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Data Article

Data on the differentiation among *Leishmania* (*Viannia*) spp., *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis* in Brazilian clinical samples using real-time PCR

Aurora Diotallevi [a], Gloria Buffi [a], Marcello Ceccarelli [a], Herintha Coeto Neitzke-Abreu [b], Laisa Vieira Gnutzmann [b], Manoel Sebastião da Costa Lima Junior [c], Alice Di Domenico [a], Mauro De Santi [a], Mauro Magnani [a], Luca Galluzzi [a, *]

[a] *Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, PU, Italy*
[b] *Programa de Pós-Graduação em Ciências da Saúde, Faculdade de Ciências da Saúde, Universidade Federal da Grande Dourados (UFGD), 79804-070, Dourados, MS, Brazil*
[c] *Fundação Oswaldo Cruz (FIOCRUZ), Instituto Aggeu Magalhães, 50740-465, Recife, PE, Brazil*

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ABSTRACT

This article contains the data regarding *Leishmania* species identification in human and canine clinical samples from a Brazilian region endemic for *Leishmania* (*Viannia*) spp., *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis*, using a previously developed approach involving two qPCR assays (qPCR-ML and qPCR-ama). The data are related to the article "Real-time PCR to differentiate among *Leishmania* (*Viannia*) subgenus, *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis*: application on Brazilian clinical samples" [1], and include also details of clinical evaluation/diagnosis of human patients and primer sequences used in the qPCR assays. The *Leishmania* species has been determined in 27 canine samples and 11 human samples, exploiting HRM analysis of qPCR-ML and Cq values of qPCR-ML and qPCR-ama, as reported previously [2]. The qPCR data were in agreement with the species characterization obtained with other methods such as conventional species-specific PCR, ITS1 PCR-RFLP or DNA sequencing. Despite the limited number of clinical samples, these data are

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* Corresponding author.
E-mail address: luca.galluzzi@uniurb.it (L. Galluzzi).

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encouraging for a potential application in regions where *L.* (*Viannia*) spp., *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis* are co-endemic.

Specifications Table

Subject	Parasitology
Specific subject area	Molecular diagnostics
Type of data	Table Figure
How data were acquired	The qPCR assays were performed using Rotor-Gene 6000 instrument (Corbett life science). The qPCR runs were analyzed with Rotor-Gene software version 1.7 to obtain Cq values and High resolution melting temperatures.
Data format	Raw Analyzed
Parameters for data collection	DNA extracted from clinical samples and strains was spotted on filter paper for storage and laboratory transfer. The qPCR assays (qPCR-ML and qPCR-ama), as well as ITS1 PCR, were performed directly from a punch of filter paper, with a pre-amplification step.
Description of data collection	HRM data obtained with qPCR-ML allowed to discriminate between subgenera *Viannia* and *Leishmania*. In case of subgenus *Leishmania*, the discrimination between *L. (L.) infantum* and *L. (L.) amazonensis* is performed through comparison of qPCR-ML and qPCR-ama Cq values. Both HRM and Cq data were collected by amplification of DNA extracted from clinical samples and spotted on filter paper.
Data source location	**Canine samples** Institution: Control Center of Zoonoses (CCZ) City/Town/Region: Campo Grande City/Mato Grosso do Sul Country: Brazil **Human samples** Institution: Hospital Universitário City/Town/Region: Dourados City/Mato Grosso do Sul Country: Brazil
Data accessibility	With the article
Related research article	Aurora Diotallevi, Gloria Buffi, Marcello Ceccarelli, Herintha Coeto Neitzke-Abreu, Laisa Vieira Gnutzmann, Manoel Sebastião da Costa Lima Junior, Alice Di Domenico, Mauro De Santi, Mauro Magnani, Luca Galluzzi. Real-time PCR to differentiate among *Leishmania* (*Viannia*) subgenus, *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis*: application on Brazilian clinical samples. Acta Tropica, volume 201, January **2020**, 105178

Value of the Data

- These data are useful to point out the potential field application of our SYBR Green-based qPCR assays to distinguish among *Leishmania* (*Viannia*) subgenus, *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis*, exploiting HRM and Cq values.
- The rapid Leishmania species identification can be particularly useful for diagnosis in regions where *Leishmania* (*Viannia*) subgenus, *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis* are co-endemic.
- These data can be considered to further develop qPCR-based assays for other species differentiation in the *Viannia* subgenus (e.g. *L.* (*V.*) *braziliensis* and *L.* (*V.*) *panamensis*) or in the *Leishmania* subgenus (e.g. *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana*).

