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Real-Time PCR Assay for Detection and Quantification of *Leishmania* (*Viannia*) Organisms in Skin and Mucosal Lesions: Exploratory Study of Parasite Load and Clinical Parameters

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Earlier histopathology studies suggest that parasite loads may differ between cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) lesions and between acute and chronic CL. Formal demonstration requires highly sensitive detection and accurate quantification of *Leishmania* in human lesional tissue. In this study, we developed a quantitative real-time PCR (qPCR) assay targeting minicircle kinetoplast DNA (kDNA) to detect and quantify *Leishmania* (*Viannia*) parasites. We evaluated a total of 156 lesion biopsy specimens from CL or ML suspected cases and compared the quantitative performance of our kDNA qPCR assay with that of a previously validated qPCR assay based on the glucose-6-phosphate dehydrogenase (*G6PD*) gene. We also examined the relationship between parasite load and clinical parameters. The kDNA qPCR sensitivity for *Leishmania* detection was 97.9%, and its specificity was 87.5%. The parasite loads quantified by kDNA qPCR and *G6PD* qPCR assays were highly correlated ($r = 0.87$; $P < 0.0001$), but the former showed higher sensitivity ($P = 0.000$). CL lesions had 10-fold-higher parasite loads than ML lesions ($P = 0.009$). Among CL patients, the parasite load was inversely correlated with disease duration ($P = 0.004$), but there was no difference in parasite load according to the parasite species, the patient's age, and number or area of lesions. Our findings confirm that CL and recent onset of disease (<3 months) are associated with a high parasite load. Our kDNA qPCR assay proved highly sensitive and accurate for the detection and quantification of *Leishmania* (*Viannia*) spp. in lesion biopsy specimens. It has potential application as a diagnostic and follow-up tool in American tegumentary leishmaniasis.

American tegumentary leishmaniasis (ATL) is a major public health problem in the New World; it is a cause of social stigma and has a considerable impact on morbidity and quality of life of the affected population. Clinical presentation and outcome of ATL are associated with the host immune response and the infecting *Leishmania* species (1, 2). Among the different parasite species causing cutaneous leishmaniasis (CL) in the New World, *Leishmania* (*Viannia*) *braziliensis* is considered the most important because of its prevalence, its difficulty to cure, its public health importance, and the risk of severe disease, i.e., disfiguring mucosal leishmaniasis (ML) (2–6).

Quantitative assessment of the *Leishmania* load in host tissues has been proposed to be useful in monitoring the response to antileishmanial therapy and for addressing gaps in the understanding of the natural history of human infection with *Leishmania* (7–9). The traditional method of quantification of *Leishmania* in host tissues is the limiting dilution assay (LDA) (10, 11). However, this assay is arduous and time-consuming; it depends on sterile conditions and highly trained personnel and can be applied only with fresh samples with relatively high parasite loads because of its low sensitivity (10, 12). Nowadays, quantitative real-time PCR (qPCR) is widely used in research and diagnostics, since it provides rapid, sensitive, and accurate detection and quantification of pathogens (13–15). As for *Leishmania*, reports on the use of qPCR have focused mostly on visceral leishmaniasis (VL) (16–18), while reports on ATL due to *Leishmania* (*Viannia*) species are scanty. One report described qPCR assays targeting the *Leishmania* glucose-6-phosphate dehydrogenase (*G6PD*) locus to identify different *Leishmania* (*Viannia*) species and quantify the parasites (19). Since *G6PD* is a single-copy gene, it is expected that the *G6PD* qPCR assay has limited sensitivity for application in tissues with low parasite loads. In contrast, multicopy kinetoplast DNA (kDNA) could boost this sensitivity. Indeed, different studies describing qPCR assays targeting kDNA proved highly sensitive for detection, species discrimination, and quantification of *Leishmania* in clinical specimens from patients with different forms of leishmaniasis (17, 20, 21).

In this study, we developed and validated a SYBR green-based qPCR assay targeting kDNA to simultaneously detect and quantify *Leishmania* (*Viannia*) parasites with high sensitivity in skin and mucosal lesion biopsy specimens. We evaluated the correlation between measurements estimated with our kDNA qPCR assay and with a *G6PD* qPCR assay validated previously (19). More-

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over, parallel quantification of the human endogenous retrovirus 3 (*ERV-3*) gene allowed normalization of the parasite number for the number of cell equivalents present in each biopsy specimen. The applicability of our assay was demonstrated by its ability to quantify low parasite loads characteristic of lesions from patients with ML and chronic CL. To our knowledge, this is the first report of the quantitative comparison of parasite loads in CL and ML lesions by means of qPCR. We also analyzed the parasite load according to parasite species and clinical parameters in patients with CL.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Institutional Review Boards of Hospital Nacional Cayetano Heredia and Universidad Peruana Cayetano Heredia (Lima, Peru) and the Hospital Nacional Adolfo Guevara Velasco (Cusco, Peru). All patients provided written informed consent for the study procedures prior to enrollment.

