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Use of the polymerase chain reaction to detect *Mycobacterium leprae* in urine

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Abstract

Leprosy is an infectious disease caused by *Mycobacterium leprae.* The polymerase chain reaction (PCR) has been applied to detect *M. leprae* in different clinical samples and urine seems to be attractive for this purpose. PCR was used to improve the sensitivity for diagnosing leprosy by amplifying a 151-bp PCR fragment of the *M. leprae pra* gene (PCR-Pra) in urine samples. Seventy-three leprosy patients (39 males and 34 females, 14 to 78 years old) were selected for leprosy diagnosis at a reference laboratory in Maringá, PR, Brazil. Of these, 36 were under anti-leprosy multidrug therapy with dapsone and rifampicin for tuberculoid (TT) and dapsone, rifampicin and clofazimine for borderline (BB) and lepromatous (LL) forms. The control group contained 50 healthy individuals without any clinical history of leprosy. DNA isolated from leprosy patients' urine samples was successfully amplified by PCR-Pra in 46.6% (34/73) of the cases. The positivity of PCR-Pra for patients with the TT form was 75% for both patients under treatment and non-treated patients ($P = 0.1306$). In patients with the LL form, PCR-Pra positivity was 52 and 30% for patients under treatment and non-treated patients, respectively ($P = 0.2386$). PCR-Pra showed a statistically significant difference in detecting *M. leprae* between the TT and LL forms of leprosy in patients under treatment ($P = 0.0033$). Although the current study showed that the proposed PCR-Pra has some limitations in the detection of *M. leprae*, this method has the potential to be a useful tool for leprosy diagnosis mainly in TT leprosy where the AFB slit-skin smear is always negative.

Key words: PCR; Leprosy; *Mycobacterium leprae*; Urine; TT leprosy; LL leprosy

Introduction

The polymerase chain reaction (PCR) has been applied to detect *Mycobacterium leprae* in different clinical samples, such as slit-skin smear (1), blood (2), nasal cavity (3,4), skin tissues (2,5), and urine (6) for the improvement of the laboratory diagnosis of leprosy. PCR targets are genes that encode protein antigens of 36 kDa, known as proline-rich antigen (*pra*) (2,6,7), of 18 kDa (8), 85 kDa (5), 65 kDa (9), 16S rRNA (1), and repetitive sequences (10) to detect *M. leprae.*

Some studies have reported the presence of mycobacterial DNA in urine (6,11), a fact that makes this clinical specimen attractive for the detection of *M. leprae* DNA. This is due to the fact that urine samples are easily collected and enhance patient adherence when compared to the distressing and traumatic slit-skin smear.

Parkash et al. (6) detected *M. leprae* DNA in the urine of leprosy patients when they used primers proposed by Hartskeerl et al. (7), which amplified a large fragment of DNA (530 bp) of the *pra* encoding gene, even though the sample studied had a limited number of individuals. As small fragments of bacterial DNA may be found in patient urine samples (10), the use of this kind of clinical samples and primer set to detect a smaller fragment of *M. leprae* should be better evaluated since the amplification of a smaller DNA fragment may improve the sensitivity of the PCR assay, as pointed out by Goulart et al. (12).

As the structure of the primers can be critical for the PCR assay, in the current study we evaluated a new primer-PCR for the detection of the *M. leprae pra* gene (PCR-Pra) in urine samples.

Material and Methods

Study population

Seventy-three patients from northwestern Paraná State with a clinical diagnosis of leprosy were selected at

Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC), Universidade Estadual de Maringá (UEM), PR, Brazil, from June 2006 to June 2007. Individuals with a clinical and laboratory diagnosis by microscopy (Ziehl-Gabett) were included. Individuals with kidney or vascular diseases were excluded. Patients were classified by physicians using clinical signs and bacteriological criteria and assigned to the following leprosy forms according to Ridley and Jopling (13): tuberculoid (TT, N = 12), borderline-borderline (BB, N = 14), lepromatous (LL, N = 45), and indeterminate (II, N = 2). Thirty-two patients were under anti-leprosy therapy with dapsone, rifampicin and clofazimine for multibacillary forms (slit-skin smear-positive BB and LL patients) and 4 were treated with dapsone and rifampicin for paucibacillary forms (slit-skin smear-negative TT and II patients). The variables age, gender, and family history of non-treated leprosy and anti-leprosy therapy were analyzed.

