Comparison between microsatellites and Ml MntH gene as targets to identify Mycobacterium leprae by PCR in leprosy *

Comparação entre microssatélites e o gene Ml MntH como alvos para a identificação do Mycobacterium leprae por PCR na hanseníase

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Abstract: BACKGROUND: The Polymerase Chain Reaction (PCR) technique has been frequently used in the molecular diagnosis of leprosy.
OBJECTIVES: To compare the results of PCR with four pairs of *Mycobacterium leprae* specific primers as well as to compare these results to multibacillary (MB) and paucibacillary (PB) leprosy according to the WHO operational classification.
METHOD: 28 DNA samples, collected from the frozen skin biopsies and biopsy imprints on filter paper of 23 patients (14 MB and PB 9), were examined for PCR using primers which amplify 131, 151 and 168bp of specific microsatellite regions and a 336 fragment of the *Ml MntH (ML2098)* gene.
RESULTS: *M.leprae* bacillus could be detected in 22 (78.6%) of the 28 samples. 9 (45%) of the 20 biopsy samples and 6 (75%) of the 8 imprints were positive to TTC. 7 (35.5%) skin biopsy specimens and 5 (62.5%) imprints were positive to AGT, and 11 (55%) biopsies and 4 (50%) were positive to AGT. 11 (55%) skin biopsies and 4 (50%) imprints were positive to AT. 8(38%) skin biopsies and 5 (62.5%) imprints were positive to the *Ml MntH gene*. In the MB group, the microsatellites detected the bacillus in 78.5% of the samples, and the *Ml MntH gene* in 57.1% of the samples, independent of the clinical material. In the PB group 55.5% of samples were positive to the microsatellite primers, while 22.2% were positive to the *Ml MntH gene*.
CONCLUSIONS: These results show that both the specific regions of microsatellites, as well as the *Ml MntH gene* fragment can be useful tools for detecting the M. leprae DNA by PCR in frozen skin biopsy samples and filter paper biopsy imprints.
Keywords: Leprosy; *Mycobacterium leprae*; Microsatellite nepeats, polymerase chain Reaction

Resumo: FUNDAMENTOS: PCR tem sido frequentemente utilizada no diagnóstico molecular da hanseníase.
OBJETIVOS: comparar os resultados da PCR com 4 pares de *primers* específicos para *Mycobacterium leprae*, bem como os resultados da PCR à classificação operacional, segundo a OMS, de multibacilar (MB) e paucibacilar (PB) da hanseníase.
MÉTODO: Vinte e oito amostras de DNA, extraído de biópsias congeladas de pele e de *imprint* de biópsias em papel de filtro de 23 pacientes (14 MB e 9 PB), foram utilizadas na PCR com *primers* que amplificam 131pb, 151pb e 168pb de regiões de microssatélites, e um fragmento de 336pb do gene *Ml MntH* (ML2098) do bacilo.
RESULTADOS: O bacilo pôde ser detectado em 22 (78,6%) das 28 amostras. Nove (45%) das 20 amostras de biópsia e 6 (75%) das 8 amostras de imprints foram positivas para TTC. Sete (35,5%) amostras de biópsias e 5 (62,5%) *imprints* foram positivos para AGT, e 11 (55%) biópsias e 4 (50%) *imprints* foram positivos para AT. Oito (38%) amostras de biópsias e 5 (62,5%) *imprints* foram positivos para o gene Ml *MntH*. Dentre o grupo MB, os microssatélites detectaram o bacilo em 78,5% das amostras, e o gene *Ml MntH*, em 57,1% das amostras, independentemente do material clínico. No grupo PB, 55,5% das amostras foram positivas para os microssatélites, enquanto que 22,2% o foram para o gene *Ml MntH*.
CONCLUSÕES: Estes resultados mostram que, tanto as regiões específicas de microssatélites quanto o gene *Ml MntH*, podem representar ferramentas úteis na detecção do *Ml MntH* por PCR em amostras de biópsias e imprint de biópsias.
Palavras-chave: Hanseníase; *Mycobacterium leprae*; Reação em cadeia da polimerase; Repetições de microssatélites

