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Real-time PCR to differentiate among *Leishmania* (*Viannia*) subgenus, *Leishmania* (*Leishmania*) *infantum* and *Leishmania* (*Leishmania*) *amazonensis*: application on Brazilian clinical samples

Running title: Leishmania species differentiation in clinical samples

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Abstract

Leishmaniasis is a complex disease caused by *Leishmania* species belonging to subgenera *Leishmania* and *Viannia*. In South America, *L.* (*L.*) *infantum* is considered the most important causative agent of visceral leishmaniasis, while *L.* (*L.*) *amazonensis* and *Viannia* subgenus species are responsible for the different cutaneous or mucocutaneous forms. In our previous work, we developed a diagnostic approach for *Leishmania* species discrimination based on two qPCRs (qPCR-ML and qPCR-ama) targeting the minicircle kDNA followed by melting analysis. This approach allowed to (i) differentiate the subgenera *Leishmania* and *Viannia*, and (ii) distinguish between *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis*. The aim of this work was to demonstrate the applicability of the approach previously described, using human and canine clinical samples and strains from a Brazilian region, where *L.* (*L.*) *infantum, L.* (*L.*) *amazonensis* and *Viannia* subgenus species coexist. After validation on New World strains, the diagnostic approach was applied blindly to 36 canine clinical samples (peripheral blood and bone marrow) and 11 human clinical samples (peripheral blood and bone marrow). The sensitivity was 95.6% (95% confidence interval 77.3-100%) and 100% (95% confidence interval 76.9-100%) in the canine bone marrow samples and human (peripheral blood and bone marrow) samples, respectively, compared to conventional PCR assays. Concerning the *Leishmania* species identification, the conventional and qPCR-based methods showed kappa value of 0.876 (95% confidence interval 0.638-1.000), indicating good agreement. Therefore, this approach proved to be useful in both veterinary and human clinical context in regions co-endemic for *L.* (*L.*) *infantum*, *L.* (*L.*) *amazonensis,* and *Viannia* subgenus, helping to provide rapid diagnosis and to allow studies of species distribution.

Keywords: *Leishmania infantum*; *Leishmania amazonensis*; *Viannia*; qPCR; HRM; minicircle kDNA; diagnostics.

1. Introduction

Leishmaniasis is a neglected tropical disease caused by *Leishmania* species and transmitted by the bite of female phlebotomine sandflies. Leishmaniasis shows a worldwide distribution and affects about 12 million people. In fact, the parasite *Leishmania* is spread in Europe, Africa, Asia (Old World) as well as in the Americas (New World) with the highest number of cases in developing countries. A total of 0.7–1 million new cases of leishmaniasis and 20,000–30,000 deaths every year has been estimated (World Health Organization, 2018).

More than 20 *Leishmania* species, belonging to subgenera *Leishmania* and *Viannia*, cause infection in humans. The *Leishmania* (*Leishmania*) species are the etiological agents of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). The *Leishmania* (*Viannia*) species, limited to tropical and subtropical America, are etiological agents of CL and mucocutaneous leishmaniasis (MCL) (Akhoundi et al., 2016). *Leishmania* (*L.*) *infantum* is the only *Leishmania* species reported in both the Old and New World. Recently, microsatellite analysis revealed that *L.* (*L.*) *infantum* in the New World (also known as *L. chagasi*) had been imported from southwest Europe (Kuhls et al., 2011). In the New World, *Viannia* subgenus, *L.* (*L.*) *amazonensis* and occasionally *L.* (*L.*) *infantum* are responsible for the different cutaneous forms in humans (da Silva et al., 2010); importantly, *L.* (*L.*) *amazonensis* can also cause visceralization (de Souza et al., 2018). The cutaneous forms can be very heterogeneous (e.g., localized, diffuse, disseminated or mucosal forms) depending on the species of *Leishmania* and the immunological and nutritional status of the human host. Therefore, species identification could be one of the prognostic factors for the possible evolution of the disease (Anversa et al., 2018; Machado et al., 2019). Concerning canine leishmaniasis in South America, *L.* (*L.*) *infantum* and *L.* (*V.*) *braziliensis* are considered the most important causative agents (Dantas-Torres, 2009), although also *L.* (*L.*) *amazonensis* was isolated from dogs with visceral leishmaniasis in Brazil (Valdivia et al., 2017). These findings highlight the importance of using proper diagnostic assays to distinguish the *Leishmania* species involved in each case of leishmaniasis and coexisting in the same geographic area (Galluzzi et al., 2018). In this view,

several PCR-based assays have been developed. In case of a generic PCR, the downstream analysis of PCR product is required to gain information about parasite species. The most affordable technique is restriction fragment length polymorphism (RFLP) analysis, where electrophoretic analysis of the DNA fragments generated by a restriction enzyme may allow the species identification. Specifically, ITS1-PCR RFLP with the enzyme *Hae*III has been developed by Schonian et al. (Schönian et al., 2003) and further assessed by in silico RFLP analysis (Van der Auwera and Dujardin, 2015).

In previous work, we developed a new and affordable diagnostic approach for *Leishmania* species discrimination. This approach, based on two qPCRs (qPCR-ML and qPCR-ama) targeting the kDNA followed by High Resolution Melting (HRM) analysis, allowed to (i) differentiate the subgenera *Leishmania* and *Viannia*, and (ii) distinguish between *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis*, exploiting the different abundance of minicircle subclasses (Ceccarelli et al., 2017) (Ceccarelli et al. Submitted manuscript). Briefly, the workflow of our method involves first a discrimination between subgenera *Viannia* and *Leishmania* based on HRM analysis of qPCR-ML amplicons. Then, to distinguish between *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis*, the qPCR-ama is performed. The qPCR-ama assay is designed to amplify a minicircle subclass predominant in *L.* (*L.*) *amazonensis*, therefore discrimination between *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis* is achieved through comparison of qPCR-ML and qPCR-ama Cq values.

