Biomédica 2019;39(Supl.2):58-65
doi: https://doi.org/10.7705/biomedica.v39i3.4347

Original article

Mucosal leishmaniasis: A forgotten disease, description and identification of species in 50 Colombian cases

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Introduction: Mucosal leishmaniasis has a progressive course and can cause deformity and even mutilation in the affected areas. It is endemic in the American continent and it is mainly caused by *Leishmania (Viannia) braziliensis.*
Objective: To describe a series of mucosal leishmaniasis cases and the infectious *Leishmania* species.
Materials and methods: We included 50 patients with a clinical diagnosis of mucosal leishmaniasis and parasitological confirmation, and we described their clinical and laboratory results. We performed species typing by PCR-RFLP using the miniexon sequence and *hsp70* genes; confirmation was done by sequencing.
Results: The median time of disease evolution was 2.9 years (range: 1 month to 16 years). The relevant clinical findings included mucosal infiltration (94%), cutaneous leishmaniasis scar (74%), total loss of the nasal septum (24%), nasal deformity (22%), and mucosal ulceration (38%). The symptoms reported included nasal obstruction (90%), epistaxis (72%), rhinorrhea (72%), dysphonia (28%), dysphagia (18%), and nasal pruritus (34%). The histopathological study revealed a pattern compatible with leishmaniasis in 86% of the biopsies, and amastigotes were identified in 14% of them. The Montenegro skin test was positive in 86% of patients, immunofluorescence in 84%, and culture in 8%. *Leishmania (V.) braziliensis* was identified in 88% of the samples, *L. (V) panamensis* in 8%, and *L. (V.) guyanensis* and *L. (L.) amazonensis* in 2% respectively.
Conclusion: In this study, we found a severe nasal disease with destruction and deformity of the nasal septum in 25% of the cases, probably associated with late diagnosis. *Leishmania (V.) braziliensis* was the predominant species. We described a case of mucosal leishmaniasis in Colombia caused by *L. (L.) amazonensis* for the first time.

Keywords: *Leishmania*; leishmaniasis, mucocutaneous; molecular typing; polymerase chain reaction.

Leishmaniasis mucosa: una enfermedad olvidada, descripción e identificación de especies en 50 casos colombianos

Introducción. La leishmaniasis mucosa tiene un curso progresivo y puede causar deformidad e incluso mutilación de las zonas afectadas. Es endémica en el continente americano y es causada principalmente por *Leishmania (Viannia) brasiliensis.*
Objetivo. Describir una serie de casos de leishmaniasis mucosa y las especies de *Leishmania* infecciosas.
Materiales y métodos. Se estudiaron 50 pacientes con diagnóstico clínico de leishmaniasis mucosa y confirmación parasitológica. Se describieron sus características clínicas y los resultados de laboratorio. La tipificación de especies se hizo mediante reacción en cadena de la polimerasa de los polimorfismos de la longitud de los fragmentos de restricción (*Restriction Fragment Length Polymorphism Polymerase Chain Reaction*, PCR-RFLP) en la secuencia del miniexon y el gen *hsp70* y se confirmó por secuenciación.
Resultados. La evolución de la enfermedad fue de un mes a dieciséis años (mediana de 2,8 años). Los hallazgos clínicos fueron los siguientes: infiltración mucosa (94 %), cicatriz de leishmaniasis cutánea (74 %), pérdida total del tabique nasal (24 %), deformidad nasal (22 %) y ulceración (38 %). Los síntomas reportados fueron: obstrucción nasal (90 %), epistaxis (72 %), rinorrea (72 %), disfonía (28 %), disfagia (18 %) y prurito nasal (34 %). La histopatología mostró un patrón compatible con leishmaniasis en 86 % de las biopsias y se identificaron amastigotes en 14 % de ellas. La prueba de Montenegro fue positiva en 86 % de los pacientes, la inmunofluorescencia en 84 %, y el cultivo en 8 %. *Leishmania (V.) brasiliensis* se identificó en 88 % de las muestras, *L. (V) panamensis* en 8 %, y *L. (V.) guyanensis* y *L. (L.) amazonensis* en 2 %, respectivamente.
Conclusión. Se encontró enfermedad nasal grave con destrucción y deformidad del tabique nasal en una cuarta parte de los casos, probablemente debido a un diagnóstico tardío. *Leishmania (V.) brasiliensis* fue la especie predominante. Se describe por primera vez un caso de leishmaniasis mucosa causado por *L. (L.) amazonensis* en Colombia.

