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Original Article

Molecular detection of *Mycobacterium leprae* in the lachrymal film of leprosy cases, health staff, and healthy individuals

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Abstract

Introduction: Leprosy is still endemic in some countries, and interruption of the infection chain is essential to reduce its burden. This article aims to verify if the lachrymal film of leprosy cases and non-leprosy can be a site of relevance in the early molecular detection of *M. leprae.*
Methodology: the quantitative polymerase chain reaction technique was used. The sample included 54 patients with leprosy compared to 49 health professionals working at a hospital specialized in leprosy (staff), and 51 healthy individuals outside the hospital without theoretical contact with patients (outsiders). The qPCR was used for the detection of *Mycobacterium leprae* in lacrimal samples of different clinical forms, where molecular detection was based on the overlap of the Specific Repetitive Element.
Results: *M. leprae* DNA was demonstrated in all three groups in significantly increasing proportions among the outsiders, staff, and patient groups, respectively, 13.7%, 37.8%, and 42.5%.
Conclusions: *Mycobacterium leprae*, in endemic countries, is present in the environment and can transmit to humans, regardless of the determination of the infection process and immune response. Known to be a direct and aerial transmission microorganism, it was also found that *M. leprae* is present in the tear film, being more prevalent in leprosy cases than in the general population. These findings may have epidemiological repercussions along the disease's transmission chain.

Key words: leprosy; eye; lachrymal film; real-time PCR (qPCR); RLEP; 85B.

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Introduction

Mycobacterium leprae is an intracellular mycobacterium and the causative agent of leprosy. It is a slow-growing microorganism that exhibits selective tropism for Schwan cells in nerves and macrophages in the skin [1]. In the interior of the cell, the bacilli proliferate at a gradual pace, inducing variable immune responses that result in alterations to the nerve structure [2]. The transmission of leprosy remains a subject of further study. However, current knowledge indicates that the transmission process occurs via the air through continued contact with an untreated individual harboring the bacilli. The hypothesis concerning the possibility of an animal reservoir within the endemic cycle has also been postulated [3]. The disease manifests in a spectrum of clinical presentations ranging from the Tuberculoid (TT) to the Virchowian (LL) pole, a phenomenon attributable to the relationship established between the cellular immune response of the host and the mycobacteria. Clinical forms are not stable, and exacerbations of the disease are frequent, suggesting a disruption in immune balance. The clinical manifestations of the condition are contingent upon the patient's immunological status. The spectrum of presentations ranges from a solitary spot with diminished sensation or localized neuritis to the presence of nodules or papules, which may or may not be accompanied by polyneuritis [4].

Leprosy is a disease that has long been of interest to ophthalmology. This is due to the fact that the eye is the site of significant changes caused by the bacteria, either directly or by way of an immunological response. Furthermore, the eye was among the first sites for *M. leprae* in vitro culture when Gerhard Hansen first discovered the bacteria [5]. It has been established that ocular involvement manifests in approximately 70% to 75% of cases, with 10% to 50% exhibiting significant symptoms. Moreover, 5% of patients may experience blindness secondary to lagophthalmos and iritis [1]. It is estimated that one million patients worldwide have

severe visual impairment or even have already lost their sight due to lesions suggestive of leprosy [6,7].

Currently, the body of knowledge concerning the presence of this bacillus in tears remains limited, as does the extent of its participation in the disease transmission chain [8]. It has been established that a variety of viruses and bacteria can be detected using molecular testing on the ocular surface [9]. The objective of this study is to determine the presence of *M. leprae* in the tear film of patients with multibacillary leprosy, healthy individuals, and health workers with constant exposure to leprosy cases. This study aims to provide novel insights into the transmission chain of leprosy.

Methodology

A cross-sectional study was conducted at the Instituto Lauro de Souza Lima (ILSL), including cases of multibacillary leprosy diagnosed according to the clinical and bacilloscopic criteria recommended by the WHO [10]. The sample set consisted of 154 participants subdivided into three groups, namely: (i) patients: 54 patients with confirmed leprosy diagnosis, already treated or under treatment; (ii) staff: 49 healthy workers at a hospital specialized in leprosy care (ILSL), including doctors, registered nurses, and nursing assistants; (iii) outsiders: 51 healthy individuals from the general population (the Control Group, outside the ILSL, without theoretical contact with patients and without no reported leprosy, and residing in various locations throughout the town).