1. Data

The presented data first describe the Clinical evaluation/diagnosis of human patients (Table 1) and the primers used in the qPCR assays targeting *Leishmania* minicircle kDNA (qPCR-ML and qPCR-ama) and host genes (Table 2). Then, the Cq and HRM values obtained from qPCR-ML and qPCR-ama used

Table 1
Diagnosis and clinical evaluation of human patients.

Sample ID	Diagnosis	Patient clinical evaluation
E1	VL	Anemia, splenomegaly
E2, E3, E4	VL	No clinical information
E5	VL	HIV patient, fever, pancytopenia, hyporexia
E6, E7	VL	Pancytopenia, hepatosplenomegaly
E8	VL	5 years old; hepatosplenomegaly, anemia, submandibular ganglia, fever, pancytopenia, abdominal distension
E9	n.a.	No clinical information
F2	CL	Nasal lesion
F3	VL	Anemia, splenomegaly

for *Leishmania* species identification in canine (n = 36) and human (n = 11) clinical samples spotted on filter paper are presented (Table 3 and Table 4, respectively). Single replicate data for canine and human samples are represented in supplementary file 1 and 2, respectively. As a further confirmation of *Leishmania infantum* species identification, ITS1 amplicons from two human clinical samples were directly sequenced (supplementary file 3 and 4) and a phylogenetic analysis was performed with ITS1 sequences from *L. (V.) braziliensis* (n = 21) *L.* (*L.*) *infantum* (n = 7) and *L.* (*L.*) *amazonensis* (n = 4) available in genbank database (Fig. 1). Moreover, the data also show the specificity of qPCR-ML and qPCR-ama assays in the conditions used to amplify DNA samples from filter paper (Fig. 2).

2. Experimental design, materials, and methods

2.1. Canine and human clinical samples

Thirty-six canine clinical samples and 11 human clinical samples were collected in Mato Grosso do Sul (Brazil), an endemic area of leishmaniasis. The canine samples consisted in 13 peripheral blood (A1-A9, B1−B4) and 23 bone marrow (B5−B9, C1−C9, D1-D9), collected by the Control Center of Zoonoses (CCZ) of Campo Grande City. Dogs were diagnosed positive for visceral canine leishmaniasis (VCL) by the immunochromatographic Dual-Path Platform (DPP™, Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil) and the ELISA test (EIE™; Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil), and direct identification of Leishmania amastigotes from Giemsa-stained smears analyzed by optical microscopy.

The human samples consisted in 7 peripheral blood (E1, E5-E8, F2, F3) and 4 bone marrow (E2-E4, E9), collected in Hospital Universitário of Dourados City from patients with a diagnosis of leishmaniasis. After evaluation of clinical signs (Table 1), patients were diagnosed using serology rapid test (rK39) (Kalazar Detect™; InBios, Washington, US) and/or direct identification of Leishmania amastigotes from Giemsa-stained smears analyzed by optical microscopy.

2.2. DNA extraction

DNA from canine and human clinical samples, positive for *Leishmania* infection, was obtained as described in [1]. Briefly, 300 μl volume of sample was added to 500 μl of 20% Sodium Dodecyl Sulfate

Table 2
Primer sequences for qPCR. Primer names are in bold.

Target	Forward primer (5′-3′)	Reverse primer (5′-3′)
B2M canine	GTCCCACAGATCCCCCAAAG	CTGGTGGATGGAACCCTGAC
GAPDH human	CCATGTTCGTCATGGGTGTG	GGTGCTAAGCAGTTGGTGGTG
kDNA (qPCR-ML)	**MLF** - CGTTCTGCGAAAACCGAAA	**MLR** - CGGCCCTATTTTACACCAACC
kDNA (qPCR-ama)	**LMi-amaF** - AAAATGAGTGCAGAAACCC	**MLR** - CGGCCCTATTTTACACCAACC

B2M canine, *Canis familiaris* Beta-2-Microglobulin.
GAPDH human, *Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase.

Table 3
qPCR-ML and qPCR-ama data in canine clinical samples.