A control group consisted of 50 healthy individuals without any clinical history of leprosy and without any cases of the disease in their families.

The study was approved by the Ethics Committee of Universidade Estadual de Maringá (protocol No. 131/2006). All participants approved the research protocol and gave informed written consent.

DNA extraction from urine samples

Two first-emission urine samples (approximately 12 mL each) were collected from all participants on consecutive days, homogenized and placed in a boiling water bath for 10 min and then centrifuged at 12,000 g for 20 min. DNA extraction was carried out as described by Sechi et al. (11).

PCR-Pra

The primers Pra1 (5'-ATCCGCTCACTGCCTAAGGA-3') and Pra2 (5'-TGCGTGATATCAGTGACGGAC-3') were designed to amplify a 151-bp segment of the *M. leprae pra* gene (GenBank accession No. X65546.1). The sequences were aligned using the Prime Premier® V. 5.0 software (Premier Biosoft International, USA). PCR-Pra assays were performed using 5 µL template in 20 µL of a reaction mixture containing 200 nM primers (Integrated DNA Technologies, USA) and PCR Master Mix (Promega Corporation, USA) according to manufacturer instructions. The amplification was carried out in an Eppendorf thermocycler (Mastercycler® gradient PCR, Germany) using an initial cycle of 5 min at 95°C, 35 cycles of 60 s at 95°C, 60 s at 54°C, 60 s at 72°C, and a final extension of 10 min at 72°C. PCR products were separated by 2% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer for 1 h at 100 V and the gels were stained with ethidium bromide. A 100-bp DNA Ladder (Invitrogen Life Technologies, Brazil) was used as a molecular marker. Positive and negative controls were included in all amplifications. Five microliters of DNA extracted (0.15 µg/mL) from an armadillo's skin lesion was added to the PCR reagents for the positive controls, and

5 µL TBE was added to the PCR reagents to serve as the negative controls.

Specificity and sensitivity of the PCR

The specificity of the PCR-Pra for the detection of *M. leprae* in urine was evaluated using DNA extracted from *M. leprae* obtained from a skin lesion of an armadillo experimentally infected and kindly supplied by the animal laboratory of Institute Lauro de Souza Lima (São Paulo, Brazil). Reference strains of *M. tuberculosis* H37Rv (ATCC 27.294), *M. bovis* AN5, *M. gordonae* (Lacen/Pr), *M. avium* (ATCC 13.950), *M. kansasii* (Lacen/Pr), *M. fortuitum* (Lacen/Pr), *M. szulgai* (Lacen/Pr), *M. flavescens* (Lacen/Pr), and *M. smegmatis* (Lacen/Pr) were also used. The DNA was extracted using a phenol-chloroform method (14). Sensitivity was determined using total DNA (*M. leprae*) obtained from a skin lesion of an armadillo (90 µg/mL) and diluted 1:100 (0.90 µg/mL), 1:300 (0.30 µg/mL), 1:600 (0.15 µg/mL), and 1:1200 (0.075 µg/mL). The detection of PCR inhibitors in urine samples was carried out by the addition of 5 µL DNA extracted (0.15 µg/mL) from a skin lesion of an armadillo to a 20-µL aliquot of each DNA extracted from urine samples.

Data analysis

Clinical samples were considered to be positive for the presence of *M. leprae* DNA when a single band of PCR product (151 bp) was observed and considered to be negative by the absence of amplification after the observation of PCR controls. In the absence of amplification of the positive controls, samples and their respective controls were amplified using diluted (1:2) DNA extracted from urine samples.

Data were analyzed with non-parametric tests for proportions, McNemar test, chi-square test, and G test, using BioEstat 5.0 (Federal University of Pará, Brazil), with the level of significance set at $P < 0.05$.

