PCR TO DETECT *MYCOBACTERIUM TUBERCULOSIS* DNA IN SPUTUM SAMPLES FROM TREATED LEPROSY PATIENTS WITH PUTATIVE TUBERCULOSIS

Abdolnasser Rafi[1] and Francois Feval[2]

[1]UCL Medical School, Department of Medical Microbiology, Division of Bacteriology, 67-73 Riding House Street, London W1P 7PN, England; [2]Baba Baghi Leprosy Hospital, Tabriz, Iran

Abstract. In this study of leprosy patients with putative tuberculosis, the polymerase chain reaction (PCR), one of the most reliable and sensitive molecular diagnostic methods, was carried out for the specific detection of *Mycobacterium tuberculosis* DNA. Sputum samples from 43 patients at Baba Baghi Leprosarium in Iran were tested. The DNA extraction method was based on the lysing and nuclease-inactivating properties of guanidinium thiocyanate (GuSCN) together with the nucleic acid-binding properties of diatoms or silica particles. Primers for a 123-base pair (bp) fragment of the repetitive DNA sequence of *M. tuberculosis* were used for the PCR assay. The results of PCR were compared with direct microscopy and culture.

In total, 14% of the patients in this study were found to be PCR positive for *M. tuberculosis*. No positive results were found by direct microscopy for acid fast bacilli (AFB) and culture. It was thought probable that the positive PCR results indicated the tuberculosis (TB) in such treated leprosy patients.

INTRODUCTION

Tuberculosis and leprosy are common disabling diseases. It is estimated that around 50 million people have, or recently have had tuberculosis and more than 12 million suffer from active leprosy or its resultant disability (Pallen, 1984). Association between tuberculosis and leprosy in individuals living in an area endemic for both diseases has been little documented although it is likely to be common. In some endemic areas the two diseases appear to coincide, whereas in others they appear to be mutually exclusive. It has been postulated that this is related to distribution of environmental mycobacterial variability influencing immunity and susceptibility. There is some evidence from the past that tuberculosis commonly afflictted institutionalised leprosy patients. One of the rare studies on this subject was done in 1895 by Armauer Hansen, who found tuberculosis to be the most common cause of death among leprosy patients in Norway (Glaziou *et al*, 1993). Some studies suggest that tuberculosis and leprosy might interact or interfere with one another (Fine, 1984). This view is no longer tenable, because it has been shown that tuberculosis is no more common in leprosy outpatients than in the general population (Jopling and Mcdougall, 1988). More recently, two studies have suggested that leprosy might encourage the development of tuberculosis (Gatner *et al*, 1980; Kumar *et al*, 1982).

In this present study, a simple PCR was carried out to detect specific IS *6110* sequences of *M. tuberculosis* DNA in sputum samples from treated leprosy patients by a set of primers with a detection limit of fewer than 10 bacilli (Eisenach *et al*, 1990; 1991). This study was done to follow up the skin testing results of the same patients that half of them had shown Koch responses to tuberculin suggestive of tuberculosis. The diagnosis of tuberculosis in such patients is not always easy, and application of the polymerase chain reaction to this problem could be extremely useful. The aim of this investigation was to find out whether PCR could detect tubercle bacilli missed by direct microscopy and culture.

MATERIALS AND METHODS

Patients and clinical samples

Forty-three treated leprosy patients in the age group 30-80, consisting of 30 males and 13 females from the Baba Baghi Leprosy Sanatorium, Iran, were studied on the basis of their skin test responses to

tuberculin and leprosin A, and about half of them (21 patients) had shown Koch responses to tuberculin. According to their clinical data, 22 had started with multibacillary (MB) leprosy, and 21 with paucibacillary (PB) leprosy.

The specimens chosen for the study were early morning sputum samples. Direct microscopy and culture examinations were performed on the same samples and the remaining sputa were transferred to small screw-capped bottles, and kept at -20°C (Rolfs *et al*, 1992) for transport to London for PCR assays.

Sputum samples from the 8 non-tuberculous patients as the control group, were also tested by the above mentioned diagnostic methods.

Direct microscopy

Smears of sputum samples treated with the protocol of Dithiothreitol-2% NaOH were prepared, fixed, and stained by Ziehl-Neelsen (ZN) method for acid fast bacilli (AFB) using strong carbolfuchsin, acid-alcohol as a decolorant, and methylene blue as the counterstain. After staining, more than 20 fields of each smear were examined carefully under the light microscope using an oil immersion (x100) lens.

