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Evaluation of Polymerase Chain Reaction (PCR) and Determination of Tuberculostearic Acid for Detection of Mycobacterium leprae in Urine and Cereprospinal fluid

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Introduction

Worldwide, more than 10 million people suffer from leprosy (3). The majority of these cases belongs to the group of paucibacillary leprosy that presents diagnostic difficulties (4). Currently, diagnosis is usually made on the basis of clinical examination and thus at a later stage of the disease when extensive and irreversible damage has already occurred. Until now, culture of the causative agent has not been possible; animal experiment attempts using armadillos or mouse foot pads have proven difficult and demanding (7). Direct determination of the agent using Ziehl-Neelsen staining is a standard procedure for multibacillary types of leprosy but succeeds only very rarely for paucibacillary types. This is why alternative methods are being sought. High expectations for specificity, sensitivity and practicability are being placed on PCR. Results from clinical biopsy material seem promising (17). Nasal swabs are easily obtainable but in endemic areas many individuals may carry *Mycobacterium (M.) leprae* in their nasal cavities without having obvious symptoms of leprosy (15).

The determination of tuberculostearic acid has become important as a rapid test for the diagnosis of tuberculosis (6). TBSA (R-10-methyl-octa-decanoic-acid) is a structural component of mycobacteria and members of the genus *Actinomyces.* It is usually not present in human tissue. In the present study the determination of *M. leprae* through tuberculostearic acid detection and PCR was evaluated and compared with the Ziehl-Neelsen staining using artificially prepared samples. Because of the above-mentioned diagnostic difficulties it still remains unclear to what extent leprosy affects the genito-urinary and central nervous systems (1, 2, 10, 12, 14). A sensitive method for detection of *M. leprae* in cereprospinal fluids (CSF) and urine samples should help to answer these questions.

Materials and methods

Materials

M. leprae was kindly provided by the IMMLEP *M. leprae*-bank (London, UK). *M. tuberculosis* (H37Rv) was obtained from the Bundesstaatliche bakteriologisch-serologische Untersuchungsanstalt (Innsbruck, Austria).

Preparation of samples

Sterile cerebrospinal fluid (CSF) of 22 patients with suspected herniated disks, who had undergone lumbar myelography, was pooled for specimen "CSF I". CSF of three patients suffering from pneumococcal meningitis and artificial CSF (lot 118, 217, Braun Austria, Maria Enzers-

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dorf, Austria) were pooled to create specimen "CSF II". Sterile urine form healthy adults was pooled to form specimens "urine I" and "urine II". All specimens were sterile-filtered using 0.45 μm pore size disposable filters (NALGE, Herford, UK). 2 mg freeze dried *M. leprae* containing approximately 2×10^{10} organisms were reconstituted in 20 ml 0.1% TWEEN 80 (SERVA, Heidelberg, Germany). CSF and urine specimens were divided into 2 ml aliquots which were spiked with *M. leprae* in various concentrations (10^4, 10^3, 10^2 and 10^1 organisms/ml, respectively). Bland samples were used as negative controls. The study protocol was approved by the Institutional Review Board at the University Hospital in Innsbruck (Austria).

Detection of tuberculostearic acid (TBSA)

The examination of TBSA was done according to the method described by FRENCH et al. (6) using gas chromatography resp. mass spectrography and selected ion monitoring.

Detection of *M. leprae* by Polymerase Chain Reaction (PCR)

