Medical Journal of the
Islamic Republic of Iran

Volume 11
Number 1
Spring1376
May 1997

THE DETECTION OF *Mycobacterium tuberculosis* BY PCR IN SPUTUM SAMPLES FROM LONG-TERM-TREATED LEPROSY PATIENTS: ASSOCIATIONS WITH SKIN TEST RESULTS AND IMMUNOTHERAPY WITH *Mycobacterium vaccae*

A. RAFI, Ph.D., A. AHMADIAN, Ph.D., M.H. MOBAYEN*, M.D., F. FEVAL*, M.D., Y. DOWLATI**, M.D., K. GHAZI SAIDI**, Ph.D., A.A. VELAYATI**, M.D. AND J.L. STANFORD***, M.D.

*From the Department of Medical Microbiology, School of Medicine, Tabriz University of Medical Sciences, Tabriz, The *Baba Baghi Leprosy Hospital, Tabriz, the **Tehran University of Medical Sciences, Tehran, and the*
****Department of Bacteriology, University College London Medical School, 67-73 Riding House Street, London W1P 7LD, UK.*

ABSTRACT

As part of a series of investigations at Baba Baghi Leprosarium in Iran, 44 long-treated leprosy patients were selected for our study. Samples of early morning sputum were obtained from each patient, examined by microscopy for acid-fast bacilli (AFB), and cultured for tubercle bacilli. These tests were negative, but the polymerase chain reaction (PCR) for an insertion sequence believed to be specific for *Mycobacterium tuberculosis* was applied to each sputum sample and those from six patients were found to be positive. Five of the six positive samples were from the 21 patients producing Koch-type responses to tuberculin, and none were from the 11 patients previously found to have skin-tissue fluid or sputum positive by PCR for *Mycobacterium leprae*. Whereas immunotherapy with killed *Mycobacterium vaccae* given nearly 2 years earlier to 23 of the patients strongly influenced PCR results for *M. leprae* ($p = 0.01$), it had no influence on results for tubercle bacilli. However, at a second sampling date 18 months later, the only 2 patients still positive by PCR for tubercle bacilli came from the placebo recipient group. The possible significance of the findings is discussed.

MJIRI, Vol. 11, No. 1, 43-47, 1997.

INTRODUCTION

Tuberculosis and leprosy are among the most common disabling diseases in the world. It is estimated that around 50 million people have, or recently have had, clinical tuberculosis. More than 2 million people suffer from active leprosy, with a larger number suffering from its resultant disabilities.[1] An association between tuberculosis and leprosy in individuals living in areas endemic for both diseases has been suggested, but little documented, although

it is likely to be common. In some endemic areas the two diseases appear to coexist, whereas in others they seem mutually exclusive. Whether this is by chance, or reflects an interaction between the two diseases remains a matter of debate.[2] It might be related also to the influence on immunity and susceptibility of variably distributed environmental mycobacteria. There is some evidence from the past that tuberculosis commonly afflicted institutionalized leprosy patients. One of the rare studies on this subject was that of Armauer Hansen in 1895, which was cited by Glaziou *et al*;[3] he found tuberculosis to be the most common cause of death among leprosy patients in Norway. Recently it has been shown that tuberculosis is no commoner in leprosy outpatients than in the general population,[4] but two studies have suggested that the presence of leprosy might encourage the development of tuberculosis.[5,6] The reality of the situation is probably that both diseases and the influence of environmental factors all interact, producing different sets of phenomena in different situations.

This investigation was carried out as part of a study of a group of long-treated leprosy patients in Baba Baghi Leprosy Sanatorium, near Tabriz in Iran. These patients had been skin tested and randomized to receive an injection of killed *M. vaccae* as an immunotherapeutic, or saline as placebo 18 months before our first samples were taken. Our aim was to search for tubercle bacilli in their sputum by the polymerase chain reaction (PCR), and relate the findings to the skin test and immunotherapy data, and to the results from our previous study[7] of PCR for *Mycobacterium leprae* (LEP-PCR).