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INTRODUCTION

Mycobacterium leprae is a facultative intracellular acid-fast bacillus (AFB) which causes leprosy, a disease that primarily affects the peripheral nerves and skin. Although leprosy is controlled effectively by multidrug therapy (MDT), new cases have been detected in areas of the world where it is still endemic. [1]Because the bacillus does not grow in artificial media, identification in clinical samples has been problematic. [2] PCR, a sensitive and specific technique for the identification of *M. leprae*, has proven to be extremely important for diagnosing leprosy, mainly of the paucibacillary (PB) type, and for identifying high-risk contacts. [3,4] Target sequences for amplification of DNA from *M. leprae,* such as genes encoding proteins of 65, 36 and 18kDa, and repetitive sequences, have been widely used for the etiological diagnosis of leprosy, and have often proved to be more sensitive and specific than the routinely-used bacilloscopic examination. [3]

In our study, four pairs of specific primers for the identification of *M. leprae,* with three specific primers for accessing microsatellite regions of the bacillus, and one specific to an internal sequence of the ions transporter *Ml MntH* gene were used in samples taken from patients with leprosy. [4] In this way we set out to determine the results of the PCR with four pairs of primers for identifying the bacillus in samples of skin biopsies and biopsy imprints, and also to compare these results to multibacillary (MB) and paucibacillary (PB) leprosy according to the WHO operational classification.

MATERIALS AND METHODS

Samples

We used 28 samples collected from 23 leprosy patients attending the dermatology outpatients clinic of the Hospital das Clinicas, Ribeirão Preto Faculty of Medicine, University of São Paulo (HC-FMRP-USP) from 2002 to 2009. 20 samples of skin biopsies were taken with a 4mm punch and frozen at -80^{0}C. 8 imprint samples of the dermal portion of the biopsy were collected on filter paper at the same time as the skin biopsies and stored at 4^{0}C. 14 of the 23 patients belonged to the multibacillary (MB) group, with positive bacilloscopes in lymph samples from the ears, knees and elbows, and in the skin biopsy (11 samples from skin biopsies and 6 imprints), while 9 in the PB group (9 biopsies and 2 imprints 2), had negative sputum smears. With the exception of two biopsy imprint samples, which were collected at the start of MDT for leprosy, the other samples were obtained from treatment naive patients. The study was approved by the HC-FMRP-USP Ethics Committee (Case No. 2609/2006). Samples collected prior to 2006 belong to the Samples Bank of the Multiuse Molecular Biology Laboratory, Department of Clinical Medicine, FMRP-USP, approved by the CEP (registration number 3605/2006).

Extraction of DNA from skin biopsies and biopsy imprints

For DNA extraction, the skin sample was digested with 1.0 mL of 1X digestion buffer [0.1 M Tris pH 8.0, 0.1 M EDTA, 1% SDS, 20 mg / ml proteinase K in PK buffer] at 55^{0}C, stirred overnight. The following day the PK was inactivated at 95°C for 10 minutes and a 1:1 mixture of phenol-chloroform was added to the lysate (inverting the tube gently) to a final volume of 1.0 mL. Centrifuged at 14,000 rpm for 2 minutes at room temperature, the supernatant was then transferred to a new tube. 500µL of chloroform was next added, gently inverting the tube, and the sample was centrifuged at 14,000 rpm for 2 minutes at room temperature. The supernatant was transferred carefully to a new tube to prevent aspiration of the white phase separation (proteins) and 1.0 mL of ice cold absolute ethanol was added, the tube gently inverted and brought to -20°C for 1 hour. The material was then centrifuged at 14,000 rpm for 30 minutes at 4 ° C and the supernatant discarded by inversion over filter paper. The sediment was washed in 500µL of ice cold 70% ethanol and the sample again centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the DNA dried by vacuum centrifugation for approximately 15 minutes. The DNA was then redissolved in 50µL of sterile deionized water (H_2Odd).

The imprint samples were cut aseptically from the filter paper, soaked in 50ml of H_2Odd, incubated at 95° C in a dry bath and stirred for 15 minutes. The supernatant obtained was used directly in the PCR.

