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Frequency of *Mycobacterium leprae and Mycobacterium lepromatosis* in patients with multibacillary leprosy identified in western Mexico during the 2019-2020 period

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1. Abstract

Leprosy, or Hansen's disease, is a chronic granulomatous disease primarily affecting the skin and peripheral nerves, caused by *Mycobacterium leprae*. In Jalisco, 44 prevalent cases and 17 new cases were reported for the year 2018; however, estimates reported by the WHO may not be entirely accurate for various reasons, notably the underreporting of cases in some countries.

For many years, *M. leprae* was considered the only mycobacterium that causes leprosy. 7 However, in 2008, a second species named *M. lepromatosis* was identified as the cause of illness and death in two Mexican patients with diffuse lepromatous leprosy.

1.2. Materials and Methods

Patients with multibacillary leprosy attending for their bacilloscopic examination were invited to participate in this study via verbal informed consent.

DNA Extraction and PCR DNA was extracted from each patient's bacilloscopic sample using a kit. PCR assays were performed using primers designed to detect *M. leprae* RLEP-7 and RLEP-8 or M. lipomatosis LPM244-F and LPM244-R

1.3. Results

A total of thirty patients were enrolled in this study; twenty-one were male (70%) and nine were female (30%), representing a 2.33:1 ratio. Among the men, 13 were identified as infected with *M. lepromatosis* (62%), one with *M. leprae* (4.7%), six had mixed infections (28.6%), and one sample failed to amplify.

2. Introduction

Leprosy, or Hansen's disease, is a chronic granulomatous disease primarily affecting the skin and peripheral nerves, caused by **Mycobacterium leprae**. Based on the bacteriological index determined via bacilloscopy or histopathology using Ziehl-Neelsen or Kinyoun staining it is classified into multibacillary (MB) cases (patients with more than 2 bacilli in 100 microscopic fields at 100X oil-immersion magnification; types BB, BL, and LL) and paucibacillary (PB) cases (patients with fewer than 2 bacilli in 100 fields at the same magnification; types BB, BT, and TT) [1-4].

2.1. Epidemiology

In Mexico, the reported prevalence is less than 0.5 per 10,000 inhabitants; 32,000 cases have been reported, predominantly in three clusters: the central-western cluster, comprising the states of Sinaloa, Nayarit, Jalisco, Colima, Michoacán, Guanajuato, Guerrero, Aguascalientes, the Federal District, Zacatecas, Durango, the State of Mexico, and Querétaro; the second cluster is the peninsular one, consisting of Yucatán and Campeche; and finally, the northeastern cluster, covering Nuevo León and Tamaulipas [5].

In Jalisco, 44 prevalent cases and 17 new cases were reported for the year 2018; however, estimates reported by the WHO may not be entirely accurate for various reasons, notably the underreporting of cases in some countries and the fact that many patients are removed from registries after two years of treatment, regardless of their clinical progress [6,7].

2.2. Clinical and Immunological Classification

Leprosy is classified according to four parameters: clinical presentation, histopathology, bacilloscopy, and immunology. The most widely used clinical classification is the Ridley-Jopling system, which is based on the patient's clinical and immunological status [8].

The disease is divided into two poles Lepromatous Leprosy (LL) and Tuberculoid Leprosy (LT) and an intermediate stage known as Borderline Leprosy (LB). Borderline cases are classified based on their proximity to either the lepromatous pole (BL) or the tuberculoid pole (BT), with the term "borderline" preceding the designation (BL, BT, and BB). There is also a group of indeterminate cases considered to represent the initial stage of leprosy. These cases are in an unstable stage that eventually progresses toward one of the poles, though this progression can be halted by treatment leading to a cure. Despite the difficulty of clinical diagnosis, this is the stage at which leprosy can be easily cured [2].