This work aimed to validate the approach previously described, using human and canine clinical samples (peripheral blood and bone marrow) previously characterized and strains from a Brazilian region, where *L.* (*L.*) *infantum, L.* (*L.*) *amazonensis* and *Viannia* subgenus species can coexist.

2. Methods

2.1 Ethical statement

This research was approved by the Committee on Ethics in Animal (protocol number 27/2016) and by the Human Research Ethics Committee (protocol number 1.662.728/2016) of the Universidade Federal da Grande Dourados.

2.2 Experimental design

To test the field applicability of our previously developed qPCRs, which differentiate the subgenera *Leishmania* and *Viannia*, and distinguish between *L*. (*L*.) *infantum* and *L*. (*L*.) *amazonensis*, we used *Leishmania* strains and clinical samples from Mato Grosso do Sul (Brazil), where *L*. (*L*.) *infantum*, *L*. (*L*.) *amazonensis* and *Viannia* subgenus species can coexist. The experimental design is depicted in Fig. 1. Briefly, after serological test and/or optical microscopy examination, human and canine clinical samples were subjected to DNA extraction. The presence of *Leishmania* DNA, as well as *Leishmania* species characterization was first assessed using well consolidated molecular methods such as conventional PCR (genus and/or species specific). Then, clinical sample DNA, as well as lysates of certified *Leishmania* spp. Brazilian strains, were spotted on filter paper and sent to a different lab for ITS1-PCR RFLP, DNA sequencing and qPCR analysis. Finally, the results of qPCR assays and conventional methods (i.e. species-specific PCR, ITS1-PCR RFLP, DNA sequencing) were compared. All methods are explained in details below.

2.3 Canine and human samples

Thirty-six canine clinical samples and 11 human clinical samples were collected in Mato Grosso do Sul State, located in the Center-West Region of Brazil, an endemic area of leishmaniasis. The dog samples, i.e., 13 peripheral blood (A1-A9, B1-B4) and 23 bone marrow (B5-B9, C1-C9, D1-D9), were collected by the Control Center of Zoonoses (CCZ) of Campo Grande City. Peripheral blood was collected by jugular venipuncture, while the bone marrow was collected from the iliac crest or sternum bone, in EDTA tubes. Dogs were referred to CCZ for euthanasia because they were

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), serologic recommended tests by the Brazilian Ministry of Health, and direct identification of *Leishmania* amastigotes from Giemsa-stained smears analyzed by optical microscopy.

The human samples, 7 peripheral blood (E1, E5-E8, F2, F3) and 4 bone marrow (E2-E4, E9), were collected in Hospital Universitário of Dourados City from patients with a diagnosis of leishmaniasis. Peripheral blood was collected by venipuncture of the upper limb, while the bone marrow was collected from sternum bone in EDTA tubes. 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, together with clinical evaluation. In details, patients E1 and F3 had anemia and splenomegaly, and they were diagnosed with visceral leishmaniasis. Samples E6 and E7 were from the same patient who had pancytopenia and hepatosplenomegaly. Sample E8 was from a 5 years old patient having hepatosplenomegaly, anemia, submandibular ganglia, fever, pancytopenia, abdominal distension. Sample E5 was from an AIDS patient with HIV dementia, fever, pancytopenia, hyporexia. Patient F2 had a nasal lesion with biopsy compatible with CL. Samples E2, E3, E4 were from the same patient who had a late diagnosis of VL and complications, which culminated in death. Diagnosis details are summarized in supplementary Table 1 (Data in Brief).

2.4 DNA extraction

DNA from canine and human clinical samples was obtained as described previously (Araújo et al., 2009), with some modifications. A 300 µl volume of blood was added to 500 µl of 20% SDS (Sodium Dodecyl Sulfate) (Sigma-Aldrich) and homogenized. Then, 400 µl of chloroform and 300 µl of protein precipitation solution (3M potassium acetate, 11% glacial acetic acid) were added. After centrifugation at 10,000 x 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 x g. The pellets were washed twice with 1 ml of 70% cold ethanol each time and centrifuged for 2 min at 10,000 x 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 qPCR, DNA samples (8 µl) were spotted on filter paper (Macherey-Nagel MN 818), air-dried and stored at room temperature until analysis.

2.5 *Leishmania* spp. Brazilian strains

L. (*L.*) *infantum* MHOM/BR/2002/LPC-RPV, *L.* (*L.*) *amazonensis* WHOM/BR/75/JOSEFA and *L.* (*V.*) *braziliensis* MHOM/BR/1987/M11272 were cultivated in 199 Medium (Invitrogen) supplemented with 1% human urine, 10% fetal calf serum (Invitrogen) and 2 mM L-glutamine (Gibco-BR), at 25 °C in a B.O.D. incubator (Logen Scientific). The parasites were washed by centrifugation at 1,600 x g for 10 min with phosphate-buffered saline (PBS) pH 7.2. Parasites were counted in a Neubauer chamber, diluted with PBS at $1x10^4$ promastigotes/µl and stored at -20 °C until DNA extraction. The DNA was obtained by incubating 100 µl at 95 ºC for 30 min in a thermal cycler (Biorad, T100 Thermal Cycler); cell lysate was centrifuged at 13,000 x g for 1 min, the supernatant was transferred to a clean tube and used directly as amplification template (Marcussi et al., 2008). For qPCR, cell lysate containing DNA was spotted on filter paper (Macherey-Nagel MN 818) in duplicates, as described above.