PCR

The sequences of the primers used in this study are listed in Table 1. The reagent solutions for the PCR included: 0.25mM of dNTP, 1U of Taq DNA polymerase (Invitrogen™), 2.0mM of $MgCl_2$, 1X PCR buffer, 40µM of each primer (Invitrogen ™) and 0.5 g of DNA sample, producing a final volume of 25µL.

For the identification of *M. leprae*

To identify the *M. leprae* bacillus in the clinical samples collected from patients we used three pairs of specific primers, encoding regions of the microsatellites TTC, AGT and AT (Young *et al*., 2004) [4], and a pair of primers that amplify an internal sequence of the manganese transporter *Ml MntH* of the bacillus. [4] The cycle used for the microsatellite was "Hot Start"

Tabela 1: Genomic location of microsatellites and the gene MntH and the respective primers used in the PCR for identification of the *M. leprae* bacillus in skin biopsy samples and biopsy imprints of leprosy patients

Repeat Motif	Location in the genome	Flanking Genes	Primer	Sequence	Fragment (bp)
TTC	2785432-2785494	ML2345 (2786390-2786807) (pseudo)[a]	TTC-F2 TTC-R2	CGTTGGGTTCGATCGAATCGA GCACGCCGACGGGAATAAGT	131
AGT	2583816-2583839	ML2173 (2584381-2584584) (pseudo)	AGT-F2 AGT-R2	ATCAACGCTGCGGTTTCGCAG ATATGCATGCCGGTGGTGTGCT	151
AT	948935-948964	ML0798 (948302-946524) (hip)[b]	AT-F1 AT-R1	CAATATGCGGGTTGGCGCTTCTG CCGTCTGGCTCGATGGCTGGATTC	168
Ml *MntH*	ML2098	--	*mntH*1 *mntH*2	CGGCTTCACGTCCAGTTTCTTC TAAGTGCCCTCGATGTAAGCGG	336

[a] Pseudo = pseudogene [b] Hip = hypothetical protein gene product. [c] The microsatellites ATC and AGT are synonymous with loci AGA and GTA respectively, both studied in strains of the bacillus in armadillos.

PCR. After initial DNA denaturation at 95°C for 10 minutes the reaction proceeded with 43 cycles consisting of: denaturation at 95°C for 10 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds, followed by final extension at 72°C for 2 minutes.

For amplification of the *Ml MntH* gene a pair of primers specific to the gene sequence of 336bp of the *Ml MntH* gene of the *M. leprae* bacillus was designed for us. Note that this pair of primers does not amplify gene sequences of tissue or human blood since they are specific (GenBank AL583924.1; gi 13093618 consulted). With the aid of the Oligo Explorer, Oligo Analyser 1.1.0 (Copyright (C) 2000-2002, Teemu Kuulasmaa) and Gene Runner 3.05 (Hastings Software, Inc. Copyright (C),1994), the 1 *MntH1* forward primers (5'-3') CGGCTTCACGTCCAGTTTCTTC and reverse *MntH2* primers (5'-3') TAAGTGCCCTCGATGTAAGCGG were designed from the complete sequence of the *Ml MntH* gene (1281 bp), obtained from the Genbank (NC_002677). The annealing temperature used was 60°C (94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 60° C for 45 seconds, 72°C for 1 minute, followed by 7 minutes at 94°C).

We used a *Mycobacterium tuberculosis* culture sample to control the specificity of the reaction. For the negative control, DNA was excluded from the reaction.

Statistical analysis

The comparison between the groups of the results of the PCRs for the four pairs of primers was carried out by *Kappa* test. This comparative analysis was done online using the Kappa Calculator [Columbia University, USA], available at: http://people.dbmi.columbia.edu/homepages/chuangj/kappa/calculator.htm).

RESULTS

The results of the PCR performed with DNA extracted from biopsy samples and biopsy imprint with primers for microsatellites and the *Ml MntH* gene are shown in Figures 1, 2 and 3.

