for 60 s. A final 7-min extension at 72 °C completed the amplification. The resulting amplicons were processed via gel electrophoresis. Of the resulting DNA solutions, 5 µl were loaded onto a gel of 2% (w/v) *UltraPure* agarose containing SYBR Safe DNA gel stain (Invitrogen, Brazil) and the gel placed into a gel box filled with 1X TBE buffer. The gel was run at 100 V for 30 min. The DNA bands were visualized by a UV transilluminator. A separate PCR procedure was designed for each of the two subgenera, based on the concentration gradient and the hybridization temperature of each primer, and with the DNA isolated from *Leishmania*-positive glass slides.

### *Sequencing*

The amplicons of samples 14 and 16 were sequenced in a genetic analyzer (Applied Bio systems, USA). The resulting sequences were analyzed by the NCBI's BLAST from a bioinformatics-analysis website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Geneious software was then used to confirm the veracity of the results from the initial analyses. Thereafter, these samples were further characterized by PCR.

### *Validation of primers and PCR on skin-positive–smear samples*

Analysis of the 16 positives-skin-smears obtained two years ago, rendered DNA from those slides suitable for use in the validation of our PCR. All the samples for the PCR described above were performed with positive and negative controls (a mix of PCR reagents without the DNA template). A positive control was made by mixing a concentration of 10 ng/µl of DNA from each of the two subgenera, which were previously quantified in a Nanodrop to create a mixed sample. Positive controls were established by previous sequencing analysis from given samples (Lesh-14 and Lesh-16), which had demonstrated a positive DNA-amplification.

### *Ethics approval*

The study protocol was approved by the Ethics Committee of CEISH-INSPI-013.

## Results

The alignment of multiple sequences performed by using the Geneious software could identify two genes—*cpb* and *nagA* (Fig. 1). Using the Primer3 algorithm, consensus sequences were extracted from the multiple sequence alignments (Fig. 2) and two pairs of primers designed were named as lCPB-F470, lCPB-R641 and lNAGA-F537, lNAGA-R836 (Table1). Amplification with above primers yielded amplicons of different sizes, the *cpb* gave a band of 172 bp for *Viannia* subgenus, whereas the *nagA* a band of 300 bp for *Leishmania* subgenus. No amplification bias was found (Fig.3).