Source	clinical sample (ID)	qPCR-ML (Cq ± SD)	qPCR-ama (Cq ± SD)	qPCR-ML (HRM T_m °C)	Species identification
Peripheral blood	A1[a]	n.d.	n.d.	n.d.	–
	A2[a]	n.d.	n.d.	n.d.	–
	A3[a]	n.d.	n.d.	n.d.	–
	A4[a]	20.58 ± 0.62	31.83 ± 0.89	84.08 ± 0.11	*L.* (*L.*) *infantum*
	A5[a]	26.51 ± 0.40	n.d.	84.12 ± 0.02	*L.* (*L.*) *infantum*
	A6[a]	n.d.	n.d.	n.d.	–
	A7[a]	n.d.	n.d.	n.d.	–
	A8[a]	n.d.	n.d.	n.d.	–
	A9[a]	27.60 ± 1.39	39.23 ± 0.81	84.03 ± 0.04	*L.* (*L.*) *infantum*
	B1[a]	n.d.	n.d.	n.d.	–
	B2[a]	n.d.	n.d.	n.d.	–
	B3[a]	23.69 ± 0.14	32.28 ± 0.66	83.64 ± 0.01	*L.* (*L.*) *infantum*
	B4[a]	18.16 ± 0.35	24.77 ± 0.16	83.95 ± 0.32	*L.* (*L.*) *infantum*
Bone marrow	B5[a]	17.96 ± 0.17	26.09 ± 0.09	83.94 ± 0.08	*L.* (*L.*) *infantum*
	B6[a]	22.90 ± 0.30	30.34 ± 0.08	84.08 ± 0.11	*L.* (*L.*) *infantum*
	B7[a]	17.39 ± 4.53	25.93 ± 3.47	83.79 ± 0.02	*L.* (*L.*) *infantum*
	B8[a]	18.18 ± 3.62	25.12 ± 2.64	84.05 ± 0.14	*L.* (*L.*) *infantum*
	B9[a]	25.58 ± 1.20	33.57 ± 0.86	84.90 ± 0.04	*L.* (*L.*) *infantum*
	C1[a]	17.41 ± 3.39	26.27 ± 2.25	83.85 ± 0.02	*L.* (*L.*) *infantum*
	C2[a]	14.80 ± 0.23	22.62 ± 0.01	83.96 ± 0.08	*L.* (*L.*) *infantum*
	C3[a]	16.02 ± 0.13	23.73 ± 0.07	84.12 ± 0.02	*L.* (*L.*) *infantum*
	C4[a]	17.53 ± 0.06	25.20 ± 0.06	83.97 ± 0.07	*L.* (*L.*) *infantum*
	C5[a]	14.57 ± 0.22	22.43 ± 0.13	83.90 ± 0.07	*L.* (*L.*) *infantum*
	C6[a]	25.95 ± 10.55	n.d.	84.86 ± 0.05	*L.* (*L.*) *infantum*
	C7[a]	20.38 ± 0.50	26.40 ± 3.57	84.10 ± 0.05	*L.* (*L.*) *infantum*
	C8[a]	22.52 ± 0.50	n.d.	84.05 ± 0.05	*L.* (*L.*) *infantum*
	C9[a]	30.81 ± 0.35	n.d.	84.10 ± 0.07	*L.* (*L.*) *infantum*
	D1[a]	n.d.	n.d.	n.d.	–
	D2[a]	28.51 ± 3.35	n.d.	84.03 ± 0.05	*L.* (*L.*) *infantum*
	D3[b]	20.04 ± 0.12	32.36 ± 0.35	80.44 ± 0.04	*L.* (*L.*) *infantum*
	D4[b]	17.65 ± 0.18	28.62 ± 0.93	80.08 ± 0.09	*L.* (*L.*) *infantum*
	D5[b]	12.14 ± 0.01	23.68 ± 0.32	79.95 ± 0.03	*L.* (*L.*) *infantum*
	D6[b]	12.17 ± 0.09	22.63 ± 0.17	80.13 ± 0.11	*L.* (*L.*) *infantum*
	D7[b]	8.72 ± 0.01	18.85 ± 0.13	80.06 ± 0.04	*L.* (*L.*) *infantum*
	D8[b]	8.38 ± 0.18	18.50 ± 0.05	80.07 ± 0.08	*L.* (*L.*) *infantum*
	D9[a]	30.75 ± 0.04	n.d.	84.10 ± 0.08	*L.* (*L.*) *infantum*

n.d. = not detectable.
[a] Samples tested with SYBR green PCR master mix (Diatheva srl).
[b] Samples tested with RT2 SYBR Green ROX FAST Mastermix (Qiagen).

