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Calmodulin Polymerase Chain Reaction–Restriction Fragment Length Polymorphism for *Leishmania* Identification and Typing

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Abstract. A precise identification of *Leishmania* species involved in human infections has epidemiological and clinical importance. Herein, we describe a preliminary validation of a restriction fragment length polymorphism assay, based on the calmodulin intergenic spacer region, as a tool for detecting and typing *Leishmania* species. After calmodulin amplification, the enzyme *Hae*III yielded a clear distinction between reference strains of *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania infantum*, *Leishmania lainsoni*, and the rest of the *Viannia* reference species analyzed. The closely related *Viannia* species: *Leishmania braziliensis*, *Leishmania panamensis*, and *Leishmania guyanensis*, are separated in a subsequent digestion step with different restriction enzymes. We have developed a more accessible molecular protocol for *Leishmania* identification/typing based on the exploitation of part of the calmodulin gene. This methodology has the potential to become an additional tool for *Leishmania* species characterization and taxonomy.

Leishmaniasis is a major neglected tropical disease affecting around 2 million people worldwide every year.[1] The infections tend to cluster among the poorest and most isolated increasing the socioeconomic burden upon these vulnerable populations. There are more than 20 species of *Leishmania* capable of infecting humans and each having distinct epidemiological/demographic patterns, and nearly all have a zoonotic transmission with one main sylvatic reservoir.[2] In the absence of vaccines and other effective preventive measures, the control of leishmaniasis is currently based on accurate diagnosis and prompt treatment.

A precise identification of the species has epidemiological, and particularly clinical importance, since the *Leishmania* species involved in human infections can influence the treatment decision and outcome.[3] Furthermore, species identification is particularly relevant now that the disease appears to be currently underestimated and on the rise in several countries.[1,4] There is also a renewed interest to evaluate the effectiveness of new drugs and/or treatment regimens, which requires accurate species identification.[5,6] In this regard, several molecular markers and protocols have been described for *Leishmania* species identification and taxonomy. However, no single genetic marker has been shown to have the sufficient discriminatory power when dealing with closely related *Leishmania* species. We have recently reported that the calmodulin locus, located on chromosome 9 of *Leishmania* species, varies in copy number (2–3) and size of the intergenic spacers.[7] The minor spacer between calmodulin copies of *Leishmania* presents sufficient variation in its sequence to allow clear discrimination between species, at least to the species/complex taxonomic level.[7]

We recognize, however, that our sequencing approach is not a feasible technology for many laboratories, particularly to those from developing countries/regions where *Leishmania* is endemic. In this sense, the aim of this study was to develop and evaluate a more practical polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) approach, based on part of the calmodulin gene for *Leishmania* identification and subsequent typing.

We initially tested the overall performance of our PCR assay using serial dilutions of DNA from different *Leishmania* species, and with the set of primers and methodology previously described.[7] The PCR conditions were designed to amplify the shortest intergenic spacer (~1,220 base pairs [bp]) between calmodulin-coding sequences. *Leishmania* DNA was extracted from cultures or skin scrapings using a commercial Kit (QIAGEN Hilden, Germany). We then performed in silico analysis of *Leishmania* calmodulin intergenic sequences deposited in GenBank–National Center for Biotechnology Information (accession numbers: JN966910 to JN966919, JQ302012, and JQ302013) using the software: "In silico simulation of molecular biology experiments" (available at the website http://insilico.ehu.es/) to search for restriction enzyme sites that allowed a clear distinction between *Leishmania* species based on the methodology previously described.[8] Selected enzymes were then evaluated in vitro according to the manufacturers' recommendations, using as the target *Leishmania* reference strains (Table 1). Finally, we validated our methodology by analyzing 20 *Leishmania* human field isolates and by performing an in silico analysis of the calmodulin intergenic spacer sequences from *Leishmania* (*Viannia*) *panamensis* isolates available in GenBank (JN966920–JN966936).