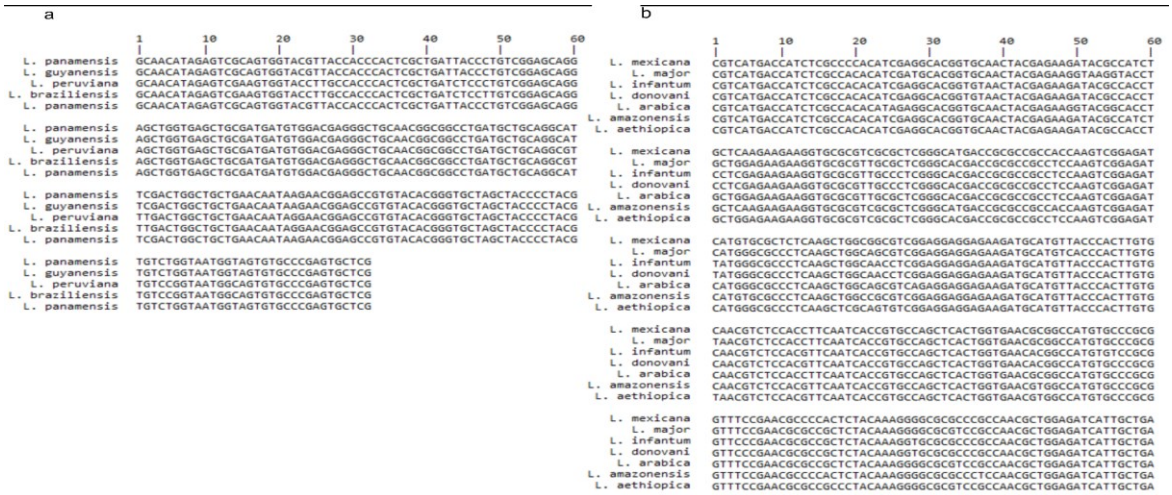


**Fig.1:** Multiplex restriction-fragment–nucleotide-sequence alignments. Panel a: *cpb* genes of *L. Viannia* species *L.* (*V.*) *panamensis*, *L.* (*V.*) *guyanensis*, *L.* (*V.*) *peruviana*, and *L.* (*V.*) *braziliensis*. Panel b: *nagA* genes of *L. Leishmania* species *L.* (*L.*) *mexicana*, *L.* (*L.*) *major*, *L.* (*L.*) *infantum*, *L.* (*L.*) *donovani*, *L.* (*L.*) *arabica*, *L.* (*L.*) *amazonensis*, and *L.* (*L.*) *aethiopica*. The numbering of the nucleotides is denoted above the columns containing the sequences.

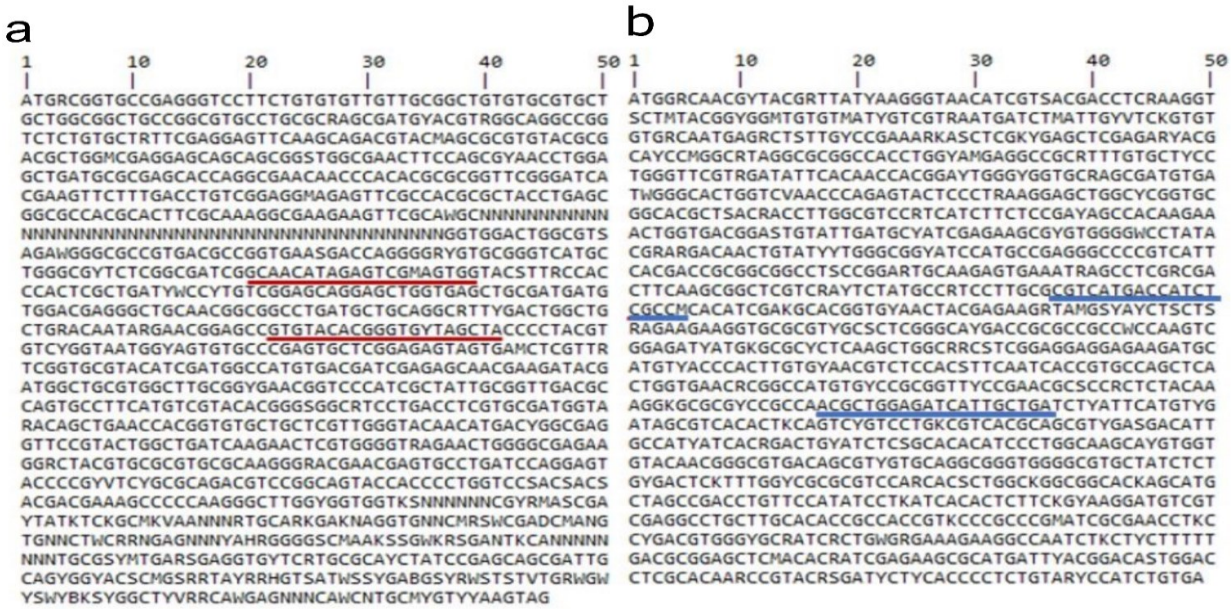


**Fig. 2:** Consensus sequence produced by exome alignments (from the GenSeq database). Panel a: Consensus sequence between the *cpb* genes of *L. Viannia* species (*L.* (*V.*) *panamensis*, *L.* (*V.*) *guyanensis*, *L.* (*V.*) *peruviana*, and *L.* (*V.*) *braziliensis*). The sequences underlined in red represent the target sites of *cpb* gene. Panel b: Consensus sequence between the *nagA* genes of *L. Leishmania* species (*L.* (*L.*) *mexicana*, *L.* (*L.*) *major*, *L.* (*L.*) *infantum*, *L.* (*L.*) *donovani*, *L.* (*L.*) *arabica*, *L.* (*L.*) *amazonensis*, and *L.* (*L.*) *aethiopica*). The sequences underlined in blue represent the target sites of *nagA* gene that were amplified by the PCR