Patient lesion biopsies and promastigote cultures. One hundred fifty-six skin and mucosal lesion biopsy specimens (2 mm in diameter) were taken before treatment from 152 Peruvian patients with clinically suspected CL, ML, or mucocutaneous leishmaniasis (MCL). In MCL, there are both CL and ML concurrent lesions. Patients were enrolled at the Instituto de Medicina Tropical Alexander von Humboldt (IMTAvH) in Lima and at the Hospital Nacional Adolfo Guevara Velasco in Cusco between 2008 and 2011. The biopsy specimens were preserved in absolute ethanol and stored at −20°C prior to further processing.

Four *Leishmania* (*V.*) *braziliensis* reference strains (MHOM/BR/75/M2903, MHOM/BR/75/M2904, MHOM/PE/93/LC2177, and MHOM/PE/91/LC2043) and 3 *Leishmania* (*V.*) *guyanensis* reference strains (MHOM/BR/75/M4147, IPRN/PE/87/Lp52, and MHOM/PE/03/LH2549) were cultured as promastigotes in Novy-MacNeal-Nicolle (NNN) medium, as reported elsewhere (22). Cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS) (pH 7.2).

Reference standards for diagnosis. We defined a lesion to be due to *Leishmania* when at least 1 of 3 tests was positive, where tests refer to direct smear (microscopy), culture, and biopsy specimen qualitative PCR targeting the kDNA minicircles. A biopsy specimen negative for the 3 tests was defined as negative. The sensitivity and specificity of the qualitative and real-time-based PCR tests targeting kDNA were evaluated (23).

Isolation of DNA from lesion biopsy specimens and cultured strains. The biopsy specimens were minced with a sterile scalpel. Then, the biopsy specimens and promastigote pellets were subjected to overnight lysis with proteinase K and processed for DNA isolation using the High Pure PCR template preparation kit (Roche), according to the manufacturer's instructions. The isolated DNA was quantified by fluorometry using the Quant-iT high-sensitivity DNA assay kit and the Qubit fluorometer (Invitrogen).

Qualitative PCR detecting *Leishmania* (*Viannia*) kDNA and species identification. *Leishmania* kDNA PCR was performed using primers and conditions described previously (24, 25). Parasites were typed according to the algorithm reported elsewhere (26).

Detection and quantification of *Leishmania* (*Viannia*) spp. by quantitative real-time PCR. A qPCR assay based on kDNA minicircle amplification (kDNA qPCR) was developed for detection and quantification of *Leishmania* (*Viannia*) DNA in biological samples. It uses the primer set described previously for qualitative, diagnostic PCR (24). To normalize the parasite load for human cell equivalents, we quantified in parallel the single-copy human gene endogenous retrovirus 3 (*ERV-3*) (27, 28).

In order to analyze the variability of the number of minicircles and its impact on the quantification results among *Leishmania* (*Viannia*)-positive samples, comparative quantification was performed for 7 reference strains and 43 clinical specimens by targeting a single-copy gene, the *Leishmania G6PD* gene (19). The relative number of kDNA copies was calculated as follows: (parasite DNA equivalents/reaction estimated by kDNA qPCR)/(parasites/reaction estimated by *G6PD* qPCR).

The qPCRs were performed in a 25-μl volume consisting of 5 μl of DNA sample (10 ng), 200 μM (each) primer (see Table S1 in the supplemental material), and 1× iQ SYBR green supermix (Bio-Rad). Reactions were run on the LightCycler 480 system (Roche). The thermal cycling conditions were as follows: 95°C for 3 min, 35 cycles (kDNA qPCR and *ERV-3* qPCR) or 36 cycles (*G6PD* qPCR) at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. Fluorescence emission was measured at the end of the elongation step. After PCR amplification, a melting curve was generated to check the amplicon specificity; it consisted of 1 cycle at 95°C for 60 s, followed by 60°C for 60 s and continuous heating at 0.02°C/s to 95°C. The PCR product of each targeted gene had a specific T_m (see Table S1). Each run included a positive-control sample (DNA from a biopsy specimen of a leishmaniasis patient), a negative control (DNA from a healthy subject), and a blank (no-template control). Each sample was tested in duplicate.

Standard curves. Genomic DNA (gDNA) of the *Leishmania* (*V.*) *braziliensis* strain MHOM/BR/75/M2904 served as the quantification standard for the kDNA qPCR assay. We considered 83.15 fg of leishmanial DNA equivalent to one parasite, based on the size of the sequenced *L.* (*V.*) *braziliensis* haploid genome (32 Mb; 70 fg for its diploid genome) (29), plus an estimated 15.8% kDNA (13.15 fg) (30). This equivalence was used for the preparation of the standard curves in the range 5×10^4 to 5×10^{-3} parasites/reaction. The *G6PD* standard curves (10^6 to 10^1 copies/reaction) were based on the pGEM-T Easy vector containing the 5′ end of the *G6PD* gene cloned from the *L.* (*V.*) *braziliensis* strain MHOM/BR/75/M2903 (19). The *ERV-3* standard curves were established using human gDNA (from peripheral blood mononuclear cells of a healthy donor) and comprised 2×10^4 to 1.6×10^2 copies/reaction (28).