Urine specimens were centrifuged at 12,000 g for 15 minutes, the supernatants discarded and the pellets reconstituted with 500 μl phosphate buffered saline (PBS). The pellets were again washed by centrifugation and reconstituted in 400 μl PBS. 100 μl of proteinase K digestion buffer (125 mM NaCl, 100 mM TRIS/HC1, 10 mM EDTA, pH 8.0) and 250 μg proteinase K were added to 400 μl CSF-specimen or 400 μl of the washed urine specimen and allowed to incubate at 60° C for 2 hours. Then the samples were incubated for 10 minutes at 94° C to inactivate the proteinase. The samples were extracted with 500 μl phenol/chloroform (1 : 1) and with 400 μl chloroform/isoamylalcohol (24 : 1). Then the DNA was precipitated with 700 μl cold ethanol and washed once with cold 70% ethanol. The DNA pellets were suspended in 30 μl H_2O. A standard amplification reaction mixture containing 10 μl of the above samples, 20 pM of each primer (5'ATTCGTCGTCGAGTTCGACTTCCT and 5'TGACAACAAACGCGTTGTTCGATTC according to WILLIAMS et al. [17]), 200 μM of each dNTP, 10 μl of amplification buffer concentrate, and 2 U of Taq polymerase (PROMEGA, Madison, WI, USA) was amplified in a Bio-med 60 thermocycler (THERES, Germany) for 45 cycles (94° C, 1 min; 60° C, 1 min; 72° C, 2 min.) The primers were designed to amplify a 360 bp region of the 18 kDa proteingene of *M. leprae*. Amplified DNA was further confirmed by hybridization using a 212 bp probe generated by amplification of the same gene by a primer pair which is located within the primer pair shown above (17). To investigate the influence of exposing urine to *M. leprae,* 10^4 organism/ml were added to urine samples and allowed to incubate at 22° C for different time intervals before washing with PBS. Then DNA preparation and PCR were done as described above. *M. tuberculosis* H37Rv was used as a control. PCR of *M. tuberculosis* was done as described earlier (16).

Microscopic analysis

Samples were stained for Mycobacteria using a Ziehl-Neelsen procedure (5).

Results

Table 1 summarizes the results of these in vitro experiments. Using PCR *M. leprae* could be detected at levels of 10^3 and 10^4 organisms/ml in specimens CSF I and CSF II, respectively. In positive samples, a 360 bp band which represents the expected size of amplification of the 18 kDa proteingene of *M. leprae* was amplified and could be verified by hybridization with an internal 212 bp DNA probe of the 18 kDa proteingene. Determination of TBSA and Ziehl-Neelsen staining gave positive results for concetrations of 10^4 organisms/ml in both CSF specimens. Despite in vitro spiking with up to 10^4 organisms/ml our PCR protocol failed to detect *M. leprae* in the two urine specimens. Also microscopic analysis using Ziehl-Neelsen staining gave negative results for both urine specimens. Determination of TBSA yielded positive results at levels of 10^3 and 10^4 organisms/ml for urine specimens I and II, respectively. No incorrect positive results were observed.

The negative results for urine specimens using the PCR protocol and Ziehl-Neelsen staining procedure were further studied for the importance of time exposure to urine. *M. leprae* and *M. tuberculosis* (as a control) were exposed to urine I for 0.5, 15 and 60 minutes, washed and then analyzed by PCR and Ziehl-Neelsen staining. Table 2 summarizes these results.

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Table 1:

Detection of *M. leprae* in two CSF and two urine specimens, spiked with *M. leprae* in various concentrations, using PCR, TBSA-determination, and Ziehl-Neelsen staining.

Specimen	Method	Inoculum (organisms/ml)				Blank
		10.000	1.000	100	10	
CSF I	PCR	+	+	–	–	–
	TBSA	+	–	–	–	–
	Ziehl-Neelsen	+	–	–	–	–
CSF II	PCR	+	–	–	–	–
	TBSA	+	–	–	–	–
	Ziehl-Neelsen	+	–	–	–	–
Urine I	PCR	–	–	–	–	–
	TBSA	+	+	–	–	–
	Ziehl-Neelsen	–	–	–	–	–
Urine II	PCR	–	–	–	–	–
	TBSA	+	–	–	–	–
	Ziel-Neelsen	–	–	–	–	–

+ positive result
– negative result

Table 2:

Detection of *M. leprae* and *M. tuberculosis* in urine using PCR and Ziehl-Neelsen staining. Bacteria were exposed to urine (10^4 organisms/ml) for time intervals of 0.5, 15, and 60 minutes.