To compare our typing results, reference strains and field isolates were also analyzed by a standard molecular methodology (*hsp*70-RFLP), performed as previously described.[9] Sequencing of the shortest calmodulin intergenic region was performed on field isolates to validate the typing results.[7] Use of clinical samples was approved by the National Review Board, (Comité Nacional de Bioética de la Investigación, Instituto Conmemorativo Gorgas de Estudios de la Salud, Panamá).

All *Leishmania* reference strains that were tested amplified a calmodulin PCR product around 1,220 bp (Supplemental Figure 1). Analytical sensitivity of our initial PCR ranged between 1.0 pg and 1.0 ng, depending on the *Leishmania* species tested (Supplemental Figure 2). This difference is likely related to the genomic organization of the calmodulin gene in different *Leishmania* species.[7] Although the set of primers used can also amplify the calmodulin gene from other trypanosomatids (data not shown), the specificity of our PCR is based on the size of the expected amplified product, which in *Leishmania* is

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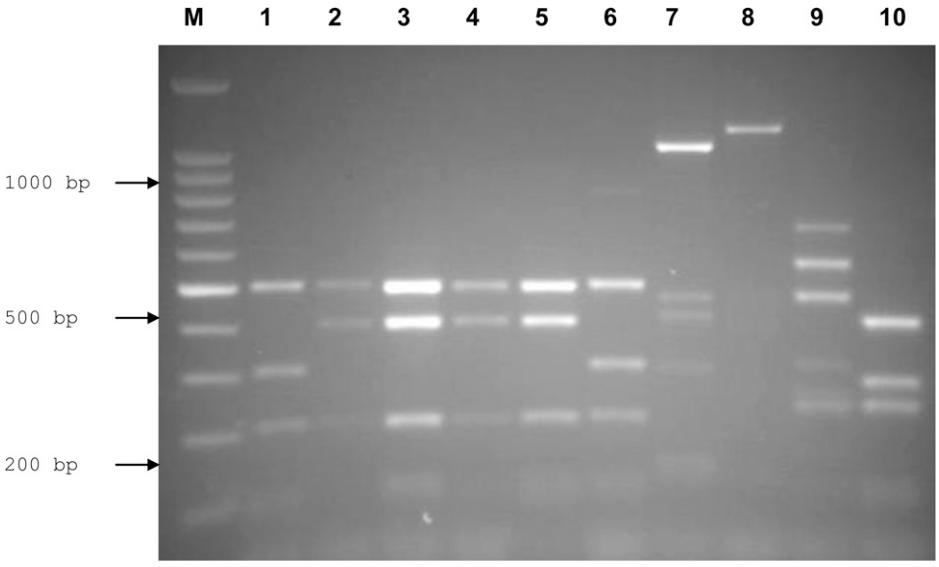
TABLE 1
Leishmania reference strains evaluated in this study

Species	International code
Leishmania (*Viannia*) *panamensis*	MHOM/PA/98/WR2306
L. (*Viannia*) *panamensis*	MHOM/PA/86/WR746C
Leishmania (*Viannia*) *peruviana*	MHOM/PE/05/WR2771
Leishmania (*Viannia*) *braziliensis*	MHOM/PA/02/WR2355
Leishmania (*Viannia*) *braziliensis*	MHOM/BR/1975/M2903
Leishmania (*Viannia*) *guyanensis*	MHOM/BR/1975/M4147
Leishmania (*Viannia*) *lainsoni*	MHOM/BR/1981/M6426
Leishmania (*Leishmania*) *infantum*	MHOM/BR/1974/PP75
Leishmania (*Leishmania*) *amazonensis*	IFLA/BR/1967/PH8
Leishmania (*Leishmania*) *mexicana*	MHOM/BZ/1982/BEL21