**Table 1:** *L. viannia* and *L. leishmania* primers for *cpb* and *nagA* genes

| *Subgenus* | *Primer* | *Sequence* | *Amplicon* |
|---|---|---|---|
| Viannia | lCPB-F470 | 5´-GCAACATAGAGTCGMAGTGG-3´ | 172 bp |
| | ICPB-R641 | 5´-TAGCTARCACCCGTGTACAC-3´ | |
| Leishmania | lNAGA-F537 | 5´-CGTCATGACCATCTCGCCM-3´ | 300 bp |
| | lNAGA-R836 | 5´-TCAGCAATGATCTCCAGCGT-3´ | |

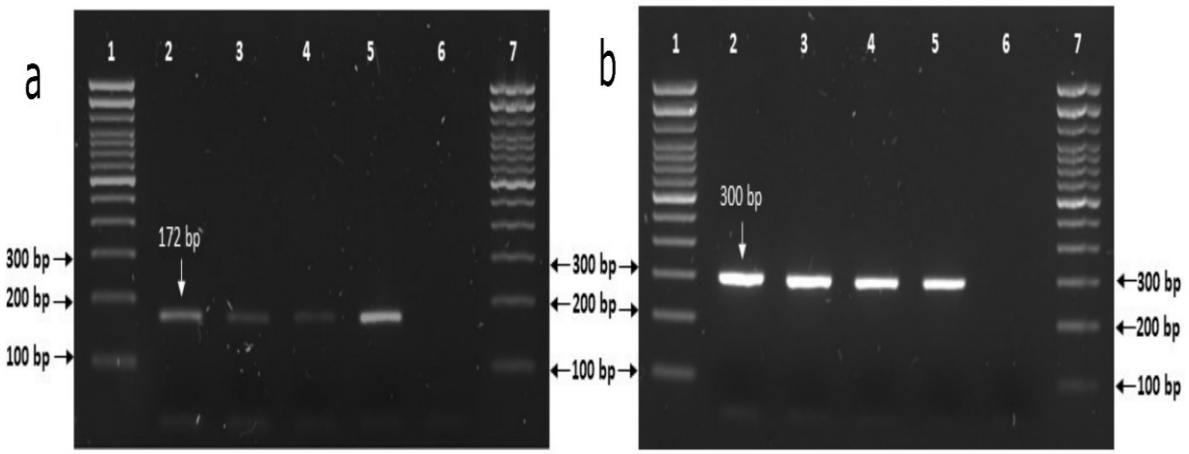


**Fig. 3:** Amplicons of *L. Viannia* and *L. Leishmania* subgenera targeting *cpb* and *nagA* genes, respectively. Panel a: Amplification of *cpb* using ICPB-F470 and ICPB-R641 primers. Lanes 2-4: *L. Viannia* clinical samples showing a band of 172 bp; lane 5: positive control of *L. Viannia* (Lesh-14); lane 6: negative control; lanes 1 and 7: MW 100 bp. Panel b: Amplification of *nagA* using INAGA-F537 and INAGA-R836 primers. Lanes 2-4: *Leishmania* clinical samples showing a band of 300 bp; lane 5: positive control of *L. Leishmania* (Lesh-16); lane 6: negative control; lanes 1 and 7: MW 100 bp

In developing the PCR assay, the temperature of the annealing phase needed to be raised to 64 °C, 4 °C higher than the average temperature of the annealing phases of the usual PCR. During PCR, we determined that the melting temperatures of the *cpb* and *nagA* primers were 62 °C and 58 °C, respectively; 14 °C higher than those expected.