To assess the impact of background human gDNA in clinical samples on assay performance (sensitivity and amplification efficiency), standard curves based on serial dilutions of the *Leishmania* DNA standard in nuclease-free water were compared to the same dilution series performed in a background of 20 ng of human gDNA per reaction. Since no interference was observed, water was used to elute the leishmanial DNA used to prepare the standard curves.

Data analysis. The "second derivative maximum" mode of the LightCycler software, v1.5.0, was used to calculate the amplification curve quantification cycle (C_q). C_q values of duplicate measurements were averaged. Replicates with a standard deviation of >0.35 in C_q values (>0.5 cycles) were retested. The "melting-curve genotyping" mode of the LightCycler software was used to generate the melting curves.

Limit of quantification, limit of detection, and expression of results. A sample was quantified when it had a C_q value falling within the range of the standard curve. The highest dilution of template of the standard curve was defined as the limit of quantification (LOQ). Samples with C_q values higher than the LOQ could be detected; they were considered positive (qualitative detection) only if their melting curves had the same profile as those of the standards included in the same experiment.

The *Leishmania* load (here called PL [parasite load]) was calculated as follows: (i) [(parasite DNA equivalents/reaction estimated by kDNA qPCR)/(*ERV-3* average copy number/2)] × 10^6 or (ii) [(*G6PD* average copy number/2)/(*ERV-3* average copy number/2)] × 10^6, expressed as the number of *Leishmania* parasites per 10^6 human cells.

Statistical analyses. Correlation analysis between the PL estimated with the kDNA qPCR and *G6PD* qPCR assays was conducted using the Spearman rank correlation test. The PL in CL and ML lesion biopsy specimens was comparatively analyzed using the Mann-Whitney U test. Among CL lesions, the association between the *Leishmania* load and the parasite species was evaluated using the Kruskal-Wallis test. The PL level was arbitrarily categorized as "low PL" (≤10,000 parasites/10^6 human cells) or "high PL" (>10,000 parasites/10^6 human cells) based on PL data distribution and then analyzed with regard to the clinical parameters studied among CL patients: the patient's age, number of lesions, duration of lesions, and total area of lesions (Mann-Whitney U test). Statistical tests were performed under a 5% significance level, using GraphPad Prism v5.02 software.

TABLE 1 Intra-assay and interassay reproducibility of the kDNA qPCR

No. of parasite DNA equivalents/reaction	Intra-assay reproducibility				Interassay reproducibility			
		No. of parasites				No. of parasites		
	Mean C_q[a]	Mean[b]	SD	% CV[c]	Mean C_q	Mean[b]	SD	% CV[c]
5.0×10^4	7.8	5.2×10^4	1.4×10^3	2.7	8.0	5.1×10^4	3.5×10^2	0.7
5.0×10^3	11.2	5.3×10^3	2.4×10^1	0.5	11.3	4.9×10^3	3.0×10^2	6.3
5.0×10^2	14.6	4.7×10^2	1.1×10^1	2.4	14.5	5.1×10^2	3.5×10^1	6.8
5.0×10^1	17.9	4.9×10^1	1.4×10^0	2.8	17.8	4.9×10^1	4.2×10^{-1}	0.9
5.0×10^0	21.1	5.1×10^0	1.3×10^{-1}	2.5	21.2	4.6×10^0	7.6×10^{-1}	16.3
5.0×10^{-1}	24.4	5.1×10^{-1}	2.2×10^{-2}	4.2	24.6	4.8×10^{-1}	5.7×10^{-2}	11.9
5.0×10^{-2}	27.4	4.8×10^{-2}	2.7×10^{-3}	5.6	28.1	5.4×10^{-2}	1.6×10^{-2}	29.3
5.0×10^{-3}	31.2	5.1×10^{-3}	1.9×10^{-3}	36.6	31.6	5.7×10^{-3}	1.3×10^{-3}	23.4

[a] C_q, quantification cycle.
[b] Mean parasite number estimated by the kDNA qPCR assay with the LightCycler 480 instrument (Roche).
[c] Coefficient of variation of the parasite number [CV = (SD/mean) × 100)]. To analyze intra-assay variation, each dilution of the standard curve was tested with 3 replicates within one LightCycler run. Interassay variation was investigated in 3 independent experimental runs.

