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Polymerase Chain Reaction Detection of *Leishmania* kDNA from the Urine of Peruvian Patients with Cutaneous and Mucocutaneous Leishmaniasis

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Abstract. We hypothesized that *Leishmania* kDNA may be present in urine of patients with cutaneous leishmaniasis (CL). Urine samples and standard diagnostic specimens were collected from patients with skin lesions. kDNA polymerase chain reaction (PCR) was performed on samples from patients and 10 healthy volunteers from non-endemic areas. Eighty-six of 108 patients were diagnosed with CL and 18 (21%) had detectable *Leishmania Viannia* kDNA in the urine. Sensitivity and specificity were 20.9% (95% confidence interval [CI] 12.3–29.5%) and 100%. Six of 8 patients with mucocutaneous involvement had detectable kDNA in urine versus 12 of 78 patients with isolated cutaneous disease ($P < 0.001$). *L.* (*V.*) *braziliensis* ($N = 3$), *L.* (*V.*) *guyanensis* ($N = 6$), and *L.* (*V.*) *peruviana* ($N = 3$) were identified from urine. No healthy volunteer or patient with an alternate diagnosis had detectable kDNA in urine. Sensitivity of urine PCR is sub-optimal for diagnosis. On the basis of these preliminary data in a small number of patients, detectable kDNA in urine may identify less localized forms of infection and inform treatment decisions.

INTRODUCTION

Polymerase chain reaction (PCR) is a highly sensitive tool for the detection of *Leishmania* DNA.[1–5] However, to date this platform has mostly been performed using invasively obtained clinical specimens such as lesion aspirates, scrapings, or biopsies in cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML). These techniques can cause considerable discomfort, require technical expertise, carry all of the risks of invasive procedures including bleeding and infection, and are especially difficult to perform in the pediatric population, in remote field settings, and in those with intercurrent bacterial or fungal superinfection. Thus, there is an ongoing need for less invasive, more simple, and sensitive diagnostic procedures.

Tegumentary forms of leishmaniasis are no longer strictly viewed as localized forms of disease.[1] Indeed, several lines of evidence support that lymphatogenous and hematogenous dissemination of the parasite may occur,[1,6–10] and that non-focal reservoirs of parasite persistence may actually protect against future reinfection.[9,11,12] Theoretically, then, parasites in both CL and ML would have access to the circulatory compartment of the host, as supported by numerous studies of CL showing *Leishmania* DNA in the blood,[7–10] and may therefore be detectable in urine. Cell-free DNA and small DNA fragments of 150–250 bp derived from human circulation can be detected in urine.[13,14] Thus, it is biologically plausible that parasite DNA could be detected from the urine of patients with what are historically thought of as more "localized" tegumentary forms of leishmaniasis. We hypothesized that kDNA may be detected in urine by PCR in patients with CL.

We herein show as proof-of-principle that while urine PCR has a sub-optimal diagnostic sensitivity in CL, it is highly specific and, on the basis of our preliminary findings, it may be useful in identifying patients with less localized forms of infection, especially those with mucosal involvement.

METHODS

Study site. Data were collected as a sub-study of a comparative diagnostic evaluation at the *Leishmania* Clinic of the Instituto de Medicina Tropical "Alexander Von Humboldt," Hospital Nacional Cayetano Heredia, in Lima, Peru, between January and April of 2009 and 2010, the results of which are reported elsewhere.[15] The study was approved by the Institutional Review Board of the Universidad Peruana Cayetano Heredia, Lima, Peru, and the University of Toronto, before initiation. Consecutive patients presenting to the *Leishmania* Clinic for the evaluation of skin lesions between January and April 2009 and 2010 were enrolled as described elsewhere.[15]

Diagnostic investigations. For the comparative diagnostic evaluation, skin scraping, aspirate specimens, and filter paper lesion impressions were obtained for Giemsa-stained smear, culture, and PCR as described elsewhere.[15–17] kDNA PCR of lesion scrapings and aspirates was performed as described using primers specific for *Leishmania* (*Viannia*) kDNA.[15–19] A single, mid-stream urine specimen was collected from each participant on enrollment before the diagnostic evaluations. In addition, urine samples were obtained from 10 healthy control volunteers living in non-endemic areas.

