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Field Validation of SYBR Green- and TaqMan-Based Real-Time PCR Using Biopsy and Swab Samples To Diagnose American Tegumentary Leishmaniasis in an Area Where *Leishmania* (*Viannia*) *braziliensis* Is Endemic

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ABSTRACT The precise diagnosis of American tegumentary leishmaniasis (ATL) is an essential task due to the disease's associated morbidity. A noninvasive, extremely sensitive, and highly specific exam is critical, particularly for mucosal leishmaniasis (ML), in which a low parasite quantity is expected. We aimed to compare the diagnostic accuracy of swab and biopsy sample analysis using SYBR Green- and TaqMan-based real-time PCR (qPCR) assays with that of a composite reference standard consisting of the Montenegro skin test, serology, histopathology, smears, culture, and conventional PCR. In total, 55 patients with ATL (ML, 18 patients; cutaneous leishmaniasis [CL], 37 patients) and 36 patients without ATL were studied. qPCR analysis of swabs was more accurate when using SYBR Green (87.88%; 95% confidence interval [CI], 77.86 to 93.73 patients) than when using TaqMan (78.79%; 95% CI, 67.49 to 86.92%) ($P = 0.031$). SYBR Green (84.72%; 95% CI, 74.68 to 91.25%) was also more accurate than TaqMan (73.61%; 95% CI, 62.42 to 82.41%) for biopsy samples ($P = 0.008$). All qPCR methods were 100% specific. Swabs and biopsy specimens had similar sensitivity when using the same chemistry ($P = 0.125$ for SYBR Green and $P = 0.625$ for TaqMan). Moreover, qPCR achieved better performance than most existing techniques used for the diagnosis of ATL and also detected the *Leishmania* parasite in a greater proportion of patients than the associated histopathology, smear, culture, and conventional PCR techniques did. Swabs therefore represent a useful diagnostic tool because they not only are noninvasive but also can achieve an accuracy similar to that of biopsy samples. The high accuracy of SYBR Green-based qPCR may also reduce the requirement for associated parasitological tests for ATL diagnosis.

KEYWORDS leishmaniasis, mucocutaneous, diagnosis, real-time PCR, kinetoplast DNA, real-time polymerase chain reaction

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Leishmaniasis is a neglected disease caused by a protozoan of the genus *Leishmania*. This disease is showing alarming growth, especially in periurban areas (1, 2). *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* both have a

tropism for the skin and mucosa and are endemic in Brazil (1). These parasites specifically stimulate an intense granulomatous reaction, resulting in disfiguring lesions in untreated patients (3).

The accurate diagnosis of American tegumentary leishmaniasis (ATL) is an essential task due to the disease's associated morbidity (1, 2) but is hindered by the lack of a gold-standard laboratory test (3–5). Currently available parasitological tests based on stained smears and culture offer low sensitivity, and other options, such as the Montenegro (leishmanin) skin test (MST), have high sensitivity but low specificity (3, 6, 7). Additionally, serological techniques may yield widely varied accuracy values (3). In contrast, although not completely sensitive, PCR can improve the accuracy of ATL diagnosis (5, 8, 9). Strategies that might improve the sensitivity of PCR include the use of reliable DNA isolation methods, primer pairs that amplify the kinetoplast DNA (kDNA) sequences of *Leishmania*, and real-time PCR (qPCR) techniques (5, 10, 11).

Swabs and cytology brush samples of localized cutaneous leishmaniasis (CL) are more sensitive than lesion aspirate and biopsy samples (12, 13). The utility of swab-based diagnosis in mucosal leishmaniasis (ML) has not been completely explored, as most studies have been conducted in highly controlled environments, usually using healthy controls for comparison (5). These shortcomings have precluded the use of this technique in clinical practice (5, 14).

In the present study, we examined the accuracy of qPCR using SYBR Green and TaqMan for the diagnosis of the clinical forms of CL and ML. In particular, we tested noninvasive (swabs) and invasive (biopsy) samples, aiming to simulate real situations experienced by clinicians (15).