The *M. leprae* bacillus could be detected in 22 (78.6%) of the 28 samples tested, and 21 (75%) could be detected by all or any of the three microsatellites, and 13 (46.42%) by the primers that amplify the *Ml MntH* gene of the *M. leprae* bacillus biological material or belonging group. Of the 20 skin biopsy samples, 9 (45%) were positive for the microsatellite TTC (8 belonging to the MB group and 1 to the PB group), 7 (35%) for the AGT microsatellite (5 samples from the MB group and 2 from the PB group) and 11 (55%) for the AT microsatellite (6 samples from the MB group and 5 from the PB group). Regarding the samples of biopsy imprints, of the 8 samples processed 6 (75%) were positive for the TTC microsatellite, 5 (62.5%) for AGT and 4 (50%) for AT. All the positive samples belonged to the MB group (see Table 2).

Regarding the primers to amplify the gene fragment *Ml MntH,* of the 20 biopsy samples 10 (50%) were PCR-positive for the gene, with 3 belonging to

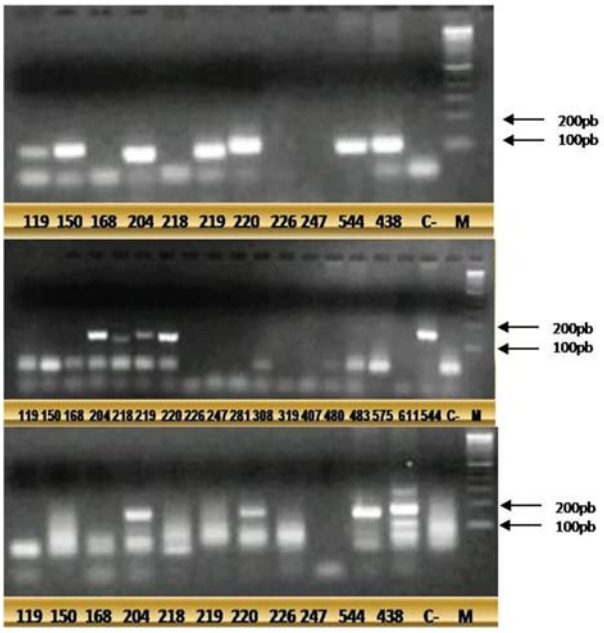

Figure 1: Agarose gel electrophoresis 2.0% for identification of the *M. leprae* bacillus in skin samples with the primer pairs for TTC, AGT and AT microsatellites. Amplification resulted in fragments of 131, 151 and 168bp, respectively. M: 100bp molecular marker. The difference between the sizes of the amplified bands is due to the difference in the annealing of the microsatellite in question

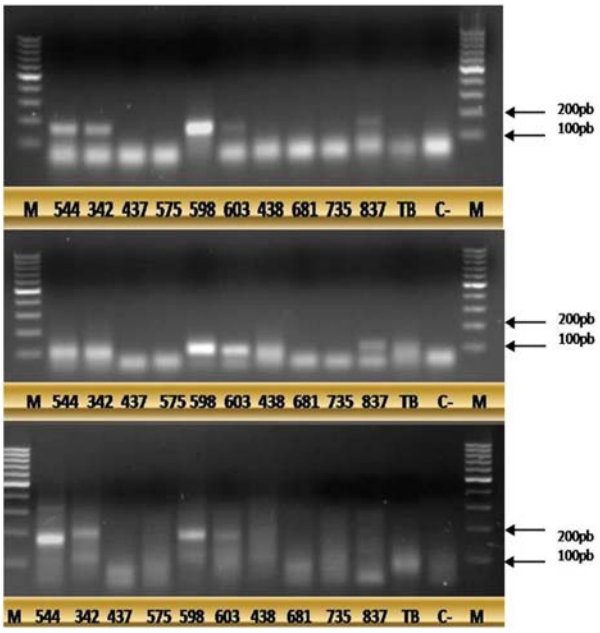

Figure 2: Agarose gel electrophoresis 2.0% for identification of the *M. leprae* bacillus in biopsy imprint samples with the primer pairs for TTC, AGT and AT microsatellites T. Amplification resulted in fragments of 131, 151 and 168bp, respectively. M: 100bp molecular marker. The difference between the sizes of the amplified bands is due to the difference in the annealing of the microsatellite in question

the PB group. 5 (62.5%) of the 8 imprints of biopsy samples were positive for the gene *Ml MntH*. No biopsy imprint sample of group PB was positive in the research of the gene *MntH* (Table 3).