RESULTS

qPCR assays for *Leishmania* quantification. (i) Dynamic range of the qPCR assays. The dynamic range of the kDNA qPCR assay encompassed at least 7 orders of magnitude (5×10^4 to 5×10^{-3} parasite DNA equivalents/reaction). The standard curves ($n = 3$) were characterized by a mean square error (MSE) of ≤0.007, correlation coefficients (r^2) of ≥0.995, and slopes of −3.20 (mean) ± 0.24 (standard deviation), indicating a high amplification efficiency (≥1.97) (2 would indicate 100% PCR efficiency). The standard curves of the G6PD qPCR assay ($n = 3$) were linear over concentrations of 10^6 to 10^1 copies/reaction, with a MSE of ≤0.005, r^2 value of ≥0.993, and slopes of −3.40 ± 0.25, corresponding to an efficiency of ≥1.95 (see Fig. S1 in the supplemental material).

The sensitivity of *Leishmania* quantification by the kDNA -qPCR or *G6PD* qPCR assay was not affected by the presence of background human gDNA, since similar C_q values were obtained in the presence versus absence of background gDNA (see Fig. S1).

For each parasite concentration of the standard curve of the kDNA qPCR assay, the coefficient of variation (CV) was calculated for one run (intra-assay reproducibility) and 3 independent runs (interassay reproducibility). The highest CV values were obtained with the smallest amount of parasite DNA equivalents in both cases (Table 1). The positive control (one clinical sample) included in 4 independent runs showed a mean of 4.78×10^3 parasites with a CV of 8.1%. Under the conditions established for each qPCR assay, no amplification of the negative-control sample or blank was detected.

(ii) Evaluation of variability of relative number of kDNA targets. We analyzed the ratio of the parasite number determined by the kDNA qPCR to the parasite number determined by the *G6PD* qPCR (further called kDNA/*G6PD* ratio). First, this was done for 7 cultured strains (promastigote stage). The ratios for the 4 tested *Leishmania* (*V.*) *braziliensis* strains were 1.1 (MHOM/BR/75/M2904, reference strain used for quantification), 1.9 (MHOM/PE/91/LC2043), 2.5 (MHOM/BR/75/M2903), and 3.3 (MHOM/PE/93/LC2177). The ratios for the 3 tested *L.* (*V.*) *guyanensis* strains were 1.2 (IPRN/PE/87/Lp52), 2.6 (MHOM/PE/03/LH2549), and 3.6 (MHOM/BR/75/M4147).

Next, the kDNA/*G6PD* ratio was evaluated directly with clinical samples ($n = 43$); ratios varied from 0.1 to 3.8 among the *Leishmania* (*Viannia*)-infected cells, with the majority of samples (38/43) showing a variation in the range from 0.7 to 2 (Fig. 1A). We then analyzed if this variability could be due to the different parasite species found in the clinical samples examined. The analysis showed that the mean variability in the relative number of minicircles was not significantly different among samples positive for *L.* (*V.*) *peruviana* (median ratio of 0.74 [interquartile range {IQR}, 0.3 to 1.4]; $n = 9$), *L.* (*V.*) *braziliensis* (median ratio of 0.46 [IQR, 0.2 to 1.0]; $n = 10$), or *L.* (*V.*) *guyanensis* (median ratio of 0.53 [IQR, 0.4 to 1.3]; $n = 15$) ($P = 0.47$, Kruskal-Wallis test).

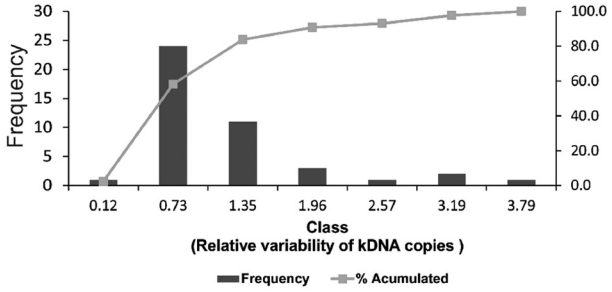
Application of qPCR assays with human biopsy specimens. (i) Detection of *Leishmania* DNA in human biopsy specimens by kDNA qPCR. The qualitative kDNA PCR detected the presence of *Leishmania* DNA with 95.5% sensitivity (95% confidence interval [CI], 90.5% to 98.5%) and 100% specificity (95% CI, 76.8% to 100%), while the kDNA qPCR assay achieved this with 97.9% sensitivity (95% CI, 94.2% to 99.6%) and 87.5% specificity (95% CI, 47.4% to 99.7%) (Fig. 2). The apparently lower specificity of the kDNA qPCR compared to the qualitative PCR reflected the fact that one tested biopsy specimen was positive by the kDNA qPCR assay while testing negative with the reference standards used here. We discarded the possibility of it being a false-positive qPCR result, since another specimen (scraping) available from the same lesion had a kDNA-positive qualitative PCR result.