(Sigma-Aldrich) and homogenized. Then, 400 μl of chloroform and 300 μl of protein precipitation solution (3 M potassium acetate, 11% glacial acetic acid) were added. After centrifugation at 10,000×*g* for 10 min, the supernatant was transferred to new tubes containing 1 ml of cold absolute ethanol, gently homogenized by inversion and centrifuged for 5 min at 10,000×*g*. The pellets were washed twice with 1 ml of 70% cold ethanol each time and centrifuged for 2 min at 10,000×*g*. The supernatant was discarded and the pellet was dried in a dry bath (AccuBlock) at 65 °C for 5 min. The DNA pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) to a final concentration of 200–500 ng/μl, stored at 4 °C for 24 h, and then frozen at −20 °C. For sample transfer, DNA samples (8 μl) were spotted on filter paper (Macherey-Nagel MN 818), air-dried and stored at room temperature until analysis.

2.3. Quantitative PCR (qPCR) assays

The approach described in Ceccarelli et al. [2], consisting in running two qPCR reactions in parallel (qPCR-ML and qPCR-ama) to amplify different classes of minicircles, has been applied on sample DNA spotted on filter paper. The workflow of this approach involves first a discrimination between

Table 4
qPCR-ML and qPCR-ama data in human clinical samples.

Source	Human clinical sample (ID)	qPCR-ML (Cq ± SD)	qPCR-ama (Cq ± SD)	qPCR-ML (HRM T_m °C)	Species identification
Peripheral blood	E1[a]	28.28 ± 0.72	34.08 ± 0.71	84.20 ± 0.05	*L.* (*L.*) *infantum*
	E5[b]	28.81 ± 2.36	n.d.	83.27 ± 0.05[d]	*L.* (*L.*) *infantum*
				83.15 ± 0.07	*L.* (*V.*) *sp*
	E6[a]	19.12 ± 0.06	27.35 ± 0.23	84.20 ± 0.01	*L.* (*L.*) *infantum*
	E7[a]	31.12 ± 0.31	34.93 ± 0.74	83.94 ± 0.08	*L.* (*L.*) *infantum*
	E8[b]	23.64 ± 0.46[d]	24.06 ± 0.86[d]	83.77 ± 0.04[d]	*L.* (*L.*) *infantum*
				82.75 ± 0.28	*L.* (*V.*) *sp*
	F2[a]	33.85 ± 4.11	n.d.	84.18 ± 0.04	*L.* (*L.*) *infantum*
	F3[a]	29.19 ± 0.18	n.d.	84.14 ± 0.06	*L.* (*L.*) *infantum*
Bone marrow	E2[c]	30.32 ± 0.96	n.d.	80.06 ± 0.08	*L.* (*L.*) *infantum*
	E3[c]	n.d.	30.35 ± 1.55	n.d.	*L.* (*L.*) *amazonensis*
	E4[c]	31.99 ± 1.31	n.d.	80.65 ± 0.05	*L.* (*L.*) *infantum*
	E9[a]	27.78 ± 1.34	n.d.	84.62 ± 0.07	*L.* (*L.*) *infantum*

n.d. = not detectable.
[a] Samples tested with SYBR green PCR master mix (Diatheva srl).
[b] Samples tested with SYBR green PCR master mix (Diatheva srl) and TB Green premix ex *Taq*II Mastermix (Takara).
[c] Samples tested with RT2 SYBR Green ROX FAST Mastermix (Qiagen).
[d] values obtained with TB Green premix ex *Taq*II Mastermix (Takara).

subgenera *Viannia* and *Leishmania* based on qPCR-ML HRM analysis, performed with primers MLF and MLR (Table 2); if this analysis indicates subgenus *Leishmania*, the discrimination between *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis* is performed through comparison of qPCR-ML and qPCR-ama Cq values. The qPCR-ama was performed using the forward primer LMi-amaF and the reverse primer MLR (Table 2). All samples were tested blindly. To evaluate the DNA integrity and amplifiability in canine and human samples, canine beta-2-microglobulin (B2M) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified under the same conditions as the qPCR-ML and qPCR-ama (described below).

