Is Leishmania (Viannia) braziliensis parasite load associated with disease pathogenesis?

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Background: Leishmania (Viannia) braziliensis is the main etiological agent of tegumentary leishmaniasis in the Americas. Parasite molecular diversity and host immune status contribute to extensive variations in its clinical presentation within endemic areas of Brazil. Pentavalent antimonials have been used for more than 60 years as the first-line drug for all cases, despite the potential for severe side effects and refractoriness. In Rio de Janeiro, Brazil, most L. (V.) braziliensis infections are benign with a scarcity of parasites, although metastasis and refractory infections can arise. In this scenario, the use of novel molecular tools can be useful for diagnosis and to assess tissue parasitism, and is of benefit to clinical and therapeutic management.

Methods: In this study, parasite load was assessed by real-time PCR based on the leishmanial small subunit ribosomal RNA gene.

Results and conclusion: The data revealed a tendency to higher tissue parasitism in the skin compared to mucous lesion sites and a reduction with disease progression. Parasite load was lower in poor compared to good responders to antimonials, and was also reduced in recurrent lesions compared to primary ones. However, parasite load became higher with sequential relapses, pointing to an immune system inability to control the infection. Therefore the parasite burden does not seem to be a good predictor of disease progression.

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Introduction

Tegumentary leishmaniasis (TL) comprises a group of infectious diseases with a broad diversity in clinical presentation. Caused by parasites of the genus Leishmania, which are transmitted by sand flies, Leishmania (Viannia) braziliensis is the main etiological agent of leishmaniasis in the Americas. Brazil has the highest incidence of TL worldwide, with autochthonic confirmed in all regions of the country. Most TL cases correspond to cutaneous leishmaniasis (CL), which is characterized by a single ulcer on the skin that may regress spontaneously. However, some patients go on to develop mucosal leishmaniasis (ML), a destructive and diffuse metastatic infiltration of the nasal/oral/nasopharyngeal mucosa that occurs months or several years after resolution of the primary lesion. Pentavalent antimonials have been used for more than 60 years as the first-line treatment for all clinical presentations of leishmaniasis. However, these drugs have several limitations, such as severe side effects and drug resistance. Most cases occurring in the state of Rio de Janeiro represent benign infections, but a poor response to treatment can occur, with a failure rate of 16% and the development of mucosal forms.

There are several arguments to explain the differences in clinical and therapeutic outcomes of TL. It is now clear that both parasite molecular diversity and the host immune response contribute to the extensive clinical polymorphism in endemic areas. Although treatment with Glucantime mostly accelerates lesion healing, it does not induce total clearance of the parasites.
and a small number are likely to persist in the cutaneous scars over the patient’s lifetime.\(^6\) The scarcity of tissue parasitism has been described as a common observation in patients infected by *L. (V.) braziliensis*,\(^7\) which underlines the need for highly sensitive molecular approaches to confirm a diagnosis.\(^8\) Additionally, for different species, parasite load could be related to the severity of the lesions. Mouse models that mimic the natural transmission of *Leishmania* (*Leishmania*) major have revealed that a high-dose inoculum leads to the development of larger lesions, while low doses of parasites lead to less severe pathologies, regardless of higher parasite titers in the chronic phase.\(^9\)

The aim of this study was to investigate the association between the parasite load measured in the lesions of patients with diverse clinical presentations and their response to therapeutic interventions in cases of *L. (V.) braziliensis* TL in Brazil.

Materials and methods

Patients

A total of 126 patients with leishmaniasis were studied. All patients were from the state of Rio de Janeiro in Brazil, an area endemic for *L. (V.) braziliensis*. Cases with any comorbidity were excluded from the study. The diagnosis was confirmed by at least one of the following methods: histopathology, Leishman-stained imprint of the lesions, culture, and PCR. In addition, all patients underwent the Montenegro skin test. TL-positive patients were treated with 10–20 mg/kg/day of *N*-methyl-glucamine (Glucantime; Rhodia Laboratories, Antony, France) for 20 to 30 days. Patients were re-evaluated 3 months after the end of treatment, and were considered as clinically cured if the lesions had reached complete epithelialization and there was an absence of erythema, induration, or papules. Patients were classified as poor responders if healing was incomplete. The response was also considered poor if reactivation or secondary metastatic lesions, either cutaneous or mucosal, appeared.