RESULTS

In total, 91 patients were included in this study. After application of the composite reference standard (CRS), 55 patients were allocated to the ATL group (ML, 18 patients; CL, 37 patients). Additionally, 36 patients who were not diagnosed with active ATL were designated non-ATL patients. Among the non-ATL patients, 32 patients with skin lesions were diagnosed with vascular ulcers ($n = 8$), basal cell carcinoma ($n = 1$), sporotrichosis ($n = 4$), bacterial infection ($n = 14$), tuberculosis ($n = 1$), or nonspecific ulcers or infiltrations without an etiological diagnosis ($n = 4$). The other 4 non-ATL patients presented mucous lesions, and the final diagnosis confirmed leprosy ($n = 1$), epidermoid carcinoma ($n = 1$), or lesions without an etiological diagnosis ($n = 2$) in these patients.

Patients with ML were older than CL patients ($P = 0.002$) and non-ATL patients ($P = 0.009$). ML patients also presented a longer disease duration than CL patients ($P < 0.001$) and non-ATL patients ($P < 0.001$) (Table 1). The subgenus *Leishmania* (*Viannia*) was identified in 38 cases of ATL, and the subgenus *Leishmania* (*Leishmania*) was identified in 1 case of CL. The case of *Leishmania* (*Leishmania*) tested negative in both qPCR assays. No adverse events were reported during the sampling procedures.

The swab-based qPCR was more accurate using SYBR Green (87.88%; 95% confidence interval [CI], 77.86 to 93.73%) than using TaqMan (78.79%; 95% CI, 67.49 to 86.92%) ($P = 0.031$). The accuracy of SYBR Green (84.72%; 95% CI, 74.68 to 91.25%) was also superior to that of TaqMan (73.61%; 95% CI, 62.42 to 82.41) for biopsy samples ($P = 0.008$). All qPCR methods were 100% specific. Swabs and biopsy specimens had similar sensitivity when using the same chemistry ($P = 0.125$ using SYBR Green and $P = 0.625$ using TaqMan) (Tables 2 and 3). The combination of index tests in parallel did not have a significant impact on diagnostic accuracy.

qPCR detected *Leishmania* in a greater proportion of ATL patients than histopathology, smears, or culture did when each of these tests was used either alone or in combination, resulting in poor diagnostic agreement (Tables 1 to 3). In contrast, SYBR Green qPCR and conventional PCR used alone had good agreement (Table 3). In 15 ATL patients (8 ML patients and 7 CL patients), there was no direct evidence for the presence of *Leishmania* using a combination of histopathology, smears, culture, and conventional PCR. However, these patients were allocated to the ATL group because

TABLE 1 Demographic characteristics and positive results from the exams used to define the composite reference standard (defined as the gold standard) for patients allocated to the mucosal and cutaneous groups of American tegumentary leishmaniasis and non-ATL patients

Characteristic or result[a]	Mucosal group	Cutaneous group	Non-ATL patients	P value[b]
Male gender	13/18 (72.22)	22/37 (59.46)	20/36 (55.55)	0.492
Age (mean [SD]) (yr)	57.39 (10.47)	42.56 (16.49)	46.58 (14.56)	0.005
Disease duration (median [range]) (mo)	48 (6–420)	3 (0.5–63)	4 (0.4–180)	<0.001
MST positivity	15/16 (93.75)	24/30 (80.00)	13/32 (40.63)	0.001
Serological positivity	12/17 (70.58)	13/32 (40.63)	6/25 (24.00)	0.004
Smear positivity	3/12 (25.00)	8/25 (32.00)	0/33 (0)	<0.001
Culture positivity	1/10 (10.00)	10/23 (43.48)	0/29 (0)	<0.001
Histopathological positivity[c]	2/16 (12.50)	13/36 (36.11)	0/35 (0)	<0.001
PCR positivity using biopsy samples	9/14 (64.29)	26/32 (81.25)	0/34 (0)	<0.001
PCR positivity using swab samples	8/14 (57.14)	14/18 (77.77)	0/34 (0)	<0.001