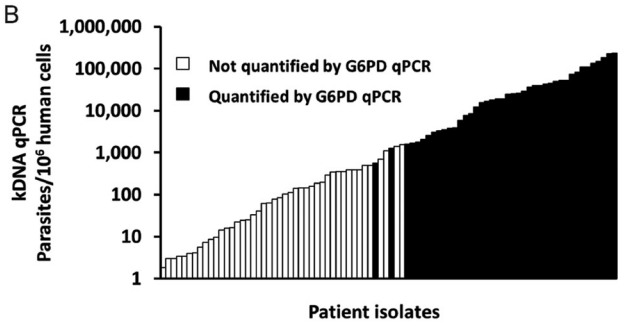
(ii) Quantification of *Leishmania* load in human biopsy specimens. The kDNA qPCR assay allowed the quantification of the parasite load in 132 out of 148 lesion biopsy specimens with positive diagnosis of ATL (89.2%; 95% CI, 83.6% to 94.3%) (Fig. 2). The PL varied from 1.8×10^0 to 1.9×10^6 parasites per 10^6 human cells (median = 5.3×10^2).

To evaluate the correlation between the kDNA qPCR and *G6PD* qPCR assays and to assess the performance of the former assay, 101 collected CL samples were quantified in parallel by both assays. In this subset of biopsy specimens, the kDNA qPCR assay allowed the quantification of the PL in 87 samples (86.1%; 95% CI, 79.2% to 92.7%), while the *G6PD* qPCR assay allowed this in 43 samples (42.2%; 95% CI, 33.3% to 52.6%), indicating that the former assay is far more sensitive than the latter ($P = 0.000$, Z test). The PL quantified by the *G6PD* qPCR varied from 2.3×10^3 to 1.5×10^6 parasites per 10^6 human cells (median = 3.3×10^4), whereas the kDNA qPCR attained lower detection limits (down to 1.8×10^0 parasites per 10^6 human cells) (Fig. 1B). As expected, when samples had a quantifiable *Leishmania* PL with both qPCR

A

Class (Relative Variability of kDNA copies)	Frequency	% Accumulated
0.12	1	2.3
0.73	24	58.1
1.35	11	83.7
1.96	3	90.7
2.57	1	93.0
3.19	2	97.7
3.79	1	100.0





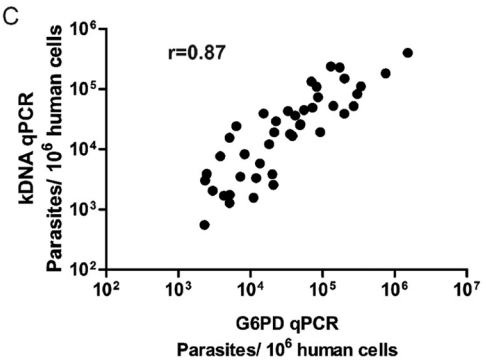


FIG 1 Variability of kDNA targets in clinical samples and comparison of parasite loads estimated by the kDNA qPCR and *G6PD* qPCR assays. (A) Variations in kDNA minicircle numbers among *Leishmania* (*Viannia*) clinical isolates. The bars (right plot) show the ratio of kDNA to *G6PD* quantification results. (B) Comparison of sensitivities of the kDNA qPCR and *G6PD* qPCR assays for quantifying the parasite load in clinical samples. The histogram shows the distribution of the kDNA qPCR results with respect to those of *G6PD* qPCR ($n = 87$ values). (C) Correlation between parasite loads estimated by the kDNA qPCR and *G6PD* qPCR assays.

assays, the measurements were highly correlated (Spearman's rho = 0.87; $P < 0.0001$) (Fig. 1C).

(iii) Comparison of parasite loads according to clinical manifestations and infecting species. The median PL in CL lesions ($n = 108$) was 1.0×10^3 parasites per 10^6 human cells, compared to 0.9×10^2 in ML lesions ($n = 21$) ($P = 0.009$, Mann-Whitney U test) (Fig. 3A). There was no significant difference in PL according to the infecting species in CL lesions ($P = 0.81$, Kruskal-Wallis test) (Fig. 3B).

(iv) Parasite load levels according to clinical parameters in CL patients. A "high PL" was characteristic of lesions of recent onset (median lesion duration of 64 days), whereas a "low PL" was observed in lesions with a higher evolution time (median lesion duration of 95 days) ($P = 0.004$, Mann-Whitney U test) (Table 2). There was no significant association between the PL level and other clinical parameters examined: the patient's age, number of lesions, or mean total area of active lesions (Table 2).

DISCUSSION

Early evidences from histopathological studies indicate that parasite abundance in lesions from ML patients is lower than that in lesions from CL patients (31, 32). Here, the power of the kDNA qPCR assay has allowed the quantification of a broad range of parasite load levels in CL and ML tissue lesions. We confirmed that parasite levels are indeed lower in ML lesions than in CL ones: the median parasite load between these groups differed remarkably, by 10-fold. To our knowledge, this is the first qPCR study that assesses parasite load in a large sample of CL and ML lesions due to *Leishmania* (*Viannia*) species and in which the parasite load is expressed as the number of parasites normalized for a fixed number of human cell equivalents.