Results

The mean age of patients with leprosy was 46 years (range: 14-78) and a higher prevalence was observed in women (51.4%) and men (58.3%) among the non-treated patients and patients under treatment, respectively. A higher proportion of patients (41.7%) with a family history of leprosy was observed among patients under treatment. The highest positivity of PCR-Pra was observed in male patients aged 31 to 60 years and in patients with a family history of leprosy. However, no significant difference was observed between patients under treatment and non-treated patients considering gender, age, family history of leprosy, and positivity of PCR-Pra ($P > 0.005$; Table 1).

PCR-Pra was specific for the detection of *M. leprae* and detected DNA up to 1:600 (0.15 µg/mL) dilution. No amplification was observed in DNA from *M. tuberculosis*, *M.*

gordonae, *M. avium*, *M. kansasii*, *M. fortuitum*, *M. szulgai*, *M. flavescens*, *M. smegmatis*, and *M. bovis*.

DNA isolated from urine samples was also successfully amplified by PCR-Pra, which was positive in 46.6% (34/73) of all patients studied. No significant difference in PCR positivity was observed between patients under treatment and non-treated patients with the TT (P = 0.1306) and LL (P = 0.2386) forms. The positivity of PCR-Pra was higher in patients under treatment with the TT form (75%) than in patients with the LL form (52%) (P = 0.0033). No significant difference in *M. leprae* detection was observed between patients with the TT and LL forms in the diagnostic phase of leprosy (P = 0.2889) (Table 2). Initial PCR-Pra inhibition in non-treated leprosy patients was observed in 24.3% (9/37) of the total samples studied. The diluted DNA extracts (1:2) showed PCR inhibitors in 13.5% (5/37) of samples.

No amplification was observed in the healthy control group (Table 2).

Discussion

An epidemiological study (15) indicated that adult males and people with a family history of leprosy are more vulnerable to leprosy. In the current study, the PCR-Pra positivity was consistent with the literature, with a higher positivity among males aged 31 to 60 years with a family history of leprosy.

Variation in *M. leprae* PCR positivity has been mainly related to different primers used (5), amplified fragment size (12), amplification techniques (16), and clinical specimens (2,6). The PCR-Pra intended to detect *M. leprae* in urine samples by amplifying a 151-bp DNA fragment of the *pra* gene, which was smaller than that described by Hartskeerl et al. (7) and other proposed PCR for improving PCR assay sensitivity.

PCR-Pra showed specific detection of *M.*

leprae among the other mycobacteria tested and showed overall higher sensitivity (46.6%) in detecting *M. leprae* in urine than that obtained by Parkash et al. (6) targeting the *pra* gene (37.5%) and Caleffi et al. (17) targeting 85 A-C intergenic region (4.10%) in previous studies, which amplified a 530- and a 250-bp DNA fragment, respectively. This result suggests that the amplification of a smaller DNA fragment (151 bp) by PCR-Pra may improve the performance of PCR-based methods for detecting *M. leprae* in urine samples, as pointed out by Goulart et al. (12). It is important to emphasize that small fragments of bacterial DNA may be found in urine samples as a result of DNA damage by antimicrobial therapy and/or by its processing and excretion (10,18).

Table 1. Distribution of non-treated leprosy patients and leprosy patients under treatment regarding gender, age, family history of leprosy, and PCR-Pra results.

	No. of non-treated patients (%)	No. of patients under treatment (%)	Positive PCR-Pra (%)
Gender			
Female	19 (51.4)	15 (41.7)	15 (44.1)
Male	18 (48.6)	21 (58.3)	19 (48.7)
Age (years)			
5-17	1 (2.7)	0 (0.0)	0 (0.0)
18-30	2 (5.4)	7 (19.4)	3 (33.3)
31-45	10 (27.0)	8 (22.2)	9 (50.0)
46-60	18 (48.6)	17 (47.2)	19 (54.3)
61-78	6 (16.2)	4 (11.1)	3 (30.0)
Total	37 (100)		14 (37.8)
Total		36 (100)	20 (55.5)
Family history of leprosy			
Yes	7 (18.9)	15 (41.7)	14 (63.6)
No	30 (81.1)	21 (58.3)	20 (39.2)

PCR-Pra = positivity *vs* gender, family history of leprosy (P > 0.05; chi-square test). PCR-Pra positivity *vs* age (P > 0.05; G test).