Material Collection and Analysis

Tear film samples were collected using a sterile swab in the lower conjunctival sac of the right eye. Afterward, the swab was immediately immersed (without homogenization) in an Eppendorf containing 1.5 ml of 70% alcohol. With the swab inside the Eppendorf, the stem was cut with a sterile scalpel, and it was placed at room temperature.

The samples were centrifuged at 14,000 rpm for 50 minutes and then all the supernatant was removed. ATL buffer (180 µL, Dneasy Blood & Tissue Kit, Qiagen®, Brazil) and proteinase K (25 µL, Dneasy Blood & Tissue Kit, Qiagen®, Brazil) were added to the pellet and incubated overnight at 56 °C. *M. leprae* DNA was extracted using a QIAcube equipment (Qiagen, Brazil) using the Dneasy Blood & Tissue Kit (Qiagen®, Brazil). After estimating the concentration (ng/uL) and purity (Nanodrop 2000, Thermo Fisher Scientific Inc.®, Wilmington, DE), the isolated DNAs were stored at -20º C until use.

Quantitative PCR (real-time PCR assays)

The PCR reaction was prepared with a final volume of 10 µL, containing 5 µL of Master Mix (qPCR SybrMaster UNG lowROX, Jena Bioscience®, Thuringia, Germany), 0.6 µL of primer mix [0.3 µL of each primer (forward and reverse) of 10 µM stock solution], 0.4 µL of nuclease-free water and 4 µL of 2.5 ng/µL of genomic DNA. The following primers were utilized: forward 5' ATTTCTGCCGCTGGTATCGGT 3' and reverse 5' TGCGCTAGAAGGTTGCCGTAT 3' to identify the RLEP gene (*Mycobacterium leprae*-specific repetitive element) according to Azevedo *et al.,* 2017 [11]. Negative controls (mixture of the PCR without DNA) and positive controls (DNA of *Mycobacterium leprae* strain Thai 53 in equal volume and concentration as the samples).

The reactions were performed in duplicate and processed in the ViiA7 real-time PCR equipment (Applied Biosystems®, USA). The cycling parameters

Table 1. Sociodemographic characteristics of the studied groups.

Characteristics	Group						Total	
	Patients		Staff		Outsiders			
	N	%	N	%	N	%	N	%
Gender								
Female	16	29.63	44	89.79	39	76.47	99	64.28
Male	38	70.37	5	10.21	12	23.53	55	35.72
Reported ethnicity								
Caucasian	47	87.04	40	81.63	45	88.23	132	85.71
Afro	2	3.7	3	6.12	1	1.96	6	3.89
Mixed	5	9.26	6	12.25	2	3.92	13	8.44
Asian	0	0	0	0	3	5.89	3	1.95
Age group (Years)								
18-27	6	11.11	0	0	11	21.57	17	11.03
28-37	5	9.26	7	14.29	11	21.57	23	14.97
38-47	7	12.96	8	16.33	5	9.8	20	12.97
48-57	11	20.37	18	36.73	8	15.69	37	24.02
58-67	15	27.78	16	32.65	11	21.57	42	27.27
68-79	10	18.52	0	0	5	9.8	15	9.74
Total	**54**	**100**	**49**	**100**	**51**	**100**	**154**	**100**

consisted of 2 minutes at 50 ºC, 2 minutes at 95 ºC, and forty cycles of 30 seconds at 95 ºC, 30 seconds at 62.5 ºC (annealing temperature), and 1 minute at 72 ºC, in addition to a final cycle of twenty minutes, with increasing temperature from 60 to 95 ºC (dissociation curve of reaction products – melting curve: used for the analysis of amplification specificity). The results were obtained according to the first fluorescence signal detected in the Limit of Detection Cycle (CT), and the sample was considered positive when it presented a CT < 40 (cutoff point = cutoff).