2.6 Genus and species-specific PCR assays

Leishmania parasites were detected by PCR using genus-specific primers 13A and 13B targeting kDNA minicircle of both old world and new world species (Rodgers et al., 1990). The reaction (25 µl) contained 0.4 µM of each primer (Sigma), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen), 1.5 U Taq DNA Polymerase (Phoneutria), 1X enzyme buffer and 2 µl extracted DNA (200-500 ng/µl).

177 The amplification was performed in a thermal cycler (Biorad, T100 Thermal Cycler) at 95 °C for 5
178 min, followed by 35 cycles: 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s.
179 *L.* (*L.*) *infantum* was detected by PCR using species-specific primers FLC2 and RLC2 targeting
180 kDNA minicircle (Gualda et al., 2015). The reaction (25 µl) contained 0.2 µM of each primer
181 (Sigma), 2 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen), 1.5 U Taq DNA Polymerase (Phoneutria), 1X
182 enzyme buffer and 2 µl extracted DNA. The amplification was performed as described above,
183 except for the annealing temperature (56 °C instead of 61 °C). DNAs from *L.* (*V.*) *braziliensis*
184 MHOM/BR/1987/M11272, *L.* (*L.*) *amazonensis* WHOM/BR/75/JOSEFA and *L.* (*L.*) *infantum*
185 MHOM/BR/2002/LPC-RPV were used as controls. Amplified fragments were visualized under UV
186 light on 3% agarose gel stained with 0.1 µg/ml ethidium bromide.
187
188 **2.7 ITS1-PCR RFLP**
189 *Leishmania* species were identified in available human clinical samples by ITS1-PCR RFLP, as
190 described by Schonian et al. (Schönian et al., 2003). Briefly, ITS1 PCR products were directly
191 digested with 10 U *Hae*III (Thermo Fisher Scientifc) at 37 °C for 3 h. The restriction fragments
192 were visualized on a 3.5% high-resolution MetaPhor (Cambrex) agarose gel stained with GelRed
193 (Biotium, Hayward, CA). *L.* (*L.*) *amazonensis* WHOM/BR/75/JOSEFA, *L.* (*V.*) *braziliensis*
194 MHOM/BR/1987/M11272, *L.* (*L.*) *infantum* MHOM/BR/2002/LPC-RPV and no template PCR
195 reaction were used as controls. ITS1 PCR products were directly sequenced as previously described
196 (Ceccarelli et al., 2018). Phylogenetic analysis was conducted using MEGA 6 software.
197
198 **2.8 Quantitative PCR (qPCR) assays**
199 The approach described in Ceccarelli et al. (Ceccarelli et al., 2017), consisting in running two qPCR
200 reactions in parallel (qPCR-ML and qPCR-ama) to amplify different classes of minicircles, has
201 been applied on sample DNA spotted on filter paper. Briefly, the workflow of our method involves
202 first a discrimination between subgenera *Viannia* and *Leishmania* based on qPCR-ML HRM

analysis, performed with primers MLF and MLR (Supplementary Table 2, Data in Brief); 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 (Supplementary Table 2, Data in Brief). 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. The primers were used as described previously (Ceccarelli et al., 2014b; Galluzzi et al., 2012) and should amplify 102 and 183 bp fragments, respectively. 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 reaction mixture containing 38 µl SYBR green PCR master mix (Diatheva srl, Fano, Italy) or RT2 SYBR Green ROX FAST Mastermix (Qiagen , Hilden, Germany) or TB Green premix ex TaqII Mastermix (Takara Bio Europe, France) and 200 nM of each primer (Supplementary Table 2, Data in Brief). 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. *Trypanosoma cruzi* and human DNA were amplified using the conditions described above to confirm qPCRs specificity. The DNA concentration of reference strains was adjusted to have qPCR-ML Cq values comparable to Cq of tested samples. Moreover, a negative control (no template control) was included for each primer pair reaction. To confirm the absence of 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 with quantification analysis of the RotorGene 6000 software.

2.9 High-resolution melt (HRM) analysis

The high-resolution melt (HRM) analysis was performed immediately after the amplification reactions in the Rotor-Gene 6000 instrument as previously described (Ceccarelli et al., 2014a). In brief, HRM analysis was conducted over the range from 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. Only samples with Cq values < 30 were considered for analysis (Ceccarelli et al., 2014a; White and Potts, 2006). 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.

2.10 Statistical analysis

The 95% confidence intervals for sensitivity in clinical samples were calculated using the modified Wald method (Agresti et al., 1998). The degree of agreement between conventional methods (i.e. *L. infantum* species-specific PCR and/or ITS1-PCR RFLP and/or DNA sequencing) and qPCR-based methods in all samples with information about *Leishmania* species was determined by calculating Kappa values with 95 % confidence intervals using GraphPad QuickCalcs (GraphPad Prism, 2018).