MATERIALS AND METHODS

Patients

A group of 279 patients with long histories (more than 10 years) of treatment for leprosy at Baba Sanatorium near Tabriz in Iran, were subjected to skin-testing with 2 new tuberculins, and randomized to receive an injection of saline as placebo or 10^9 killed *M. vaccae* plus tuberculin as immunotherapy. The patients investigated in the present study, and that previously reported,[7] were selected from among them.

Skin-testing

The 2 new tuberculins used were Tuberculin (T) and Leprosin A (LA). These were prepared in the Medical Microbiology Department of UCL Medical School, London from *M. tuberculosis* and *M. leprae*, respectively. The reagents were injected, 10 cm apart, two on the volar surfaces of each forearm. Doses injected were 0.2 μg of T and 1.0 μg of LA. Reactions were read as longitudinal and transverse diameters of the areas of induration 72 hours after injection. The mean diameter was recorded for each reaction and sizes of 2 mm or greater were taken as positive responses with these reagents.[8] Reactions to Tuberculin showing qualitative evidence of incipient necrosis were recorded as Koch-type responses.

Immunotherapy

All the patients skin tested were randomized to receive immunotherapy or placebo as a single intradennal injection of 0.1 mL, given high up over a deltoid muscle. The immunotherapy consisted of a suspension of autoclaved *Mycobacterium vaccae* strain NCTC 11659, 10 mg wetweight/mL in borate buffered saline (pH 8.0), to which Tuberculin was added to a final concentration of 0.2 μg/mL shortly before injection. The placebo used was saline.[9]

Selection of patients and collection of samples

Forty-four patients in the age range 30-80 years (mean 57.2 years), comprising 31 men and 13 women representative of the two major forms of leprosy, were selected for our study partly on the basis of the quality of their skin test response to Tuberculin. About half of them (23 cases) had received immunotherapy and the remainder (21 cases) had received placebo. Nineteen had scars of past BCG vaccination. According to their clinical records, 22 had initial diagnosis of multibacillary (MB) leprosy, and 22 of paucibacillary (PB) leprosy. A group of 8 healthy members of staff of the Sanatorium volunteered to provide control samples for our study. Early morning sputum was collected from each participant.

At the time of second sampling, to make up for those from the first selection who were not available, 10 additional patients agreed to give samples. They were 3 women and 7 men, with an average age of 51.3 years, two of whom had BCG scars. Eight had original diagnosis of MB and 2 of PB leprosy, and 6 had received immunotherapy with *M. vaccae*. The details of the patients are included in the Table.

Culture

All collected sputum samples were decontaminated and concentrated by a method employing Dithiothreitol and 2% NaOH. Deposits of treated samples were inoculated onto Loewenstein-Jensen (LJ) medium and incubated at 37°C for 6-8 weeks. The slopes were examined for growth at weekly intervals.

Sputum microscopy

Smears of the treated sputum deposits were prepared, fixed, and stained by the Ziehl-Neelsen (ZN) method for acid-fast bacilli (AFB). After staining, more than 20 fields of each smear were examined carefully under the light microscope using an oil immersion (× 100) lens.

Isolation of DNA from sputum

After bacteriological examination, remaining sputum was transferred to small screw-capped bottles, and kept at

Table 1. Results of prior skin testing and randomized immunotherapy with *M. vaccae* in relation to PCR for tubercle bacilli (TB-PCR) and leprosy bacilli (LEP-PCR) at both times of taking samples.