The nucleotide sequences of the amplicons as determined by BLAST (Fig. 4) indicated that the clinical sample *Leish-14* matched 97% with the *cpb* consensus sequence (Fig. 4, Panel A), while the *Leish-16* sample corresponded 96% to the *nagA* consensus sequence (Fig. 4, Panel B). When a coinfection was simulated by mixing the DNAs from both subgenera, the resulting admixture was successfully amplified in the same reaction. When the resulting amplification was chromatographed on a 2% (w/v) UltraPure agarose gel, the run generated major bands at 172 bp and 300 bp corresponding to the respective amplicons of the *cpb* and *nagA* loci (Fig. 5 and 6).

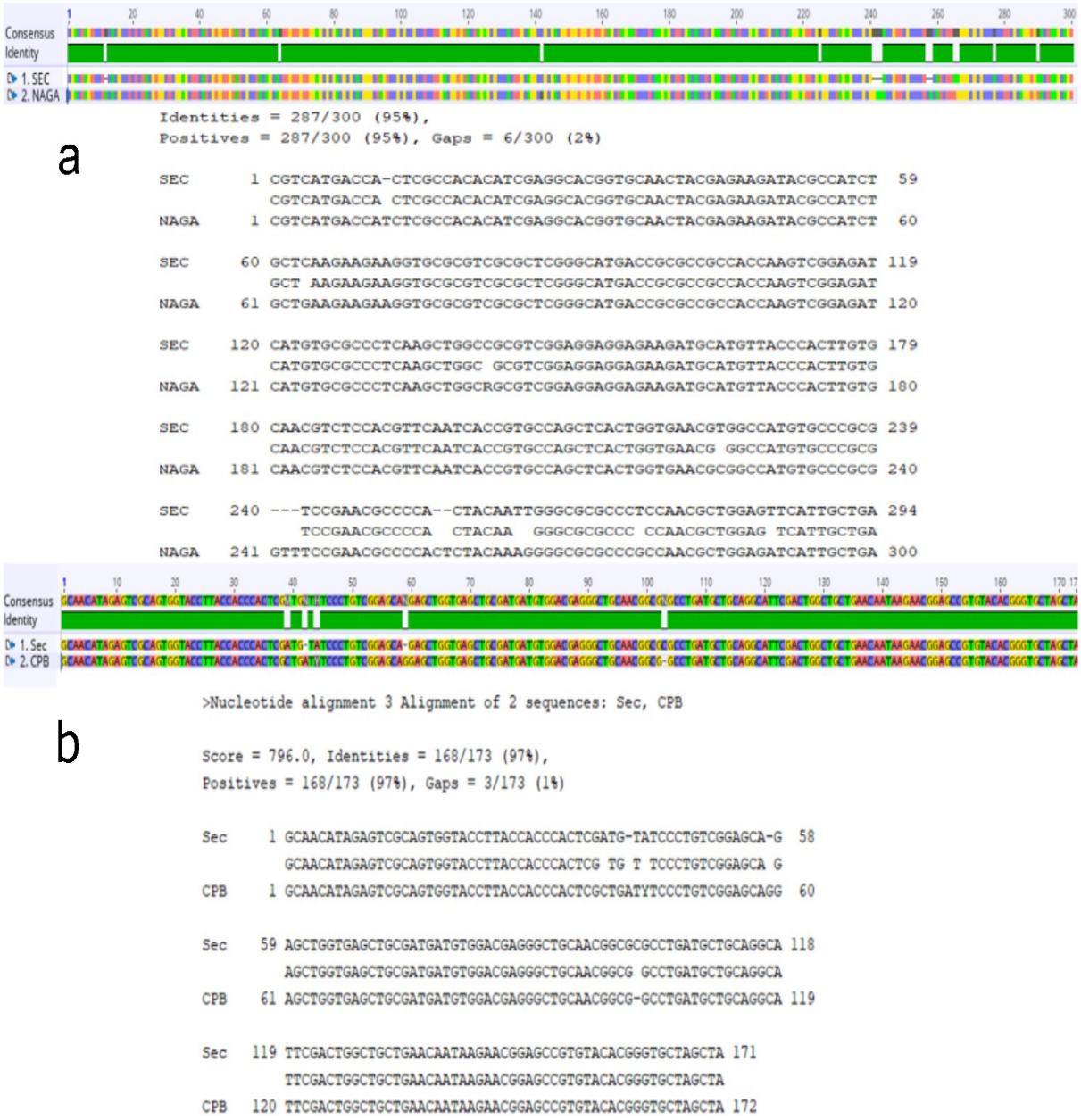


**Fig. 4:** Alignment between sequenced samples and consensus sequences by Geneious program. Panel a: *nagA* alignment for the *Leishmania*-subgenus–positive control (Leish-16; Fig. 1). Panel b: *cpb* alignment for the *Viannia*-subgenus–positive control (Leish-14; Fig. 1). The consensus sequences were compared and were cleared using the IUPAC nomenclature for degenerate sequences. In