3. Results

3.1 Brazilian strains characterization

The approach based on two qPCRs to distinguish between *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis* exploiting the different abundance of minicircle subclasses, previously developed with European *L.* (*L.*) *infantum* strains, was evaluated with additional New World strains. In particular, one New World *L.* (*L.*) *infantum* strain, one *L.* (*L.*) *amazonensis* strain and one *L.* (*V.*) *braziliensis* strain were tested blindly in duplicates. The contextual evaluation of qPCR-ML HRM peaks and qPCR-ML/qPCR-ama Cq values allowed to correctly identify the subgenus (*Leishmania* or *Viannia*) and the species (*infantum* or *amazonensis*) in each sample (Table 1). Samples F4 and F7 showed Cq (qPCR-ama) <Cq (qPCR-ML) and were classified as *L.* (*L.*) *amazonensis*. Samples F5, F6, F8, F9 showed Cq (qPCR-ML) <Cq (qPCR-ama) and were classified as *L.* (*L.*) *infantum* (F6, F9) and *Viannia* subgenus species (F5, F8), depending on the Tm of qPCR-ML amplicons determined by HRM analysis (Fig. 1).

3.2 Canine clinical samples characterization

Once confirmed the feasibility on New World strains, the approach described above was used to analyze 36 clinical sample DNAs, spotted on filter paper, from dogs with a diagnosis of leishmaniasis. Thirteen DNA samples (A1-A9, B1-B4) were from peripheral blood and 23 from bone marrow (B5-B9, C1-C9, D1-D9). All peripheral blood samples were positive using conventional PCR with primers specific for genus *Leishmania*, and all bone marrow samples were positive using conventional PCR with primers specific for *L.* (*L.*) *infantum*.

Concerning the qPCR analysis, the amplifiability of DNA on filter paper was confirmed for all samples using canine B2M as a target. Regarding DNA samples isolated from peripheral blood, 8 out of 13 samples were not detectable with both qPCR-ML and qPCR-ama assays. The remaining 5 samples (A4, A5, A9, B3, B4) resulted positive with both qPCR-ML and qPCR-ama assays, with

281 Cq (qPCR-ML) <Cq (qPCR-ama) (Supplementary Table 3, Data in Brief). Regarding DNA samples
282 isolated from bone marrow, only one sample (D1) was not detectable with both qPCR-ML and
283 qPCR-ama assays; 5 samples (C6, C8, C9, D2, D9) were positive with the qPCR-ML only; the
284 remaining 17 samples were detected with both the assays, with Cq (qPCR-ML) <Cq (qPCR-ama)
285 (Supplementary Table 3, Data in Brief).
286 After evaluation of HRM analysis, all samples were identified as *L.* (*L.*) *infantum* (Table 2).
287 Compared to conventional PCR results in bone marrow samples, the sensitivity was 95.6% (95%
288 confidence interval 77.3-100%). On the other hand, the sensitivity in peripheral blood samples was
289 38.5% (95% confidence interval 17.6-64.6%).
290
291 **3.3 Human clinical samples characterization**
292 In conventional PCR, 4 peripheral blood samples (E6-E8 and F3) and 3 bone marrow samples (E2-
293 E4) were positive for the *Leishmania* genus. Samples E1, E9 and F2 were not detectable, while
294 genus-specific PCR for sample E5 was not available. ITS1-PCR RFLP indicated the presence of *L.*
295 (*L.*) *infantum* in samples E6, F2, F3, while sample E5 was characterized as *Viannia* subgenus
296 species. The DNA sequence of F2 and F3 ITS1 amplicons confirmed the identification of those
297 samples as *L.* (*L.*) *infantum* (Table 3) (Supplementary Fig. 1, Data in Brief).
298 Concerning the qPCR assays, the amplifiability of DNA on filter paper was confirmed for all
299 samples using human GAPDH as a target. Regarding DNA samples isolated from peripheral blood,
300 the samples E1, E6, E7 and E8 were positive with both qPCR-ML and qPCR-ama assays, with Cq
301 (qPCR-ML) <Cq (qPCR-ama) (Supplementary Table 4, Data in Brief). After HRM analysis,
302 samples E1, E6 and E7 were identified as *L.* (*L.*) *infantum*, while in sample E8 both *L.* (*L.*) *infantum*
303 and *Viannia* subgenus species were identified. The samples E5, F2 and F3 were positive with the
304 qPCR-ML only. After HRM analysis, samples F2 and F3 were identified as *L.* (*L.*) *infantum*, while
305 in sample E5 both *L.* (*L.*) *infantum* and *Viannia* subgenus species. were identified (Table 3).
306 Regarding DNA samples isolated from bone marrow, the samples E2, E3 and E4 were identified as

L. (*L.*) *infantum*, *L.* (*L.*) *amazonensis* and *L.* (*L.*) *infantum,* respectively (Table 3) (Supplementary Table 4, Data in Brief). The sample E9 was not detectable in conventional PCR but it was identified as *L.* (*L.*) *infantum* in qPCR. The sensitivity of the qPCR assays in both blood and bone marrow samples was 100% (95% confidence interval 76.9-100%).

The specificity of qPCR-ML and qPCR-ama with pre-amplification conditions from filter paper was confirmed using *Trypanosoma cruzi* and human DNA as template (Supplementary Fig. 2, Data in Brief).

3.4 Agreement between conventional and qPCR-based methods

The agreement between conventional and qPCR-based methods was evaluated among all samples having species information (including *Leishmania* strains) by calculating Kappa values. The samples were classified in 3 categories: *L.* (*L.*) *infantum*, *L.* (*L.*) *amazonensis* and *Viannia* subgenus. The strength of agreement was considered to be "very good" (Kappa=0.876; standard error=0.122; 95% confidence interval 0.638 - 1.000).

4. Discussion

In the New World, *L.* (*L.*) *infantum*, *L.* (*L.*) *amazonensis* and several *Viannia* subgenus species coexist in the same geographic area and can infect both humans and dogs (Tolezano et al., 2007). In particular, in Mato Grosso do Sul, *L.* (*L.*) *infantum*, *L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis* were identified as the etiologic agents of Leishmaniasis (Souza Castro et al., 2018).