BCG scar	Skin-test-results T	LA	IT/P	First sampling TB-PCR	LEP-PCR	Second sampling TB-PCR	LEP-PCR
MB patients							
-ve	20K	0	IT	+ve	-ve	-ve	-ve
-ve	22K	0	P	+ve	-ve	+ve	-ve
+ve	14	0	P	+ve	-ve	-ve	-ve
+ve	16K	0	P	-ve	+ve	-ve	+ve
-ve	12	0	P	-ve	+ve	-ve	-ve
-ve	5	10	P	-ve	+ve	nd	nd
-ve	14K	0	IT	-ve	-ve	-ve	-ve
+ve	20K	0	P	-ve	-ve	-ve	-ve
-ve	25K	0	IT	-ve	-ve	-ve	-ve
+ve	14K	0	P	-ve	-ve	-ve	-ve
-ve	13K	0	IT	-ve	-ve	-ve	-ve
-ve	13K	0	P	-ve	-ve	-ve	-ve
+ve	14K	0	P	-ve	-ve	-ve	-ve
-ve	20	0	P	-ve	-ve	-ve	-ve
-ve	8	0	P	-ve	-ve	-ve	-ve
+ve	17	0	IT	-ve	-ve	-ve	-ve
-ve	9	0	IT	-ve	-ve	-ve	-ve
-ve	3	0	P	-ve	-ve	-ve	-ve
-ve	11	0	IT	-ve	-ve	-ve	-ve
+ve	17	0	IT	-ve	-ve	-ve	-ve
+ve	0	0	IT	-ve	-ve	-ve	-ve
-ve	0	0	IT	-ve	-ve	-ve	-ve
-ve	15	0	IT	nd	nd	-ve	-ve
-ve	4	0	IT	nd	nd	-ve	-ve
-ve	4	0	IT	nd	nd	-ve	-ve
-ve	0	4	IT	nd	nd	-ve	-ve
-ve	0	0	IT	nd	nd	-ve	-ve
-ve	0	0	P	nd	nd	-ve	-ve
+ve	0	0	P	nd	nd	-ve	-ve
-ve	0	0	P	nd	nd	-ve	-ve
PB patients							
-ve	20K	0	P	+ve	-ve	+ve	-ve
-ve	16K	8	IT	+ve	-ve	-ve	-ve
-ve	12K	16K	IT	+ve	-ve	-ve	-ve
+ve	15K	10	IT	-ve	+ve	-ve	+ve
-ve	21K	6	P	-ve	+ve	nd	nd
+ve	15K	11	P	-ve	+ve	-ve	+ve
+ve	12	4	IT	-ve	+ve	-ve	+ve
-ve	6	10	P	-ve	+ve	-ve	+ve
+ve	10	15	P	-ve	+ve	-ve	+ve
+ve	17	6	P	-ve	+ve	nd	nd
-ve	9	0	P	-ve	+ve	-ve	-ve
+ve	12K	0	IT	-ve	-ve	-ve	-ve
-ve	17K	0	P	-ve	-ve	-ve	-ve
-ve	16K	15	IT	-ve	-ve	-ve	-ve
+ve	15K	0	IT	-ve	-ve	-ve	-ve
+ve	18K	0	IT	-ve	-ve	-ve	-ve
+ve	14	0	P	-ve	-ve	nd	nd
+ve	13	8	IT	-ve	-ve	-ve	-ve
+ve	10	0	IT	-ve	-ve	-ve	-ve
-ve	0	12	IT	-ve	-ve	-ve	-ve
-ve	0	0	P	-ve	-ve	-ve	-ve
-ve	0	0	IT	-ve	-ve	-ve	-ve
-ve	12	10	IT	nd	nd	-ve	-ve
+ve	0	0	P	nd	nd	-ve	-ve

Abbreviations used in the Table:
IT: immunotherapy, P: placebo, T: Tuberculin, LA: Leprosin A, TB-PCR: PCR for *M. tuberculosis*, LEP-PCR: PCR for *M. leprae*, MB: multibacillary, PB: paucibacillary, K: Koch-type response, -ve: negative, +ve: positive, nd: not done.

-20°C[10] and transported to London for PCR assays.