around 1,220 bp. Moreover, in the case of cutaneous leishmaniasis, the most common clinical form of the disease, the clinical sample sources are usually skin scrapings or biopsies that are unlikely to contain trypanosomatids other than *Leishmania*. However, the presence of other trypanosomatids circulating in *Leishmania*–endemic regions and the reports of the opportunistic presence of monogenetic trypanosomatids in immunosuppressed patients indicate that other trypanosomatids must be taken into account in any approach aiming to identify and characterize *Leishmania*. To address this situation, we performed an in silico analysis to observe the predicted restriction profile after cutting with different restriction enzymes the shortest intergenic region of calmodulin in different trypanosomatids. After amplification, several enzymes were capable of producing restriction patterns with different levels of discrimination between *Leishmania* species and/or complex. The best broad resolution was obtained with the enzyme *Hae*III which yielded a clear distinction between reference strains from *Leishmania* (*Leishmania*) *mexicana*, *Leishmania* (*Leishmania*) *amazonensis*, *Leishmania* (*Leishmania*) *infantum*, *Leishmania* (*Viannia*) *lainsoni*, and the rest of the *Viannia* reference species analyzed (Figure 1). Interestingly, *Leishmania peruviana* (MHOM/PE/05/WR2771) presented a digestion profile different from the rest of the reference strains, but identical to the one presented by *Leishmania* (*V.*) *braziliensis* (MHOM/PA/02/WR2355) (Figure 1). To further investigate this result, we analyzed this *L.* (*V.*) *braziliensis* strain by *hsp*70-RFLP and by hsp70 sequencing.[9,10] *Leishmania* (*V.*) *braziliensis* (MHOM/PA/02/WR2355) was classified as *L.* (*V.*) *braziliensis* by *hsp*70-RFLP, but was grouped by hsp70 sequencing as a "*L.* (*V.*) *braziliensis* outlier," closer to the *L.* (*V.*) *peruviana* cluster (results not shown). In this regard, the classification of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* has generally been problematic. Previous isoenzyme and genetic analyses have grouped *L.* (*V.*) *peruviana* as a subcluster in the *L.* (*V.*) *braziliensis* complex; however, discrimination between these two strains has been hindered because many parasites of this complex seem to have a composite genotype from both species.[11,12] Our finding reinforces the need to evaluate a larger sample of reference strains from both species.

The closely related *L.* (*V.*) *braziliensis*, *L.* (*V.*) *panamensis*, and *Leishmania* (*V.*) *guyanensis*, presented the same digestion pattern after *Hae*III digestion (Figure 1). However, if for medical or epidemiological reasons a distinction between these three *Viannia* species is needed, this can be readily accomplished in a subsequent digestion with the enzyme *Bcc*I (Figure 2A). Alternatively, due to the absence of a restriction site in *L.* (*V.*) *panamensis*, the enzyme *Ava*1 can differentiate



1: *L. braziliensis* ((MHOM/PA/02/WR2355); 2: *L. braziliensis* (MHOM/BR/1975/M2903);
3: *L. guyanensis* (MHOM/BR/1975/M4147); 4: *L. panamensis* (MHOM/PA/86/WR746C);
5: *L. panamensis* (MHOM/PA/98/WR2306); 6: *L. peruviana* (MHOM/PE/05/WR2771);
7: *L. amazonensis* (IFLA/BR/1967/PH8); 8: *L. mexicana* (MHOM/BZ/1982/BEL21);
9: *L. infantum* (MHOM/BR/1974/PP75); 10: *L. lainsoni* (MHOM/BR/1981/M6426)

FIGURE 1. Calmodulin intergenic spacer restriction pattern from *Leishmania* reference strains with *Hae*III enzyme. 1 = *Leishmania braziliensis* (MHOM/PA/02/WR2355); 2 = *L. braziliensis* (MHOM/BR/1975/M2903); 3 = *Leishmania guyanensis* (MHOM/BR/1975/M4147); 4 = *Leishmania panamensis* (MHOM/PA/86/WR746C); 5 = *L. panamensis* (MHOM/PA/98/WR2306); 6 = *Leishmania peruviana* (MHOM/PE/05/WR2771); 7 = *Leishmania amazonensis* (IFLA/BR/1967/PH8); 8 = *Leishmania mexicana* (MHOM/BZ/1982/BEL21); 9 = *Leishmania infantum* (MHOM/BR/1974/PP75); 10 = *Leishmania lainsoni* (MHOM/BR/1981/M6426).