The comparative analysis of the PCRs with primers amplifying microsatellite and the gene *Ml MntH* was concordant in 14 (50%) samples assayed (K=0.428, P <0.001; $CI_{95\%}$ = 0.277 ≤ K ≤ 0.529).

As for the MB group, the performance of the AGT and *MntH* primers was similar in both in the skin biopsy specimens and in those of the biopsy imprint. The best detection for the MB group in the biopsies and biopsy imprints was obtained with the use of primers that amplify the microsatellite TTC (72.7% and 100%, respectively). PCR with primers that amplify the gene *Ml MntH* was positive in 45.4% of biopsy specimens and in 5 (83.3%) of the 6 imprint samples tested. The test was reasonably consistent for all primer pairs tested in the skin biopsies (36.4%) (K = 0.290; P = 0.019; $CI_{95\%}$ = 0.049 ≤ K ≤ 0.531), and in 66.7% of the imprint samples (K = 0.411; P = 0.014; $CI_{95\%}$ = 0.084 ≤ K ≤ 0.737).

In the PB group, the best performance results were obtained from the primers that amplify the AT microsatellite (44%). The PCR with the pair of primers that amplify the gene *Ml MntH* was positive in 2 (22%) samples, matching the sensitivity of the primer AGT. The comparison of PCR results with the primers that amplify microsatellite regions and primers that amplify the gene *Ml MntH* was fairly consistent (22%), according to the classification of Landis and Koch (1997) (K=0.16; P= 0.238, $CI_{95\%}$ = -0.106 ≤ K ≤ 0.437).

Table 2: Results of the PCR survey of microsatellites of the *M. leprae* bacillus in skin biopsies and biopsy imprints according to the multi- and paucibacillary leprosy groups

	Multibacillary						Paucibacillary					
Grupos	**BIOPSY**			**IMPRINT**			**BIOPSY**			**IMPRINT**		
PCR	TTC(%)	AGT(%)	AT(%)	TTC(%)	AGT(%)	AT(%)	TTC(%)	AGT(%)	AT(%)	TTC(%)	AGT(%)	AT(%)
Positive	8 (72.7)	5 (45.4)	7 (63.6)	6 (100)	5 (83.3)	4 (66.7)	1 (11.1)	2 (22.2)	4 (44.4)	0 (0)	0 (0)	0 (0)
Negative	3 (27.3)	6 (54.6)	4 (36.4)	0 (0)	1 (16.7)	2 (33.3)	8 (88.9)	7 (77.8)	5 (55.6)	2 (100)	2 (100)	2 (100)
TOTAL	11	11	11	6	6	6	9	9	9	2	2	2

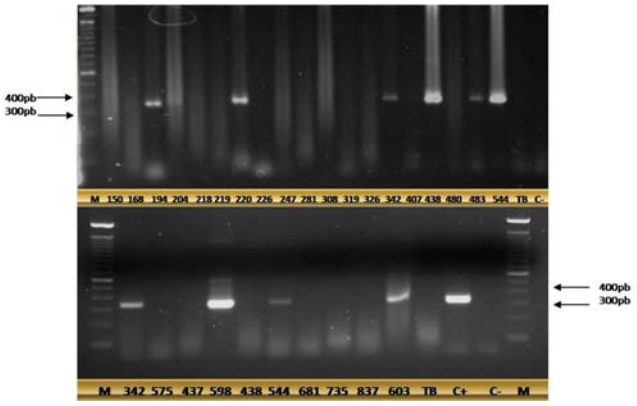

Figure 3: Agarose gel electrophoresis 2.0% to identify the gene *Ml MntH* in biopsy specimens and biopsy imprints. Amplification resulted in a 336bp fragment. Sample 544 was used as positive control in this reaction. C: reaction with exclusion of DNA; C +: plasmid pBADp16LC containing the gene *MntH* . TB: culture of *M. tuberculosis*, M: molecular marker 100bp

DISCUSSION

No specific and sensitive test is available to date for diagnosing asymptomatic leprosy or to predict the progression of leprosy among exposed individuals. [5] There is an urgent need therefore to find molecular tools not only to differentiate between the mycobacteria but also to identify the *M. leprae* bacillus in view of the high detection rates of new cases. [1]