both panels, the colors in the schematic sequences in the rows directly above the nucleotide arrays (as indicated in Panel a) correspond to the bases as follows: violet, C; yellow, G; green, T; red, A; while the gaps with missing nucleotides are left black; and in the written nucleotide sequences below, the nucleotides A and T are marked in boldface. The uppermost colored schema in each panel is a summary of the consensus from the bottom two, and the green band in between illustrates only the locations of the lacunae in correspondence *i. e.*, different or absent nucleotides simply as spaces.

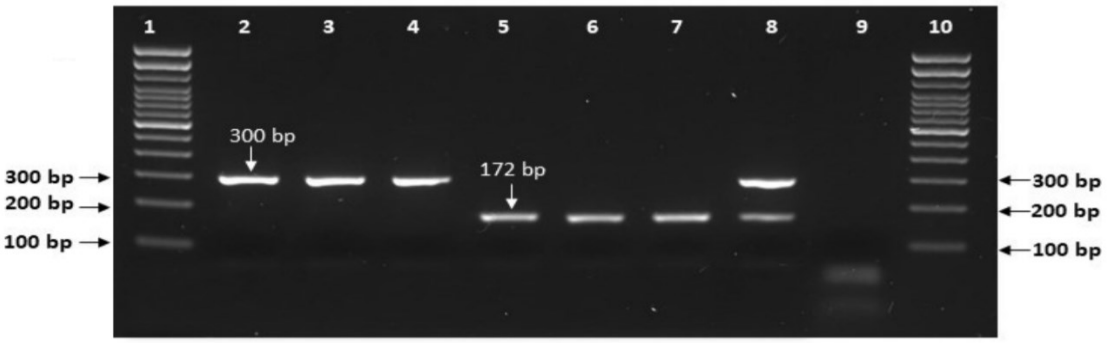


**Fig. 5:** PCR amplification together *nagA* and *cpb* genes. Lanes 2-4: *L. Leishmania* clinical samples showing a band of 300 bp. Lanes 5-7: *L. Viannia* clinical samples showing a band of 172 bp; lane 8: positive control of mixed DNA samples from *L. Viannia* (Leish-14) and *L. Leishmania* (Leish-16) revealing the presence of both the 300-bp and the 172-bp bands, respectively; lane 9: negative control; lanes 1 and 10: MW 100 bp.