Culture

All collected sputum samples were treated with the protocol of Dithiothreitol-2% NaOH, inoculated onto Lowenstein-Jensen (LJ) medium, and incubated at 37°C for 6-8 weeks and the slopes were examined every week.

Isolation of DNA from sputum samples

Isolation of DNA was based on the lysing and nuclease-inactivating properties of guanidinium thiocyanate (GuSCN) combined with the nucleic acid-binding properties of diatoms or silica particles.

Sputum samples were liquefied by the method described by Victor *et al* (1992) and then 50 µl of each liquefied sample was prepared as follows:

Diatom suspension (40 µl) was mixed with 900 µl of lysis buffer (containing GuSCN 120 g; 0.1 M Tris-HCl at pH 6.4, 100 ml; 0.2 M EDTA at pH 8.0, 22 ml; and Triton X-100, 2.6 g) in a 1.5 ml Eppendorf microcentrifuge tube, and briefly vortex-mixed. The sputum sample (50 µl) was added to the above, vortex-mixed for 5 seconds, and allowed to stand at room temperature for 10 minutes. It was vortex-mixed again, and spun at 12,000*g* for 15 seconds. The supernatant was discarded, and the nucleic acid (NA)-pellet was washed twice with washing buffer (containing GuSCN 120 g; and 0.1 M Tris-HCl at pH 6.4, 100 ml), twice with 70% ethanol and once with acetone. The acetone was removed and the NA-pellet was dried at 56°C for 10 minutes. 100 µl of TE buffer (Tris-HCl at pH 8.0, 10 mM; and EDTA at pH 8.0, 1 mM) was added to the NA-pellet, vortex-mixed, and incubated for 10 minutes at 56°C. It was mixed vigorously again, spun at 12,000*g* for 2 minutes, and 5 µl of the supernatant was used for PCR assay (Boom *et al*, 1990).

Preparation of chromosomal DNA

DNA of *Mycobacterium tuberculosis* was purified from fresh culture of *M. tuberculosis* by a boiling method (McFadden, 1990). A colony of *M. tuberculosis* was placed in 500 µl of strile distilled water, boiled for 10 minutes, spun at 12,000*g* for 2 minutes, and 5 µl of the supernatant used as a positive control in every PCR assay.

Selection of primers

The primers used for the specific amplification were originally designed by Eisenach *et al* (1990) from sequences which are repeated several times in the chromosome of *M. tuberculosis*. The sequences of the primers (synthesized by Oswel DNA Service, Edinburgh, UK) from 5' to 3' were CCTGCGAGC-GTAGGCGTCGG and CTCGTCCAGCGCCGC-TTCGG, which amplify a 123-bp fragment of the repetitive DNA sequence IS*6110*.

PCR procedure

Briefly, 5 µl of each prepared sputum sample was incubated in a 45 µl reaction mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 1 µM each of primers, 0.2 mM each of deoxynucleotides dATP, dTTP, dGTP and dCTP (Pharmacia) and 1.25 units of Taq polymerase (purchased from Perkin-Elmer Cetus). The reaction mixture were covered with 40 µl of sterile mineral oil (Eisenach *et al*, 1990). A control tube containing no target DNA as a negative control, with another tube

containing chromosomal DNA of *M. tuberculosis* as a positive control were included with every set of tests. Precaution were taken to avoid contamination with extraneous DNA. PCR negative samples were retested after adding 2 µl of the chromosomal DNA to the amplification mixture to control for the presence of PCR inhibitors (De Wit *et al*, 1991).

The reaction was performed using an automated thermal cycler. The samples were denatured at 94°C for 5 minutes, and then 30 amplification cycles were done. Each cycle consisted of denaturation at 94°C for 2 minutes, annealing of primers at 68°C for 2 minutes, and primer extension at 72°C for 2 minutes. After the 30th cycle, the extension reaction was continued for another 5 minutes at 72°C (Eisenach *et al*, 1990). The presence of the 123-bp amplification product was sought by electrophoresis of 5 µl of the amplified mixture at 88 V for 45 minutes on an agarose gel (1%). The DNA was stained with ethidum bromide (0.5 µg/ml) and visualized on a 302-nm UV transilluminator. The molecular size marker used was 123-bp DNA Ladder (Sigma).