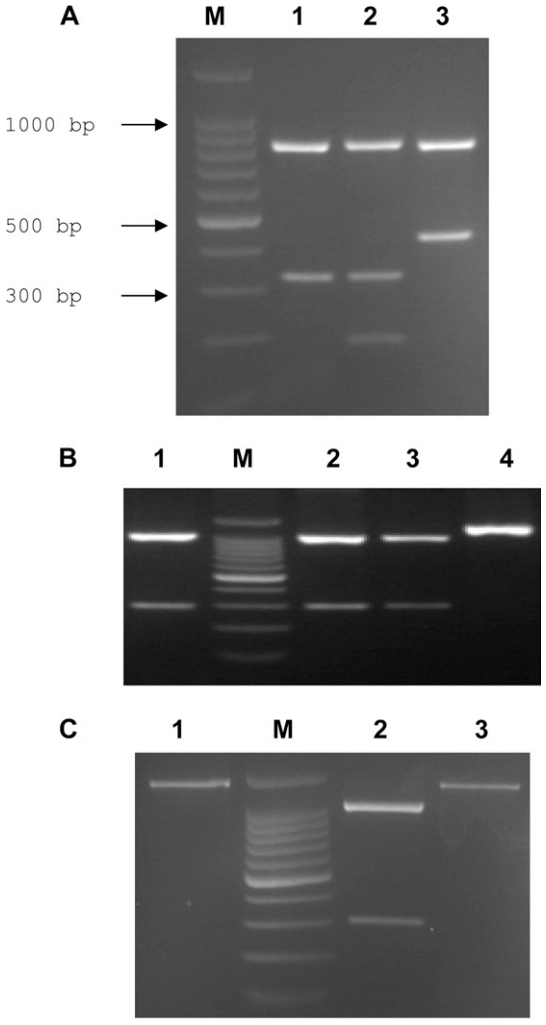


FIGURE 2. Calmodulin intergenic spacer restriction pattern from *Leishmania* (*Viannia*) reference strains with (**A**) *Bcc*I enzyme. M = Molecular size marker (100-bp ladder); 1 = *Leishmania panamensis* (MHOM/PA/98/WR2306); 2 = *Leishmania guyanensis* (MHOM/BR/1975/M4147); 3 = *Leishmania braziliensis* (MHOM/BR/1975/M2903). (**B**) *Ava*1 enzyme. M = Molecular size marker (100-bp ladder); 1 = *Leishmania peruviana* (MHOM/PE/05/WR2771); 2 = *L. guyanensis* (MHOM/BR/1975/M4147); 3 = *L. braziliensis* (MHOM/BR/1975/M2903); 4 = *L. panamensis* (MHOM/PA/98/WR2306). (**C**) *Bsu*36I enzyme M = Molecular size marker (100-bp ladder); 1 = *L. guyanensis* (MHOM/BR/1975/M4147); 2 = *L. panamensis* (MHOM/PA/98/WR2306); 3 = *L. braziliensis* (MHOM/BR/1975/M2903).

this *Viannia* species from *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis*, which have the same digestion pattern (Figure 2B). On the other hand, the enzyme *Bsu*36I makes a single cut in the *L.* (*V.*) *panamensis* calmodulin sequence, but does not recognize *L. braziliensis*/*guyanensis* (Figure 2C). All field isolates were identified as *L.* (*V.*) *panamensis* by the calmodulin-RFLP and *hsp*70-RFLP approaches. These typing results were further confirmed by sequencing the calmodulin minor spacer as described.[7] In silico analysis of calmodulin intergenic spacer sequences available in GenBank (JN966920–JN966936) confirmed that these isolates were *Leishmania* (*V.*) *panamensis*. The enzyme *Hae*III also clearly separates *Leishmania* from trypanosomatids of the genus *Crithidia*, *Endotrypanum*, *Leptomonas*, and *Trypanosoma* (Supplemental Figure 3). Therefore, trypanosomatids other than *Leishmania* that might be found in clinical samples can be easily distinguished by the calmodulin-RFLP approach itself.