In a previous investigation, a new diagnostic approach based on two qPCR assays (qPCR-ML and qPCR-ama) to differentiate the subgenera *Leishmania* and *Viannia* and to discriminate *L.* (*L.*) *infantum* from *L.* (*L.*) *amazonensis* exploiting differences in minicircle subpopulations has been developed (Ceccarelli et al., 2017). This work represents a validation of the previously developed approach, using strains and clinical samples (canine and human) from Brazil, tested blindly.

The qPCR-ML and qPCR-ama results obtained with the Brazilian strains corroborated the results obtained previously. In particular, the *L.* (*L.*) *infantum* MHOM/BR/2002/LPC-RPV strain showed the same results obtained with European strains, i.e. Cq (qPCR-ML) <Cq (qPCR-ama), in agreement with previous studies showing identity between *L.* (*L.*) *infantum* from south Europe and *L.* (*L.*) *infantum* from New World (Kuhls et al., 2011). Importantly, these results suggest a homology between Old and New world strains also at the level of minicircle subclass composition. Concerning canine samples, all samples with positive results were characterized as *L.* (*L.*) *infantum*, and the species identification was confirmed with the species-specific conventional PCR in all bone marrow samples. This was expected since all canine samples were from VCL cases. The qPCR sensitivity varied between peripheral blood (38.5%) and bone marrow (95.6%). The decreased sensitivity observed in blood samples could be due to the low parasite load in the peripheral blood compared to bone marrow or lymph node (Manna et al., 2008; Solano-Gallego et al., 2007) along with the low amount of template DNA, due to amplification of DNA from a punch of filter paper. While dogs are mainly infected by *L.* (*L.*) *infantum*, and occasionally by *L.* (*V.*) *braziliensis* (Carvalho et al., 2015) or *L.* (*L.*) *amazonensis* (Sanches et al., 2016; Tolezano et al., 2007), humans can be infected by different *Leishmania* species giving heterogeneous clinical manifestations. The human samples E1, E2, E4, E6, E7, E9, F2 and F3 were characterized as *L.* (*L.*) *infantum*, E3 as *L.* (*L.*) *amazonensis*, E5 and E8 as co-infection *Viannia* subgenus species/*L.* (*L.*) *infantum*. The species characterization was in agreement with the clinical history of the patients. The fact that *L.* (*L.*) *infantum* was identified also in sample F2 (a CL patient) is not surprising since *L.* (*L.*) infantum has been identified also as a causative agent of cutaneous leishmaniasis in the state of Mato Grosso do Sul (Castro et al., 2016). Samples E2, E3, E4, which were from the same patient, were collected at different time points and were characterized as *L.* (*L.*) *infantum*, (E2 and E4) and as *L.* (*L.*) *amazonensis* (E3). The different species characterization could be due to the presence of a co-infection with two *Leishmania* species or to infection with *L.* (*L.*) *infantum* and *L.* (*L.*) *amazonensis* occurring at different times in the same patient. This is plausible also because some *Lutzomyia* spp.

358 are supposed to transmit more than one *Leishmania* spp. In particular, *Lu. longipalpis*, the main
359 vector of *L.* (*L.*) *infantum* in Latin America, has also been found infected by *L.* (*V.*) *braziliensis* and
360 *L.* (*L.*) *amazonensis* in Brazil (Guimarães-e-Silva et al., 2017).
361 On the whole, the agreement between conventional methods and qPCR-based methods for
362 *Leishmania* species identification, determined by Kappa test, was "very good". The agreement was
363 not perfect due to a possible co-infection [*L.* (*L.*) *infantum* and *Viannia* subgenus species] revealed
364 by qPCR in human sample E5, but not detected with ITS1-PCR RFLP. Importantly, concerning the
365 methodology, the species characterization in canine or human samples was reached regardless the
366 qPCR master mix used (Diatheva, Qiagen or Takara) -provided that reference strains are included in
367 each run as internal controls- demonstrating the robustness of this approach (Supplementary Table 3
368 and 4, Data in Brief). Since there is no PCR-based method considered as gold standard for species
369 determination, DNA sequencing was performed on two representative samples (F2 and F3) to
370 confirm the results obtained with qPCR-based assays. However, due to limited sample size, caution
371 is still needed and the results should be considered as preliminary for the South American clinical
372 context.
373 Nevertheless, the fact that the closed-tube format of the assays helps to minimize contamination and
374 accelerate the workflow, could make this approach a potential alternative to conventional PCR
375 and/or PCR-RFLP methods (Souza Castro et al., 2018). For example, this has been demonstrated in
376 endemic areas of Iran, where qPCR assays have shown highest sensitivity, specificity, positive and
377 negative predictive values both in canine and human samples (Mohammadiha et al., 2013b, 2013a).
378 On the other hand, high costs of qPCR reagents and equipment could still impair the use of this
379 approach in low-income settings.
380 In conclusion, the applicability of the method developed in (Ceccarelli et al., 2017) was
381 demonstrated in clinical samples from a Brazilian endemic area. Our data indicate that this
382 approach could be useful in a clinical context in regions co-endemic for *L.* (*L.*) *infantum*, *L.* (*L.*)

383 *amazonensis,* and *Viannia* subgenus, helping to provide rapid diagnosis and to allow studies of
384 species distribution.
385
386
387 **Acknowledgments**
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392

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Legends to figures

Fig. 1. Flow diagram of experimental design. See text for details.