RESULTS

Sputum samples obtained from 43 treated leprosy patients were tested by direct microscopy for AFB, and culture on LJ medium and none was found to be positive. In contrast, PCR detected the presence of the 123-bp DNA fragment specific for tubercle bacilli in 6 (14%) of the sputum samples. The results of direct microscopy, culture and PCR were negative in sputum samples from the 8 non-tuberculous control group (Table 1). No PCR inhibitors were detected in the PCR negative specimens.

Amongst male patients, 4/30 were PCR TB-positive, and amongst females, 2/13 were PCR TB-positive.

The distribution according to type of the original disease, is shown in Table 2. There was no significant difference for the positivity rate of PCR between treated MB patients (3/22, 13.6%) and treated PB patients (3/21, 14.3%).

The distribution of PCR results according to age of both leprosy types are shown in Table 3. Apparently, the positivity rate of TB-PCR in older age group (51 years and over) was higher than younger age group.

The results of PCR according to tuberculin testing are summarized in Table 4. Amongst patients with Koch response to tuberculin 5/21 (23.8%) were PCR tuberculosis positive, whereas amongst patients without Koch response to tuberculin 1/22 (4.5%) were PCR TB-positive. All these six PCR TB-positive patients had previously shown strong responses to tuberculin (defined as more than 12 mm of induration), but no responses or a weak one to leprosin A.

DISCUSSION

The PCR used in this study shows the efficiency of this technique for the rapid diagnosis of paucibacillary situations of tuberculosis, considering the

Table 1

Results of direct microscopy, culture, and PCR in sputum samples from treated leprosy patients with putative TB and control group.

Samples	No. tested	Positive by:		
		Direct microscopy	Culture (LJ)	TB-PCR
Sputum from treated leprosy patients	43	0	0	6 (14%)
Sputum from non-tuberculous patients (control group)	8	0	0	0

Table 2

Results of TB-PCR in treated leprosy patients with putative TB according to type of leprosy.

*Type of leprosy	No. of treated leprosy patients	No. (%) PCR TB-positive patients
MB	22	3 (13.6%)
PB	21	3 (14.3%)
Total	43	6 (14%)

*MB, multibacillary leprosy
PB, paucibacillary leprosy

Table 3

Results of TB-PCR in treated leprosy patients with putative TB according to age group.

Age group	No. of treated leprosy patients	No. (%) PCR TB-positive patients
30-50	11	1 (9.1%) *
51 and over	32	5 (15.6%)
Total	43	6 (14%)

*Not significant

conventional methods gave negative results. The accuracy of this study is demonstrated by the fact that the control group did not give positive results (Table 1). The repetitive nature of the target sequence amplified by the PCR described here, probably contributes to the sensitivity, and fewer than 10 bacilli can be detected by application of this PCR. The detection limit of this PCR with application of a DNA extraction method that has successfully shown its efficacy in isolation of *M. tuberculosis* and *M. leprae* DNAs from ancient bones (Spigelman *et al*, 1993; Rafi *et al*, 1994), provide a powerful tool for the rapid and specific diagnosis of difficult cases of tuberculosis.

Table 4

Results of TB-PCR in treated leprosy patients with putative TB according to tuberculin testing.

Tuberculin testing	No. of treated leprosy patients	No. (%) PCR TB-positive patients
With Koch response	21	5 (23.8%) *
Without Koch response	22	1 (4.5%)
Total	43	6 (14%)

*Not significant

It seems that this finding of 6/43 treated leprosy patients to be positive by PCR for *M. tuberculosis*-specific DNA is important since one of the causes of death in leprosy is tuberculosis. The increased incidence of pulmonary tuberculosis observed in leprosaria, gives rise to the presumption that leprosy patients are susceptible to tuberculosis.

In this study, a higher proportion of PCR TB-positive patients among treated MB patients rather than treated PB patients, was not found (Table 2). However, a study found a negative correlation between the tuberculoid form of leprosy and tuberculosis (Kaklamani *et al*, 1991). A cause of this negative association could be a defective cellular immunity against mycobacteria in lepromatous patients, but this has never been demonstrated. Another study suggested that lepromatous patients could share factors of susceptibility to mycobacterial diseases with patients developing tuberculosis (Glaziou *et al*, 1993). An explanation for the mentioned finding, may be the leprosy treatment of the patients of this study. It seems that all types of leprosy patients after treatment of leprosy, might be at risk from tuberculosis in the equal proportion.

