

# *In silico* and *in vitro* evaluation of primers for molecular differentiation of *Leishmania* species

## Avaliação *in silico* e *in vitro* de iniciadores para a diferenciação molecular de espécies de *Leishmania*

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### Abstract

Leishmaniasis is a zoonotic disease caused by over 20 species of protozoan parasites of the genus *Leishmania*. Infection is commonly spread by sandflies and produces a wide spectrum of clinical signs and symptoms. Therefore, from an epidemiological and therapeutic standpoint, it is important to detect and differentiate *Leishmania* spp. The objective of this study was to combine *in silico* and *in vitro* strategies to evaluate the analytical specificity of primers previously described in the literature. According to electronic PCR (e-PCR) analysis, 23 out of 141 pairs of primers selected through literature search matched their previously reported analytical specificity. *In vitro* evaluation of nine of these primer pairs by quantitative PCR (qPCR) confirmed the analytical specificity of five of them at the level of *Leishmania* spp., *L. mexicana* complex or *Leishmania* and *Viannia* subgenera. Based on these findings, the combination of e-PCR and qPCR is suggested to be a valuable approach to maximize the specificity of new primer pairs for the laboratory diagnosis of infections with *Leishmania* spp.

**Keywords:** Leishmaniasis, differential diagnosis, e-PCR, qPCR.

### Resumo

As leishmanioses são zoonoses causadas por mais de 20 espécies de protozoários do gênero *Leishmania*. As infecções são comumente disseminadas por flebotomíneos e causam um amplo espectro de manifestações clínicas. Portanto, a detecção e diferenciação de espécies de *Leishmania* são importantes do ponto de vista epidemiológico e terapêutico. O objetivo deste estudo foi combinar estratégias *in silico* e *in vitro* para avaliar a especificidade analítica dos *primers* descritos anteriormente na literatura. De acordo com a PCR eletrônica (e-PCR), 23 dos 141 pares de *primers* selecionados por meio de pesquisa da literatura estavam de acordo com a especificidade analítica anteriormente relatada. A avaliação *in vitro* de nove desses pares de *primers*, por PCR quantitativa (qPCR), confirmou a especificidade analítica de cinco deles ao nível de espécie de *Leishmania*, do complexo *L. mexicana* ou dos subgêneros *Leishmania* e *Viannia*. Com base nos resultados, sugere-se que a combinação de e-PCR e qPCR é uma abordagem valiosa para a validação e maximização da especificidade de novos pares de *primers* para o diagnóstico laboratorial de infecções com *Leishmania* spp.

**Palavras-chave:** Leishmaniose, diagnóstico diferencial, e-PCR, qPCR.

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## Introduction

Trypanosomatidae (Trypanosomatida) is a family of parasites that includes monoxenous (infect only a single invertebrate host) and dixenous (infect both a vertebrate and invertebrate host) pathogens (Maslov et al., 2019). Leishmaniasis is a zoonotic disease caused by a dixenous trypanosomatid of the genus *Leishmania*, transmitted to mammals by the bites of sand flies, and is a major global health problem (Akhoundi et al., 2017; Galluzzi et al., 2018; Lukeš et al., 2018). More than 20 *Leishmania* species produce a wide range of clinical manifestations of leishmaniasis (WHO, 2020).

In the Americas, *Leishmania infantum* (syn. *Leishmania chagasi*; subgenus *Leishmania*) is the main species involved in Visceral Leishmaniasis, while species of the subgenera *Leishmania* and *Viannia* are responsible for Cutaneous Leishmaniasis and Mucocutaneous Leishmaniasis. Apart from *L. infantum*, the *Leishmania amazonensis*, *Leishmania mexicana* and *Leishmania venezuelensis* species are also of clinical relevance within the subgenus *Leishmania*, whereas *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis* and *Leishmania peruviana* are among the major species of the *Viannia* subgenus (PAHO, 2012a, b).

The detection and differentiation of *Leishmania* species contribute to the determination of patient prognosis and treatment and can be an invaluable tool for epidemiological surveillance (Schriefer et al., 2008; Medley et al., 2015).