Palabras clave: *Leishmania*; leishmaniasis mucocutánea; tipificación molecular; reacción en cadena de la polimerasa.

Received: 12/02/18
Accepted: 12/12/18
Published: 20/12/18

Citation:
Ovalle-Bracho C, Muvdi-Arenas S. Mucosal leishmaniasis: A forgotten disease, description and identification of species in 50 Colombian cases. Biomédica. 2019;39(Supl.2):58-65. https://doi.org/10.7705/biomedica.v39i3.4347

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Funding:
This study was completely developed and funded by the *Centro Dermatológico Federico Lleras Acosta*, *E.S.E.*, project code 4000 4000-16-1P

Conflicts of interest:
The authors declare that there is no conflict of interest.

Mucosal leishmaniasis occurs mainly in the Americas affecting mostly the nasal mucosa. It has a progressive course and can cause destruction, deformity, and mutilation in severe cases (1,2). Mucosal leishmaniasis is caused by *Leishmania* species of the subgenus *Viannia*. Most of the cases reported in the literature are associated with *L. (V.) braziliensis* followed by *L. (V.) panamensis* and *L. (V.) guyanensis* (3).

This is a rare disease and the scientific literature is scarce on the topic. It is often underdiagnosed or confused with other diseases. In addition, most of the case series reported are small and they lack information on clinical findings, confirmatory laboratory tests, and species typing (4,5). In the literature reviewed for the present study, we found only one study in Colombia collecting clinical information on this form of the disease (6).

We describe here a series of 50 cases of mucosal leishmaniasis treated at the *Centro Dermatológico Federico Lleras Acosta* in Bogotá, D.C., Colombia, focusing on their clinical characteristics, diagnostic tests, and the *Leishmania* species identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the miniexon sequence and the *hsp70* gene.

Materials and methods

Study population

We studied 50 patients from the *Centro Dermatológico Federico Lleras Acosta*'s leishmanisis registers who were diagnosed with mucosal leishmaniasis and had tested positive for the miniexon gene by PCR. *Leishmania* parasites were isolated in four patients.

The data collected included the time of disease evolution, a history of having been in an endemic area, and clinical signs and symptoms suggestive of the disease such as rhinorrhea, pruritus, nasal obstruction, epistaxis, ulcer, and perforation or destruction of the septum, dysphagia, dysphonia, and mucosal infiltration. Diagnostic tests included specific antibody titers measured by indirect immunofluorescence, Montenegro skin test, and histopathological study.

Type of study

We conducted an observational descriptive study (case series).

Reference strains

The following reference strains were included*: L. (V.) braziliensis* (MHOM/BR/75/M2903), *L. (V.) panamensis (MHOM/PA/71/LS94), L. (V.) guyanensis* (MHOM/BR/75/M4147), *L. (L.) amazonensis* (IFLA/BR/67/PH8), and *L. (L.) mexicana* (MNCY/BZ/62/M379). We used the electrophoretic and restriction patterns of the reference strains to define the parasite species.

DNA extraction

DNA extraction of both the reference strains and the fresh biopsies was performed using the DNeasy Blood and Tissue Kit™ (Qiagen, Maryland, USA) following the manufacturer's instructions. The DNA was eluted in 100 µl of elution buffer solution and stored at -20°C until use. For the samples included in paraffin, DNA extraction was performed with Chelex-100™ (Bio-Rad, Hercules, USA) using a previously reported methodology (7). Finally, the DNA was rehydrated with 250 µl of distilled water and stored at -20°C until use.