Time of exposition to urine:		0.5 min	15 min	60 min
M. leprae	PCR	+	–	–
	Ziehl-Neelsen	+	–	–
M. tuberculosis	PCR	+	+	+
	Ziehl-Neelsen	+	+	+

While urine exposure did not influence the detectability of *M. tuberculosis,* samples gained after exposure for 15 and 60 minutes proved to be negative for *M. leprae.* Samples with a short exposure time, gained and washed immediately after inoculation, gave positive results using PCR as well as Ziehl-Neelsen staining.

Discussion

The diagnosis of leprosy can sometimes be as difficult today as it was 100 years ago. For third world countries which are primarily affected, determination of the disease by means of animal experiments is impractical and too demanding. For this reason new molecular biological methods like PCR which might detect *M. leprae* quickly, economically and reliably have raised great expectations. The PCR determination of *M. leprae* from skin smears is currently being tested (17). The aim of this work was to examine whether *M. leprae* could be detected by PCR in CSF and urine and to compare the sensitivity of this procedure to determination of TBSA and a Ziehl-Neelsen staining method. CSF and urine were used as sample materials to establish analytical conditions for investigating potential genito-urinary and central nervous systems involvement in leprosy. Our experiments show that *M. leprae* can be detected in CSF using PCR although sensitivity is not substantially better than TBSA determination and the simple and inexpensive Ziehl-Neelsen staining. In 1993, YOON et al. (18) examined 102 biopsy specimens by PCR and Ziehl-Neelsen staining and found that the difference in overall results between the two methods was not statistically significant (18). However, PCR offers the advantage of high specificity for *M. leprae* wheras Ziehl-Neelsen staining and TBSA test don't allow differentiation from other mycobacteria.

The conditions are different for urine; detection of *M. leprae* was neither possible with PCR nor with Ziehl-Neelsen staining, not even in the highest concentration of 10^4 organisms/ml which were used. Only the determination of TBSA allowed detection of the causative agent at this concentration of organisms. For the diagnosis of *M. leprae* the use of TBSA tests means accepting lower specificity; other mycobacteria like *M. smegmatis* also lead to positive results (6). Problems, like the presence of unspecific inhibitors were noted repeatedly while testing urine samples with PCR (11). However, we suggest that the failure to detect *M. leprae* in urine with PCR and Ziehl-Neelsen staining indicates a direct disintegrative effect on *M. leprae* by urinary components. If the genito-urinary tract becomes affected by *M. leprae* the causative agent naturally gets into the bladder which is why we examined the kinetics of the inactivation of *M. leprae* in urine. *M. tuberculosis,* which can be detected in the urine even after prolonged incubation, was used as a control. We obtained positive results with PCR and Ziehl-

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Neelsen staining only when the samples were washed immediately after inoculation with *M. leprae*. After only 15 minutes incubation time, however, both tests proved negative. As DNA is more susceptible to degradation after cell death than are other cell components (8), the discrepancy between TBSA positivity and PCR negativity is not surprising.

In summary, we conclude that although PCR does not yet fulfil the expectations concerning sensitivity for the detection of *M. leprae* in urine and CSF it seems worthwhile to work on improving this molecular genetic determination because of its high specificity. With PCR, SANTOS et al. were recently able to detect 0.1 fg *M. leprae*-DNA, a quantity equal to about 1/10 of the bacterial genome, using *M. leprae*-specific repetitive sequence as template. These results, however, were obtained with pure DNA, not with clinical samples (13). The problem of false positive PCR results is aggravated by the use of repetitive sequences which are usually evolutionally highly conserved. In a study, NISHIMURA et al. found false positive amplifications in 9 out of 24 non-leprosy control samples (paraffin-embedded skin biopsies) (9). At present the use of PCR to detect *M. leprae* in the genito-urinary tract does not seem promising. An improved in vitro determination of *M. leprae* in CSF specimens should help to establish the analytical conditions for investigating clinical material as to the role of *M. leprae* in CSF.

