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ORIGINAL ARTICLE

Identification of *Mycobacterium leprae* and *Mycobacterium lepromatosis* in Formalin-Fixed and Paraffin-Embedded Skin Samples from Mexico

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Background: The causative agents of leprosy are the well-known *Mycobacterium leprae* and the newly discovered *Mycobacterium lepromatosis*. This agent was found in 2008, and it was found to be the cause of diffuse lepromatous leprosy in two Mexican patients. **Objective:** The objective of this work was to determine if *M. leprae* and *M. lepromatosis* were present in formalin-fixed and paraffin-embedded skin samples from cases from different regions in Mexico. **Methods:** A total of 41 skin samples were obtained from 11 states of Mexico. All patients' samples were diagnosed by clinical and histopathological analyses. Total DNA was isolated using a Qiagen-DNeasy blood and tissue kit and molecular identification was achieved by two semi-nested polymerase chain reactions. **Results:** The 41 patient included 33 samples from men and 8 samples from women; 29 samples were polymerase chain reaction (PCR)-positive to *Mycobacterium* and 12 samples were PCR-negative. From those 29 samples, 13 were PCR-positive to *M. leprae*, 8 to *M. lepromatosis* and 8 were positive to both species. The histopathological diagnosis included; Nodular lepromatous leprosy (NLL); Diffuse lepromatous leprosy (DLL); and Borderline leprosy (BL). The 29 PCR-positive samples were classified as follow: 14 NLL, 4 DLL, and 11 BL. In the 12 samples negative to *Mycobacterium*, 7 showed the NLL, 2 DLL and 3 BL. **Conclusion:** These findings add evidence to the *M. leprae* and *M. lepromatous* distribution, clinical forms and participation of dual infections in Mexico.
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-Keywords-
Diffuse lepromatous leprosy, Leprosy, *Mycobacterium leprae*, *Mycobacterium lepromatosis*, Nodular lepromatous leprosy

INTRODUCTION

Leprosy is a mycobacterial infection, which affects primarily the skin, peripheral nerves, eyes, and mucous membranes of the upper respiratory tract[1]. This disease has plagued humans for millennia and remains a significant public health problem[2]. Worldwide, it is an outstanding cause of morbidity due to physical handicaps and social stigma. Until 2013, the World Health Organization reported a cases-rate of ＞10 per 100,000 population in India and Brazil; 1～10 per 100,000 in Africa and Far East; ＜1 per 100,000 in Latin America, United States,

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China, Middle East and Australia[3]. In Mexico, leprosy is an endemic disease in 28 states, with 166 new cases reported at the end of 2016[4].

The causative agents of leprosy are the well-known *Mycobacterium leprae* and the newly discovered *Mycobacterium lepromatosis*[2]. This new agent was discovered in 2008, and it was found to be the cause of diffuse lepromatous leprosy (DLL) in two Mexican patients. The *Mycobacterium bacilli* differs at least 9.1% and diverged ~10 million years ago from their last common ancestor[5]. The *M. lepromatosis* genome matched ~87% overall with the *M. leprae* genome (3,268,071 bp)[6-8].

The causative agent is transmitted via airborne droplets or by prolonged skin direct contact with a multibacillary leprosy patient. This disease manifests a wide spectrum of clinic and pathological forms (depending of immune host response) ranging from tuberculoid leprosy (TT), passing the borderline leprosy (BL) forms, to lepromatous leprosy (LL), and an initial stage (indeterminated leprosy). A remarkable geographic variation of clinical aspects also exists; in India and Africa, 90% are TT, in Southeast Asia, the two forms are equally distributed[3], whereas in Mexico, over 60% of cases are LL. The objective of this work was to determine if *M. leprae* and *M. lepromatosis* were present in formalin-fixed and paraffin-embedded tissue samples from cases from different regions in Mexico.