Miniexon gene PCR

The miniexon gene was amplified following the protocol reported by Marfurt, *et al.* in 2003 (8). Four microliters of DNA at a concentration of 10 ng/µl were combined with 16 µl of a reaction mixture containing 1X amplification buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl; 2 mM $MgCl_2$; 200 µM of each deoxynucleoside triphosphate; 0.5 µM of each primer (Fme 5´-TAT TGG TAT GCG AAA CTT CCG-3´ and Rme5´-ACA GAA ACT GAT ACT TAT ATA GCG-3´); 0.05 U/µL Platinum Taq Polymerase™ (Invitrogen, São Paulo, Brazil), and 6% DMSO in a final volume of 20 µl.

The amplification profile was as follows: 1 cycle of DNA denaturation at 95°C for 8 minutes; 40 amplification cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s, as well as one final extension cycle at 72°C for 5 minutes in the Mastercycler EP gradient S thermal cycler™ (Eppendorf AG, Hamburg, Germany). Each round of amplification contained a negative control, which had no DNA, and three positive controls from DNA of the reference strains of *L. (V.) braziliensis*, *L. (L.) amazonensis,* and *L. (L.) mexicana*. PCR products were visualized and digitized with the Gel Doc XR gel documentation system™ (Bio-Rad, California, USA).

Restriction fragment length polymorphism

The samples that showed an amplification profile of the miniexon gene compatible with the subgenus *Viannia* were subjected to restriction with the enzyme Hae III (Invitrogen, São Paulo, Brazil). The products were purified using the QIAquick PCR Purification Kit™ (Qiagen, Duesseldorf, Germany). For the restriction, we used 12.5 µl of the purified PCR product, 1.4 µl of reaction buffer, 0.5 µl of Hae III (10 u/µl) and then incubated at 37°C for 120 min. We analyzed restriction products by 2.5% high-resolution agarose gel electrophoresis (Sigma-Aldrich, St. Louis, Missouri, USA) at 2.5 V/cm for 1.5 hours using a 100-bp marker (Bioline, London, England).

We identified *L. (V.) braziliensis* in samples exhibiting digestion by the Hae III enzyme and a pattern of two bands close to 108 and 118 bp. The amplicons that did not present restriction fragments were identified as *L. (V.) panamensis / L. (V.) guyanensis*. In these samples, the *hsp70* gene was amplified following the protocol published by García, *et al.* in 2004. The reaction mixture contained 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 2 mM $MgCl_2$; 200 µM dATP, dCTP, dTTP, and dGTP; 0.4 µM *HSP70* for 5'-GAC GGT GCC TGC CTA CTT CAA-3' and *HSP70* rev 5'-CCG CCC ATG CTC TGG TAC ATC-3'; 10% DMSO; 2 µl of DNA at a concentration of 10 ng/µl, and 0.05 U of Platinum Taq Polymerase™ (Invitrogen, São Paulo, Brazil) in a final volume of 50 µl.

The amplified product was purified and subjected to restriction with 10u/µl BccI enzyme™ (New England Biolabs, Ipswich, England). The restriction products for the reference strains *L. (V.) panamensis* (MHOM/PA/71/LS94) and *L. (V.) guyanensis* (MHOM/BR/75/M4147) exhibited two bands of 428 and 890 bp and three bands of approximately 346, 428, and 544 bp. The species in the biopsies were determined based on the restriction patterns obtained with the reference strains.

Sequencing

The amplified fragments were sent for sequencing to confirm the species identified by PCR and PCR-RFLP. The BigDye Terminator, version 3.1, Cycle Sequencing Kit™ (Applied Biosystems, Foster City, USA) and the

3730xl DNA Analyzer™ (Applied Biosystems, Foster City, USA) were used. For the analysis, we used the Geneious Pro software, version 5.5.6, where alignments were made with the gene sequences of the species of interest available in NCBI.

Statistical analysis

We recorded PCR-RFLP-based miniexon and *hsp70* gene typing and sequencing in a database. We calculated Cohen's kappa coefficient with Stata™, version 13 (Stata Corp LP, College Station, Texas, USA) to determine the agreement between the two methodologies.