Isolation of DNA from urine and PCR. After collection, urine specimens were allowed to sediment for 10 minutes before two 1,000-μL aliquots were obtained using a sterile transfer pipette, and stored in Eppendorf tubes at –20°C before DNA extraction. Before extraction, 1,000-μL urine samples were thawed at room temperature, centrifuged at 8000 *g* for 10 minutes, and supernatant was discarded. Samples were resuspended in 500 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA) and incubated at room temperature for 5 minutes. Specimens were then centrifuged at 8000 *g* for 10 minutes and supernatant was discarded. DNA isolation was performed as described elsewhere.[15–18] Final DNA pellets were resuspended in 50 μL of TE buffer. Amplification by

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PCR of a 70-bp fragment of multicopy minicircle kDNA with primers specific for *Leishmania* (*Viannia*) (MP1-L (fwd) 5′-TACTCCCCGACATGCCTCTG-3′ and MP3-H (rev) 5′-GAACGGGGTTTCTGTATGC-3′)[19] was performed on diagnostic specimens and urine samples using the HotStar Taq DNA Polymerase kit (QIAGEN, Germany), as described elsewhere.[15] Sequences of control primers, which amplified a region of the human beta hemoglobin gene, were HBBL (fwd) 5′-GGCAGACTTCTCCTCAGGAGTC-3′ and HBBR (rev) 5′-CTTAGACCTCACCCTGTGGAGC-3′, and generated a product with a length of 197 bp.[15] PCR conditions were as described elsewhere.[15] Negative extraction and reagent controls were also run along with clinical samples. Amplicons were visualized and stained as described elsewhere.[15]

Species identification by PCR and PCR-restriction fragment length polymorphism (PCR-RFLP). In Peru, the causative agent of CL is predominantly *Leishmania* (*Viannia*) *braziliensis*, though other related New World species such as *Leishmania* (*Viannia*) *peruviana* and *Leishmania* (*Viannia*) *guyanensis*, and more rarely, *Leishmania* (*Viannia*) *lainsoni* and *Leishmania* (*Leishmania*) *amazonensis*, can be isolated as well.[20,21] Three PCR assays employing nuclear genomic targets were used for species identification following initial kDNA PCR of diagnostic samples.[15] The first assay, targeting the mannose phosphate isomerase gene (MPI), used allele-specific primers in two separated reactions with the following sequences, which differentiates *L.* (*V.*) *peruviana* from non-*L.* (*V.*) *peruviana* species (such as *L.* (*V.*) *braziliensis* or *L.* (*V.*) *guyanensis*), and generated a product 312 bp long: MPI (fwd) 5′-GCTCTTCCTGTCGGACAGCGAGC-3′ (common to the three species) and MPI (rev) 5′-GTCGGCAGCGTCACGG AGGTCC-3′ (specific for *L.* (*V.*) *peruviana*) or MPI (rev) 5′-GTCGGCAGCGTCACGGAGGTCG-3′ (specific for *L.* (*V.*) *braziliensis and L.* (*V.*) *guyanensis*).[15,22] MPI PCR conditions were as follows: 95°C for 15 min, followed by 32 cycles of denaturation at 94°C for 30 s; primer annealing at 69°C for 30 s; extension at 72°C for 30 s, and a final extension step at 72°C for 5 min (iCycler iQ, Bio-Rad, Hercules, CA).[15,22] All PCR products were visualized and stained as described elsewhere.[15]

The second assay, targeting the cysteine proteinase B (*Cpb*) gene, used primers with the following sequences, which distinguish between *L.* (*V.*) *braziliensis* and non-*L.* (*V.*) *braziliensis* species, and generated a product 1,170 bp long: *Cpb* (fwd) 5′-TGTGCTATT CGAGGAGTTCAA-3′ and *Cpb* (rev) 5′-TTACCCTCAGGAATCACTTTGT-3′.[15,23] *Cpb* PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s; primer annealing at 60°C for 30 s; extension at 72°C for 60 s, and a final extension step at 72°C for 6 min (iCycler iQ, Bio-Rad).[15,23]