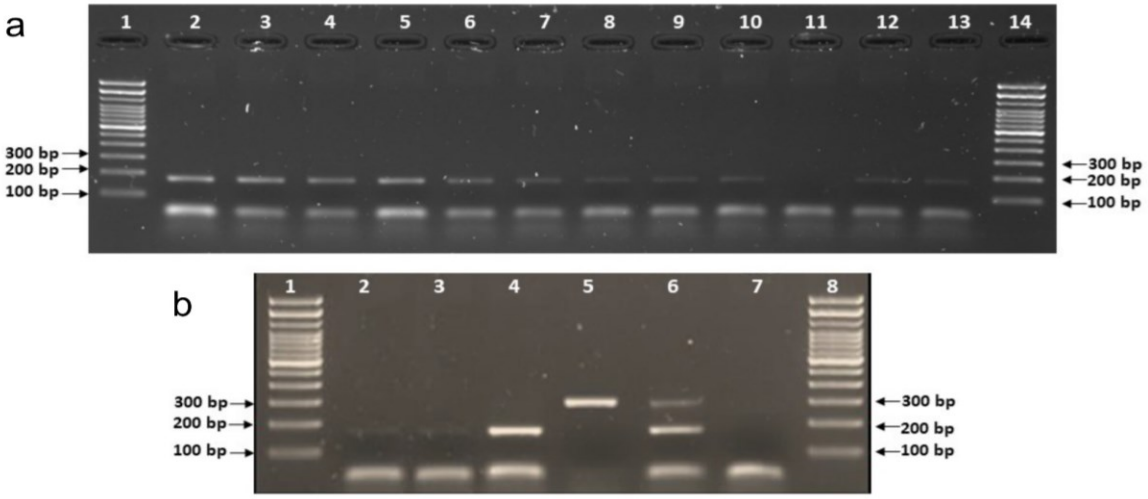


**Fig. 6:** PCR validation assay. Panel a**:** Lanes 2-13: bands of the DNA from the positive clinical samples, showing a 172 bp band corresponding to *L. Viannia*; lanes 1 and 14: MW 100 bp. Panel b: Lanes 2-5: bands of the DNA from the positive samples (lane 5 show a band of 300 bp; lane 6: positive control of mixed DNA samples from *L. Viannia* (Leish-14) and *L. Leishmania* (Leish-16). Lane 7: Negative Control. Lines 1 and 8: MW 100 bp

The DNA concentrations of the 16 clinical samples ranged from 39.1 to 153 ng/µl, had to be diluted to a final concentration of 10 ng/µl. The A260/A230-nm absorbance indicated a contamination with organic compounds and salts. Contamination with salts in the DNA, resulted from the extraction method used, can cause interference. Nevertheless, those contaminants did not interfere with the PCR amplification as showed in Fig. 5 and 6. The PCR resulted in products of the expected size of 172 bp for *L. viannia* and 300 bp for *L. leishmania*. Fifteen corresponded to *L. viannia* and one to *L. leishmania* (Fig. 6).

## Discussion

The present study demonstrated that the newly designed primers lCPB-F470 and lCPB-R641 for *L. Viannia* and, lNAGA-F537 and lNAGA-R836 for *L. leishmania* targeted to the corresponding *cpb* and *nagA* genes, exhibited a high sensitivity for the detection of and differentiation between the two subgenera in clinical samples. Furthermore, we designed a PCR protocol to detect the *Leishmania* genus circulating throughout the American continent and simultaneously discriminate between the two *Leishmania* subgenera (*Viannia* and *Leishmania*) without requiring two separate reactions. All 20 species infecting humans and reservoirs in the New World leishmaniasis are included in both subgenera. The high sensitivity of this PCR assay was confirmed by the detection of parasites in clinical samples of dermal scrapings from affected individuals. The high copy number per cell of the *cpb* and *nagA* genes of the various *Leishmania* parasites indicates these primers as being appropriate templates for PCR (11,13,14).

The primers designed detected and differentiated between the two *Leishmania* subgenera from 16 *Leishmania*-positive Giemsa-stained smears from the Ecuadorian Amazon. Of those sixteen, 15 were identified as members of the *L. Viannia* subgenus, corresponding to previous studies where *Viannia*-species predominated in the Ecuadorian Amazon (6). The results of this study further support the several previous studies (8,9,15–17) that PCR exhibits a high sensitivity and specificity, demonstrating that such PCR could be applied to diagnose all cases of New World leishmaniases.

