

CLINICAL REPORT

Oral Lesion in Leprosy: Borderline Tuberculoid Diagnosis Based on Detection of *Mycobacterium leprae* DNA by qPCR

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Oral lesions are rarely reported in paucibacillary forms of leprosy. We report here a case with an erythematous hyposensitive lesion in the palate and no skin lesions. In addition to routine tests, biopsies of the lesion in the palate and of clinically normal surrounding areas were performed and subjected to real-time PCR for detection of *Mycobacterium leprae* DNA. The biopsy of the oral lesion was positive for bacilli DNA, followed by positive serum anti-PGL-1 and Mitsuda test, but with negative histopathology. The patient was diagnosed with a borderline tuberculoid form. After multidrug therapy the lesion had significantly regressed and the bacilli DNA detection in the former lesion was negative. The bacilli DNA detection in an oral lesion by real-time PCR not only improved leprosy diagnosis, but also helped in the classification of clinical form, and in the establishment of the appropriate therapeutic regime. **Key words:** leprosy; *Mycobacterium leprae*; diagnosis; PCR; mouth mucosa.

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Leprosy, an infectious disease caused by *Mycobacterium leprae*, is a relevant public health issue in Brazil, which has the second highest prevalence worldwide, with 37,610 new cases detected in 2009 (1).

An operational classification distinguishes multibacillary (MB) from paucibacillary (PB) forms of leprosy, according to the bacterial index (BI) and the number of lesions (2); for clinical form classification, Mitsuda's test and PGL-1 antibody testing are reported in addition to the BI (3).

As a result of effective antimicrobial therapy, advanced lesions of the face, nasopharynx and oropharynx are now rarely seen, but the presenting signs and symptoms of leprosy still arise occasionally in the nasal and oral mucosa (4). A case of oral lesion associated with leprosy has been described elsewhere in a borderline tuberculoid

(BT)-MB patient with papulo-nodular lesions in the centre of the hard palate, by histopathological findings (5). However, oral mucosa lesions are not normally found in PB patients (5, 6), and when they are present, manifestations have been described exclusively in the tuberculoid and borderline forms in the reactional state, with few scientific studies and poorly characterized descriptions (7).

Oral lesions of leprosy occur more frequently in areas of the mouth with a lower surface temperature (8). The main oral cavity sites of leprosy include the gingival in the anterior portion of the maxilla, the hard and soft palate, the uvula and the tongue (9).

An earlier study detected *M. leprae* DNA by PCR in oral mucosa samples of patients with MB forms of leprosy (10), but literature is scarce regarding detection of bacilli DNA in PB leprosy forms.

MATERIAL AND METHODS

Mitsuda's intradermal tests for evaluation of the cellular immune response to *M. leprae*, and the serum enzyme-linked immunoassay (ELISA) that evaluates the response of IgM antibodies against the PGL-1 bacillus antigen were performed, together with *M. leprae* DNA detection in nasal swab, buccal swab, dermal swabs, peripheral blood, biopsies of the inferior nasal turbinate and of the sensory nerve, a research protocol adopted at CREDESH under the UFU Ethics Committee approval number 499/2008.

Real-time PCR (qPCR) was performed for *M. leprae* DNA detection in all samples. The set of primers and probe (sense 5'-TGACATGAATACGCAAGGGCAGGA-3'; antisense 5'-TCATCTCCTGCACCCCGA-3'; probe TaqMan 5'-CACATAGGGTCCAGTCGTGCCGC-3') were designed to amplify and detect a 69-bp fragment of the ML0024 *M. leprae* gene, a highly specific region. The genomic region was confirmed by dideoxy sequencing, which aligned only with the *M. leprae* genome in a Basic Local Alignment Search Tool (BLAST) search (www.ncbi.nlm.nih.gov/BLAST). The sensitivity of the biomarker was close to 60%, detecting all BB, BL and LL clinical forms. Other *Mycobacteria* spp. and negative controls were used, and confirmed 100% specificity. Interestingly, bacilli DNA were detected by the ML0024 primers in 25% of the patients' samples with negative bacilloscopy.