Fig. 2. Example of high resolution melting curve analysis for qPCR-ML amplicons. The plot of the negative derivative of fluorescence (dF/dT) vs temperature is presented, showing melting transitions as peaks. Replicates are grouped for clarity. **a)** Curves obtained from reference strains *L.* (*V.*) *braziliensis* MHOM/BR/75/M2904 (Bin A), *L.* (*L.*) *amazonensis* MHOM/BR/00/LTB0016 (Bin B) and *L.* (*L.*) *infantum* MHOM/FR/78/LEM75 (Bin C); **b)** same curves including sample F8, assigned to *Viannia* subgenus (blue arrow); **c)** same curves including sample F6, assigned to *Leishmania* subgenus.

528 **Table 1. qPCR-ML and qPCR-ama results in Brazilian strains**

Species/strain	Sample ID	qPCR-ML (Cq ± SD)	qPCR-ama (Cq ± SD)	qPCR-ML (HRM T_m °C)	Species identification
L. (*L.*) *amazonensis* WHOM/BR/75/JOSEFA	F4	n.d.	18.38±0.17	n.d.	*L.* (*L.*) *amazonensis*
L. (*V.*) *braziliensis* MHOM/BR/1987/M11272	F5	29.67±0.15	35.54±0.02	83.56±0.01	*Viannia* subgenus
L. (*L.*) *infantum* MHOM/BR/2002/LPC-RPV	F6	20.37±0.06	31.39±0.31	84.30±0.04	*L.* (*L.*) *infantum*
L. (*L.*) *amazonensis* WHOM/BR/75/JOSEFA	F7	36.39±1.26	17.80±0.49	u.	*L.* (*L.*) *amazonensis*
L. (*V.*) *braziliensis* MHOM/BR/1987/M11272	F8	28.80±0.33	33.84±1.15	83.46±0.04	*Viannia* subgenus
L. (*L.*) *infantum* MHOM/BR/2002/LPC-RPV	F9	24.45±0.18	37.94±0.12	84.37±0.04	*L.* (*L.*) *infantum*

529 n.d. = not detectable

530 u. = unreliable since qPCR-ML Cq>>30

531

532 **Table 2. Summary of results from canine samples**

Source	sample ID	Serology[1]	Microscopy Identification[2]	Conventional PCR	Species identification (qPCR-ML and qPCR-ama)
Peripheral blood	A1	+	+	genus *Leishmania*[3]	-
	A2	+	+	genus *Leishmania*[3]	-
	A3	+	+	genus *Leishmania*[3]	-
	A4	+	+	genus *Leishmania*[3]	*L.* (*L.*) *infantum*
	A5	+	+	genus *Leishmania*[3]	*L.* (*L.*) *infantum*
	A6	+	+	genus *Leishmania*[3]	-
	A7	+	+	genus *Leishmania*[3]	-
	A8	+	+	genus *Leishmania*[3]	-
	A9	+	+	genus *Leishmania*[3]	*L.* (*L.*) *infantum*
	B1	+	+	genus *Leishmania*[3]	-
	B2	+	+	genus *Leishmania*[3]	-
	B3	+	+	genus *Leishmania*[3]	*L.* (*L.*) *infantum*
	B4	+	+	genus *Leishmania*[3]	*L.* (*L.*) *infantum*
Bone marrow	B5	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	B6	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	B7	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	B8	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	B9	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*

	C1	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C2	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C3	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C4	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C5	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C6	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C7	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C8	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	C9	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D1	+	+	*L.* (*L.*) *infantum*[4]	-
	D2	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D3	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D4	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D5	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D6	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D7	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D8	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*
	D9	+	+	*L.* (*L.*) *infantum*[4]	*L.* (*L.*) *infantum*

[1]immunochromatographic Dual-Path Platform and the ELISA test

[2]direct identification of suggestive forms of *Leishmania* amastigotes from Giemsa-stained smears analyzed by optical microscopy

[3]conventional PCR with primers specific for genus *Leishmania* (Rodgers et al., 1990)

[4]conventional PCR with primers specific for *L.* (*L.*) *infantum* (Gualda et al., 2015)

Table 3. Summary of results from human samples

Source	sample ID	Serology[1]	Microscopy identification[2]	Conventional PCR[3]	ITS1-PCR RFLP[4]	ITS1 DNA sequencing	Species identification (qPCR-ML and qPCR-ama)
Peripheral blood	E1	+	n.a.	-	n.a.	n.a.	*L.* (*L.*) *infantum*
	E5	-	+	n.a.	*Viannia* subgenus	n.a.	*L.* (*L.*) *infantum* *Viannia* subgenus
	E6	+	n.a.	genus *Leishmania*	*L.* (*L.*) *infantum*	n.a.	*L.* (*L.*) *infantum*
	E7	+	n.a.	genus *Leishmania*	-	n.a.	*L.* (*L.*) *infantum*
	E8	+	n.a.	genus *Leishmania*	-	n.a.	*L.* (*L.*) *infantum* *Viannia* subgenus
	F2	n.a.	+*	-	*L.* (*L.*) *infantum*	*L.* (*L.*) *infantum*	*L.* (*L.*) *infantum*
	F3	n.a.	n.a.	genus *Leishmania*	*L.* (*L.*) *infantum*	*L.* (*L.*) *infantum*	*L.* (*L.*) *infantum*
Bone marrow	E2	-	+	genus *Leishmania*	n.a.	n.a.	*L.* (*L.*) *infantum*
	E3	-	+	genus *Leishmania*	-	n.a.	*L.* (*L.*) *amazonensis*
	E4	-	+	genus *Leishmania*	n.a.	n.a.	*L.* (*L.*) *infantum*
	E9	n.a.	n.a.	-	n.a.	n.a.	*L.* (*L.*) *infantum*