To ensure applicability with samples on filter paper, a pre-amplification step was introduced as follows. A punch of filter paper (2 mm in diameter) was placed in 40 μl SYBR green reaction mixture containing 200 nM of each primer. Three different PCR master mix were tested: SYBR green PCR master mix (Diatheva srl, Fano, Italy), RT2 SYBR Green ROX FAST Mastermix (Qiagen, Hilden, Germany), TB Green premix ex *Taq*II Mastermix (Takara Bio Europe, France). Tubes were placed in a thermal cycler (GeneAmp PCR System 2700), and pre-amplified under the following conditions: 94 °C for 5 min, 10 cycles at 94 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s. At the end of this pre-amplification step, the tubes were centrifuged for few seconds and placed in ice; the filter paper was removed and the reaction was split into two PCR tubes (20 μl each tube). Then, the tubes were placed in the Rotor-Gene 6000 instrument and amplified as follows: 45 cycles at 94 °C for 30 s, 60 °C for 20 s and 72 °C for 20 s. As reference, PCR mixtures containing template DNA isolated from *L. (L.) infantum* MHOM/FR/78/LEM75, *L. (L.) amazonensis* MHOM/BR/00/LTB0016 and L. *(V.) braziliensis* MHOM/BR/75/M2904 were included in each run. A no template control (NTC) was included for each primer pair reaction. To monitor non-specific products or primer dimers, a melting analysis was performed from 79 to 95 °C at the end of each run, with a slope of 1 °C/s, and 5 s at each temperature. The Cq values were evaluated using the quantification analysis of the RotorGene 6000 software.

Trypanosoma cruzi and human DNA were amplified using the conditions described above to confirm qPCR_ML and qPCR-ama specificity with the three master mix used and including the pre-amplification step. The absence of specific amplicons was confirmed by visualization on 2% agarose gel electrophoresis.

2.4. High-resolution melt (HRM) analysis

The high-resolution melt (HRM) analysis was performed immediately after the amplification reactions in the Rotor-Gene 6000 instrument. HRM analysis was conducted over the range from