Six-hundred nanograms of DNA (4 µl) from clinical samples were used in a 25-µl PCR reaction, which consisted of 2× TaqMan® Universal PCR Master Mix (Applied Biosystems), 200 nM each primer, and 100 nM probe. The PCR reactions were carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles

of 95°C for 15 s and 60°C for 1 min. All tissues were weighed before DNA extraction to allow the absolute quantification per milligram of biopsied tissues. The absolute quantification of each DNA sample was calculated based on a standard curve constructed for the gene. All samples were run in triplicate.

ELISA followed the procedures described elsewhere (11). Briefly, microtitre plates (Maxisorp®, NUNC, Rochester, NY, USA) were coated with 50 µl native PGL-I diluted in absolute ethyl alcohol at a concentration of 10 µg/ml. Serum samples were added in duplicate using a dilution of 1:100 (native PGL-I) in phosphate buffered saline/bovine serum albumin (PBS/BSA) 1%, and incubated for 1 h at 37°C, followed by washing. The anti-human IgM-peroxidase conjugate (Sigma Chemical Co., St Louis, MO, USA) was added to the plates at a dilution of 1:10,000 (PGL-I ELISA) in PBS/BSA 1%. The substrate o-phenylenediamine dihydrochloride (OPD, Sigma-Aldrich, Inc., St Louis, MO, USA) enzyme substrate was added to the plates and incubated at room temperature for 10 min in the dark. The reaction was stopped by the addition of 25 µl H₂SO₄ 4N. The optical density (OD) was obtained in a microplate reader (Thermo Plate, TP-Reader, Rayto Life and Analytical Sciences C. Ltd, Germany) at 492 nm. Two positive and three negative controls were included in each plate. The cut-off level for positivity was a standard deviation above the mean OD obtained for the three negative controls. The antibody titres were expressed as the ELISA index (EI) according to the following formula: $EI = OD_{\text{sample}} / OD_{\text{cut-off}}$ in which the cut-off point was determined by the mean OD of the negative controls plus 3 standard deviations. Values of EI > 1.1 were considered positive.

CASE REPORT

A 46-year-old Brazilian woman attended the National Reference Center in Leprosy and Sanitary Dermatology (CREDESH), of the Hospital das Clínicas, at the Federal University of Uberlândia, in January 2009, referred by her neurologist physician, presenting hypoesthesia at the bottom of the right foot, high-stepping gait and an electroneuromyography demonstrating an axonal mononeuropathy with moderate to severe intensity of the right peroneal nerve, leading to suspicious leprosy. Although the referred patient had no known contact with other patients, it is worth noting that this is an

endemic region for leprosy, and that the possible source of transmission could not be determined. Clinical evaluation did not reveal any skin lesions in any part of the patient's body, but did present an important hypoesthesia and pain at the bottom of the right foot, and paresis due to difficulty dorsiflexing the right foot, in the region innervated by the right fibular.

For diagnosis, BI of dermal swabs was performed, collected at seven skin sites of the patient (two ear lobes, two elbows, two knees and a suspected area at the bottom of the right foot) as well as a biopsy of the intermediate cutaneous branch of the right superficial fibular nerve during surgical decompression of the right common fibular, due to impairment of this nerve, with swelling and tenderness, associated with sensory loss and muscle paresis.

Other laboratory tests, such as liver and kidney function, blood sugar, thyroid hormones, antinuclear antibodies (ANA), rheumatoid factor (RF) and urinalysis, were requested to rule out other causes of neuropathies, including autoimmune diseases and metabolic disorders. These tests were all negative.

During the intraoral examination, a hypoesthetic erythematous lesion was observed, without elevated borders, in the region of the right hard palate, involving the medial line, of approximately 2.0 cm diameter (Fig. 1A). Biopsies were collected from the lesion and from the clinically normal adjacent region (control), sparing the central region of the lesion in order to avoid the area of the greater palatine nerve.