For the analysis of parasite load, patients were divided into five different groups according to their clinical presentation: cutaneous (CL, *n* = 71), recurrent CL (REC, *n* = 15), mucosal lesion (ML, *n* = 18), mucocutaneous lesion (MCL, defined as cases presenting concomitant active mucous and cutaneous lesions, *n* = 14), and cutaneous scars (*n* = 8). When appropriate, the total CL group was divided according to the treatment response and duration of disease: (1) good response to treatment (GR, *n* = 54) or poor response to treatment (PR, *n* = 17), and (2) duration of disease up to 3 months (early, *n* = 43), between 3 and 12 months (intermediate, *n* = 18), and more than 12 months (late, *n* = 10).

Biopsies were taken with a punch at the border of the lesions and submitted to diagnostic procedures, including nucleic acid isolation for real-time PCR quantification assays. All specimens were taken before treatment, except in recurrent cases. Dermatitis cases were included as negative controls.

Parasites and human cell cultures

The *L. (V.) braziliensis* reference strain (MHOM/BR/1975/M29903) was obtained from the *Leishmania* Collection of the Oswaldo Cruz Institute (CLOC). Promastigotes were grown at 25 °C in Schneider’s *Drosophila* medium containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 IU/ml penicillin, and 50 μg/ml streptomycin (Sigma, St. Louis, MO, USA).

The human acute monocyte leukemia cell line (THP-1), obtained from the Rio de Janeiro Cell Bank (ATCC TIB-202; American Type Culture Collection, Rockville, MD, USA) was maintained at 37 °C and 5% CO\(_2\) in RPMI 1640 medium (Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Cultilab), 2 mM l-glutamine (Pharmacia Biotech, Piscataway, NJ, USA), 100 IU/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, and 0.05 mM 2-mercaptoethanol (Sigma Chemicals, St. Louis, MO, USA).

Nucleic acid purification

DNA was purified from parasites, human cultured cells, and biopsy specimens using an Illustra Tissue and Cells Genomic Prep Mini Spin Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) following the manufacturer’s instructions. Extracted DNA was quantified by NanoDrop (Thermo Scientific, Waltham, MA, USA), diluted in Tris–ethylenediaminetraacetic acid (TE) buffer to 25 ng/μl if necessary, and stored at −20 °C.

Parasite quantification assay

An absolute quantification real-time PCR (qPCR) was performed using the StepOne Real Time System (Applied Biosystems, Foster City, CA, USA) with Power SYBR-Green PCR Master Mix (Applied Biosystems). The thermal profile was 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 75 °C for 30 s as the reading step.\(^10\) An additional melt curve was performed to check amplification product specificity from 65 °C to 95 °C. The *Leishmania* small subunit RNA gene (SSR) (accession number M80292) was used to assess the parasite load,\(^11,13\) and human β-actin (ACT) (accession number NM000101) was included to correct DNA content variations and inhibitor interference among samples. Oligonucleotides at 500 nM were used (5’ to 3’): ACT, TAATGTACCCCGACAGATTCCC and TCACCGACCGCCGCT,\(^14\) and SSR, TACTGGGGCGTCAGAG and GGTTGTCTCATGTTGCG.\(^11\) Standard curves were prepared with log dilutions of the number of cultured *Leishmania* and of the DNA mass (ng) of human cells (immortalized THP-1 human monocyte lineage), for SSR and ACT analyses, respectively. Parasite load was defined as the number of parasite equivalents per mass of DNA host cells (ng). No more than 50 ng of total DNA was analyzed per reaction. Negative samples were classified as having an undetermined SSR quantification cycle (Cq) if values that corresponded to ACT Cq were above the limit of detection (LOD). Data analyses were performed using Excel.

Ethical issues

This research study was approved by the Research Ethics Committee of Fiocruz (protocol 0033.0.011.346-11). Written informed consent was obtained from all patients. Diagnostic tissue fragments were obtained from the border of the ulcers by biopsy, under sterile conditions after local anesthesia. Normal skin samples were obtained from esthetic surgery patients. The *Leishmania* Collection of the Oswaldo Cruz Institute (CLOC, http://cloc.fiocruz.br/) is registered with the World Federation for Culture Collections (WFCC-WDCM 731) and is recognized as a Depository Authority by the Brazilian Ministry of the Environment (D.O.U. 05.04.2005).