Ethical considerations

This study was approved by the Research Ethics Committee of the *Centro Dermatológico Federico Lleras Acosta*. The use of cryopreserved samples and the access to patient data were performed in accordance with the guidelines established by the committee, which are based on the Colombian regulations and the Declaration of Helsinki.

Results

Seventy-six percent of the patients were men and 34% were women; their age range was 4-84 years with a median of 32 years. The time of disease evolution ranged from one month to 16 years with a median of 2.8 years. The patients came from 15 Colombian departments belonging to five geographical regions.

One of the patients had oral mucosal involvement and the others had nasal mucosal lesions. A scar suggestive of cutaneous leishmaniasis was identified in 74% of cases and four cases had cutaneous leishmaniasis concomitant with mucosal lesions. The most frequent symptoms of nasal involvement included nasal obstruction (90%), rhinorrhea (72%), epistaxis (72%), and pruritus (34%). On the physical examination, infiltration of the affected mucosa was identified in 94% of the cases, septal perforation in 58%, ulceration in 38%, and total loss of the nasal septum in 24% with an associated deformity in 22%. Additionally, 28% of the cases had dysphonia and 18% reported dysphagia.

All cases were confirmed by miniexon gene PCR and the parasite was obtained by culture in four of 50 patients. A histopathological pattern compatible with leishmaniasis was observed in 86% of the biopsies and only in 14% of them, amastigotes were identified.

The Montenegro skin test was positive in 46 patients (86%). Specific indirect immunofluorescence was negative in 14% of the cases, titers were 1/32 or 1/64 in 32% of them, and 1/128 or 1/512 in 54% was positive in 84% of the patients.

Leishmania (V.) braziliensis was the predominant species, as it was identified in 88% of the cases, followed by *L. (V.) panamensis* (8%), *L. (V.) guyanensis* (2%), and *L. (L.) amazonensis* (2%).

The patient in whom *L. (L.) amazonensis* was identified was a 62-year old farmer who lived in Caquetá, a department on the eastern region of the country. He had had cutaneous lesions for seven months and nasal symptoms for five months. On the physical exam, he showed disseminated papules, nodules, and infiltrated ulcerated plaques on face, trunk, and

extremities, some of which had lymphangitic involvement. On the nasal mucosa, he had erythema, infiltration, and ulceration. He was treated with pentavalent antimonials; his mucosal lesions responded well but had a cutaneous relapse. He was HIV negative.

The agreement in species typing between the miniexon and *hsp70* genes by PCR-RFLP was “very good” according to the Landis and Koch scale, and a kappa value of 1 was obtained.

Discussion

If the scale of mucosal leishmaniasis severity, proposed by Lessa and Lessa in 2012, is applied, 82% of our cases had severe mucosal disease: 58% of them would be on stage IV of the disease with irreversible lesions (septal perforation), and 24% would be on stage V with deformities secondary to severe tissue destruction and total loss of the septum (2).

The characteristics of age, gender, and associated symptoms were similar to those described on the epidemiological reports of the Colombian *Instituto Nacional de Salud* (INS) for the last five years. In our study, we observed an increased frequency of previous scarring (74%) compared with the epidemiological reports of the INS (50%).

In contrast, the frequency of septal perforation (58%) was considerably increased in our series compared with other reports, which ranged from 11% to 22% (2,6).

The advanced stage of the disease is typically associated with late diagnosis, which was the case in our series (median time to diagnosis, 2.8 years). The delay in the diagnosis could be explained by the following reasons: Lack of access to health services, the poor performance of diagnostic methods, and health professionals not familiarized with the disease, which leads to wrong diagnoses, inadequate management, and disease progression.

In Brazil, Pereira Diniz, *et al.,* described a series of 21 patients with a period until the diagnosis ranging from 5 months to 20 years with an average of 6 years (9). The duration of the disease is a factor that determines the spread of the parasites and is one of the main determining factors in the severity of mucosal lesions (9-11). Boaventura emphasizes that it is rare to access to clinically-diagnosed early stages of mucosal disease (12), which coincides with our findings.