Notably, the scarcity of parasites in lesional tissue of *Leishmania* (*Viannia*)-infected patients, particularly in lesions from patients with ML and chronic CL, contrasts with the severe tissue damage observed in ATL. On the one hand, this suggests that *Leishmania* might alter local tissue homeostasis, promoting tissue damage. On the other hand, some reports show that the immune system response, rather than the parasites *per se*, causes ulceration and tissue destruction in ATL (33, 34).

Our finding of low parasite loads in ML lesions is consistent with the reported difficulty in visualizing *Leishmania* parasites in Giemsa-stained smears of lesion biopsy specimens from patients with ML (31, 32) and with the lesser success in isolating the parasites through culture of ML lesion samples than with CL (35). The differences in parasite load between CL and ML lesions could be associated with the differential immunopathological manifestations documented in ATL (36). CL is characterized by a moderate T-cell hypersensitivity, whereas ML represents the extreme expression of the T-cell hypersensitivity pole with an exacerbated Th1-type immune response. In a previous study, complex links between New World *Leishmania* infection and immune responses in the skin and mucosa were evidenced; for instance, the leishmanin skin test (LST) responses showed bigger induration sizes in ML than in CL, consistent with higher levels of inflammatory cytokine mRNAs found in ML (37).

Another relevant observation was that among the CL lesions analyzed here, the parasite load level was inversely correlated with disease duration. This is in line with reports documenting that the