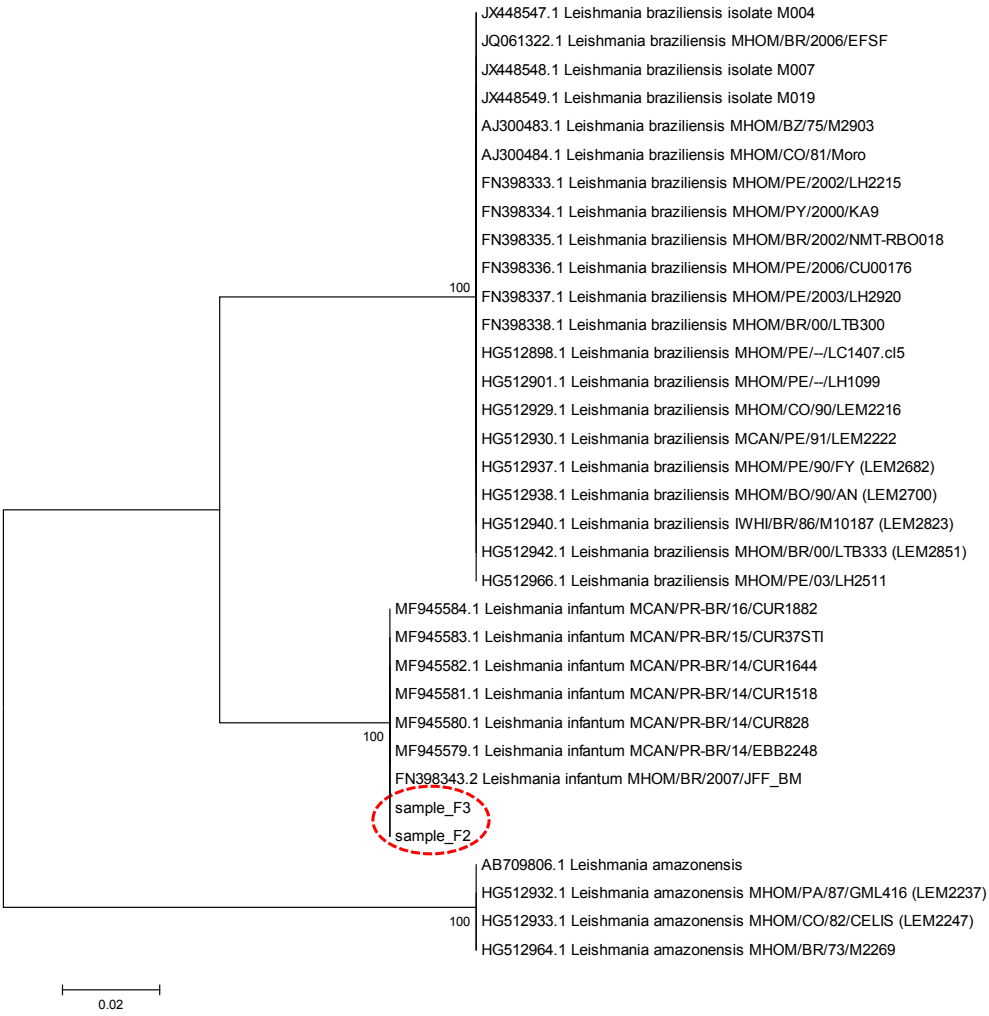

Fig. 1. Phylogenetic analysis of ITS1 fragments of F2 and F3 samples (red dotted line). The phylogenetic tree was constructed by using the maximum likelihood method and Tamura-Nei model. Numbers below branches represent bootstrap support.

79 °C to 90 °C, rising at 0.1 °C/s and waiting for 2 s at each temperature. Each sample was run in duplicate, and the gain was optimized before melting on all tubes. HRM curve analysis was performed with the derivative of the intensity of fluorescence at different temperatures (dF/dT), after smoothing, with the Rotor-Gene 6000 software. Template DNA isolated from *L. (L.) infantum* MHOM/FR/78/LEM75 and L. *(L.) amazonensis* MHOM/BR/00/LTB0016 were used as reference for *Leishmania* subgenus, while template DNA from *L. (V.) braziliensis* MHOM/BR/75/M2904 was used as reference for *Viannia* subgenus. Bins were set to define Tm of amplicons for each species. Automated classification of genotypes (i.e. subgenus *Leishmania* or *Viannia*) of unknown samples was performed by the Rotor-Gene software according to the presence of a derivative peak located within a defined temperature bin.