Mucosal lesions caused by *Leishmania* species of the subgenus *Viannia* are severe and progressive and can cause deformity and mutilation. Hence, a delay in diagnosis has aesthetic, functional, and emotional impact in patients (13). The majority of cases of mucosal leishmaniasis reported in the Americas are caused by *L (V.) braziliensis* followed by *L. (V.) panamensis* and *L (V.) guyanensis*. Previous studies have demonstrated that mucocutaneous leishmaniasis caused by *L. (V.) panamensis* is less destructive in most patients (6,14). In contrast, mucosal leishmaniasis caused by *L. (V.) braziliensis* presents more severe forms of the disease (1,14-16) and patients have increased frequency of septal perforation (17).

In our series, almost 90% of the cases were caused by *L.* (*V.*) braziliensis, so we were unable to analyze differences in severity according to species. In one of our patients with nasal mucosal disease, we identified *L. (L.) amazonensis* as the infecting species. He had concomitant disseminated

cutaneous lesions and responded to pentavalent antimonials but relapsed. This is the first time that a case of mucosal leishmaniasis caused by this species has been described in Colombia; however, it has been reported in other countries (3).

The pathogenicity of mucosal leishmaniasis depends on several factors that involve the virulence of the parasite and the immune response of the host (2,16). In recent years, the presence of a virus of the Totiviridae family, *Leishmania* RNA virus (LRV1), has been described in some species of *Leishmania*, mainly *L. (V.) braziliensis* and *L. (V.) guyanensis.* This virus is capable of altering the immune response of the host via TLR 3 enhancing the inflammatory response and may be a determinant of disease severity (18).

The appearance of mucosal lesions in minors is rare. However, five (10%) of the cases in this series were under 18 years of age and two of these were under 5 years of age. In Tunisia, Kharfi, *et al.,* described 106 cases of cutaneous leishmaniasis in children under 16 years of age, of which 6.8% also had mild mucosal lesions (19). In 2007, De Oliveira Guerra reported 147 cases of American tegumentary leishmaniasis in children less than 15 years of age in Brazil, of which one had concomitant skin and mucosal lesions (20).

In Colombia, there are few studies on this clinical form of the disease. In 1998, Osorio described a series of 23 patients with mucosal leishmaniasis caused by *L. (V.) panamensis* in the Pacific region of Colombia (6). The distribution by age and gender, as well as the most frequent location of the lesion, was similar to that of the patients included in this study. In contrast, in Osorio's study, differences in the frequency of septal perforation (reported in 15% of patients) and the presence of previous scarring indicative of cutaneous leishmaniasis (only reported in 17% of patients) were observed. The presence of simultaneous mucosal and cutaneous lesions was reported in 78% of patients but was observed only in 8% of the patients in this study. The time of evolution of the disease in that study ranged between 1 month and 10 years with an average of 23 months, which indicated an earlier diagnosis compared with our series.

An important difference in the two study groups is the infecting species. Approximately 90% of the species in this series corresponded to *L. (V.) braziliensis* unlike Osorio's series in which 77% of the species identified were *L. (V.) panamensis*. The difference in the severity of the lesions between the two studies could be explained by the differences in the infecting species or differences in the time until diagnosis.

It is pertinent to ask whether mucosal leishmaniasis patients in Colombia are being diagnosed in a timely manner, whether there are barriers to access the health system, and whether there is a lack of specific training in health professionals about this disease. It is fundamental to strengthen the education of general practitioners in this area to improve the diagnosis in our patients including an adequate mucosal evaluation and a more frequent follow-up in patients with cutaneous leishmaniasis caused mainly by *L. (V) braziliensis*.

We report the largest series of mucosal leishmaniasis patients in Colombia where the predominant species was *L. (V.) braziliensis* and one of the cases was caused by *L. (L.) amazonensis*.

Acknowledgements

We thank the Editorial Committee of the *Centro Dermatológico Federico Lleras Acosta* for its constructive comments that helped to improve the manuscript.

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