Data analysis

In analytical statistics, Fischer's Exact Test was selected, considering the relatively small numbers of discrete quantitative variables in the case of positive and negative cases across the three groups, with a significance level set at 5%. Demographic data were collected in a specific spreadsheet and analyzed regarding the frequencies of the variables involved, presented in tables in absolute numbers and their percentages. Statistical analyses were conducted using IBM SPSS® statistical software version 28.0.1 (IBM, Inc, Chicago, IL).

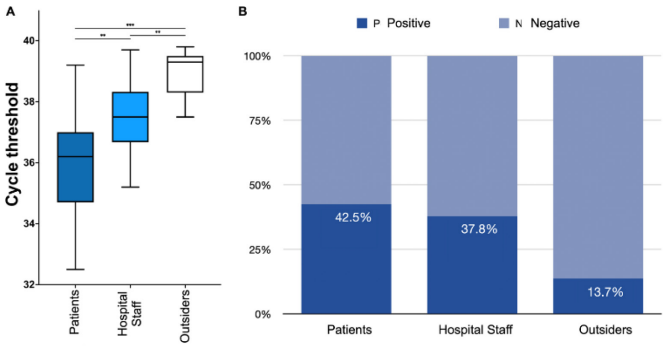
Results

The demographic characteristics of the studied groups are presented in Table 1. The material obtained from the three studied groups was submitted to the DNA identification test of *M. leprae* in the target gene RLEP by the qPCR technique. The value above and including 40 cycles was taken as a cutoff, i.e., cycle threshold (CT) for negativity, therefore, positivity was identified for values of less than 40 cycles.

The CT results across the various groups are illustrated in Figure 1A, which showcases the mean and standard deviation of CT in positive cases (38.9) that closely align with the cutoff value, exhibiting a relatively narrow standard deviation (0.77). The mean and standard deviation shift away from the CT cutoff value, widening the standard deviation towards the group of patients.

The distribution of positive and negative cases among groups is illustrated in Figure 1B. As the analysis progresses from the group of patients to the group of outsiders (the general population), there is a marked increase in negativity, as would be expected.

Figure 1. Box-chart showing a significant statistical difference of prevalence of positive qPCR for *M. leprae* DNA among the three groups.



A. qPCR Cycle threshold comparison; **B.** Difference of *M. leprae* DNA prevalence among the study groups.

The results of qPCR as related to the clinical forms in the patient's group are presented in Table 2. Fischer's exact test reveals that there is no statistical difference between the clinical forms regarding positivity or negativity for the qPCR test, for $p < 0.05$.

Discussion

In 1982, the World Health Organization (WHO) introduced the use of multidrug therapy (MDT) as a treatment for leprosy, significantly altering the management of a disease that had been considered incurable for millennia [12]. Among the changes in the strategies of leprosy control programs, the interruption of the transmission chain stands out. Early and adequate treatment appears as a key action since contagious cases become non-contagious after a few days of starting MDT. However, data available so far indicates that MDT does not have a marked role in the interruption of the transmission chain [13]. Confounding factors such as the BCG vaccine [14] and chemoprophylaxis [15] can hinder this interruption or reduction of transmission. In this regard, further research is needed to elucidate the mechanisms of transmission, as there is currently limited understanding of the complete process and its underlying details Thus, the identification of *M. leprae* in tears is of interest to expand knowledge about the transmission route.

Table 2. Distribution of leprosy patients by clinical form of leprosy and result of qPCR. Fisher Exact Probability test $p = 0.35$.