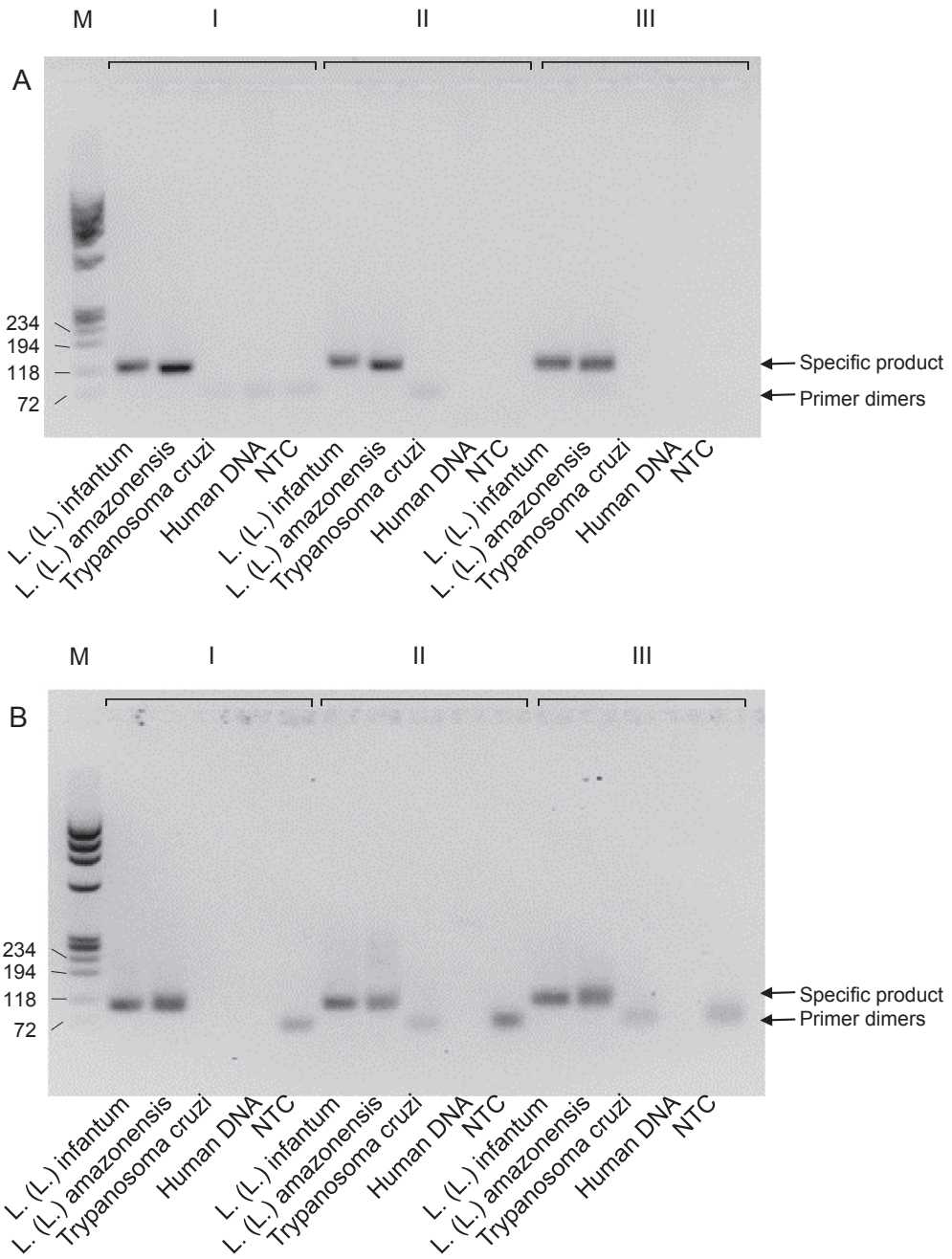

Fig. 2. Specificity of qPCR-ML (A) and qPCR-ama (B). The qPCR amplicons were run on a 2% agarose gel. Both qPCR assays were performed using three different PCR master mix: SYBR green PCR master mix, Diatheva (I); RT2 SYBR Green ROX FAST Mastermix, Qiagen (II); TB Green premix ex *Taq*II Mastermix, Takara Bio (III). For each master mix, DNA from *L.* (*L.*) *infantum* (0,006 ng/μl), *L.* (*L.*) *amazonensis* (0,15 ng/μl), *Trypanosoma cruzi* (0,1 ng/μl) and human DNA (30 ng/μl) were tested with the condition described in the manuscript. As negative control, a no template control was used for each qPCR run. M: ΦX174 DNA/*Bsu*RI (*Hae*III) Marker, 9 (ThermoFisher Scientific); NTC: no template control.

2.5. ITS1 DNA sequencing and phylogenetic analysis

To confirm *Leishmania* species identification, ITS1 fragments, obtained by ITS1-PCR RFLP [3], were sequenced in two human samples (F2 and F3) as follows. The ITS1 amplicons were directly digested with 10 U *Hae*III (Thermo Fisher Scientifc) at 37 °C for 3 h. The restriction fragments were visualized on a 3.5% high-resolution MetaPhor (Cambrex) agarose gel stained with GelRed (Biotium, Hayward, CA). The larger fragment was excised from gel, purified using MinElute Gel Extraction kit (Qiagen) and directly sequenced. DNA sequencing was performed using the BigDye Terminator v. 1.1 Cycle Sequencing Kit on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The F2 and F3 sequences were aligned with ITS1 sequences from *L.* (*L.*) *infantum* (n = 7), *L. (V.) braziliensis* (n = 21) and *L.* (*L.*) *amazonensis* (n = 4) available in GenBank database, using BioEdit Sequence Alignment Editor using default options. The phylogenetic analysis of aligned ITS1 fragments was conducted with MEGA 6 software. Phylogenetic relationships were inferred by using the maximum likelihood method and Tamura-Nei model. Bootstrap values were calculated from 100 replications.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dib.2019.104914.

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