Table 3 shows that older leprosy patients are more likely to have detectable *M. tuberculosis* DNA in their sputum samples than are younger patients. This may be due to the decrease of the innate immunity in older patients, that is followed by the weakening of both external defensive factors and internal defensive factors in older leprosy patients.

The interesting finding, of more PCR positive

results from patients who had previously shown Koch responses to tuberculin than from those who had not previously shown Koch response to tuberculin (Table 4), indicates the usefullness of performance of tuberculin testing among treated leprosy patients in order to ascertain Koch responses, and guess tuberculous infections among such patients. Considering that tuberculin testing studies cost cheaper than PCR investigations, therefore it will be wise that skin testing is done prior to PCR on such patients. It is evident that PCR will confirm the results of skin testing, and find out the suspected TB cases in a leprosarium.

The results of this study illustrate the potential value of PCR in the investigation of difficult cases of tuberculosis, and particularly indicate that it may monitor an incidence of tuberculosis among treated leprosy patients. We are hoping to extend our studies to active leprosy patients, and find out whether PCR detects any proportion of suspected TB cases among active leprosy patients.

ACKNOWLEDGEMENTS

The authors would like to thank Dr MH Molayen, the Director and his staff from Baba Baghi leprosy Hospital and Sanatorium, Iran and Mr Ali Akhavan, the Assistant from the Research Department of the Tabriz Unversity of Medical Sciences, for their valuable help throughout this study. The Bacteriology Laboratory staff of 7-Tir Hospital of Tabriz, Iran are also much appreciated for their co-operation in the conventional bacteriology.

REFERENCES

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-Van Dillen PME, Van Der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; 28 : 495-503.

De Wit MYL, Faber WR, Krieg SR, *et al.* Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues. *J Clin Microbiol* 1991; 29 : 906-10.

Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis* 1990; 161 : 977-81.

Eisenach KD, Sifford MD, Cave MD, Bates JH, Crawford JT. Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction. *Am Rev Respir Dis* 1991; 144 : 1160-3.

Fine P. Leprosy and tuberculosis-an epidemiological comparison. *Tubercle* 1984; 65 : 137-53.

Gatner EMS, Glattharr E, Imkamp MFJH, Kok SH. Association of tuberculosis and leprosy in South Africa. *Lepr Rev* 1980; 51 : 5-10.

Glaziou P, Cartel JL, Moulia-Pelat JP, *et al.* Tuberculosis in leprosy patients detected between 1902 and 1991 in French Polynesia. *Int J Lepr* 1993; 61 : 199-204.

Jopling WH, McDougall AC. Handbook of leprosy 4th ed. London: Heinemann, 1988; p36.

Kaklamani E, Koumandaki Y, Katsouyani K, Trichopoulos D. BCG, tuberculosis and leprosy. *Lancet* 1991; 337 : 304.

Kumar B, Kaur S, Kataria S, Roy SN. Concomitant occurrence of leprosy and tuberculosis-a clinical bacteriological and radiological evaluation. *Lepr India* 1982; 54 : 671-76.

McFadden J. Molecular biology of mycobacteria. UK, Surrey: University Press, 1990: pp 139-72.

Pallen MJ. The immunological and epidemiological significance of environmental mycobacteria on leprosy and tuberculosis control. *Int J Lepr* 1984; 52 : 231-45.

Rafi A, Spigelman M, Stanford J, Lemma E, Donoghue H, Zias J. *Mycobacterium leprae* DNA from ancient bone detected by PCR. *Lancet* 1994; 343 : 1360-1.

Rolfs A, Schuller I, Finckh U, Weber-Rolfs I. PCR: clinical diagnostics and research. Germany: Springer-Verlag, 1992; p 75.

Spigelman M, Lemma E. The use of the polymerase chain reaction to detect *Mycobacterium tuberculosis* in ancient skeletons. *Int J Osteoarchaeol* 1993; 3 : 137-43.

Victor T, Du Toit R, Van Helden PD. Purification of sputum samples through sucrose improve detection of *Mycobacterium tuberculosis* by polymerase chain reaction. *J Clin Microbiol* 1992; 30 : 1514-7.