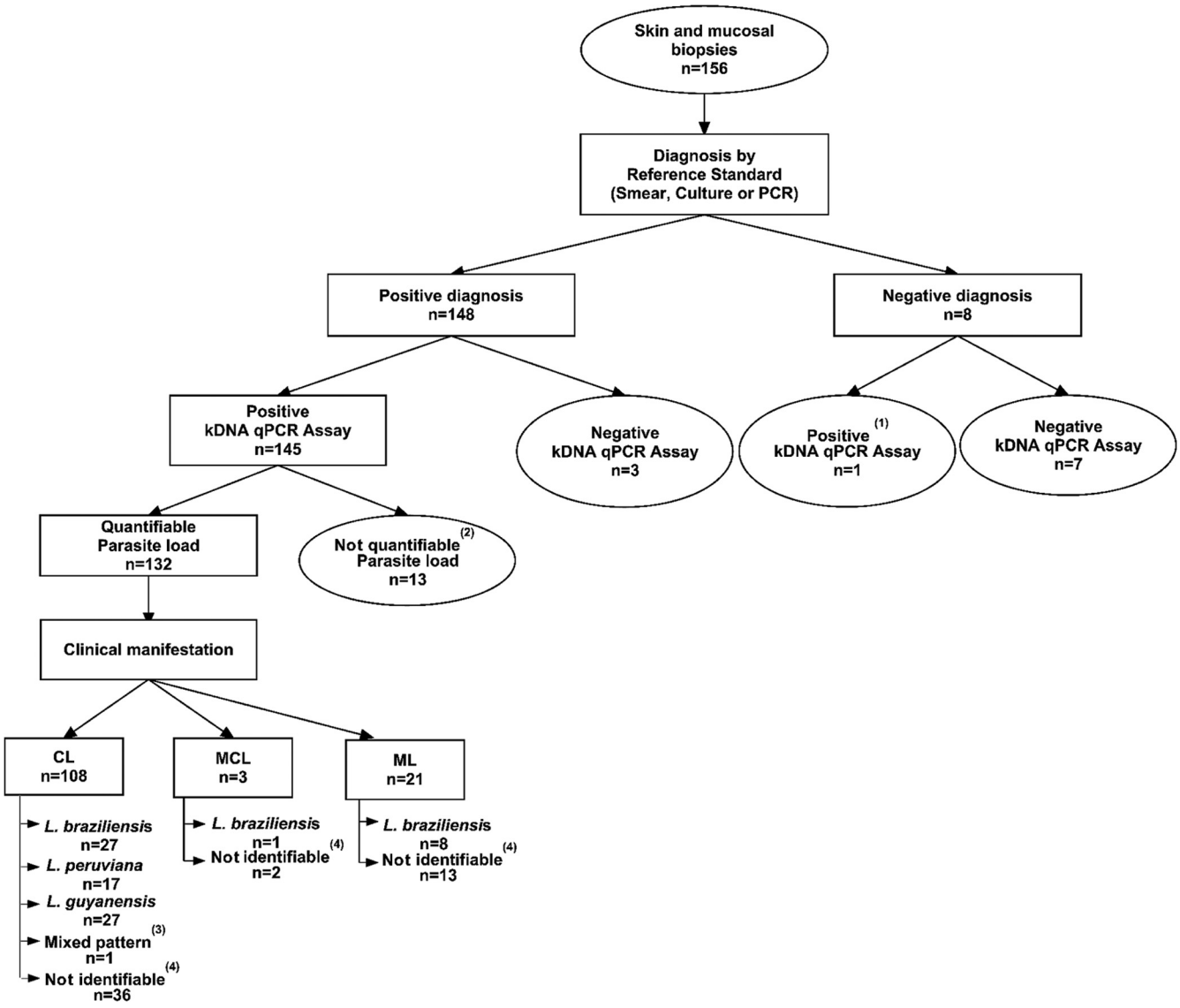


FIG 2 Flow diagram of the study procedures (diagnosis, parasite load determination, and species identification) performed on lesion biopsy specimens from patients with ATL. Superscript numbers indicate the following: (1) another specimen (scraping) available from the same lesion had a kDNA-positive qualitative PCR result; (2) these samples could not be quantified because they fell out of the limit of quantification; (3) mixed pattern for *L.* (*V.*) *peruviana* and *L.* (*V.*) *braziliensis*; (4) the species could not be assigned due to a lack of recognizable patterns common to reference *Leishmania* strains included in the species identification algorithm used herein, or the species could not be determined because of a very low parasite load or insufficient concentration of amplifiable genomic DNA.

diagnosis of CL caused by *Leishmania* (*Viannia*) spp. is more challenging for lesions of greater than 6 months' duration than for lesions of more recent onset (38). Our finding is also consistent with findings of a previous study that focused on Old World CL due to *Leishmania* (*L.*) *tropica* (39), pointing to a high parasite load in acute cutaneous disease.

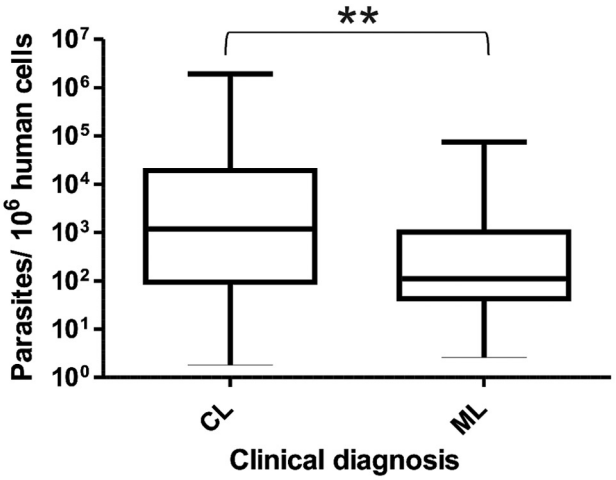
Concerning pathogenicity differences according to *Leishmania* species, a study performed in Brazil showed that the diseases caused by *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis* are different with regard to the number, size, and location of skin lesions and the characteristics of lymphatic involvement (40). The analysis performed here revealed no significant differences in parasite load according to the infecting *L.* (*Viannia*) species or with regard to the number or size of the skin lesions. These findings suggest that the degree of clinical pathology in CL is not associated with the parasite load.

Reports of the use of qPCR assays based on repetitive sequences, like the *Leishmania* kDNA minicircles, to simultaneously detect and quantify the parasite load in clinical specimens have mostly focused on VL (7, 16–18). As for ATL, a few qPCR assays that amplify multicopy DNA targets have been described (20, 41, 42). In terms of diagnostic sensitivity, our kDNA qPCR assay performed similarly to the qualitative PCR test based on the same primers. The achieved analytical sensitivity for the quantification of *Leishmania* (*Viannia*), i.e., 5×10^{-3} parasite DNA equivalents/reaction, was the same as that of the most sensitive reported qPCR assay that also targets kDNA (20).

In order to accurately quantify parasites in clinical specimens,

A

	Parasite load[1]	
	CL	ML
Number of values	108	21
Minimum	1.5×10^0	2.2×10^0
Median	1.0×10^3	0.9×10^2
Maximum	1.6×10^6	6.3×10^4
Interquartile range	1.6×10^4	7.1×10^2



B

	Parasite load[1]		
	L.(V.) braziliensis	*L.(V.) guyanensis*	*L.(V.) peruviana*
Number of values	27	27	17
Minimum	1.6×10^1	3.4×10^0	3.4×10^0
Median	5.7×10^3	2.5×10^3	1.2×10^3
Maximum	1.9×10^6	4.0×10^5	1.5×10^5
Interquartile range	3.1×10^4	2.9×10^4	4.5×10^4