Several authors have used PCR-based techniques to differentiate *Leishmania* species (Akhoundi et al., 2013; Morais et al., 2016; Conter et al., 2018). These molecular techniques, which include real-time quantitative polymerase chain reaction (qPCR), have provided an alternative to multilocus enzyme electrophoresis (MLEE) (Galluzzi et al., 2018), which has been considered the “gold standard” technique for the differentiation of *Leishmania* species (Schönian et al., 2011), and enables testing of a wide range of biological samples (blood, lymph node, bone marrow, skin, etc.). These techniques are also highly sensitive and specific, and are fast to perform (Galluzzi et al., 2018). However, their success and specificity depend on prior knowledge of target sequences (Tsokana et al., 2014).

Today, the availability of genomic information and bioinformatic tools enables one to predict PCR efficiency (Ivens et al., 2005; Cantacessi et al., 2015). In addition, *in silico* analyses such as electronic PCR (e-PCR) can be performed before molecular assays, saving time at no additional cost (Schuler, 1997).

In this study, we evaluated the specificity of primers described in the literature using an *in silico* strategy based on the genomic information available in public databases. We then conducted qPCR assays using reference strains of the main American *Leishmania* spp. to confirm our *in silico* results.

## Material and Methods

### Selection and *in silico* evaluation of primers specific for *Leishmania* spp.

Subgenus-specific, complex-specific and species-specific primers were selected based on previous reports (Supplementary file -Table S1). *In silico* evaluation was performed using the e-PCR command line tool version 2.3.12 (Schuler, 1998) and the R (R Development Core Team, 2020) statistical computing language for data handling and inspection. The dataset used in the electronic PCR step included the genomic sequences of the hosts (i.e., dog and human), vector (i.e., sand fly) and Trypanosomatidae, including dixenous and some monoxenous trypanosomatids (Supplementary file -Table S2). The data were retrieved among the available genomes present in the National Center for Biotechnology Information-NCBI (NCBI, 2018) and in TriTrypDB (TriTrypDB, 2018) databases. All the data were collected by April 2018 and combined with additional genomic sequences of the genus *Leishmania* obtained by our research group (SRA accession: PRJNA521679) (NCBI, 2019) also included in the Supplementary file (Table S2).

The e-PCR results consisted of all the *in silico* amplified sequence information in the dataset. In order to select potential candidates for further *in vitro* evaluation, a filtering procedure was applied using scripts developed in-house in the R environment, where a primer was retained if: i) it amplified a species-specific region that matched those previously reported in the literature; ii) the amplified fragment size ranged from 80 to 250 base pairs (bp) in the target species of *Leishmania*; iii) alignment gaps or mismatches were absent in targeted species; and iv) amplification occurred in non-targeted species, its alignment presented five or more gaps or mismatches.

### *In vitro* evaluation of primers by real-time PCR (qPCR)

Primer sets filtered by the aforementioned parameters were subjected to qPCR in order to evaluate *in vitro* specificity. In particular, nine reference strains of *Leishmania* were used: *L. amazonensis* (IFLA/BR/1967/PH8), *L. braziliensis* (MHOM/BR/1975/M2903), *L. infantum* (MHOM/BR/1972/LD46), *L. guyanensis* (MHOM/BR/1975/M4147), *L. major* (LV 39 MRHO/Sv/59/P), *L. mexicana* (MHOM/BZ/1982/BEL21), *L. panamensis* (isolated strain in Panama in 2018), *L. shawi* (MCEB/BR/1984/M8408) and also a local *L. infantum* strain isolated in the city of Araçatuba, SP, Brazil.

DNA was extracted from cultured strains using a DNeasy Blood & Tissue kit (Qiagen®) according to the manufacturer's instructions. After optimizing the qPCR reaction conditions (data not shown), the assays were standardized and performed in triplicate in a Real Time IQ™5 (Bio-Rad®) thermocycler. The final reaction volume was 20 µl and contained between 30-50 ng of sample DNA (evaluated by a NanoDrop® ND-1000 spectrophotometer), 10 µM of each primer and 10 µl of Power SYBR™ Green PCR Master Mix (Applied BioSystems®). After activation of AmpliTaq Gold® enzymes (95°C for 10 min), amplification was performed at 95°C for 15 sec, and 60°C for 60 sec, in a total of 40 cycles.