All borderline or indeterminate cases eventually progress toward one of the poles, most commonly LL [2,8]. The WHO divides the disease into only two broad groups, based on the treatment regimen to be administered. For public health control purposes, cases are classified as: Multibacillary (MB) including lepromatous, dimorphous, and diffuse infiltration forms and Paucibacillary (PB) including tuberculoid and indeterminate forms [9]. The clinical manifestations of leprosy appear in the skin, mucous membranes, and peripheral nervous system; their presentation depends on the specific clinical variant exhibited by the patient [10]. Nodular Lepromatous Leprosy (LL) predominantly affects the face specifically the supraciliary and intercalary regions, cheeks, and nose as well as the earlobes, trunk, buttocks, and extremities; multisystem involvement may also occur. The dermatosis consists of firm nodules of varying sizes, which may appear as isolated lesions or coalesce. They may be skin-coloured, exhibit surface telangiectasias, or appear erythematous. Upon involution, these lesions leave behind atrophic areas or scars if ulceration occurs [9]. In diffuse (LL) also known as Lucio-Latapí leprosy-there is generalized skin involvement, yet no distinct macules, nodules, or infiltrated plaques are observed. The condition progresses through two phases: initially, the skin becomes smooth and turgid (*facies suculenta*); subsequently (following treatment or over time), it becomes folded, scaly, and atrophic, presenting with telangiectasias and milia cysts (*facies atrophica terminalis*). Loss of eyebrows, eyelashes, and body hair (madarosis) occurs as a result of the formation of perifollicular inflammatory infiltrates [9].

For many years, *M. leprae* was considered the only mycobacterium that causes leprosy.7 However, in 2008, a second species named *M. lepromatosis* was identified as the cause of illness and death in two Mexican patients with diffuse lepromatous leprosy (LL) [3]. Both *M. leprae* and *M. lepromatosis* are acid-fast bacilli (AFB) and obligate intracellular Gram-positive organisms. Taxonomically, they are classified within the order Actinomycetales and the family Mycobacteriaceae [10]. They are slightly curved bacilli measuring 1–8 µm in length and 0.3 µm in diameter. Like other mycobacteria, they reproduce via binary fission. Macrophages are the preferred host cells for both bacilli; within them, the bacteria cluster to form intracellular aggregates known as "globi" (Figure 4 and 5). Although they cannot be cultured in artificial media, the nine-banded armadillo served as the first animal model for the study of leprosy [11].

3. Materials and Methods

Patients with multibacillary leprosy attending for their bacilloscopy examination were invited to participate in this study via verbal informed consent. For patients who agreed to participate, a bacilloscopy sample was collected from the earlobe and/or skin lesion(s) via scraping with a No. 15 scalpel blade to obtain tissue (with or without lymph). The sample obtained via scraping was used to prepare a smear on a slide (pre-labeled with an ID number) for the institution's diagnostic purposes, while a second scraping was performed at the same sites for molecular analysis. Patients with a positive bacilloscopy result (1–6+) were included in the study. Transport containers holding the tissue samples were moved in a thermal cooler to the microbiology laboratory for PCR analysis. Subsequently, the data were entered into an Excel spreadsheet and the Microsoft SPSS statistical software program Figure 6a and 6b.

DNA Extraction and PCR DNA was extracted from each patient's bacilloscopy sample using a kit (High Pure PCR Template Preparation Kit; Ref. 11796828001). Sections of the sample obtained via bacilloscopy were digested with proteinase K and loaded onto a silica-based minicolumn. Following a washing step, the DNA was eluted in a buffer for testing. PCR assays were performed using primers designed to detect *M. leprae* (RLEP-7 [5'-3' TGAGGCTTCGTGTGCTTTGC] and RLEP-8 [5'-3' ATCTGCGCTAGAAGGTTGCC]) or *M. lepromatosis* (LPM244-F [5'-3' GTTCCTCCACCGACAAACAC] and LPM244-R [5'-3' TTCGTGAGGTACCGGTGAAA]).

The latter primer pair amplifies a 244-base pair (bp) fragment of the HemN gene, which is absent in *M. leprae* and specific to *M. lepromatosis* [12].

Samples that did not react with any of the aforementioned primers were amplified using TB11 and TB12, which target the hsp65 gene. Amplification begins with an initial denaturation step at 95°C, followed by 40 cycles consisting of annealing at 58°C for 40 seconds and extension at 72°C for 30 seconds, and concludes with a final extension at 72°C for 10 minutes [13].

4. Statistical Analysis

Data capture and statistical testing were performed using Excel to create the database, and SPSS v. 20 and GraphPad v. 6 for statistical analysis. Descriptive statistics were employed, using measures of central tendency (such as the mean) and dispersion (such as the standard deviation) for quantitative variables. Statistical significance was defined as a p-value of 0.05 or less in the Student's t-test.