The third and final assay, targeting heat shock protein 70 (*hsp70*), used primers with the following sequences, which distinguish between *L.* (*V.*) *guyanensis* and non-*L.* (*V.*) *guyanensis* species, and generated a product 1,422 bp long: *hsp70* (fwd) 5′-GACGGTGCCTGCCTACTTCAA-3′ and *hsp70* (rev) 5′-CCGCCCATGCTCTGGTACATC-3′.[15,24] *Hsp70* PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s; primer annealing at 60°C for 60 s; extension at 72°C for 60 s, and a final extension step at 72°C for 6 min (iCycler iQ, Bio-Rad).[15,24]

RFLP analysis of *Cpb* and *Hsp70* PCR products. Following *Cpb* and *Hsp70* PCR amplification as described,[15] products were separately digested overnight at 65°C for the *Cpb* assay, or 37°C for the *Hsp70* assay, in a total volume of 20 μL, with 5 U of each restriction enzyme.[15] The following enzymes were used in each reaction: *Cpb* (*Taq*I) and *Hsp*70 (*Hae*III) (Fermentas, Glen Burnie, MD). The RFLP products were then analyzed separately using 12% polyacrylamide gel electrophoresis (MiniProtean III, Bio-Rad), and stained with silver stain (Promega, Madison, WI).[15] MPI PCR was used to identify *L.* (*V.*) *peruviana, Cpb* PCR-RFLP to differentiate *L.* (*V.*) *braziliensis* from non-*L.* (*V.*) *braziliensis*, and *Hsp70* PCR-RFLP to specifically identify *L.* (*V.*) *guyanensis and L.* (*V.*) *lainsoni*.[15] The kDNA or other assays used would not have detected members of the *Leishmania* (*Leishmania*) complex such as *L.* (*L.*) *amazonensis*.

Statistical analysis. Primary outcomes were sensitivity and specificity. The composite reference standard against which urine PCR was compared for sensitivity and specificity analysis was 2/4 tests positive, where tests referred to lesion smear, culture, PCR of lesion samples, or leishmanin skin test, as described in the diagnostic evaluation.[15] Descriptive statistics (mean, SD, median, range) were calculated for continuous variables, and differences were compared using two-tailed *t* testing. Categorical variables were quantitated by proportions, and differences between the groups were compared using Yate's corrected χ^2 analysis. Statistical analyses were performed using SigmaStat 2.03 software (SPSS Inc., Chicago, IL). The level of significance was set at $P < 0.05$.

RESULTS

One hundred eight patients were enrolled over the study period, 86 of whom fulfilled criteria for a diagnosis of CL, and 22 received other diagnoses including myiasis, sporotrichosis, paracoccidioidomycosis, and malignancy. Clinical and demographic characteristics of the 2009 patient cohort have been summarized previously.[15] Sixty-six males (61%) and 42 females (39%) were enrolled over the study periods. Median age was 34 years (range 5–82 years). Median duration of exposure in the risk area for the cohort was 1.4 years (range 1 day–78 years), and work in agriculture (31%), or residence (11%), tourism (11%), or schooling (11%) in an endemic area were the principal risk occupations. Median age of presenting lesions was 2.5 months (range 0.5–624 months). The majority of lesions were ulcers (84%), with 8% and 7% having a nodular or verrucous appearance. Lesions were located on the upper extremity in 35%, the lower extremity in 32%, and the face in 28% of the cohort.

Of 86 patients who fulfilled reference standard criteria for a diagnosis of CL, 42% ($N = 36$) had positive cultures of lesion aspirates, 52% ($N = 45$) had positive smears, 57% ($N = 49$) had positive leishmanin skin tests, and 98% ($N = 84$) had lesion scrapings, aspirates, or filter paper impressions that were positive by PCR (87% had positive filter papers, and 88% had positive scrapings or aspirates). Of the 86 patients with CL, 18 had enrollment urine samples that were positive for *L.* (*Viannia*) kDNA by PCR, yielding a diagnostic sensitivity of 20.9% (95% confidence interval [CI] 12.3–29.5%), and specificity of 100% for urine PCR (Table 1).