The ELISA anti-PGL-1 serum test was positive, with an EI of 2.37. The Mitsuda test was strongly positive, with a 10 mm-diameter nodule. BI and qPCR were negative in all the dermal swabs samples, nerve and inferior nasal turbinate biopsies, buccal swab, nasal swab, and peripheral blood. The histopathological examination of nerve biopsy for the cutaneous branch through the right superficial peroneal nerve showed no inflammatory process of the neural tissue fragment and presented

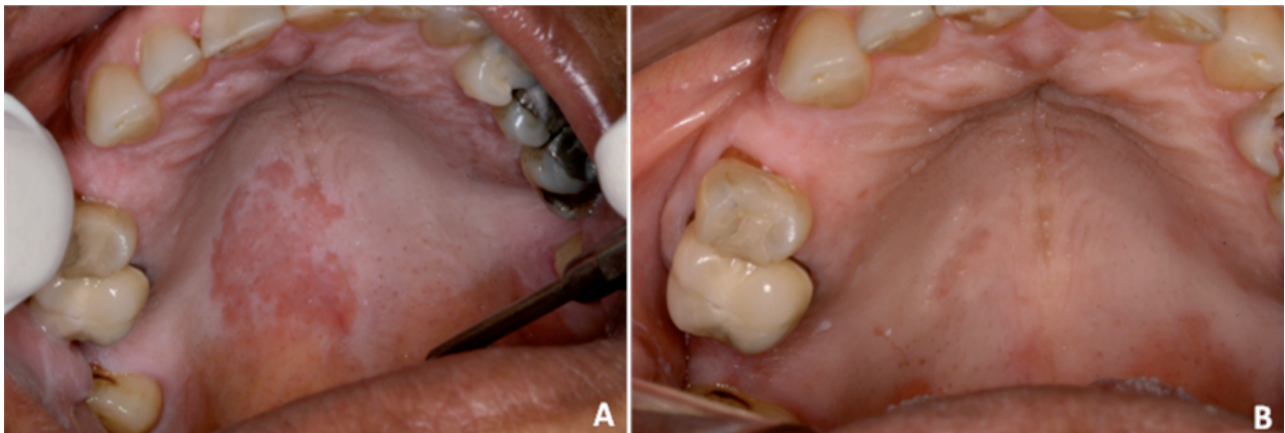


Fig. 1. Lesion before and after multidrug therapy. (A) Erythematous lesion of the hard palate involving the midline. (B) Regression of the hard palate lesion.

negative acid-fast bacilli by Ziehl-Neelsen staining. The buccal lesion biopsy of the hard palate displayed negative bacilloscopy, without apparent pathological changes (Fig. 2); however, the qPCR presented an amplification curve of the bacillus DNA and detected 1,126 copies of the *M. leprae* DNA per mg of biopsied tissue. The clinically healthy mucosa of the hard palate had negative results, both in the histopathological and the qPCR tests.

The possibility of contamination of the sample from the buccal mucosa lesion was excluded with the undisputed demonstration that qPCR for the detection of *M. leprae* DNA was negative in the clinically healthy area adjacent to the lesion, in the buccal swab, the nasal swab, the blood, and the biopsy of the nasal turbinate, a fact that precludes any possibility of contamination by saliva, nasal secretion and/or blood at the site as a result of the invasive biopsy procedure. Furthermore, the biopsy was split into three samples, which were submitted to normal extraction protocols with the other sampling replications prior to PCR reactions.

Considering the positive ELISA for serum PGL-1, the strong positive Mitsuda test, and the positive PCR result in the oral lesion, the patient was classified as borderline tuberculoid, a PB leprosy form, and received multidrug therapy (MDT) for 6 months, with a daily dose of 100 mg dapsone, and a monthly dose of 600 mg rifampicin. Successful treatment was evidenced by the regression of the hard palate lesion after the treatment (Fig. 1B), by reversed results of qPCR with total absence of *M. leprae* DNA (negative), and by the reduction in EI from 2.37 to 1.16 (normal ≤ 1.1).

DISCUSSION

Despite diagnosis and classification of leprosy patients being based on the identification of the bacillus in cutaneous smears and the histopathological examination of skin lesion biopsies, PB forms are not easily detected by

these methods (3) and, as yet there is no gold-standard laboratory test for diagnosis of the disease (12). However, PCR has been slowly incorporated as an auxiliary diagnostic tool, due to its higher sensitivity, and has been demonstrated to be useful in early diagnosis and prevention of disability in leprosy (13, 14).

An earlier publication identified *M. leprae* DNA by conventional PCR in normal oral mucosa biopsies in patients with multibacillary leprosy, suggesting the use of this test for investigation of infection by *M. leprae* and for follow-up of cases since it is easy, quick, and reliable (10).

Although less frequent, oral lesions may contribute to the correct diagnosis of the disease clinical and operational classification of the patient, as in this case, and to the therapeutic regimen to be adopted, since the bacillus was identified only in the hard palate oral mucosa.

