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A N   I N T E R N A T I O N A L   J O U R N A L

Detection of Lsr2 Gene of *Mycobacterium leprae* in Nasal Mucus

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ABSTRACT

In the present study, nasal mucus from patients with leprosy were analyzed by PCR using specific primers for Lsr2 gene of Mycobacterium leprae*. The presence of Lsr2 gene in the nasal mucus was detected in 25.80% of patients with paucibacillari leprosy, and 23.07% of contacts. Despite the absence of clinical features in the contact individuals, it was possible to detect the presence of Lsr2 gene in the nasal mucus of these individuals. Therefore, PCR detection of* M. leprae *targeting Lsr2 gene using nasal mucus samples could contribute to early diagnosis of leprosy.*

Key words: *Mycobacterium leprae*, leprosy, hanseniase, nasal mucus, PCR.

INTRODUCTION

Although a long time has elapsed since the discovery of *Mycobacterium leprae* by Gerhard Armauer Hansen in 1873 (De Zubiria et al. 2003; Fernandes et al. 2004), the understanding of the biology of this microorganism is still incomplete. For instance, the reasons for the inability of *M. leprae* to grow in media and conditions commonly used for bacteria is still not known. Recent studies have presented interesting data about the relationship between *M. leprae* and its host (Zanazzi et al. 2000; Nurse 2003), but many relevant questions about it remain unanswered (Siddiqui et al. 2003). An important aspect of mycobacterial pathogenesis is the ability of the pathogen to establish long lasting latent infections in the host. Leprosy (or Hanses's Disease) is a millenary chronic infectious granulomatous disease, which attacks skin, peripheral nerves and mucous membranes and is associated with immune response and secondary infections involving the status from the host. The World Health Organization estimates that more than 12 million people are affected by leprosy in the world (Braga 2002). Leprosy remains highly endemic in several countries such as Angola, Brazil, Central African Republic, Democratic Republic of Congo, India, Madagascar, Mozambique, Nepal, and the United Republic of Tanzania. It has been a problem in the countries which received a large number of immigrants from the endemic areas (Chae et al. 2002; Jianping et al. 2004). (Wiwanitkit 2005) performed a database search in order to find the genes of *M. leprae* recorded with complete sequences and compared their homology

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to human genomes using BLAST method. It is known that B cell responses to specific sequences within the Lsr antigen have been shown to be associated with the immunopathological responses in leprosy patients with erythema nodosum leprosum. It has been observed that some B and T cell epitopes localized to the regions with amino acid substitutions may account for the putative differential responsiveness to this antigen in tuberculosis and leprosy (Oftung et al. 2000). (Misra et al. 1995) found that the patients who did not show acid-fast bacilli in the tissues by the conventional methods presented positive for *M. leprae* DNA. PCR was initially used for the detection this pathogen in 1989 (Hartskeerl et al. 1989; Woods et al. 1989) and since them it has been used in most different clinical specimens as skin (De Wit et al. 1991; Yoon et al. 1993), nasal swab and hair bulb (Santos et al. 1995) and oral swab (Goulart et al. 2001). A proteomic approach was undertaken to identify the proteins present in the soluble/cytosol and membrane subcellular fractions obtained from the armadillo derived from *M. leprae*. Proteins from each fraction were separated by two-dimensional gel electrophoresis (2-DE) and identified by mass spectrometry. A total of 147 protein spots were identified from 2-DE patterns and shown to comprise the products of 44 different genes, 28 of them corresponding to new proteins (Marques et al. 2004).

Multidrug treatment has contributed to disease control, but new cases are being reported, showing that the development of early detection methods is imperative. Knowledge of the genomic sequence of *M. leprae* (Cole et al. 2001) is an important step for understanding this bacterium and its interactions with its host, as well as for developing the detection tests such as those based on PCR (Meima et al. 2004) or helicase-dependent amplification (HDA) (Young 2001; Vincente et al. 2004). Several PCR-based methods for the detection and evaluation of leprosy have been reported (Zanazzi et al. 2000; Santos et al. 2001; Guerrero et al. 2002; Torres et al. 2003; Sakamuri et al. 2009). The aim of this work was to evaluate the PCR detection of lsr2 gene of *M. leprae* in nasal mucus.

MATERIAL AND METHODS

With the approval at the Human Ethics Committee of Londrina State University and Philantropic Society Humanitas, Parana, Brazil, 130 Brazilian patients were included in thisn study. DNA sequences of *M. leprae* were analyzed by BLAST (http://ncbi.nlm.nih.gov/BLAST) and the rate of repetition in the genome was checked searching for a unique sequence copy, as well as for similarity with the genome of other microorganisms. Lsr2 gene was analyzed in the PRIMER3 program (http://frodo.wi.mit.edu/primer3/) to draw the primers and these were analyzed by the BLAST program, checking its specificity to *M. leprae*. The 130 patients enrolled in this study were grouped as following: 22 samples (~16,9%) from multibacillary leprosy, 31 samples (~23,8%) from paucibacillari leprosy, 52 samples (40%) from asymptomatic household contacts of patients with leprosy and 25 samples (~19,2 %) from people without history of contact with leprosy. *Mycobacterium fortuitum* (11 isolates), *Mycobacterium scrofulaceum* (1 isolate), *Mycobacterium tuberculosis* (29 isolates), *Mycobacterium avium* (4 isolates), which were kindly provided by Dra. Halha Ostrensky Saridakis, (Centro de Ciências Biológicas, Departamento de Microbiologia – Universidade Estadual de Londrina - Brasil), were used as controls.