[1]Rapid Test (rK39) for visceral leishmaniasis

[2]direct identification of suggestive forms of *Leishmania* amastigotes from Giemsa-stained smears analyzed by optical microscopy

[3]convencional PCR with primers specific for the genus *Leishmania* (Rodgers et al., 1990)

543 [4]ITS1-PCR RFLP according to Schonian et al (Schönian et al., 2003)

544 *from lesion of patient

545 n.a. not available

546

Figure 1

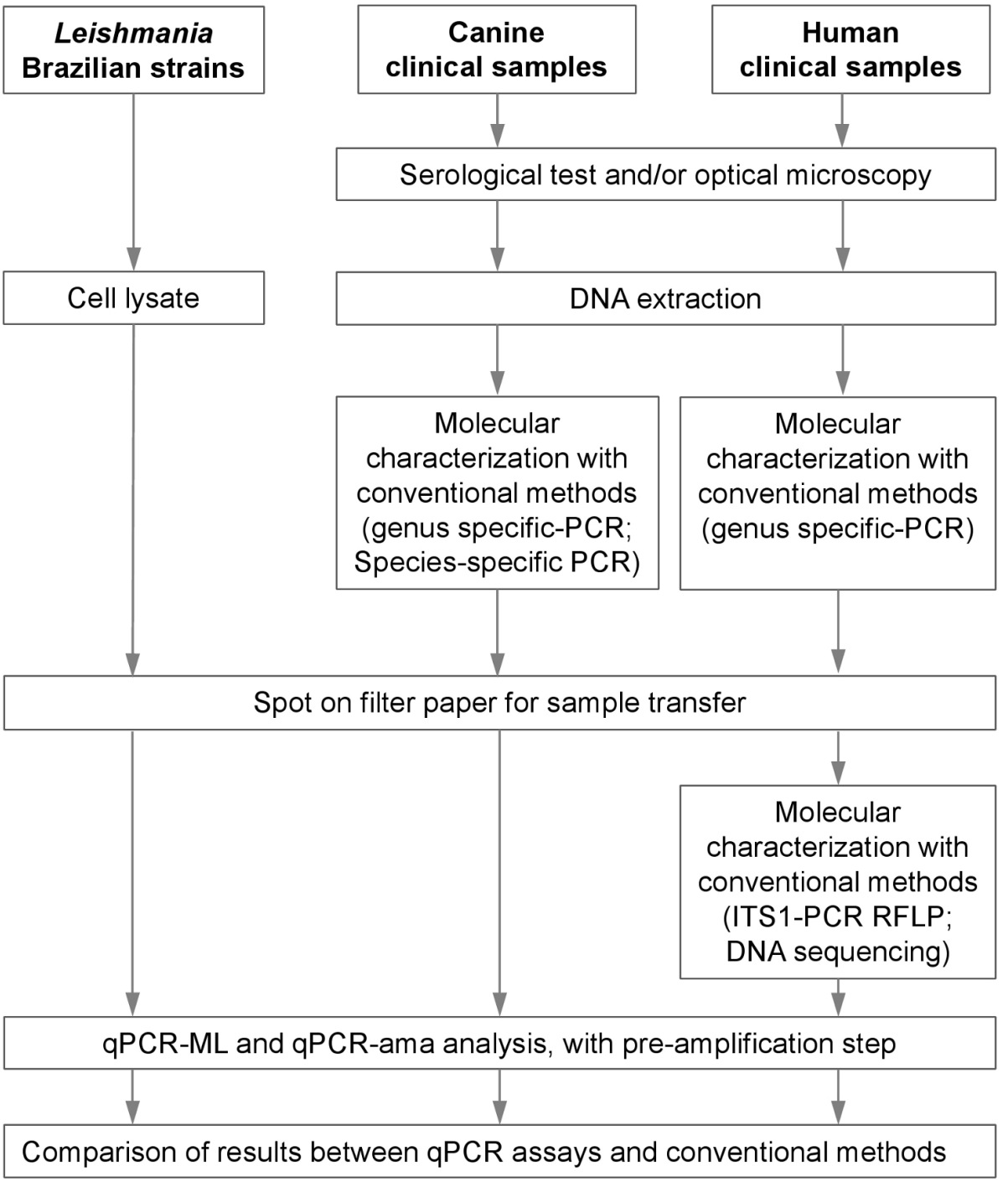
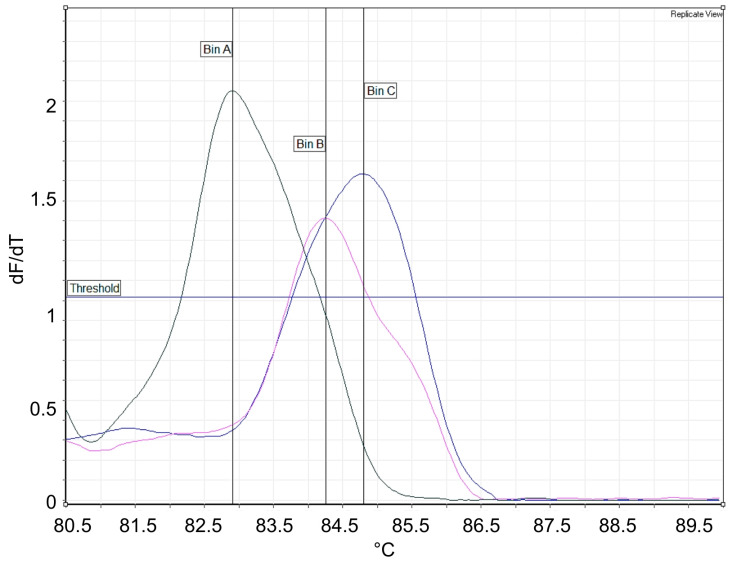