[a]Data are shown as the no./total no. (%), unless otherwise indicated.
[b]Comparison of the three groups simultaneously. The assessment of significant differences is presented in the Results.
[c]For comparison purposes, positivity in histopathology was considered to be the presence of amastigote forms.

they presented a positive MST or indirect immunofluorescence (IIF) value, along with histopathology showing granuloma formation, as well as an absence of any other identifiable causative agent. In this group, SYBR Green qPCR was positive for one case of ML when using a biopsy sample and for another case of ML when using a swab sample. However, TaqMan qPCR yielded negative results for this group of patients.

DISCUSSION

Reliable diagnostic examinations of ATL have been extensively studied (3, 16). A noninvasive, extremely sensitive, and highly specific exam is essential, particularly for ML, in which a low parasite quantity is expected (17, 18). In the current study, SYBR Green and TaqMan were 100% specific. High specificity is the most important characteristic of any ATL diagnostic test, as the unjustified prescription of pentavalent antimonials or amphotericin B for patients without ATL can be extremely harmful (19, 20).

After the high specificity of qPCR was established, its sensitivity was analyzed. SYBR Green qPCR exhibited greater sensitivity values (Table 2). Although TaqMan qPCR is generally more specific, proper standardization suppresses unspecific amplification in SYBR Green qPCR (21). Additionally, in the present study, swabs and biopsy samples had similar sensitivity values when the same chemistry was used for qPCR. These findings are in contrast to the higher accuracy of swab analysis in CL reported in previous studies (12, 13, 18).

The highest achieved sensitivity for ML in our study, or 69.23% (95% CI, 42.37 to 87.32%), was achieved using SYBR Green qPCR to analyze biopsy samples (Table 2) and is an important milestone (22). A recent meta-analysis stated that the sensitivity of conventional PCR in ML was approximately 70%; however, at least half of the analyzed

TABLE 2 Test properties for ATL forms and controls

Sample type/test	Mucosal leishmaniasis		Cutaneous leishmaniasis		American tegumentary leishmaniasis[a]		
	Positivity (no./total no. tested)	Sensitivity (% [95% CI])	Positivity (no./total no. tested)	Sensitivity (% [95% CI])	Sensitivity (% [95% CI])	Specificity (% [95% CI])	Accuracy (% [95% CI])
Biopsy/SYBR Green	9/13	69.23 (42.37–87.32)	22/29	75.86 (57.89–87.78)	73.81 (58.93–84.70)	100 (86.65–100)	84.72 (74.68–91.25)
Biopsy/ TaqMan	6/13	46.15 (23.21–70.86)	17/29	58.62 (40.74–74.49)	54.76 (39.95–68.78)	100 (88.65–100)	73.61 (62.42–82.41)
Swab/SYBR Green	7/14	50 (26.80–73.20)	17/18	94.44 (74.24–99.01)	75 (57.89–86.75)	100 (89.85–100)	87.88 (77.86–93.73)
Swab/TaqMan	6/14	42.86 (21.38–67.41)	12/18	66.67 (43.75–83.72)	56.25 (39.33–71.83)	100 (89.85–100)	78.79 (67.49–86.92)

[a]CI, confidence interval; the American tegumentary leishmaniasis group represents test properties calculated for mucosal leishmaniasis patients and cutaneous leishmaniasis patients together.

TABLE 3 Agreement between real-time PCR assays of biopsy samples and tests used to define the composite reference standard[a]