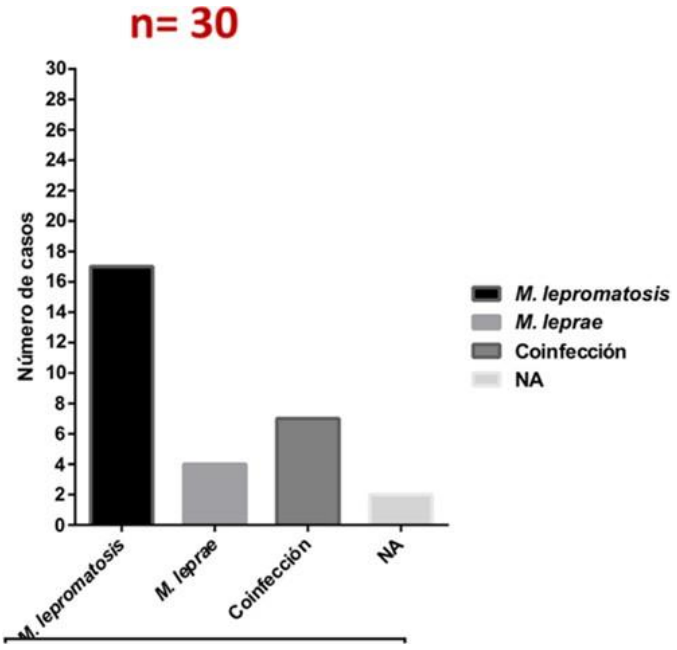


Figure 4: Like other mycobacteria, they reproduce via binary fission. Macrophages are the preferred host cells for both bacilli; within them, the bacteria cluster to form intracellular aggregates known as "globi.

5. Result

Sampling and identification Between June 1, 2019, and June 1, 2020, 30 patients with multibacillary leprosy and positive bacilloscopy results were included; molecular identification was successfully achieved for 28 (93.33%) of them. Among these, 4 (13.3%) were identified as *M. leprae* using specific primers RLEP 7 and 8, while 17 (56.7%) were identified as *M. lepromatosis* using the specific primer LPM-244. Species Graph 1. Identification of *Mycobacterium leprae* and *Mycobacterium Lepromatosis* species via PCR. Of the 30 bacilloscopy samples collected from patients with a clinical and microbiological diagnosis of leprosy, PCR analysis revealed a clear predominance of *M. lepromatosis* infections totalling 17 samples (56.7%) compared to *M. leprae* infections, where amplification occurred in 4 cases (13.3%). This reinforces the hypothesis that *M. lepromatosis* is the primary causative agent of lepromatous leprosy in Mexico, although further, larger-scale studies are needed to confirm these conclusions. A significant number of co-infections was also observed (7 cases, or 23.3%), considering the limited sample size; additionally, 2 specimens tested negative for both species (Table 1).

***NA: No Amplification**

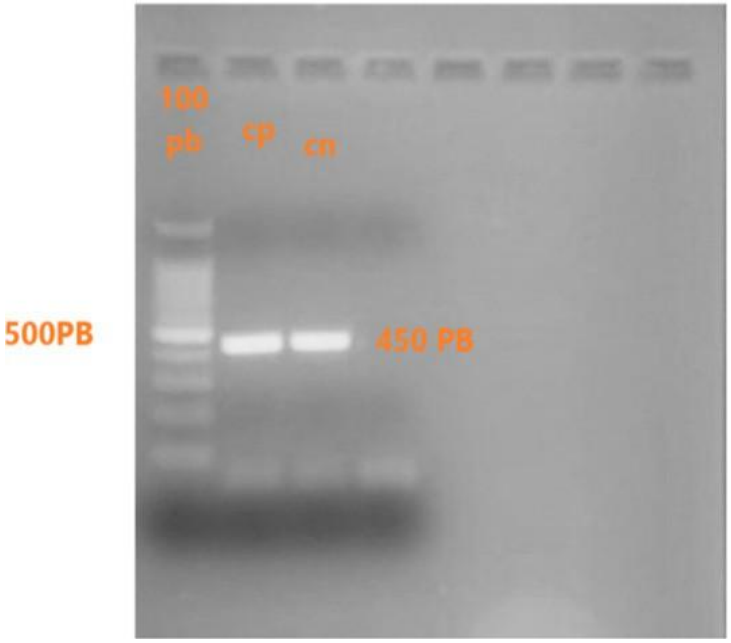


Figure 5: Electrophoresis gel, positive sample, amplification using RLEP primers 7 and 8 for the identification of *Mycobacterium leprae* (bp: base pairs, cp: positive control, cn: negative control). Lanes from left to right: molecular weight marker (100 bp), positive control: sample from an armadillo infected with *Mycobacterium leprae*, sample from a patient with a clinical diagnosis of *lepromatous leprosy* (amplicon size 440 bp, consistent with M. leprae) and negative control.