Results

Clinical profile of the patients

The distributions of age, Montenegro skin test results, number of lesions, and duration of disease are compared in Figure 1. ML and MCL represent a reactivation process of CL that often occurs in patients with a compromised immune system. These scenarios are frequently related to older patients, and thus ML and MCL showed a higher mean patient age than CL, which exhibited a wider age range (Figure 1A). The intradermal Montenegro skin test revealed higher responsiveness for ML patients than CL and MCL patients.
(Figure 1B), MCL patients presented higher numbers of cutaneous lesions compared to CL and REC patients (Figure 1C). The longest duration of disease was observed for ML and MCL (Figure 1D). Additionally, the same criterion was longer for reactivation cases than for primary CL cases (Figure 1E). These results showed a relationship between a poor prognosis and a longer healing time after treatment.

Parasite quantification assay

Standard curves for both targets (quantification cycle versus logarithm of the *Leishmania* cell number or mass of human DNA) showed similar efficiencies (96.66% for SSR and 99.25% for ACT), good linear correlation ($r^2 = 0.99$ for both), and a linear dynamic range. The efficiency for parasite curves was not affected by the presence of human DNA. Melting temperatures ($T_m$) were 78.0 ± 0.5 °C for the SSR assay and 81.0 ± 0.5 °C for the ACT assay. Negative controls provided undetectable SSR amplification values. Undetected parasite loads were not plotted.

Tissue parasitism in the different clinical forms and treatment outcomes

There was a tendency towards lower tissue parasitism for mucous cases (ML) compared to cutaneous lesions (CL), although the difference was not statistically significant (Figure 2A). This observation is in agreement with a previous study from Peru reporting *Leishmania* (Viannaia) cases. Nevertheless, the data diverged from the histopathological consensus that mucous lesions present significantly lower parasite levels than cutaneous ones. Scars showed very low and mostly undetectable values (Figure 2A), indicating that treatment and the patient’s immune system had been able to reduce the parasite number. Skin samples from mucocutaneous lesions (MCL) ($n = 11$) presented significantly higher parasite loads than mucous samples from MCL ($n = 7$), following the same pattern observed for CL and ML, according to the tissue localization (Figure 2B). When the total CL group was classified into good and poor responders, the latter group showed statistically lower parasite levels (Figure 2C). However, on analyzing the relapse group separately from the poor responders, a different scenario was found. Only the first reactivation group (1stREC) presented statistically lower tissue parasite levels than all of the others (Figure 2D), compared to primary lesions from good responders (GR) and poor responders (primPR) and first (1stREC) and second recurrences (2ndREC). Lesions from multiple relapses showed a high parasite load, indicating immune system exhaustion, possibly as a result of chronic disease. Additionally, further analysis of a small number of samples, including paired primary and first recurrent lesions from the same patient, was conducted ($n = 8$) (data not shown). However, no statistical difference and no correlation was found, corroborating parasite persistence.

Parasite load is inversely correlated to the duration of disease progression

Recurrent infections showed significantly longer durations of disease progression than primary lesions (Figure 1E). An inverse and slight but significant correlation was observed between the duration of disease progression and parasite load when all cutaneous lesions were assessed, with recurrences (Spearman’s $r = -0.35$, $p = 0.002$) (Figure 3A). Also when the CL group was classified according to the duration of disease progression into early ($n = 43$, up to 3 months), intermediate ($n = 18$, between 3 and 12 months), and late CL ($n = 10$, older than 12 months), a longer disease duration led to a significant reduction in parasite load (Figure 3B).

Discussion

*Leishmania (V.) braziliensis* infections correspond to lesions with a scarcity of parasites. In this scenario, molecular tools could be useful to confirm the diagnosis, identify the species, and also to assess the parasite load, which would be of benefit in the clinical and therapeutic management. In the present study, qPCR was used...
to investigate parasite loads in TL lesions from the different clinical forms, the response to treatment, and the duration of disease progression.