Reference test	Biopsy				Swab			
	SYBR Green		TaqMan		SYBR Green		TaqMan	
	Overall agreement	Cohen's kappa	Overall agreement	Cohen's kappa	Overall agreement	Cohen's kappa	Overall agreement	Cohen's kappa
MST	61.29 (48.85 to 72.42)	0.26 (0.04 to 0.49)	54.84 (42.53 to 66.58)	0.19 (0.00 to 0.39)	53.47 (40.70 to 65.98)	0.12 (−0.11 to 0.34)	50 (37.33 to 62.67)	0.07 (−0.14 to 0.28)
Serology	53.33 (40.89 to 65.37)	0.06 (−0.18 to 0.31)	48.33 (36.17 to 60.69)	−0.06 (−0.31 to 0.18)	50.98 (37.68 to 64.14)	−0.004 (−0.31 to 0.24)	60 (46.18 to 72.39)	0.12 (−0.16 to 0.39)
Smear	81.36 (69.62 to 89.26)	0.52 (0.30 to 0.74)	85 (73.89 to 91.9)	0.52 (0.28 to 0.77)	85.42 (72.83 to 92.75)	0.52 (0.27 to 0.77)	89.58 (77.83 to 95.47)	0.61 (0.35 to 0.87)
Culture	84.62 (72.48 to 91.99)	0.58 (0.33 to 0.83)	88.46 (77.03 to 94.6)	0.60 (0.33 to 0.87)	87.5 (73.89 to 94.54)	0.55 (0.28 to 0.83)	92.50 (80.14 to 97.42)	0.69 (0.39 to 0.98)
Histopathology[b]	73.91 (62.49 to 82.81)	0.41 (0.22 to 0.60)	73.91 (62.49 to 82.81)	0.29 (0.07 to 0.50)	75.81 (63.85 to 84.75)	0.38 (0.16 to 0.60)	80.33 (68.69 to 88.37)	0.38 (0.14 to 0.63)
Biopsy sample/PCR	95.83 (88.45 to 98.57)	0.92 (0.68 to 1.14)	87.5 (77.92 to 93.28)	0.74 (0.52 to 0.96)	91.38 (81.36 to 96.26)	0.81 (0.56 to 1.06)	86.21 (75.07 to 92.84)	0.69 (0.44 to 0.94)
Swab sample/PCR	90.38 (79.39 to 95.82)	0.79 (0.53 to 1.05)	86.54 (74.73 to 93.32)	0.69 (0.42 to 0.96)	90.91 (81.55 to 95.77)	0.80 (0.56 to 1.04)	90.91 (81.55 to 95.77)	0.78 (0.55 to 1.02)
Biopsy sample/SYBR Green			88.89 (79.58 to 94.26)	0.77 (0.54 to 0.99)	92.31 (81.83 to 96.97)	0.84 (0.57 to 1.10)	88.46 (77.03 to 94.6)	0.75 (0.49 to 1.01)
Biopsy sample/TaqMan					96.15 (87.02 to 98.94)	0.91 (0.64 to 1.18)	90.48 (77.93 to 96.23)	0.61 (0.32 to 0.91)
Swab sample/SYBR Green							90.91 (81.55 to 95.77)	0.79 (0.56 to 1.02)
Swab sample/TaqMan								

[a]All values are shown with the 95% confidence interval in parentheses.
[b]For comparison purposes, positivity in histopathology was considered to be the presence of amastigote forms.

references were case-control accuracy studies and did not use a blinded evaluator (5). As the methodology of the present study was based on a cross-sectional/cohort design and used blinded evaluators, we expected a different outcome from that of other methodologies.

Cultures, smears, and detection of amastigote forms via histopathology are clinically recognized to be highly specific (3, 23) and were defined as 100% specific in the current study once visualization of parasites ensured allocation to the ATL group. Although certain technique optimization methods, such as press imprint smears from tissue fragments (24) and microculture (16), can improve the positivity rate, these exams are frequently used in parallel to enhance sensitivity (3), which can also elevate costs. In the present study, in addition to its high specificity, qPCR yielded positive results in certain patients in whom none of the parasitological exams could detect *Leishmania*. qPCR was thus able to substitute for the use of parasitological tests alone or in parallel. Although SYBR Green qPCR detected 2 ATL cases among 15 cases in which *Leishmania* was not found using the CRS, conventional PCR and qPCR performed similarly (Table 3). The main advantage of qPCR is its greater automation, which results in time savings (25–27).