Figure 6a. Figure 6b.

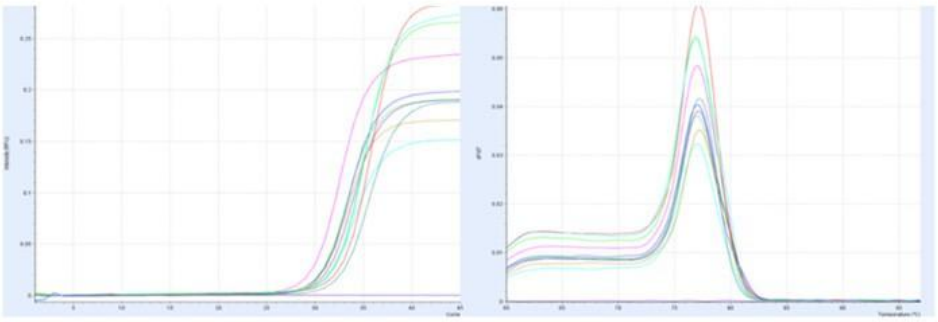
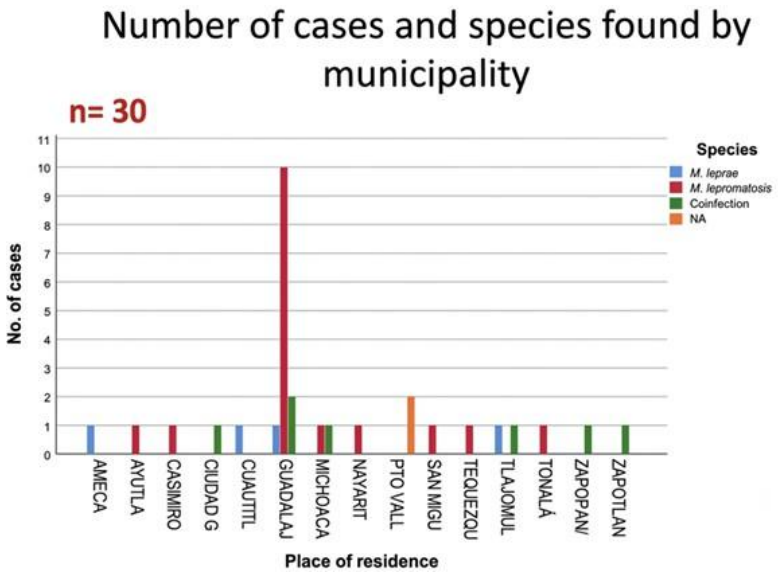


Figure 6a. Amplification curve Figure 6b: Melting curve Figure 6. Identification of *Mycobacterium lepromatosis* by qPCR using LPM-244 Figure 6a: Amplification curve of the PCR products. Figure 6b: Melting curve showing a single amplification peak, thereby confirming the reaction's specificity for the expected product.

A total of thirty patients were enrolled in this study; twenty-one were male (70%) and nine were female (30%), representing a 2.33:1 ratio. Among the men, 13 were identified as infected with *M. lepromatosis* (62%), one with *M. leprae* (4.7%), six had mixed infections (28.6%), and one sample failed to amplify (4.7%)

Regarding the female patients, three infections with *M. leprae* were identified (33.3%), four with *M. lepromatosis* (44.4%), one patient had a mixed infection (11.1%), and one sample failed to amplify (11.1%).



Graf 1.