Leishmania Brazilian strains
Canine clinical samples
Human clinical samples
Serological test and/or optical microscopy
Cell lysate
DNA extraction
Molecular characterization with conventional methods (genus specific-PCR; Species-specific PCR)
Molecular characterization with conventional methods (genus specific-PCR)
Spot on filter paper for sample transfer
Molecular characterization with conventional methods (ITS1-PCR RFLP; DNA sequencing)
qPCR-ML and qPCR-ama analysis, with pre-amplification step
Comparison of results between qPCR assays and conventional methods

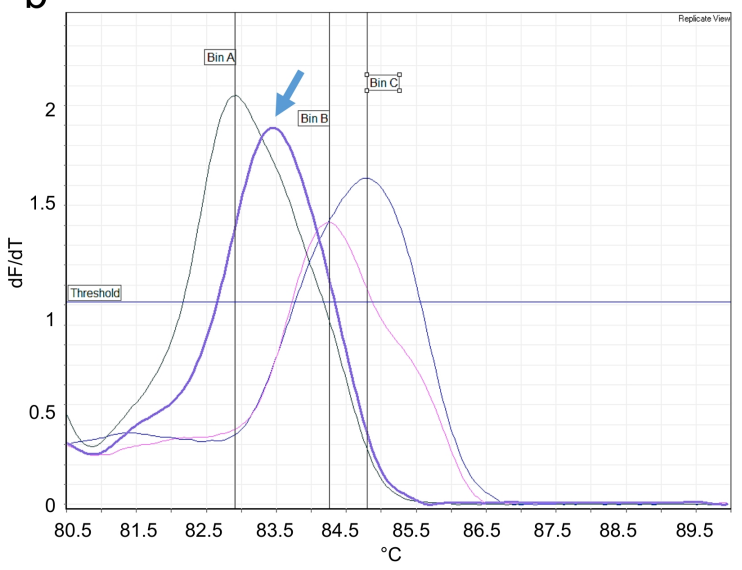
Figure 2

a



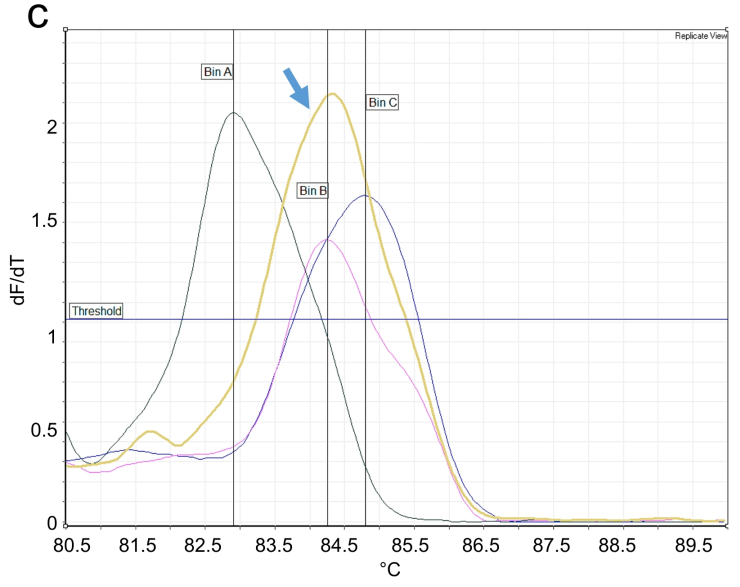
Name	Genotype	Peak 1
MHOM/FR/78/LEM75	Leishmania	84.83 (Bin C)
MHOM/FR/78/LEM75	Leishmania	84.73 (Bin C)
MHOM/BR/00/LTB0016	Leishmania	84.30 (Bin B)
MHOM/BR/00/LTB0016	Leishmania	84.20 (Bin B)
MHOM/BR/75/M2904	Viannia	82.93 (Bin A)
MHOM/BR/75/M2904	Viannia	82.88 (Bin A)

b



Name	Genotype	Peak 1
MHOM/FR/78/LEM75	Leishmania	84.83 (Bin C)
MHOM/FR/78/LEM75	Leishmania	84.73 (Bin C)
MHOM/BR/00/LTB0016	Leishmania	84.30 (Bin B)
MHOM/BR/00/LTB0016	Leishmania	84.20 (Bin B)
MHOM/BR/75/M2904	Viannia	82.93 (Bin A)
MHOM/BR/75/M2904	Viannia	82.88 (Bin A)
F8	Viannia	83.50 (Bin A)
F8	Viannia	83.42 (Bin A)

c



Name	Genotype	Peak 1
MHOM/FR/78/LEM75	Leishmania	84.83 (Bin C)
MHOM/FR/78/LEM75	Leishmania	84.73 (Bin C)
MHOM/BR/00/LTB0016	Leishmania	84.30 (Bin B)
MHOM/BR/00/LTB0016	Leishmania	84.20 (Bin B)
MHOM/BR/75/M2904	Viannia	82.93 (Bin A)
MHOM/BR/75/M2904	Viannia	82.88 (Bin A)
F6	Leishmania	84.30 (Bin B)
F6	Leishmania	84.33 (Bin B)