The MST is clinically recognized to be sensitive but not sufficiently specific to diagnose ATL alone (28). Here, the use of immunological exams in the CRS was crucial for the inclusion of patients in whom the parasite was not detected. This important group of patients is frequently neglected because most studies use only parasitological or molecular exams for CRS definition (5). In fact, these patients would most benefit from highly sensitive qPCR. The qPCR assessed here provided high sensitivity rates similar to those of the MST, but it was more specific. IIF detected 70% of ML patients but was positive for fewer than 50% of CL patients and for 24% of controls, confirming its controversial role in ATL diagnosis (Table 1) (3, 7).

A limitation of the present study is that the primer pairs used in both the SYBR Green and TaqMan assays were not capable of detecting *Leishmania* (*L.*) *amazonensis* (Fig. 1). Clinicians should be aware that in South America, approximately 3% of CL cases are caused by this species (29, 30). The standardization of multiplex qPCR capable of detecting both species might improve the sensitivity of qPCR. The primers used also target different, but adjacent, regions of the *Leishmania* (*Viannia*) kDNA. The present results do not indicate that SYBR Green is superior to TaqMan for ATL; the greater sensitivity observed for SYBR Green might be due to a better DNA target for the local *Leishmania* strains rather than the chemistry itself.

Missing data is another limitation that can jeopardize results. In the present study, not all patients were subjected to all the tests partly because of difficulty in accessing certain laryngeal ML lesions and mainly because of an insufficient quantity of the extracted DNA for multiple PCR and qPCR tests. However, this limitation did not greatly affect the CRS because parasite detection by more than one technique was possible. With respect to the missing values for the index tests, this limitation was minimized by performing the agreement comparison for only those patients who were tested using all exams (Table 3).

We conclude that the performance of qPCR was superior to that of most of the techniques used for the detection of *Leishmania*. However, qPCR is not sufficiently accurate to be used as a single technique because its sensitivity is less than 100%. The inclusion of clinical data and the MST thus remained necessary in compatible cases in which the qPCR yielded negative results in our study. Nevertheless, the combination of swabs with qPCR is emerging as a powerful diagnostic tool because of its noninvasive yet simple collection method.

MATERIALS AND METHODS

Study location. Patients were consecutively included at the Brasilia University Hospital, Brasilia, Federal District, Brazil, and at the University Hospital of Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo State, from 1 January 2012 to 30 August 2015.

Study population. Patients who had clinical lesions consistent with ATL were included. All patients were tested using the MST, indirect immunofluorescence (IIF) analysis of *Leishmania* (*L.*) *amazonensis* culture (31), histopathologic examination of biopsy samples, culture of aspirate (Novy-MacNeal-Nicolle

medium), smears from the lesion, conventional PCR of biopsy and swab samples, as previously described (8, 31), and qPCR of biopsy and swab samples.

Composite reference standard. A composite reference standard (CRS) was defined. An ATL case was defined by detection of the parasite in at least one of the following examinations: histopathology, culture, smears, and conventional PCR of biopsy or swab samples, as described elsewhere (8). In the absence of parasite detection, ATL was diagnosed based on the MST or IIF positivity in combination with the presence of granulomas on histopathologic examination and a lack of identification of another etiological agent by special staining methods. The *Leishmania* subgenus was identified using conventional PCR coupled with restriction enzyme analysis, as described elsewhere (8).

After application of the CRS, the patients were prospectively allocated to two groups, consisting of ATL patients and patients without ATL. The ATL patients were grouped into ML and CL groups depending on the presence or absence, respectively, of mucous lesions, as previously described (8). In contrast, patients without ATL were not divided according to the presence of cutaneous or mucous lesions because of the small number of patients with mucous involvement.

Patients who were under 18 years old; who belonged to an indigenous community; or who presented any sign of immunosuppression, such as a positive HIV test, decompensated diabetes mellitus, or use of systemic corticosteroids or immunosuppressants, were not included.