The sputum samples were liquified by the method of Victor et al.[11] and 50 µL aliquots of each were taken for extraction of DNA by the method of Boom et al.[12] A simple PCR for *M. tuberculosis* was carried out to detect specific IS6110 DNA sequences using a set of primers with a detection limit of fewer than 10 bacilli.[13,14]

Preparation of *M. tuberculosis* DNA as a positive control

Chromosomal DNA of *M. tuberculosis*, prepared and purified from a fresh culture of tubercle bacilli by a boiling method,[15] was used as a positive control in every PCR assay.

Selection of primers and PCR

The primers used for the specific amplification were originally designed by Eisenach et al.[13] from sequences 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:

CCTGCGAGCGTAGGCGTCGG and CTCGTCCAGCGCCGCTTCGG which amplify a 123-bp fragment of the repetitive DNA sequence IS 6110. PCR was performed on each sample using the method of Eisenach et al.[14]

Statistical analysis of results

Where appropriate, Fisher's exact test and Student's t-test were used to determine the likely statistical significance of our findings.

RESULTS

Initial parameters

The individual skin test results for the forty-four studied patients are shown in the Table. Twenty one (10 MB and 11 PB patients) were selected because they were recorded as having Koch-type responses (mean reaction size 16.6±3.5 mm) to Tuberculin at skin-testing prior to immunotherapy or placebo 18 months before our first set of samples were collected. Twelve of those producing Koch responses received *M. vaccae* and 10 received placebo. Twenty three patients were randomly selected from the 258 other patients in the immunotherapy study. Eighteen of them were tuberculin positive (10 MB and 8 PB patients) with a mean positive reaction size of 11.5±4.4 mm, and 5 had zero responses ($p<0.001$ for the difference in sizes between Koch and non-Koch responses to Tuberculin). Thirteen patients made positive responses to Leprosin A (1 MB and 12 PB cases). Eleven PB patients were positive to both reagents.

Eighteen months after our first sampling, repeat samples were obtained from the 40 of our patients still at the sanatorium. Of the additional 10 patients (8 MB and 2 PB cases) first sampled at this time, four had positive tuberculin tests (88±4.9 mm), though not of Koch type, two responded to leprosin A (1 MB, 1 PB), and four were negative to both skin tests.

Of all the patients sampled for our study, 2/30 MB and 13/24 PB patients were positive to Leprosin A ($p=0.00013$), and the 12 responders to both two reagents were PB patients. Otherwise skin testing showed no difference between the groups.

Bacteriology and molecular results

None of the first sputum samples from the forty-four

treated leprosy patients or eight healthy controls were positive by direct microscopy or culture for AFB. In contrast, PCR detected the presence of the 123-bp DNA fragment specific for tubercule bacilli in 6 (13.6%) of the sputum samples from leprosy patients (Table). None of the PCR negative specimens were found to be inhibitory. Four of the positive samples were from men and two were from women. There were equal numbers of positive results among patients with MB or PB disease, and amongst those who had received immunotherapy or placebo 18 months earlier.

The results of PCR according to tuberculin testing are shown in the Table. Amongst patients with Koch responses to Tuberculin 5/21 (23.8%) were positive for *M. tuberculosis* by PCR in comparison with only 1/23 (4.3%) in those with non-Koch responses (p= 0.074). None of the patients PCR-positive for *M. tuberculosis* were amongst the 11 patients found PCR-positive for *M. leprae* in the previous study,[7] although this exclusion was not significant (p = 0.16). There was no association between PCR-positivity for tubercle bacilli and reaction to Leprosin A. Similarly, there was no relationship between PCR-positivity for *M. leprae* and tuberculin positivity, but there was quite a strong association between a positive PCR for leprosy bacilli and a positive response to Leprosin A (6/13 compared with 3/31; p = 0.012).

Samples were collected at the second visit from all but four of the original patients (including all six who were positive for *M. tuberculosis* by PCR), and from the additional 10 patients. All these second series of samples were tested by PCR and all were negative except for two (1 MB and 1 PB patient) of the original six positives, both of them having received placebo rather than immunotherapy.