	BT		BB		BL		LL		Total	
	n	%	n	%	n	%	n	%	n	%
Positive	0	0.0	1	1.8	4	7.4	18	33.4	23	42.6
Negative	2	3.7	5	9.2	5	9.3	19	35.2	31	57.4
Total	2	3.7	6	11.0	9	16.7	37	68.6	54	100.0

The presence of the causative agent of leprosy, detected by a highly sensitive and specific method, in the three groups, at first glance, seems contradictory once only the patient group consisted of individuals recognized as gross carriers of leprosy bacilli. Therefore, it should not be expected that *M. leprae* would be found in the staff group and, even less, in the outsider's group, which are healthy individuals who do not have daily contact with leprosy cases. However, the ubiquity of microorganisms can be extended to *M. leprae*, a fact that has already been reported in several studies [16,17]. Certainly, the connection between the causative agent and the presence of disease in humans is not being discussed here, but the presence of the agent in the environment, even though it may come from the most likely reservoir, humans. Consequently, it is not surprising that, by examining the specificity and sensitivity of the qPCT/RLEP, *M. leprae* was identified in these three groups, each with different temporal and quantitative exposure to the causative agent. It is noteworthy that the bacillus, once eliminated by a carrier, has been shown to remain viable in the environment for up to 46 days in moist soil and at room temperature [16].

Data revealed a higher incidence of *M. leprae* positive cases among the patient group, with a statistically significant decrease observed across the other groups. The control group, comprising healthy individuals, exhibited the lowest number of positive cases for mycobacteria. Despite the low positivity rate in this group, it can be explained by the ability of *M. leprae* to remain viable in the environment, the possibility of asymptomatic carriers, and unidentified patients, conditions that are not rare in tropical countries [18].

In the patient group, all cases were determined to be multibacillary, a condition characterized by a pronounced bacillary load. It is acknowledged that, in such cases, the number of bacilli in the body tends to decrease gradually even after the start of MDT treatment. The initial bacilloscopic index (BI) has been observed to decrease in proportion to one BI unit for each year of treatment, indicating that the presence of bacilli, even in treated cases, is common [19]. Indeed, viable bacilli were observed in 23.5% of cases treated up to 1 year, 7.1% at 2 years, and 3.84% at 3 years, as evaluated by inoculation in mouse paws [20].

The transmission of *M. leprae* occurs directly via airborne droplets, known as Pflüger droplets, which facilitate the dissemination of the bacillus [21]. In such contexts, individuals engaged in occupations involving the treatment of such cases are exposed to a greater extent than those in other roles, represented in this study by the outsider's group. This finding may offer a potential explanation for the higher proportion of health workers (staff group) who were qPCR positive for the bacillus when compared with the control group. It is imperative to reiterate that the scope of this study is limited to the detection of the causative agent in the tear, rather than the identification of the disease itself, given that the disease manifests only in individuals who are susceptible to it. The hypothesis that a genetic determinant exists, enabling an individual who has been exposed to the bacillus and subsequently infected to develop the disease, is currently under investigation [22].

Although the armadillo (*Dasypus novencinctus*) may assume some relevance in the chain of transmission of the disease, so far, evidence indicates that its epidemiological participation is questionable, therefore, humans and the environment are the main sources of transmission [3,23–25]. Additionally, *M. leprae* can remain viable in three common and ubiquitous species of Acanthamoeba and two strains of *Hartmannella*. Although, it is not possible to prove that this endocytobiotic in nature indicates these organisms as vehicles or transmission vectors for the *M. leprae* [26]. Water is also mentioned as a potential source of these mycobacteria [27–29]. Triatomids, a type of insect, also seem to harbor *M. leprae*, however, the epidemiological significance of these findings remains unclear [30]. Soil analysis around a leprosy hospital in India and soil collected around the homes of patients who had already been treated clearly demonstrated the presence of viable *M. leprae* [31]. As for arthropods, they can acquire *M. leprae* from blood or nasal mucus from bacilliferous patients and later deposit it at a distance, still viable [32]. However, the vector transmission of leprosy is not considered to be epidemiologically relevant.

Findings indicate that *M. leprae* may be present in the environment under different conditions, and humans are the most evident reservoir. Furthermore, *Pflüger* droplets constitute the most important mechanism in the transmission process. Consequently, the presence of healthy individuals with a positive result for *M. leprae* DNA in an urban area of an endemic country, even in a region of low endemicity, should not be regarded as an exceptional or implausible occurrence.