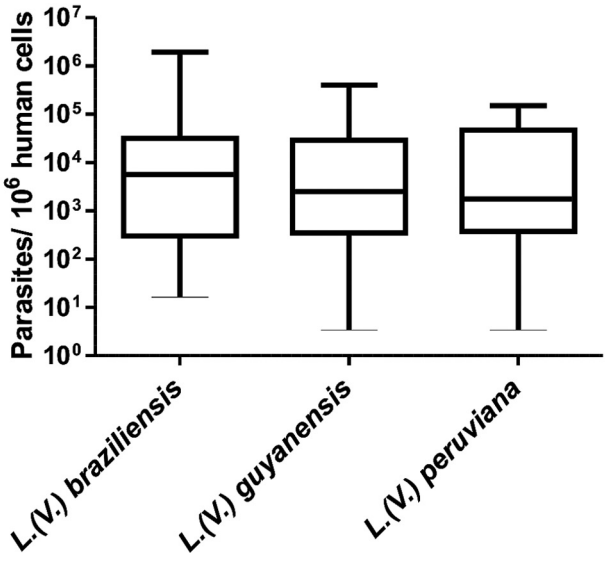


FIG 3 Parasite load levels in human biopsy specimens. (A) Parasite load levels in clinical samples according to clinical manifestations. **, a significant difference was found in parasite loads between cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) lesions ($P = 0.009$). (B) Parasite load levels in CL lesions according to the infecting species. 1, the number of parasites per 10^6 human cells is indicated.

we investigated the variability of the relative number of kDNA targets (adjusted to the single-copy *G6PD* gene) in the promastigote stage (7 strains) and the amastigote stage (43 clinical samples) of *L.* (*Viannia*)-positive samples. In agreement with previous reports on Old World *Leishmania* (17, 20), the relative number for kDNA differed moderately (up to 4-fold) among the strains/specimens of a same *L.* (*Viannia*) species analyzed here. However, in this study, there were no statistically significant differences in the relative number of kDNA minicircle targets among the three *L.* (*Viannia*) species found in clinical samples, which indicates that there is no quantitative bias related to the parasite species. Therefore, our data suggest that quantification of *L.* (*Viannia*)-infected samples could be performed by comparison with a standard curve generated using only one reference strain DNA. Altogether, our observations support the validity of using kDNA minicircle targets to quantify *Leishmania* in tissues from human lesions. Absolute parasite numbers would likely be most accurate if the quantification is based on a single-copy target, but it has to be taken into account that single-copy targets present in chromosomes might be dependent on variation in ploidy, which has been shown to occur extensively in *Leishmania* (43, 44). Last, any amplification assay based on a single-copy target will have very low sensitivity in application for survey (e.g., follow-up of patient's response to therapy) and epidemiologic purposes.

Further work using well-documented clinical samples covering different regions of endemicity is needed to replicate the present finding of an association between the parasite load and clinical manifestations of ATL with an independent sample. The kDNA qPCR assay developed here has potential diagnostic and prognostic application value in the clinical management of ATL. Since *Leishmania* (*Viannia*) infection can still sometimes be detected after treatment (45, 46), a highly sensitive quantitative technique can be employed not only for diagnostic purposes but also for monitoring the parasite load in patients during treatment and

TABLE 2 Clinical characteristics of CL patients and the level of parasite load

Clinical characteristic	Value for patients with parasite load classification[b]		*P* value[d]
	Low ($n^c = 57$)	High ($n^c = 28$)	
Age (yrs)			
Mean ± SD	31.8 ± 15.7	34.4 ± 14.2	0.362
Median (IQR[a])	28.0 (20.5–39.0)	32.5 (22.8–47.0)	
No. of lesions			
Mean ± SD	2.2 ± 1.8	3.3 ± 5.7	0.984
Median (IQR)	2.0 (1–3)	1.5 (1–3)	
Lesion area (cm^2)			
Mean ± SD	9.9 ± 9.5	7.3 ± 7.0	0.170
Median (IQR)	7.5 (3.6–13.2)	4.8 (2.3–10.1)	
Duration of disease (days)			
Mean ± SD	362.1 ± 1,555.6	79.5 ± 63.0	0.004
Median (IQR)	95.0 (73.5–159.5)	64.0 (40.5–96.0)	

[a] IQR, interquartile range.
[b] The parasite load was determined by the kDNA qPCR assay and categorized as "low" (≤10,000 parasites/10^6 human cells) or "high" (>10,000 parasites/10^6 human cells).
[c] *n*, no. of patients.
[d] *P* values were determined by the Mann-Whitney U test.

follow-up as a way to assess or predict the outcome of therapy. Such an application of qPCR has been clearly demonstrated in VL (7, 18, 47). Last, our kDNA qPCR assay will allow evaluating the association of parasite load with the human immune response in ATL, which could be helpful in defining the prognosis of this disease.

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V.A. and J.A. conceived the study. B.M.V., D.M., and M.C. contributed to data collection and were responsible for enrolling patients. M.J., M.A., and C.C. conducted molecular analyses. J.A., I.C., G.V.D.A., J.-C.D., and A.L.-C. contributed to study design, implementation, and data interpretation. M.J. and V.A. contributed to study design, data collection, analysis, and interpretation and were primarily responsible for writing the manuscript. All authors critically appraised the manuscript.

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