The purpose of this study was to evaluate the PCR method to detect the *M. leprae* bacillus in clinical samples of skin biopsy and biopsy *imprints*. The results of PCR, using specific primers for the microsatellites TTC and AT in samples from the MB group showed a better performance when compared to the *Ml MntH* gene. The latter,in turn, displayed a similar performance to the AGT primers in this group. Despite there being no significant difference in the statistical correlation between the PCRs of the MB group, 9 of the 11 samples from skin biopsies were positive for 1 or 3 microsatellites, while the pair of primers that amplify the gene *Ml MntH* was able to detect the presence of bacilli in 5 of the 11 samples tested, one of which was not identified by the primer TTC. Contrary to what occurred in the biopsy specimens, the pair of primers of the *Ml MntH* gene was able to amplify 5 of 6 biopsy imprint samples, which were positive for the pair of primers TTC, showing better performance than the pair of primers AT, which amplified only 4 of the samples tested.

The diagnosis of leprosy is currently based on clinical examination findings and the detection of AFB (bacilloscopy) in smears obtained from leprosy lesions and other sites selected for collection such as ear lobes, elbows and knees. These results are expressed in bacterial index, which is nowadays considered to be the most accurate, fast and usual quantitative assessment method, for reading of leprosy smears (Ministry of Health/SPS, Brazil). In order to apply the PCR technique for diagnosing leprosy in clinical terms, a careful comparison needs to be made between the results of this test with those obtained by both bacilloscopy of the lymph as well as of the skin biopsy. [6]

The diagnostic primers tested in this study were previously used for epidemiological mapping of the *M. leprae* bacillus in *inter alia* India, Indonesia and the Philippines. [4,7,8] However, these primers have not been routinely used given the difficulty of obtaining samples of genomic DNA from clinical material. [1]

On the other hand the use of repetitive sequences as targets of DNA in the PCR enhances the sensitivity of the test, since these sequences are found in several sites of genomic DNA. [2]

In summary, the PCR with primers for microsatellites diagnosed bacilli in 18 of 23 (78.3%) patients, whereas the PCR with primers for the gene *Ml MntH* was able to diagnose 10 out of 23 (43%) patients. Among the patients in the MB group, the microsatellites detected the bacillus in 11 out of 14 samples (78.5%), and the gene *Ml MntH* detected the bacillus in 8 out of 14 (57.1% of the samples, independent of the clinical material. The positivity of the PCR for the microsatellites in the biopsy specimens

Tabela 3: Results of the PCR to study gene *Ml MntH* of the bacillus M. leprae in skin biopsies and biopsy imprint according to the multi-and paucibacillary leprosy groups (MB and PB)

	Groups			
	Multibacillary		Paucibacillary	
PCR	BIOPSY (%)	IMPRINT (%)	BIOPSY (%)	IMPRINT (%)
Positive	5 (45,4)	5 (83,3)	2 (22,2)	0 (0)
Negative	6 (54,6)	1 (16,7)	7 (77,8)	2 (100)
TOTAL (n)	11	6	9	2

collected from the MB group is similar to that reported for targets such as the gene *pra* or 16S rRNA, which showed positive values of around 70-80%. However, it was higher than (microsatellites) or equal to (*Ml MntH* gene*)* some studies using RLEP repetitive sequences as a target for diagnosis, which showed 54-74% of positivity in the tests. [2, 6,.9-14]

In the PB group 5 out of 9 samples (55.5%) were positive for microsatellites while 2 out of 9 (22.2%) were positive for the *Ml MntH* gene. These biopsy samples had higher positivity compared to the literature. [9, 15, 16]

With regard to the biopsy imprint samples from the MB group, the PCR with primers for microsatellites was able to identify the bacillus in 66.7-100% of the specimens, but was not positive for 2 samples from the PB group. These data were higher than those reported for PCR results in lymph samples. [2, 9, 10, 15, 17]

CONCLUSIONS

This study shows that the primers for microsatellite sequences are more effective for the amplification of DNA samples from skin biopsy than the primers for the gene *Ml MntH* in the MB group. However, the results were similar with respect to the PB group. These findings suggest that both associated targets could become useful tools for identifying, in clinical samples such as biopsies and frozen biopsy imprints, the bacillus which causes leprosy. ❑

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