The tear excretory system begins at the superior and inferior lacrimal points, the common duct, and the lacrimal sac (situated beneath the internal palpebral ligament), and flows into the inferior nasal meatus,

through the lacrimo-nasal duct [33], it is possible that Hansen's bacillus reaches the eye from this nasal area or reaches the meatus and consequently the oral cavity, coming from the eyes through the tear. The presence of the agent in different parts of the oral cavity has been previously studied [34]. This suggests the existence of a communication relationship between these three anatomical levels, which could have implications for the dispersion of *M. leprae* along these spaces. Moreover, the tear film contains immunoglobulins and enzymes, such as lysozyme, beta-lysine, and lactoferrin, along with free radicals in the mucin layer and the action of cleaning the eyelids during blinking, which collectively form a local defense mechanism against bacterial infection. This set of factors contributes to the unfavorable ocular surface environment for bacterial growth. However, disruption of the corneal epithelium and a loss of tear balance (quantitatively or qualitatively) alters this defense mechanism. In leprosy, the initial clinical ocular manifestations are prominent corneal nerves, conjunctivitis, superficial punctate keratitis, scleritis, and uveitis. Secondarily, neural damage may occur with a decrease in the iridian response, indicating injury to the autosomal nerves [1].

Due to the high level of environmental exposure of the eye as well as to pathogens, and given the knowledge that Hansen's bacillus can cause inflammatory lesions of the conjunctiva and keratitis, it is possible to consider the confirmed observation of the pathogen in the tear as a causal factor of direct changes in the anterior segment of the eye, as well as its presence within the anterior chamber, facilitated by the loss of defense of the corneal epithelium, with the subsequent access to the ciliary body and blood pathways. Consequently, the study by Silva *et al.* [35] hypothetically suggests that, during subclinical infection and the early stages of leprosy, respiratory epithelial cells may serve as a reservoir for bacterial retention and survival, potentially acting as a portal of entry for *M. leprae*. It is noteworthy that the majority of the nasal cavity is lined by the typical respiratory system epithelium, characterized by pseudostratified columnar ciliated epithelium with goblet cells. However, it should be noted that, from an ophthalmological perspective, the corneal epithelium is of the squamous, stratified, non-keratinized type, resting on a basement membrane composed of type IV collagen and extracellular matrix proteins. The cornea is transparent, without vascularization, and does not have goblet cells [36].

In the present study, we can only confirm that *M. leprae* is clearly present in the tear film and on the external surface of the eye. However, continuing the parallelism to the interesting finding by Silva *et al.* [35] they state that *M. leprae* has an arsenal of adhesins involved in its interaction with epithelial cells. Ng *et al.* [37] recall that these cell surface glycolipids, such as PGL-I, play a role as adhesins, but may also contribute to the interaction of *M. leprae* cells and the epithelium.

Thus, even considering the differences between the corneal and respiratory epithelium, one cannot fail to suggest as relevant the potential possibility of the same interaction of adhesion of *M. leprae* to the surface of the corneal epithelium and its internalization to the interior of the eye, even without epithelial damage [1]. Consequently, further exploration into this phenomenon may unveil novel insights into the infection pathway and pathogenicity of *M. leprae* within the ocular environment. This, in turn, could significantly impact our understanding of the transmission chain and, consequently, the epidemiology of leprosy.

The main limitation of the study is the inclusion of patients with varying treatment durations, which may have affected the quantification of DNA detection. To mitigate this limitation, it is crucial to note that even cases that have undergone complete treatment require a substantial period after treatment to achieve a reduction in bacilloscopy [38].

Conclusions

The presence of *Mycobacterium leprae* was detected in the tears of all the study groups. However, a significant increase in positivity was observed from the outsider's group to the patient group. Quantitatively, the outsider’s group had the lowest number of positive individuals with the highest average of cycles, an average with a value close to the cut-off for test negativity, indicating a very low indirect bacillary load. The present findings suggest a potential involvement of the ocular adnexa in the mechanism of infection installation by *M. leprae*. This is based on the premise that there is significant ocular exposure to the environment and contiguity with the nasal cavity. Further studies are necessary to understand its epidemiological repercussions in the chain of transmission of the disease.

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Conflict of interests

No conflict of interests is declared.

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