The present assay targeted at the *cpb* and *nagA* genetic repeats of *Leishmania* genus that code for different-sized products in each subgenus. No primers targeting the *cpb* and *nagA* genes in PCR have been reported in the literature. Certain PCR protocols for identifying the *Leishmania* genus, individual complexes, or species-specific involving minicircle kDNA or a multiplex assay that targeted the spliced leader RNA (mini-exon) of the gene, have been described (9,10). One study reported a single-step PCR that identified the *Viannia* subgenus (8), but did not simultaneously detect and differentiate between the two subgenera. In the present study, we considered the *cpb* gene to be the ideal candidate for identification of the *Viannia* subgenus because a single locus may contain a great number of copies of the *cpb*, making that gene more suitable for identification via PCR. In addition *cpb* has been shown in previous studies to have polymorphic properties, such as in the C-terminals (11). We performed multiple sequence alignments demonstrating that the gene also has highly conserved regions, such as the coding sequences. In contrast, for the identification of the *Leishmania* subgenus, we selected the *nagA* gene because that locus is highly conserved and present in both chromosome 36 of Old-World species and chromosome 20 of those of the New-World (14).

It is also important to mention that *Leishmania* DNA was successfully extracted from dried scrapings of the Giemsa-stained smears on glass slides even after two years of storage. Thus, the protocol for the DNA extraction used here involving a Chelex-100 resin gave good results, even with such dried samples, as has been tested in previous studies from different sources of samples (18,19).

The primers designed in combination with the PCR protocol also detected experimental coinfections and differentiated between them with respect to the subgenus constituting the etiologic agent in a single PCR reaction. This capability was tested via an admixture of DNA that we prepared from separate clinical samples that were positive for *Viannia* and *Leishmania* subgenera. These results are both relevant and significant because the method can detect coinfections such as those that

have been reported in the literature *e. g.*, both *L. (V.) braziliensis* and *L. (L.) amazonensis* were found in the same lesion of cutaneous leishmaniasis (9) while coinfections were reported in regions where the two subgenera coexisted, as occurs in the Americas (3). Another application would be in determining the geographic distribution of the *L.* (*Viannia*) and *L.* (*Leishmania*) and detecting autochthonous cases (20). In several countries of South America the *Viannia* subgenus is implicated in producing mucosal metastasis to the nasal and oropharyngeal tissues, whereas the *Leishmania* subgenus is involved in the anergic diffuse cutaneous leishmaniasis as well as in the visceral leishmaniasis (2). At the present time no multiplex PCR exists that is able to detect all 20 species in a single-step PCR or even subspecies or strains present among species within the Americas (21).

Hence, the present assay, detecting all *Leishmania* species assayed together but differentiating between subgenera, would guide the correct drug treatment and prognosis of the disease in parasitized patients. With an aim at minimizing costs, an optimized PCR mix of just 10 µl was tested and successfully used in this study.

Limitations of this study are: (a) present new primers will detect and differentiate only at *Leishmania* subgenera, not at species level; (b) limited clinical samples were used to determine the specificity that can affect the results; (c) did not use other parasites' DNA that are genetically related to *Leishmania* spp. as *Trypanosoma cruzi* that may cross-react in the PCR reaction, and (d) not using samples from other endemic places than Amazonian regions. Therefore, it is suggested that in future studies testing a greater number of clinical samples, sources such as filter paper or skin biopsies, testing together to compare related parasites, bacteria, and fungi DNA, and from different origin and countries should be considered.

## Conclusion

We have developed new primers targeting different two genes and standardized a PCR assay that will be useful for both the diagnosis and the differentiation between *Leishmania* parasites at subgenus level, all species are within both subgenera in the American tegumentary leishmaniasis. A prompt and accurate diagnosis along with the identification of the specific parasite would be of enormous advantage to physicians, epidemiologists, and decision makers regarding therapeutic approaches and prognoses as well as in determining the geographic distribution of the *Leishmania*.

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## Conflicts of interest

The authors declare that there is no conflict of interest.

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