Sampling (index tests). Samples were collected in the following sequence: a sterile cotton swab (Absorve, São Paulo, Brazil) was rotated 360° 10 times against the internal border of the lesion, with slight pressure applied. The stem of the swab was then cut off using a sterile scalpel blade and stored in a 1.5-ml sterile microtube. A biopsy using a 4-mm punch was acquired from the border of a cutaneous or mucous lesion after proper cleansing and anesthesia and was then stored in a 1.5-ml sterile microtube. All samples were stored at −80°C before and after DNA extraction.

DNA extraction and quantification (index tests). DNA extraction was performed using the NucleoSpin tissue XS kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany), according to the manufacturer's instructions. After extraction, the samples were quantified in a NanoVue Plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom) and diluted with ultrapure water to a concentration of 10 ng/μl for use as a DNA template.

Standardization tests (index tests). Primer pair choices were based on previously described sequences that amplify fragments of the *Leishmania* (*V.*) *braziliensis* minicircle kDNA (5, 32–36). We evaluated primer and probe specificity for *Leishmania* (*V.*) *braziliensis* using NCBI BLAST. Two primer pairs were ultimately selected: MP1L/MP3H (for SYBR Green qPCR) (34) and *L. braziliensis* kDNA3 (for TaqMan qPCR) (33). For the *L. braziliensis* kDNA3 primer pair, we opted to use the described probe to enhance specificity. Standard curves were generated using *Leishmania* (*V.*) *braziliensis* (MHOM/BR/1994/M15176) DNA from 6×10^5 to 6×10^{-5} parasite DNA equivalents/reaction for MP1L/MP3H and from 2.4×10^5 to 2.4×10^{-5} parasite DNA equivalents/reaction for *L. braziliensis* kDNA3, as described elsewhere (33, 34).

Tests with *Leishmania* cultures and negative controls were performed prior to the processing of clinical samples, with the aim of improving the qualitative performance. This step was based on a comparison of *Leishmania* (*V.*) *braziliensis* (MHOM/BR/1994/M15176), *Leishmania* (*Viannia*) *guyanensis* (MHOM/BR/75/M4147), *Leishmania* (*L.*) *amazonensis* (IFLA/BR/67/PH8), and *Trypanosoma cruzi* (Y strain) cultures with negative controls, consisting of 10 healthy skin samples and controls in which the DNA template was omitted. The primer concentrations and annealing temperatures were optimized to reduce background signals, primer dimerization, nonspecific amplification, and false-positive results (Fig. 1).

Real-time qualitative PCR (index tests). SYBR Green qPCR was performed with primers targeting the sequences of the kDNA of *Leishmania* of the subgenus *Viannia*, namely, MP1L-5′-TACTCCCCGACAT GCCTCTG-3′ and MP3H-5′-GAACGGGGTTTCTGTATGC-3′, resulting in an amplified product of 70 bp (34). Reactions were performed in a final volume of 50 μl, consisting of 1× SYBR Green PCR master mix (Life Technologies, Carlsbad, CA, USA), 30 nM each primer, 19.7 μl of ultrapure water, and 50 ng of DNA template.

Amplification was performed at an initial holding temperature of 95°C for 10 min, followed by 40 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 20 s and melting curve analysis in 1°C increments from 60°C to 99°C. All samples with a threshold cycle (C_T) value greater than 35 and those with a melting curve not compatible with the expected pattern were considered negative. The SYBR Green qPCR resulted in a detection limit ranging from 6×10^5 to 6×10^{-2} parasite DNA equivalents/reaction ($R^2 = 0.999$, efficiency = 90.238%, slope = −3.58).

TaqMan qPCR was performed with primers targeting sequences of the kDNA of *Leishmania* (*Viannia*) and a hydrolysis probe specific for *Leishmania* (*V.*) *braziliensis*, namely, primers 5′-TGCTATAAAATCGTAC CACCCGACA-3′ and 5′-GAACGGGGTTTCTGTATGCCATTT-3′, and probe FAM-TTGCAGAACGCCCCTACCCA GAGGC-TAMRA (FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine), resulting in an amplified product of 83 bp (33). The reaction was performed in a final volume of 20 μl, consisting of 1× TaqMan Universal master mix (Life Technologies, Carlsbad, CA, USA), 400 nM each primer, 250 nM probe, 2 μl of ultrapure water, and 20 ng of DNA template.