A comparison of characteristics of CL patients with and without detectable kDNA in the urine is provided in Table 2. There were no differences in age, sex, duration of exposure, or duration of illness between CL patients with and without detectable

TABLE 1

Performance characteristics of urine polymerase chain reaction (PCR) for detection of *Leishmania Viannia* kDNA in 108 patients suspected to have cutaneous leishmaniasis

Test	Number positive	Number negative	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Urine PCR	18	90	20.9	100.0	100.0	24.4

kDNA in the urine. Seventy-eight percent of CL patients with detectable kDNA in the urine had skin lesions that were culture positive, and 50% had skin lesions that were smear positive, which did not differ significantly from patients without detectable kDNA in the urine (Table 2). Patients with detectable *Leishmania* kDNA in the urine appeared to have a higher mean number of skin lesions (2.4 versus 1.9), although this difference was not statistically significant ($P = 0.28$) (Table 2). Of 8 patients with intercurrent mucosal and CL, 6 (75%) had detectable kDNA in the urine compared with 12 of 78 (15%) patients with isolated CL ($P < 0.001$). Similarly, 33% (6/18) of patients with detectable kDNA in the urine had mucocutaneous leishmaniasis versus only 3% (2/68) of those without kDNA in the urine. Thus, patients with intercurrent mucosal disease were more likely to have detectable kDNA in the urine than those with CL only, and those with detectable kDNA in the urine were more likely to have mucocutaneous leishmaniasis.

TABLE 2

Comparison of demographic and clinical characteristics of 86 patients with cutaneous leishmaniasis who did and did not have detectable *Leishmania Viannia* kDNA in the urine

Characteristic	kDNA undetectable in urine by PCR ($N = 68$)	kDNA detectable in urine by PCR ($N = 18$)	P value
Sex			
Male	44 (64.7%)	12 (66.7%)	0.903*
Female	24 (35.3%)	6 (33.3%)	
Age (years)			
Mean ± SD	35.1 ± 2.4	38.4 ± 5.1	0.544†
Median, range	34.5, 5–82	31, 9–75	
Duration of exposure in endemic area (months)			
Mean ± SD	149.5 ± 26.9	132.6 ± 59.7	
Median, range	23, 0.03–900	5, 0.03–900	0.798†
Duration of skin lesion(s) (months)			
Mean ± SD	22.2 ± 9.0	18.1 ± 11.4	
Median, range	2.5, 0.5–624	2.5, 0.5–180	0.850†
Multiple lesions			
Yes	29 (42.7%)	9 (50%)	0.59*
No	39 (57.4%)	9 (50%)	
Number of lesions			
Mean ± SD	1.9 ± 0.2	2.4 ± 0.5	
Median, range	1, 1–10	1.5, 1–10	0.282†
Intercurrent cutaneous and mucosal leishmaniasis			
Yes	2 (2.9%)	6 (33.3%)	
No	66 (97.1%)	12 (66.7%)	< 0.001*
Smear result			
Positive	37 (54.4%)	9 (50%)	
Negative	31 (45.6%)	9 (50%)	0.944*
Culture result			
Positive	44 (64.7%)	14 (77.7%)	0.442*
Negative	24 (35.3%)	4 (22.2%)	
Species Identification‡			
L. (*V.*) *braziliensis*	8 (28.6%)§	3 (25.0)	0.99*
L. (*V.*) *guyanensis*	4 (14.3%)	6 (50.0%)	0.027*
L. (*V.*) *peruviana*	8 (28.6%)	3 (25.0%)	0.99*
Hybrid *L.* (*V.*) species	2 (7.1%)	–	–
Species not identifiable	6 (21.4%)	1 (8.3%)	0.52*
Unknown¶	40	5	0.038*

* Compared by Yates' corrected χ^2 analysis.

† Compared by two-tailed t test.

‡ Species identification was performed only for those patients who likely had sufficient concentration of amplifiable genomic DNA for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as determined by band thickness of kinteoplast DNA PCR of lancets and filter paper lesion impressions as described previously.[15]

§ Percentage of those that underwent species identification analysis by PCR and PCR-RFLP.

¶ Species identification was not performed on these specimens because of insufficient concentration of amplifiable genomic DNA based on kDNA PCR band thickness.