Table 2. Performance of PCR-Pra for the detection of *Mycobacterium leprae* in urine according to the clinical form of non-treated patients and patients under treatment.

	PCR positivity (%)										Total	Control group
	Non-treated patients					Patients under treatment						
	II	TT	BB	LL	Total	II	TT	BB	LL	Total		Total
PCR-Pra	(0/2)	75.0 (6/8)	28.6 (2/7)	30.0 (6/20)	37.8 (14/37)	(0/0)	75.0 (3/4)	57.1 (4/7)	52.0 (13/25)	55.5 (20/36)	46.6 (34/73)	(0/50)

The number of individuals is given in parentheses. II = indeterminate leprosy; TT = tuberculoid leprosy; BB = borderline leprosy; LL = lepromatous leprosy. P = 0.1306 (McNemar test), PCR results *vs* patients with the TT form. P = 0.2386 (McNemar test), PCR results *vs* patients with the LL form. P = 0.2889 (McNemar test), PCR results *vs* non-treated patients with the TT form and the LL form. P = 0.0033 (McNemar test), PCR results *vs* patients with the TT and LL forms under treatment.

Parkash et al. (6) observed high PCR positivity in patients under anti-leprosy treatment (66.7%) compared to non-treated patients (20.0%). In the present study, in which PCR-Pra was applied to a larger number of patients, the difference between these groups of patients was not statistically significant.

In the case of patients under treatment, PCR-Pra showed a significant difference between the TT and LL forms of leprosy (P = 0.0033). This result may be associated with the action of the antimicrobial, cell immune response, increased excretion of dead bacilli by the kidneys in the TT form of leprosy, and a higher sensitivity of the PCR-Pra by amplification of a smaller DNA fragment (12). Since the cell immune response, predominant in the TT form, is more efficient in combating the bacilli than the humoral immune response, predominant in the LL form, the excretion of a greater amount of free DNA by the kidneys would be expected to occur in the TT form as reported by Parkash et al. (6) and in the previous study targeting the 85 A-C intergenic region using the same clinical samples from non-treated patients (diagnostic phase) (16). However, PCR-Pra positivity showed no statistically significant difference among patients with the TT and LL forms in the diagnostic phase (P = 0.2889).

PCR-Pra proved to be highly useful to confirm the diagnosis of leprosy, especially in the TT form (75% PCR-Pra positivity in patients under treatment and in non-treated patients), where microscopic detection of AFB in slit-skin smears is normally negative owing to the low number of bacilli present in clinical samples (19).

In the current study, certain interfering elements were present in the urine samples, which resulted in PCR inhibi-tion. Urine normally contains substances such as insoluble amorphous salts, nitrate, urea, proteins, blood, and even high ratios of human genomic DNA, which can inhibit PCR (5). Thus, the effect of PCR inhibitors in urine may have been reduced when the DNA samples were diluted. A significant reduction (10.8%) in PCR inhibition rate was consequently observed in patients in the diagnostic phase. Since the dilution of *M. leprae* DNA probably occurred simultaneously, detection of the bacillus turned out to be difficult in some cases.

It is noteworthy that the nucleic acids in urine or other clinical specimens detected by PCR do not necessarily indicate the presence of viable *M. leprae* in the human organism (20). PCR results are slow to turn negative after the start of antimicrobial therapy. This is due to the presence of DNA fragments, which remain in circulation for several weeks after death of the bacilli (11). The time period during which *M. leprae* DNA may be excreted in urine is unknown since the bacilli have already been reported to last up to 8 years after the completion of anti-leprosy therapy (3).

PCR-Pra showed some potential as a useful assay for the detection of *M. leprae* DNA in urine samples, with a further asset for the diagnosis of leprosy mainly in the TT form or in inconclusive cases where the AFB slit-skin smear is always negative. PCR-Pra should be evaluated in an extended number of patients from endemic and non-endemic regions to address limitations such as performing a multiplex-PCR in urine with an internal control.

Acknowledgments

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