Amplification was performed at two consecutive holding temperatures, or 50°C for 5 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The TaqMan qPCR resulted in a detection limit ranging from 2.4×10^5 to 2.4×10^2 parasite DNA equivalents/reaction ($R^2 = 0.993$, efficiency = 90.782%, slope = −3.565).

Amplifications were performed in a Rotor-Gene Q (Qiagen, Hilden, Germany). The Dynamic Tube normalization option was selected to determine the average background of each sample. The threshold point of each reaction was defined manually in the region immediately above the nonspecific signal
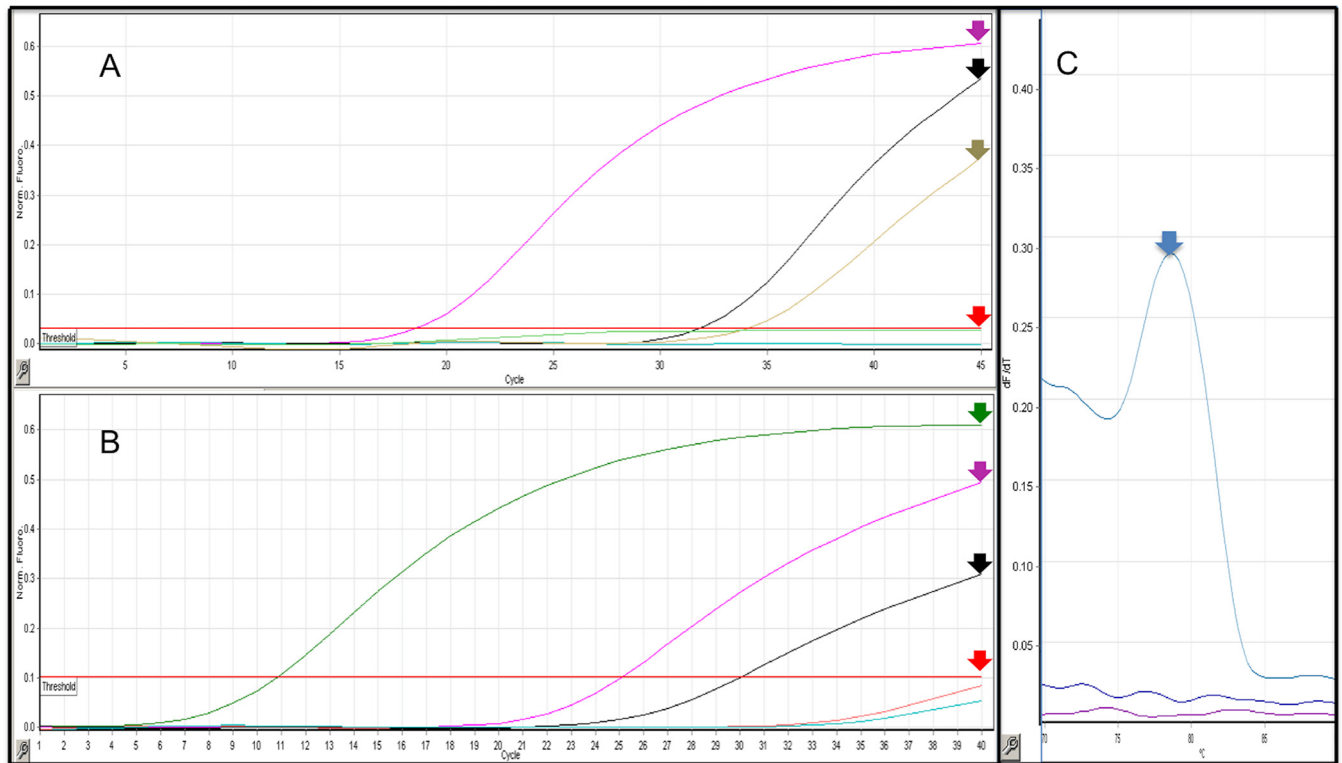
FIG 1 (A) Linear amplification graph for the TaqMan-based assay. *Leishmania* (*Viannia*) *braziliensis* culture (pink arrow) (C_T = 18.62), *Leishmania* (*Viannia*) *guyanensis* culture (black arrow) (C_T = 31.86), *Trypanosoma cruzi* culture (brown arrow) (C_T = 34.09). *Leishmania amazonensis*, DNA from healthy human skin, and a nontemplate control remained under the threshold line (red arrow) (C_T = 0). *y* axis, normalized fluorescence; *x* axis, number of cycles. (B) Linear amplification graph for the SYBR Green-based assay. *Leishmania* (*Viannia*) *braziliensis* culture (green arrow) (C_T = 10.84), *Leishmania* (*Viannia*) *guyanensis* culture (pink arrow) (C_T = 25.13), and *Trypanosoma cruzi* culture (black arrow) (C_T = 30.05). *Leishmania amazonensis*, DNA from healthy human skin and a nontemplate control remained under the threshold line (red arrow) (C_T = 0). *y* axis, normalized fluorescence; *x* axis, number of cycles. (C) Melting curve for the SYBR Green-based assay. Expected melting curve analysis for *Leishmania* (*Viannia*) *braziliensis* culture (blue arrow). *y* axis, normalized fluorescence; *x* axis, temperature (°C). The images' scales were adjusted to fit the images in the panels.