The distribution of oral lesions has been attributed to the preference of the bacillus for temperatures below 37°C (15). The clinical case presented here and in other papers corroborates this fact, which explains the greater frequency of lesions along the midline of the palate, since this is a structure traversed by two air currents, nasal and oral, and therefore, remains 1–2°C below body temperature (16, 17).

In reviews of oral leprosy, diagnosis was based on cutaneous lesions (15), unlike our case, which presented no skin lesions. The regression of the oral lesion after conclusion of treatment and the non-identification of the *M. leprae* DNA by qPCR at the time of clinical discharge, make this lesion, if not characteristic, at least directly related to the presence of the bacillus.

The possibility of contamination of the sample from the buccal mucosa lesion was excluded with the undisputed demonstration that qPCR for the detection of *M. leprae* DNA was negative in the area adjacent to the lesion, in the buccal swab, the nasal swab, the blood, and the biopsy of the nasal turbinate, a fact that precludes any possibility of contamination by saliva, nasal secretion and/or blood

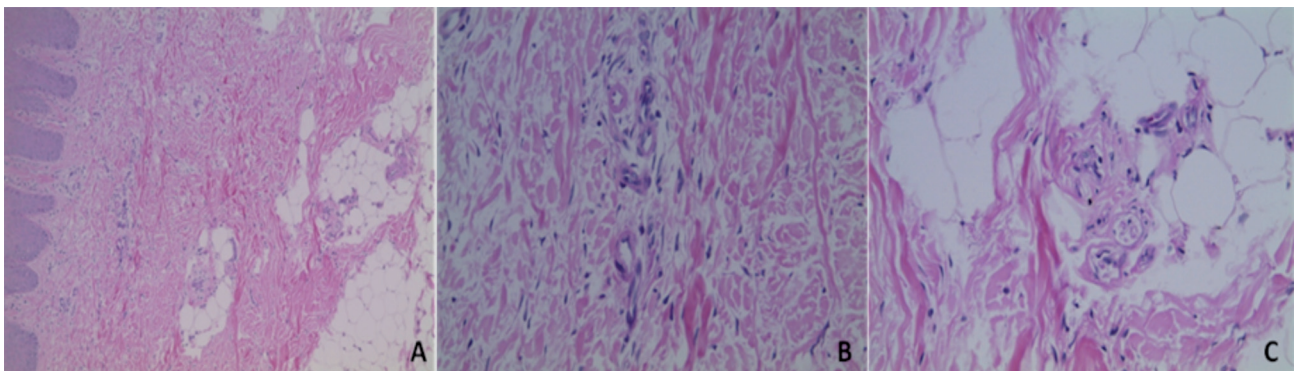


Fig. 2. Histological aspects of biopsy collected from the oral lesion in the hard palate. (A) The histological sections stained with haematoxylin and eosin (H&E $\times 400$) showed palatal fragment, showing the deep portion of the epithelial tissue lining and lamina propria of the mucosa. The lamina propria consists of connective tissue, not modelled, evidencing collagen bundles of various thicknesses and areas of neurovascular structures with common aspects of normality. At depth, there is fatty tissue in the sub-mucosa without apparent pathological changes. (B) and (C) Magnified view of the vascular-neural structures observed in the lamina propria and submucosal tissue (H&E $\times 400$).

at the site as a result of the invasive biopsy procedure. Furthermore, the use of qPCR, with two primers and one internal probe, avoids excessive manipulation of the sample, and supports a highly sensitive and specific detection system.

Even though the histopathological examination in this clinical case did not suggest or provide evidence for the disease, the diagnosis of borderline tuberculoid leprosy was determined by detection of the bacilli DNA in the oral lesion, which was supported by other indirect evidence, such as: positive ELISA (meaning subclinical infection, once only 22% of patients with PB present positive ELISA), positive Mitsuda (commonly detected in paucibacillary patients that present a cellular response), and resolution of disease symptoms and oral lesion after MDT, with absence of DNA post-treatment.

Our results demonstrated the great relevance of the qPCR detection system in oral samples of PB patients, which may be used not only for leprosy diagnosis, but also for clinical classification, especially for the limited sensitivity and specificity of the conventional diagnostic tools (18).

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