Thirteen of 18 (72.2%) CL patients with detectable kDNA in the urine had a sufficient concentration of *Leishmania* DNA present in scrapings, aspirates, or filter paper impressions[15] for species identification by PCR and PCR-RFLP, compared with 28 of 68 CL patients with PCR-positive clinical specimens, but without detectable kDNA in the urine ($P = 0.038$). Represented species among those CL patients with detectable kDNA in the urine included *L.* (*V.*) *guyanensis* ($N = 6$), *L.* (*V.*) *braziliensis* ($N = 3$), and *L.* (*V.*) *peruviana* ($N = 3$). *Leishmania* (*V.*) *guyanensis* was over-represented among those CL patients with detectable kDNA in the urine where species identification was successful, compared with those without detectable kDNA in the urine ($P = 0.027$). Of the 8 CL patients with intercurrent mucosal involvement, species identification occurred in 3: two had *L.* (*V.*) *guyanensis* isolated from clinical specimens, and one had *L.* (*V.*) *peruviana* isolated. None of the 10 healthy volunteers residing in non-endemic areas had detectable *L.* (*Viannia*) kDNA in the urine, nor did any of the 22 patients with alternate diagnoses.

DISCUSSION

We have demonstrated that *Leishmania* kDNA can be detected from the urine of ~21% of patients with CL in Lima, Peru, and in 75% of patients with intercurrent cutaneous and mucosal involvement. Although the overall sensitivity of urine PCR for the diagnosis of CL was sub-optimal compared with the very sensitive molecular assays on invasively obtained scrapings and biopsies of cutaneous and mucosal lesions,[1–5] the presence of detectable kDNA in the urine may suggest a potential role in categorizing patients as candidates for different therapies, especially given the over-representation of mucosal disease in the group with detectable kDNA in the urine. Given the toxicity associated with currently used parenteral therapies for CL (such as pentavalent antimonials and amphotericin B),[1,25] there is a movement toward use of topical therapies such as paromomycin,[26–30] cryo- or thermotherapy,[31–33] or intralesional antimonials.[32–34] However, patients with detectable kDNA in urine may represent a group of patients with less localized forms of disease that may be most amenable to systemic therapy. Indeed, of the 8 patients with intercurrent mucosal and cutaneous leishmaniasis, 75% had detectable kDNA in the urine versus only 15% of those with isolated CL. In areas endemic for *L.* (*V.*) *braziliensis*, the most common causal agent of ML, evidence of parasitemia

and parasite dissemination may have important prognostic implications, or may even portend clinical failure. Evaluation of these hypotheses in a prospective fashion is most certainly indicated.

Urine may be a suitable specimen for detection of parasite DNA in tegumentary leishmaniasis and is supported by a number of lines of evidence. First, lymphatic spread and lymph node involvement are common in localized CL, and may actually precede lesion development.[1,6] Second, ML is characterized by the ability of the *Leishmania* organism to spread to mucosa by lymphatic or hematogenous dissemination.[1,35] Third, lifelong protection against reinfection may be caused by live parasite persistence in the host. *Leishmania* subgenus *Viannia* DNA can be detected in healed scars and blood of patients clinically cured of CL.[9,11,12] Moreover, recurrences caused by reactivation of persistent parasites or secondary to trauma have also been reported.[36,37] And, some patients may develop a second skin ulcer at a different site after their primary lesion has healed,[1] suggesting a non-focal reservoir of persistent parasites. Coupled with our findings, these studies suggest that parasitemia and/or systemic involvement even within the "cutaneous" leishmaniases may occur variably. Further investigation of the host-parasite biological factors that influence the degree of localization in CL would be informative.