Summary

To what extent leprosy affects the genito-urinary and central nervous systems remains unclear. Because *M. leprae* is not cultivable, high expectations are placed on identifying the causative agent directly through polymerase chain reaction (PCR). Cerebrospinal fluids (CSF) and urine-specimens were spiked in vitro with up to 10^4 *M. leprae*/ml and used to evaluate PCR, Ziehl-Neelsen staining and determination of tuberculostearic acid (TBSA) for detection of *M. leprae*. The PCR based on the selective amplification of a 360 bp fragment of the 18 kDa proteingene of *M. leprae* was able to detect 10^3 to 10^4 organism/ml in CSF. Determination of TBSA and Ziehl-Neelsen staining showed positive results for concentrations of 10^4 organisms/ml. In contrast, PCR and Ziehl-Neelsen staining showed negative results for the urine specimens, whereas determination of TBSA, a structural component of all mycobacteria, yielded positive results at levels of 10^3 to 10^4 organisms/ml. To study the importance of time exposure to urine, *M. leprae* was exposed to urine for various time periods. Only samples washed immediately after exposure showed positive results using PCR or Ziehl-Neelsen staining. Our findings suggest that the use of PCR to detect *M. leprae* from the genito-urinary tract is not promising. However, an improved in vitro determination of *M. leprae* in CSF samples should help to provide the means to establish the role of *M. leprae* in CSF.

Key words

Leprosy, tuberculostearic acid, PCR.

Zusammenfassung

Beurteilung der diagnostischen Wertigkeiten von Polymerasekettenreaktion (PCR) und Bestimmung von Tuberkulostearinsäure zum Nachweis von Mycobacterium leprae in Harn und Liquor

In welchem Ausmaß der Urogenitaltrakt und das Zentralnervensystem bei Lepra betroffen sind, ist derzeit noch unklar. Da *M. leprae* nicht kultivierbar ist, werden große Erwartungen in die Polymerasekettenreaktion (PCR) zum direkten Erregernachweis gelegt. Liquor- und Harnproben wurden in vitro mit bis zu 10^4 *M. leprae*/ml beimpft und zur Beurteilung der diagnostischen Wertigkeiten von PCR, Ziehl-Neelsen-Färbung und Tuberkulostearin-Säure (TBSA)-Nachweis herangezogen. Die PCR, die auf der selektiven Vervielfachung eines 360 bp Fragmentes aus dem 18 kDa Proteingen von *M. leprae* basiert, war in der Lage zwischen 10^3 und 10^4 Organismen/ml im Liquor nachzuweisen. Bestimmung der TBSA und Ziehl-Neelsen-

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Färbung waren ebenso in der Lage, 10^4 Organismen/ml nachzuweisen. Im Gegensatz dazu ergaben PCR und Ziehl-Neelsen-Färbung negative Ergebnisse bei den Harnproben, während der Nachweis von TBSA, die ein struktureller Bestandteil aller Mykobakterien ist, im Bereich von 10^3 und 10^4 Organismen/ml positiv war. Um die Bedeutung der Expositionszeit im Harn zu untersuchen, wurde *M. leprae* für verschiedene Zeitintervalle im Harn exponiert. Nur Proben, die unmittelbar nach der Inokulation gewaschen wurden, waren in der PCR und in der Ziehl-Neelsen-Färbung positiv. Unsere Ergebnisse zeigen, daß die PCR zum Nachweis von *M. leprae* im Urogenitaltrakt nicht erfolgversprechend ist. Im Gegensatz dazu scheint eine Verbesserung des in vitro-Nachweises von *M. leprae* in Liquorproben zur Klärung der Beteiligung des Zentralnervensystems bei Lepra geeignet zu sein.

Schlüsselwörter Lepra, Tuberkulostearinsäure, PCR.

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