The results of this study showed that skin from the cutaneous and mucocutaneous clinical forms exhibited the highest parasite loads compared to mucosal sites. This suggests that the microenvironment of the different anatomical sites might be the key to the specific host response. The present study data corroborate the findings of Jara et al. (2013) in Peru, in spite of the use of a different target. In the present work, the small subunit
ribosomal RNA gene (SSU or SSR) was used\textsuperscript{11,12,17} and not kDNA, since its accuracy might be compromised by mini-circle heterogeneity and copy number variation among species, strains, and isolates.\textsuperscript{18,19} Moreover, the SSR used herein is very conserved among members of the Trypanosomatidae family, enabling its use with other trypanosomatid clinical samples to assess variations in parasitism.

Sparse or undetectable parasite levels were observed in scars, indicating a role for the immune response in the healing process. Parasitological cure, however, does not seem to occur for \textit{L. (V.) braziliensis} cases, since kDNA is present in almost 80\% of scar tissue from treated patients.\textsuperscript{20} In the present work, although a different and less sensitive target than kDNA was used, it was possible to confirm parasite persistence in the scars.

In endemic areas of Rio de Janeiro, a good response to treatment and spontaneous cure is often observed, but a few cases may have a poor response, corresponding to a delay in healing or recurrence. Poor responders showed lower tissue parasitism than those with a good response. When infection doses were evaluated in a murine model for \textit{L. (L.) major}, the low dose group were found to have chronic infections that required longer periods to heal, in spite of the minor pathology observed in the acute phase.\textsuperscript{21} These results suggest that reduced parasite titers could be related to chronic disease outcomes. However, when primary poor response lesions were analyzed separately from recurrences, a new scenario was revealed. Parasite loads from primary good and poor responders were indistinguishable, but loads were significantly lower in the first episode of recurrence, indicating that the host has some control over parasite growth. A Th1 response is important for the clinical resolution process, and is indeed expressed in both cutaneous and mucosal lesions.\textsuperscript{22–25} However, this response does not seem to be sufficient to induce parasitological cure, since parasites remain at a very low quantity within the scars.\textsuperscript{6,24} Additionally secondary recurrences showed parasite loads indistinguishable from those of primary lesions, reflecting parasite persistence and indicating an inability of the host immune system to resolve the infection and/or possible drug refractoriness. On the other hand, recurrent and mucosal diseases both have a long duration as compared to single cutaneous disease. An inverse and slight negative correlation was observed between tissue parasitism and the duration of the disease. Also, when all cutaneous cases were categorized according to the duration of disease, lower parasite titers were observed for the oldest lesions.

The present authors, in studies performed in the Rio de Janeiro endemic area, have previously demonstrated that poor responders exhibit high proinflammatory cytokine ratios (high ratio of interferon gamma (IFN-\textgamma) to transforming growth factor beta (TGF-\textbeta)) and metalloproteinase activity characteristic of poor wound healing, in contrast to the anti-inflammatory good responders profile (low ratio of IFN-\textgamma to interleukin (IL)-10), associated with lower gelatinase activity in the lesions.\textsuperscript{22,23} This proinflammatory cytokine balance could also explain the tendency of recurrent lesions to have long durations regardless of lower parasite titers. Furthermore, disease refractoriness resulting in recurrent infection could be related to immune system exhaustion and parasite persistence.

Finally, the data from this study make an important contribution to the previous statement that chronic \textit{L. (V.) braziliensis} infections are associated with scarce parasitism.\textsuperscript{26} Furthermore, parasitism in the mucosal forms was statistically indistinguishable from that in cutaneous lesions, despite the tendency to lower values. These observations are in contrast to the histopathological consensus that mucosal disease has scarce parasitism. Ultimately, the synergistic effect of treatment and the host immune system often result in a good response and parasite reduction. In this endemic area, the majority of cases end in cure. However, a few cases may exhibit cutaneous recurrence or mucous lesions, corresponding to metastatic events. In this study, it was demonstrated that patients with multiple relapses had high tissue parasitism, suggesting treatment refractoriness and/or immune system exhaustion leading to parasite persistence.

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**Conflict of interest**

We have no competing interests to declare.

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