Much of the literature supporting the potential use of urine PCR for diagnosis of human tegumentary leishmaniasis comes from clinical cases of canine leishmaniasis, arguably a more "disseminated" entity than either CL or ML. Published studies have also reported the detection of *Leishmania* DNA in urine of patients with visceral leishmaniasis,[38,39] though given the *de facto* dissemination of the parasite in visceral disease, these data are less comparable to our patients. Recently, Franceshi and others[40] investigated 40 dogs with canine leishmaniasis (confirmed by lymph node biopsy and immunofluorescent antibody testing), and found that 16 were PCR positive from blood, and 10 were PCR positive from urine. None of the dogs had abnormal urinalysis or urine microscopy, and only 3 of 40 had elevated creatinine levels. Thus, there was no demonstrable correlation between positive urine PCR and renal damage. In another series, 35% of dogs with *Leishmania infantum* infection and no detectable renal impairment or proteinuria had urine that was positive for parasite DNA by PCR.[41] Furthermore, Manna and others[42] found demonstrable levels of *Leishmania* DNA in the urine of 22 dogs with only cutaneous manifestations of canine leishmaniasis, and no evidence of nephropathy. Collectively, these studies suggest that the presence of parasite DNA in urine could simply be a function of normal physiological renal activity, and that renal involvement is not a prerequisite for detectable parasites or parasite DNA in the urine. Although the patients in our study were enrolled as part of a separate diagnostic evaluation,[15] and did not undergo testing of renal function as part of their diagnostic work-up, we have no evidence that any of the patients with detectable kDNA in the urine had nephropathy or predisposing comorbidities such as hypertension or diabetes mellitus. Furthermore, dipstick urinalyses of kDNA-positive patients were normal, without evidence of glycosuria, proteinuria, or hemoglobinuria. However, prospective evaluation of a potential contribution of nephropathy to urine kDNA-positivity in the setting of CL is warranted.

This study has several limitations. First, that urine PCR was positive in 21% of patients in whom a diagnosis of CL was made may simply be a function of long-term residence/exposure in endemic areas, rather than a reflection of active disease. However, that urine PCR was negative in patients with skin lesions not deemed to be CL who also had resided in or been exposed to risk areas (i.e., our "true" negatives), counters this supposition. This group of "true" negatives includes individuals without evidence of active leishmaniasis, with evidence of another cutaneous process (such as skin cancer or sporotrichosis), and with a significant exposure history in an area endemic for CL. Thus, this group of patients along with healthy human volunteers served as controls for lack of specificity of the assay. Future studies, which evaluate urine PCR in larger groups of individuals, particularly family clusters, with and without skin lesions, with and without an exposure history are justified. In addition, evaluating the use of urine PCR over the course of treatment and follow-up of CL patients is also indicated.

A limitation of this analysis is the small number of patients who were urine positive, which limits our ability to draw conclusions about true or suggested demographic and clinical predictors of which patients may have detectable kDNA in the urine. Although we used a multicopy target specific for *Leishmania Viannia* minicircle kDNA (up to 10,000 copies per parasite) in a qualitative assay, it is possible that our processing methods led to poor detection rates, and that more prolonged sedimentation of the initial specimen, or a higher aliquot volume may have increased the diagnostic yield of urine PCR. With respect to species identification, all the targets used for PCR-RFLP (i.e., *Mpi*, *Cpb*, and *hsp70* PCR) are genomic rather than kinetoplastid sequences with a total number of copies per cell between 2 and 6. Thus, the rate of species identification was lower than the rate of detection of kDNA in urine. Moreover, our assays would not have detected members of the *Leishmania* (*Leishmania*) complex such as *L.* (*L.*) *amazonensis*. Investigation of further optimization strategies warrants additional evaluation.

In summary, we have demonstrated as proof-of-principle that *Leishmania* kDNA can be detected from the urine of certain patients with tegumentary leishmaniasis, particularly in those with mucosal disease. That the overall sensitivity of urine PCR was inadequate for diagnostic purposes, does not diminish its potential role in identifying those with potentially high-burden or less-localized forms of disease. On the contrary, that this specimen is completely non-invasive, easily obtained in pediatric and remote populations, and requires no technical laboratory expertise to acquire, supports that efforts to further elucidate the potential role of urine PCR in the diagnosis, treatment stratification, and management of patients with CL should be encouraged. Patients with mucosal disease were over-represented among those with detectable kDNA in the urine and this is a significant finding that may help to inform further studies of ML pathogenesis and host-parasite interaction. That *L.* (*V.*) *guyanensis* was over-represented among CL patients with detectable kDNA in the urine may indicate a species-specific predilection for parasitemia or parasituria, or may simply indicate that this species is more detectable from clinical samples, the reasons for which could be manifold. Our preliminary results reinforce that *Leishmania* syndromes occur along a clinical spectrum and conventional notions of tegumentary leishmaniasis existing under the umbrella of fully localized disease are, perhaps, dated.

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