Samples were run in triplicates and Ct values for each species of *Leishmania* and combinations of primer pairs were compared with the Ct values of the positive controls. Wilcoxon Rank-Sum test was applied, and p-values lower than the Bonferroni-corrected significance level (0.0056) were defined as negative (i.e., no amplification), while the opposite was defined as positive (i.e., amplification).

DNA samples of the aforementioned strains and the 13A/13B genus-specific primers (Rodgers et al., 1990) were used as positive controls.

## Results and Discussion

This study combined *in silico* (e-PCR) and *in vitro* (qPCR) analyses to evaluate the specificity of primer pairs for molecular differentiation of *Leishmania* spp. Before testing primers *in vitro*, researchers have often used Primer-BLAST (NCBI) to predict the outcome of PCR assays. However, Primer-BLAST allows for testing only one pair of primers at a time. Therefore, e-PCR is a more powerful strategy, given that it allows for the simultaneous screening of primers, which enabled us to narrow the test list down.

A total of 141 pairs of primers mined from the literature and described as species-, complex-, subgenus- or genus-specific were evaluated for their specificity while amplifying *Leishmania* DNA (Table S1). e-PCR evaluations resulted in positive *in silico* amplifications for 94 of these primer pairs (66.7%), of which only 36 (25.5%) matched their expected specificity. After filtering, 23 primer pairs (16.3%) met the selection criteria described in the material and methods section. Out of these, we prioritized the test of nine pairs covering species of primary interest in Brazil for *in vitro* testing by qPCR. The results of five of these pairs (55.5%) were in agreement with previous reports (Table 1) (Supplementary file - Figure S1).

In our study, the *in silico* and *in vitro* test results confirmed that ISVB-ISVC primers are specific for *L. braziliensis* (Castilho et al., 2003). Also, the specificity of primers 11H, 6F and B6F was confirmed for the subgenus *Viannia*, corroborating the findings of Oddone et al. (2009).

Primers G6PD-LLF/G6PD-LLR, previously mentioned as specific for the subgenus *Leishmania* (Castilho et al., 2008), showed specificity for the *L. mexicana* complex, amplifying a DNA fragment present in *L. mexicana* and *L. amazonensis*.

Primers b1-b2 amplified *L. braziliensis* DNA by e-PCR and by qPCR, as previously reported (Mimori et al., 1998). However, in the qPCR test *L. shawi* DNA was also amplified. As *L. shawi* is one of the species belonging to the subgenus *Viannia*, this pair of primers was not considered species-specific but only subgenus specific.

MLF-MLR primers (Ceccarelli et al., 2014) did not succeed in differentiating the subgenera *Leishmania* and *Viannia* by either e-PCR or qPCR. However, Ceccarelli et al. (2014) reported that these primers enable subgenus differentiation when amplification is followed by high resolution melting (HRM) analysis, which we did not perform. Thus, without HRM analysis, we suggest the use of this primer set in qPCR solely for genus-specific analysis.

Although the electronic PCR amplification of LEISH1-LEISH2 primers corroborated the literature in indicating the primer pair as specific for *L. infantum* (Francino et al., 2006), by qPCR we observed DNA amplification of *L. infantum*, *L. guyanensis*, *L. major*, *L. mexicana* and *L. shawi* species.

Primers Mary-f/Mary-r, which were developed to quantify *L. infantum* in biological specimens using kinetoplast DNA (kDNA) as the molecular target (Mary et al., 2004), showed a non-specific pattern in this study. This lack of

**Table 1.** Specificity of primers according to the literature search and the results of *in silico* (e-PCR) and *in vitro* (qPCR) analysis.