MATERIALS AND METHODS

Samples

A total of 41 formalin-fixed and paraffin-embedded skin biopsy samples were obtained from eleven states of Mexico; 16 samples from Yucatan, 8 from Guerrero, 6 from Michoacán, 3 from Guanajuato, 2 from Morelos and one sample from Campeche, Ciudad de Mexico, Estado de Mexico, Oaxaca, Puebla and Quintana Roo. The samples were collected at the Hospital General "Dr. Manuel Gea Gonzalez" and Centro Dermatológico de Yucatan "Dr. Fernando Latapi" from 1994 to 2014. All patients' samples were diagnosed by clinical and histopathological analyses. The clinical and pathological information included the patient's age, sex, localization, biopsy site and date, and histopathological diagnostic. This study was approved by the Institutional Review Board of Hospital General Dr. Manuel Gea González, Mexico City, Mexico (IRB no. 06-54-2015). For DNA extraction, eight to ten sections of five micron thickness of tissue were used from each sample.

DNA extraction and polimerase chain reaction (PCR)

The formalin-fixed and paraffin-embedded tissue samples were process to remove paraffin using xylene protocol. Total DNA was isolated using a DNeasy blood and tissue kit (Qiagen, Ventura, CA, USA) according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry at 260 nm. Molecular identification was achieved by two semi-nested PCR, the first PCR used primers AFBFO (5-gcgtgcttaacacatgcaagtc-3) and MLER4 (5-ccacaagacatgcgccttgaag-3). The amplification fragment (171 bp) was used for two separate second-round PCRs using MLER4 and LPMF2 (5-gtctcttaatacttaaacctattaa-3) for *M. lepromatosis* (142-bp) and MLER4 and LERF2 (5-ctaaaaaatctttttttagagatac-3) for *M. leprae* (135-bp)[2,9]. The PCR reactions contained 25 μl of Top Taq master mix (Qiagen), 100 ng of DNA (10 μl), and 25 μM of each primer (2 μl) in a total volume of 50 μl. Amplification conditions for the first PCR were: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturing (94°C, 30 s), annealing (57°C, 30 s) and extension (72°C, 30 s), followed by a final extension at 72°C for 5 minutes. For the second round of PCR's, we use the same protocol with annealing at 53°C. A sample of 10 μl of product from each PCR was electrophoresed in a 3% agarose gel with 0.5 μg of ethidium bromide/ml and 1X Tris-acetate-EDTA buffer for 1 hour. DNA bands were visualized on a UV transilluminator and documented with The Gel Logic 212 Pro Software (Carestream, Woodbridge, CT, USA).

RESULTS

Forty-one patients were diagnosed with leprosy by clinical and histopathological analysis; twenty-nine samples were PCR-positive to *Mycobacterium* (70.73%) and twelve samples were PCR-negative (29.27%). From those twenty-nine samples, thirteen were PCR-positive to *M. leprae* (44.83), eight to *M. lepromatosis* (27.58%) and eight were positive to both species (27.58%).

The forty-one patients included thirty-three samples from men and eight samples from women. The average age was 52 years, range from 23 to 78 years-old. The 29 PCR-positive samples to *Mycobacterium*; included 13 *M. leprae* positive (11 men and 2 women), and 8 *M. lepromatosis* positive (7 men and a woman). The eight patients positive for both species, six were men and two women. From the twelve negative samples, nine were men and three women. Regarding the geographic region; *M. leprae* was found in six samples from Yucatan, 3 from Michoacán, and one from Mexico City, Mexico state, Guerrero and Puebla (13 samples). *M. lepromatosis* was found in two samples from Guerrero, 2 from Michoacán, 2 from Yucatan, 1 from Guanajuato, and 1 from Quintana Roo (8 samples). The dual infection was present in six samples from Yucatan,