We have developed and performed a preliminary validation of a molecular methodology (PCR-RFLP) based on the calmodulin gene for detecting and typing *Leishmania* species. Although we observed a relatively high experimental sensitivity for detecting *Leishmania* with our PCR approach, we recognize that the experimental method we used to evaluate the sensitivity does not mirror what happens biologically. Many factors such as the molecular target characteristics, the presence of PCR inhibitors in the clinical sample, and/or DNA extractions/PCR protocols can all significantly influence the overall PCR performance. Only 2–3 copies of calmodulin are present on chromosome 9 of the *Leishmania* genome, depending of the species. Since we designed the calmodulin-RFLP based on the amplification of the minor spacer between two copies of the calmodulin arrangement, only one copy from the *Leishmania* genome is targeted in this PCR approach. Other molecular markers such as the kinetoplastid or ribosomal genes are present in multicopies, whereas the heat shock protein gene is present in two tandem copies in chromosome 28 and a single copy in chromosomes 34 or 35. This fact, related to differences between the number of PCR targets in the *Leishmania* genome clearly influence the potential sensitivity of the test. In this sense, our methodology for species determination performed well with *Leishmania* DNA extracted from cultures, but the results were inconsistent when trying to detect and type *Leishmania* from DNA extracted directly from the cutaneous lesion samples (skin scraping or biopsies), probably due to the low number of parasites and calmodulin spacer targets in these biological samples.

The calmodulin-RFLP methodology was capable of distinguishing between most of the *Leishmania* reference strains and trypanosomatids so far tested. After amplification, an initial digestion with only one enzyme (*Hae*III) allowed identification of most *Leishmania* species. Using different enzymes in a second digestion step, it is also possible to separate the other closely related *Viannia* species. The *hsp*70 gene is probably the molecular marker more widely used for *Leishmania* species identification, and currently the *hsp*70-RFLP approach has become a reference molecular method in many laboratories for these purposes.[10] In this regard, an almost perfect agreement was observed between our calmodulin-RFLP methodology and the *hsp*70-RFLP protocol previously described.[9] All field samples were typed as *L.* (*V.*) *panamensis* by both molecular methods. However, a bias due to the geographical origin of the samples, where *L.* (*V.*) *panamensis* predominates is likely.[7,13] We also recognize that so far we have only tested a small number of

reference strains and field isolates from a particular region. Some *Viannia* species such as *Leishmania* (*Viannia*) *naiffi* and *Leishmania* (*Viannia*) *shawi* were not evaluated in this study due to the lack of reference strains in our laboratory or the absence of calmodulin sequences from these two species in GenBank to perform an in silico analysis. This is an important limitation of our study considering that in some areas, such as the Amazon, sympatric circulation of *Viannia* species is very common. Thus, the potential of this marker as a complementary tool for *Leishmania* species identification and taxonomical analysis requires further field studies using a larger number of strains from different geographical areas.

Several molecular markers and PCR protocols have been developed for *Leishmania* species identification, strain typing, and consequent phylogenetic analysis.[14–17] Despite the molecular progress, the taxonomy and phylogenetic status of closely related *Leishmania* species within complexes and the classification of interspecies hybrids is still problematic. The evaluation of other variable genes has been recommended to address this issue. In this sense, the calmodulin-RFLP technique might provide additional information to understand the genetic complexity of *Leishmania* parasites.

Recent studies have demonstrated that multilocus sequence typing (MLST) based on sequence analysis of several internal control genes presents one of the highest discriminatory typing capacities for *Leishmania* species.[18–20] However, the establishment of MLST or any combination of molecular markers as a global and acceptable typing system requires a detailed evaluation of the number and type of *Leishmania* genes needed to achieve consistent typing results, as well as the validation of the methodology analyzing a wider range of *Leishmania* species from different geographical regions. In light of our results, the calmodulin intergenic spacer could be an interesting molecular marker to be considered in the standardization of a global system to type *Leishmania*. In conclusion, we have developed a more accessible molecular protocol for *Leishmania* identification/typing based on the exploitation of part of the calmodulin gene. This methodology has the potential to become an additional tool for characterizing *Leishmania* field isolates.

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