using a logarithmic amplification plot. All reactions were compared with a positive control, consisting of *Leishmania* (*V.*) *braziliensis* culture (MHOM/BR/1994/M15176), and negative controls, consisting of DNA extracted from a healthy skin sample and ultrapure water.

All reactions were performed twice. Divergent results were always considered negative in TaqMan assays and SYBR Green-based assays if the mean C_T value of both reactions was greater than 35 cycles, without a compatible melting curve. All negative samples were analyzed for an endogenous gene by performing a qPCR assay using the C18X 343-bp 5′-GAAAGTGCCAGACCCGCCCCC-3′ and 5′-GCTGAAG CCACCGCCATAG-3′ primer pair specific for human keratin, as described elsewhere (8, 37). The reactions were performed without previous knowledge of the CRS, and the results were tabulated by a responsible party blinded to the final diagnosis of each patient.

Sample size. An ATL prevalence of 60% was expected for the target population (patients attending the dermatology ambulatory clinic who had clinical lesions consistent with ATL). As this prevalence was >50%, we first estimated the number of controls based on an expected specificity of 95% (8, 38). Tables proposed by Flahault et al. were applied (39). Considering a minimal acceptable lower confidence limit of 0.75 (1 − α), the inclusion of 34 controls was recommended. Application of the following formula proposed by the same authors, or number of controls = number of cases [(1 − disease prevalence)/disease prevalence], indicated that the minimal number of ATL cases had to be 51 (39).

Statistical analysis. Sensitivity was defined as the percentage of positive results in patients with ATL, and specificity was defined as the percentage of negative results in patients without ATL. Additionally, accuracy was defined as the sum of true-positive and true-negative results divided by the total number of patients tested. We assessed agreement between the qPCR and CRS exams based on the total agreement percentage, Cohen's kappa agreement, and McNemar's test. Missing variables were ignored, and only patients who underwent all exams were tested for agreement. We used the program SPSS 19.0 (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as a *P* value of <0.05, and confidence intervals (CIs) were set at 95%.

Ethics. Patients were included after signing an informed consent form. This study complied with the Declaration of Helsinki (40) and was approved by the Committee for Ethics in Research of the School of Medicine at the University of Brasilia and of the University Hospital of Ribeirão Preto Medical School, University of São Paulo (35611714.7.1001.5558,036/2011 and 35611714.7.2001.5440).

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