Table 1. Histopathological findings and geographical distribution

Region	NLL				DLL				BL				Total
	Ml	Mlm	B	-	Ml	Mlm	B	-	Ml	Mlm	B	-	
Yucatán	1	1	1	2	0	0	2	0	5	1	3	0	16
Guerrero	1	1	0	4	0	1	0	1	0	0	0	0	8
Michoacán	3	2	0	0	0	0	0	0	0	0	0	1	6
Guanajuato	0	0	0	0	0	1	0	1	0	0	0	1	3
Morelos	0	0	0	1	0	0	0	0	0	0	0	1	2
Campeche	0	0	0	0	0	0	0	0	0	0	1	0	1
Mexico City	1	0	0	0	0	0	0	0	0	0	0	0	1
Mexico State	1	0	0	0	0	0	0	0	0	0	0	0	1
Puebla	1	0	0	0	0	0	0	0	0	0	0	0	1
Oaxaca	0	0	0	0	0	0	0	0	0	0	1	0	1
Quintana Roo	0	1	0	0	0	0	0	0	0	0	0	0	1
Total	8	5	1	7	0	2	2	2	5	1	5	3	41

NLL: nodular lepromatous leprosy, DLL: diffuse lepromatous leprosy, BL: borderline leprosy, Ml: *Mycobacterium leprae*, Mlm: *Mycobacterium lepromatosis*, B: both infections, −: negative.

one from Campeche and one from Oaxaca. The twelve negative samples were; five from Guerrero, two from Morelos, two from Guanajuato, two from Yucatan, and one from Michoacan.

The histopathological diagnosis observed included; Nodular lepromatous leprosy (NLL) (twenty-one samples); DLL (six samples); and Borderline leprosy (BL) (fourteen samples). In the twenty nine PCR-positive samples, fourteen showed the NLL form, four showed the DLL form, and eleven the BL form. In the twelve samples negative to *Mycobacterium*, seven showed the NLL form, two showed the DLL form and three the BL form.

In sum, the twenty-nine positive samples to *Mycobacterium* showed a distribution profile of eight samples with the NLL form positive to *M. leprae*, five to *M. lepromatosis* and one positive to both. The DLL form was present in two positive samples to *M. lepromatosis*, and two samples positive to both. The BL form was shown in five positive samples to *M. leprae*, one to M. lepromatosis, and five positive to both (Table 1).

DISCUSSION

M. leprae was the only known cause of leprosy until 2008, when the long-elusive *M. lepromatosis* was identified as the second agent in leprosy patients from 12 Mexican states; Tamaulipas, Sonora, Sinaloa, Nayarit, Colima, Michoacan, Guerrero and Queretaro[6]. Currently, it is the dominant cause of leprosy and considered endemic in the western and central part of Mexico. Globally, the male/female leprosy rate is male dominated 3/2[10]. In Mexico, this rate is 2/1[3,11]. In this work, the male dominance was greater up to 4.12/1 (33/8 cases).

Dual infections due to *M. lepromatosis* and *M. leprae* also had been reported[12], its frequency may be up to 16.1%[6]. In our study, it was greater, up to 19% (8/41) (Table 1). Interestingly, the origin of most of the dual infections was the Yucatan peninsula, an area known to have frequent leprosy cases[11], except for a case from Oaxaca[6-8].

M. lepromatosis has been related as the specific cause of the severe DLL form[13,14]. Since its discovery, its prevalence and significance has raised the scientific interest. According to our findings, *M. lepromatosis* may cause the NLL and BL (Table 1). Given that *M. lepromatosis* is not geographically restricted to Mexico as it has been identified in America and Asia; Brazil, Myanmar, Canada, and Singapore[12,15-19], and that it can participates in dual infections in Leprosy endemic areas, *M. lepromatosis* should be taken on account for diagnosis worldwide. However as it has been observed in another papers DLL is related only with *M. lepromtosis* alone or with dual infection, but not only with *M. leprae*, it could explain the severity of these cases[13].

Finally, these findings add evidence to the *M. leprae* and *M. lepromatous* distribution and clinical forms in the Mexican territory, and may aid to clarify the Lucio's phenomenon etiological agent[20,21].

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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