Primer	References	Forward	Reverse	Minimum target size by ePCR	Maximum target size by ePCR	Literature specificity	e-PCR specificity	qPCR specificity
ISVB-ISVC*	Castilho et al. (2003)	TACTGGCCATGTCGGAGG	ATCACAATGATGGTCAACGCAC	235	235	Species-specific ( <i>L. braziliensis</i> )	<i>L. braziliensis</i>	<i>L. braziliensis</i>
11H*	Oddone et al. (2009)	CACACCTGCTACTGGTCCTC	TCTGTTTCATGACATGCCTTT	86	160	Subgenus-specific ( <i>Viannia</i> complex)	<i>L. guyanensis</i> ; <i>L. braziliensis</i> ; <i>L. panamensis</i>	<i>L. shawi</i> ; <i>L. braziliensis</i> ; <i>L. guyanensis</i> ; <i>L. panamensis</i>
6F*	Oddone et al. (2009)	CAACAGCAAAAGCACAAAGAA	CAGCAATGCCGATAAGAAAC	83	112	Subgenus-specific ( <i>Viannia</i> complex)	<i>L. braziliensis</i> ; <i>L. panamensis</i>	<i>L. shawi</i> ; <i>L. braziliensis</i> ; <i>L. guyanensis</i> ; <i>L. panamensis</i>
B6F*	Oddone et al. (2009)	CACCTCTTGCCCTGCACCT	TTTAAACGTCGGTCTGTGTG	81	187	Subgenus-specific ( <i>Viannia</i> complex)	<i>L. braziliensis</i>	<i>L. shawi</i> ; <i>L. braziliensis</i> ; <i>L. guyanensis</i> ; <i>L. panamensis</i>
G6PD-LLF/ G6PD-LLR*	Castilho et al. (2008)	CTTGTGGCCTCCGGCTAC	GGCCATGTAAGCATCCTCAT	101	101	Subgenus-specific ( <i>Leishmania</i> complex)	<i>L. mexicana</i>	<i>L. mexicana</i> ; <i>L. amazonensis</i>
b1-b2	Mimori et al. (1998)	GTGGGGTATCTGCTGATGAC	CAAAAAGCGAGGGACTGCGGA	103	103	Species-specific ( <i>L. braziliensis</i> )	<i>L. braziliensis</i>	<i>L. braziliensis</i> ; <i>L. shawi</i>
MLF-MLR	Ceccarelli et al. (2014)	CGTTCTGCGAAAACCGAAA	CGGCCCTATTTTACACCAACC	110	112	Subgenus-specific ( <i>Leishmania</i> and <i>Viannia</i> complex)	<i>L. braziliensis</i> ; <i>L. infantum</i> ; <i>L. major</i> ; <i>L. panamensis</i> ; <i>L. tarentolae</i>	<i>L. infantum</i> ; <i>L. major</i> ; <i>L. mexicana</i> ; <i>L. shawi</i> ; <i>L. braziliensis</i> ; <i>L. guyanensis</i> ; <i>L. panamensis</i> ; <i>L. chagasi</i>
LEISH1/ LEISH2	Francino et al. (2006)	AACTTTTCTGGTCTCCGGGTAG	ACCCCCAGTTTCCCGCC	119	119	Species-specific ( <i>L. infantum</i> )	<i>L. infantum</i>	<i>L. major</i> ; <i>L. infantum</i> ; <i>L. mexicana</i> ; <i>L. shawi</i> ; <i>L. guyanensis</i> ; <i>L. chagasi</i>
Mary-f/ Mary-r	Mary et al. (2004)	CTTTTCTGGTCTCCGGGTAGG	CCACCCGGCCCTATTTTACACCAA	140	140	Species-specific ( <i>L. infantum</i> )	<i>L. infantum</i>	<i>L. major</i> ; <i>L. infantum</i> ; <i>L. mexicana</i> ; <i>L. shawi</i> ; <i>L. braziliensis</i> ; <i>L. guyanensis</i> ; <i>L. panamensis</i> ; <i>L. chagasi</i>

\*Primers which resulted in specificity at the species, complex or subgenus level.

specificity confirms the findings reported by Ceccarelli et al. (2014), who evaluated the same primers and observed DNA amplification of the species *L. amazonensis*, *L. braziliensis*, *L. guyanensis*, *L. infantum* and *L. panamensis*. Although kDNA is a good target for sensitive detection of *Leishmania* spp. its specificity is low due to the fact that kDNA are small circular molecules of DNA presenting different subclasses, with high conserved regions and copy number (Ceccarelli et al., 2017; Kocher et al., 2018). PCR and qPCR techniques using kDNA targets have already shown to result in amplification of DNA fragment in more than one species of *Leishmania* (Ceccarelli et al., 2014, 2017; Losada-Barragán et al., 2016).