Regarding the patients' places of origin, our study population spanned three states-Jalisco, Michoacán, and Nayarit with the vast majority concentrated in the Guadalajara metropolitan area (13 cases, or 43.33%). In this area, we identified ten cases positive for **M. lepromatosis** (76.92%), two coinfections (15.38%), and a single case of **M. leprae** (7.69%). It should be noted that the two specimens we were unable to amplify via PCR came from patients residing in Puerto Vallarta: a 24-year-old woman who visited the institute for clinical and bacilloscopic assessment-diagnosed with lepromatous leprosy of 1.5 years' duration and a bacillary load of 4+ (four crosses), and who was not yet under treatment-and a 50-year-old man with a clinical diagnosis of lepromatous leprosy of 5 years' duration, untreated, and with a bacillary load of 2+. Similarly, regarding Michoacán, two positive cases were found: a 60-year-old woman with a clinical diagnosis of lepromatous leprosy (2 years' duration, untreated, bacillary load of 3+) and a 42-year-old man with a clinical diagnosis of nodular leprosy (3 years' duration, one month of multidrug therapy, bacillary load of 4+); notably, the latter patient presented a coinfection with both species. The identified specimen from Nayarit belonged to a 43-year-old woman with a clinical diagnosis of lepromatous leprosy of eight years' duration, undergoing multidrug therapy, with a bacillary load of 4+ (Graph 1).

Patient ages ranged from 24 to 76 years, with a mean of 56; the highest bacillary load was observed in the 41–76 age group, with the exception of Patient 3, who was 24 years old at the time of sampling and presented a 4+ load (Ridley-Jopling scale)-notably, this patient had been ill for 1.5 years and had not yet started the

WHO-recommended multidrug therapy (MDT). The duration of the disease ranged from 6 months to 20 years; of the 30 patients enrolled, 20 (66.6%) were already on the WHO-recommended MDT regimen. Nevertheless, these treated patients continued to show persistent bacilli after months or even years of therapy, including alternative antibiotic regimens-as seen in patients 7, 17, 28, and 30, who received additional antibiotics such as clarithromycin, levofloxacin, ofloxacin, minocycline, and clofazimine alongside MDT, all of which have proven effective as adjuncts in leprosy treatment. It should be noted that these patients were infected with *M. lepromatosis*, except for Patient 7, who presented a mixed infection; these findings suggest that *M. lepromatosis* may be associated with multidrug-resistant forms of the disease.

Regarding the clinical forms of the disease among the patients, we found a predominance of lepromatous leprosy (LL), with a total of 22 cases representing 73.33% of all cases included in the study. Of these: 12 (54.54%) tested positive for **M. lepromatosis*, 3 (13.63%) for *M. leprae*, 5 (22.72%) showed mixed infections (P = 0.860; two-tailed p-value based on significant 2x2 χ^2 tests regarding the relationship between clinical form and identified species), and 2 samples (9.1%) failed to amplify. Among the remaining clinically diagnosed cases, there was 1 case of borderline lepromatous leprosy that tested positive for *M. lepromatosis*, 1 case of diffuse lepromatous leprosy where the causative agent was again *M. lepromatosis*, and six cases of nodular leprosy; of the latter, 1 tested positive for *M. leprae*, 3 for *M. lepromatosis*

and 2 were identified as mixed infections (Table 6). Based on the foregoing, we conclude that *Mycobacterium Lepromatosis* is associated with lepromatous leprosy in patients diagnosed by the Dermatological Institute of Jalisco.

6. Conclusions

Leprosy is a disease primarily affecting the skin and peripheral nerves; its causative agent was traditionally identified as *M. leprae*. However, in 2008, a new causative mycobacterium *M. lepromatosis* was discovered in two patients presenting with diffuse lepromatous leprosy (LL) and the Lucio phenomenon; it was confirmed to be genetically distinct from *M. leprae* Following the report of those two cases, molecular studies have been conducted worldwide to determine the frequency with which *M. lepromatosis* causes the disease. Few studies on this subject have been conducted in our country; two took place in northwestern Mexico, where the type of leprosy observed differs from that seen in the western region. In Monterrey, *M. leprae* predominated, whereas in Sinaloa and Jalisco, the prevalence of *M. lepromatosis* infection was higher. The present study confirms that *M. lepromatosis* is the predominant species in Mexico and appears to be associated with lepromatous leprosy, although bacilloscopy remains a useful tool for the diagnosis and monitoring of Hansen's disease. Furthermore, it is important to implement molecular biology tools to correctly identify the causative agent and to conduct testing for multidrug therapy (MDT) resistance.

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