Swab samples of nasal mucus were collected using sterile cotton swabs wet in phosphate buffer (pH 7.0), maintained at 4°C until   transferred to Eppendorf® tubes containing 1.0 mL of ultra pure water and stored at –20°C until DNA extraction.

DNA extraction

Genomic DNA was isolated following the method proposed by (Torres et al. 2003) with modification. Briefly, 200μL of the sample was centrifuged 15 minutes at 12.000 g and the pellet was treated at 100°C for 1 minute and freezing in nitrogen for 3 minutes. It was resuspended in 100 μl of Tris- HCl 50 mM buffer (pH 8.0), containing lysozime 10 mg/mL, incubated at 37°C for30 minutes, added 10 μL of proteinase K 50 mg/mL, followed by the addition of 1.0 μl of Triton X-100 (10%). The mixture was kept at 55°C for two hours and after the addition of absolute ethanol centrifuged at 10.000 g for five minutes. The DNA was suspended in ultra pure water and stored at –20°C until use.

Amplification conditions

PCR products were analyzed by the electrophoresis on acrylamide gel (10%) and detected by a nonradioisotopic technique using a commercially available silver staining method (data not shown).

RESULTS AND DISCUSSION

The Lsr2 gene of *M. leprae* codifies a protein of 15 kDa with capability of stimulating T lymphocytes. Table 1 shows the results of amplifications by PCR from 130 samples of nasal mucus of leprosy patients, asymptomatic household contacts of patients with leprosy and people without contact history. Samples with negative PCR results were tested for the presence of inhibitors in the reaction, by adding standardized DNA to the extracted sample. To test the specificity of PCR-probing with the primers for *M. leprae* Lsr2 region to Lsr2, the gene of other mycobacterias as *M. avium, M. fortuitum, M. scrofulaceum and M. tuberculosis* were tested in the same region. In this context, all the strains were negative in PCR test.

Table 1 - Detection of Lsr2 gene of *M. leprae* in smear of nasal mucus from patients.

Class	PCR-probing		
	Positive	Negative	Total
Multibacillary	19	03*[1]	22
Paucibacillari*	08	23	31
Contact**	12	40	52
No contact**	0	25	25
Total	39	91	130

[1]Three were discharged from treatment. *p<0,0001. **p<0,0227

In Brazil, despite actions for the treatment and control of leprosy, the number of new cases is highly threatening, and considering the existence of infected and undiagnosed people as a true reservoir of *M. leprae* and their contacts (Braber 2004) as infection sources, the development of methods for early diagnosis become imperative. PCR is a useful tool for the diagnosis of leprosy and its recent developments such as helicase dependent amplification point to the possibility of developing methods of detection with better sensitivity and specificity. (Bang et al. 2009) evaluated the sensitivity and utility of polymerase chain reaction (PCR) to detect *M. leprae* in comparison with other conventional methods for the diagnosis such as split skin smears, histopathology and serodiagnosis. They observed that the PCR detection of *M. leprae* targeting 16S ribosomal RNA was specific and more sensitive than the conventional methods, and could contribute to early and accurate diagnosis of leprosy. The present work standardized a PCR technique to amplify the single copy gene Lsr2 of *M. leprae*. This technique presented 100% of specificity for *M. leprae* relative to other mycobacteria, in agreement with previous reports (Santos et al. 2001; Guerrero et al. 2002; Torres et al. 2003; Willams et al. 2003; Braber 2004; Cortes-Herrera et al. 2008). Therefore, it seemed possible to use this gene as a target for large-scale screening of human reservoirs of *M. leprae,* Despite the small number of patients with multibacillary leprosy, the majority of the inhabitants of endemic areas showed the signs of exposure to *M. leprae*, which could be explained by the presence of subclinical bacilliferous infections in the community. (Guerrero et al. 2002) studied the use of a polymerase chain reaction (PCR) test to detect LSR/A15 gene that coded for the 15 kDa *M. leprae* antigen in the samples of nasal mucus from asymptomatic household contacts of patients with leprosy. Their finding demonstrated that this method could be used to monitor the high-risk populations and also for the elimination programs in the countries where the disease's prevalence has been significantly reduced. Recent advances in the molecular epidemiology of leprosy through the genotyping of variable number tandem repeats (VNTRs) and single nucleotide polymorphisms (SNPs) have been described. VNTRs with a broad range of diversity are useful genotyping tools for analyzing the transmission in community areas, and SNPs and VNTRs with a small degree of variation are favorable for investigating the global transmission of leprosy (Matsuoka 2009). The use of PCR for the early diagnosis to detect *M. leprae* in leprosy patients as well asymptomatic household contacts

of patients with leprosy with a high risk for developing or transmitting the disease would be of clinical and predictive value. While the prevalence of leprosy has declined around the world, there has not been a corresponding decrease in its incidence, thus indicating that it has not been possible to prevent the transmission of the disease.

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