Until recently, maxicircle kDNA of many species of *Leishmania* were not completely sequenced. Although next-generation sequencing has increasingly being used for nuclear genome assembly, few works used this technology to study *Leishmania* mitochondrial genomes (Simpson et al., 2015; Camacho et al., 2019; Kaufer et al., 2019; Urrea et al., 2019) This lack of kDNA genomic information may be a limitation for *in silico* analysis.

It should be noted that primers that showed specificity by e-PCR, as reported in the literature, but were not selected for further qPCR evaluation because they did not meet the criteria established in this study, should not be discarded. For instance, we selected the optimal DNA product size range to run qPCR (Tevfik Dorak, 2006). However, primers with product sizes larger than the chosen upper limit could work in conventional PCR. The number of gaps and mismatches may also influence the success of the analysis. In this study, the absence of gaps or mismatches in the alignments by e-PCR with the target species of *Leishmania* was chosen to limit the results to more specific alignments. Indeed, a single mismatch near the 3' end positions could have a severe impact on amplification (Bru et al., 2008). Primers with less than five gaps and mismatches in alignment with other sequences of species of *Leishmania* were excluded to avoid possible non-specific alignment during qPCR.

Although the e-PCR was conducted using genomes collected until April 2018, we searched for updates in August 2020 and no new species have been added to the database used here (NCBI and TriTrypDB). In addition, the lack of genomic information in the public and private databases influences the rate of false positive (i.e., no amplification on non-target species) and false negative (i.e., no amplification on target species) results. However, increasing the availability of genomic information in public databases (Akhoundi et al., 2017) should favor future studies. This study shows that e-PCR is an extremely useful tool for both time and resources optimization in the design of hybridization- and amplification-based assays.

Another important fact is that, although qPCR is known to be highly sensitive and specific (Galluzzi et al., 2018), there are some conditions that cannot be controlled to the same extent as in e-PCR, and some clear examples of this are the number of gaps and mismatches.

We conducted qPCR assays using only the main American *Leishmania* reference strains to evaluate our *in silico* results. However, additional strains should also be tested to ensure the specificity of species present in other regions.

The results obtained in this study demonstrate the advantage of evaluating a set of primers *in silico* prior to *in vitro* analysis. This evaluation enabled us to rapidly identify numerous primers with the desired specificity. However, the *in silico* and *in vitro* analysis showed divergent results, which may be explained by the threshold definition in the filtering step, by the current lack of genomic information in the databases, and/or by the small number of *Leishmania* strains tested. Nevertheless, compared to the time and resources needed to test the full set of primers *in vitro*, the new approach proposed herein greatly increases efficiency in the design of candidate primers, even if false positive and false negative primers may occasionally occur.

Primers previously reported and analyzed in this study to identify *Leishmania* at subgenus-, complex- and species-specific level could be used for differentiating *Leishmania* spp. not only to improve diagnosis performance but also in biodiversity analysis. Currently, DNA metabarcoding has been used for the detection of targeted species in environmental samples. Since metabarcoding protocols include PCR-based enrichment of specific DNA fragments that are found in the species of interest (D'Avila-Levy et al., 2015), the set of primers here evaluated could be also used in DNA metabarcoding given they can differentiate a genus and/or species.

Given the fact that the genus *Leishmania* has many species and specific diagnosis may be essential the strategy used in this study, i.e., performing *in silico* analysis by e-PCR followed by qPCR, drastically reduced the cost and time needed to identify primers with the desired specificity. As the volume of genomic information grows at an astonishing speed, this methodology is also recommended for the optimization of molecular analysis for any species.

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### Supplementary files

Supplementary material accompanies this paper.

**Table S1.** Table list of pairs of species-, subgenus-, complex- or genus-specific primers described in the literature and details of their amplification.

**Table S2.** Table of information about the dataset used in the electronic PCR step including the genomic sequences information of dog, human, sand fly and trypanosomatids.

**Figure S1.** Box plot of the DNA fragment amplifications of *Leishmania* spp. by qPCR according to Ct values. Positive controls are represented in blue, negative controls in black, samples considered positive in green, and negative in red.

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