DISCUSSION

The results obtained are surprising in that 6/44 long-treated leprosy patients have sputum apparently positive for *M. tuberculosis* by the PCR technique, though negative by smear and culture. The association between positive PCR results and a Koch response to Tuberculin performed 18 months earlier, together with the negative findings in the staff members suggest that the results are meaningful. There was no relationship between the PCR results for *M. leprae* reported before[7] and tuberculin responses, but there was a relationship with Leprosin A, probably reflecting the association of positivity to both with PB leprosy.

The PCR used in this and our preceding study[7] apparently shows the efficacy of the technique for the identification of paucibacillary situations for both tubercle and leprosy bacilli, in comparison with the negative results achieved by conventional microscopy and culture.

Although immunotherapy with *M. vaccae* did not appear to have an effect on the PCR for tubercle bacilli at the first time, the only two patients still positive at the second test both came from the placebo recipient group. Immunotherapy did have a significant negative effect on PCR positivity for *M. leprae*, only 2/23 being positive, both PB patients, among immunotherapy recipients compared with 9/21 in the placebo group (p = 0.01). This observation suggesting clearing of the tissues of residual *M. leprae* deserves further investigation.

Just what do our results mean? If 6 out of 44, admittedly selected, leprosy patients really have bacilli in their sputum, are they failing to cause tuberculosis in the same way that *M. intracellulare* and *M. scrofulaceum* seem not to cause disease when they are cultivable from the tissues of MB patients? Are they present as cell wall defective organisms as described for M. *scrofulaceum*[16] and perhaps unable to induce pathology because of their lack of cell-wall-associated adjuvant? Are our results wrong or is the PCR picking up some other resource of the supposed *M. tuberculosis*-specific insertion sequence in the patients' sputum? If the DNA of tubercle bacilli is present, immunity would seem to have little relationship to its cryptic nature, since PCR-positivity was equally divided between patients originally with MB and PB leprosy. This was not the case for detection of cryptic leprosy bacilli which were associated with PB patients.[7]

Thus our data asks more questions than it answers, but it does suggest a previously unexpected relationship between leprosy patients and tubercle bacilli.

ACKNOWLEDGEMENT

The authors would like to thank the staff of Baba Baghi Leprosy Hospital and Sanatorium of Tabriz, Iran. The Bacteriology Laboratory staff of 7th-Tir Hospital of Tabriz, Iran are also much appreciated for their cooperation in the conventional bacteriology.

REFERENCES

1. Pallen MJ: The immunological significance of environmental mycobacteria on leprosy and tuberculosis control. Int J Lepr 52: 231-245, 1984.
2. Fine P: Leprosy and tuberculosis--an epidemiological comparison. Tubercle 65: 137-153, 1984.
3. Glaziou P, Cartel JL, Moulia-Pelat JP, Ngoc LN, Chanteau S, Plichart R, Groasset JH: Tuberculosis in leprosy patients detected between 1902 and 1991 in French Polynesia. Int J Lepr 61: 199-204, 1993.
4. Jopling WH, McDougall AC: Handbook of leprosy. Great Britain: Heinmann, 4th edition, p. 36, 1988.
5. Ganter EMS, Glatthaar E, Imkamp MFJH, Kok SH: Association of tuberculosis and leprosy in South Africa. Lepr Rev 51: 5-10, 1980.
6. Kumar B, Kaur S, Kataria S, Roy SN: Concomitant

occurrence of leprosy and tuberculosis–a clinical, bacteriological and radiological evaluation. Lepr India 54: 671-676, 1982.

7. Rafi A, Donoghue HD, Stanford JL: Application of polymerase chain reaction for the detection of *Mycobacterium leprae* DNA in specimens from treated leprosy patients. Int J Lepr 63: 42-47, 1995.
8. Stanford JL, Stanford CA, Ghazi-Saidi K, Dowlati Y, Weiss F, Farshchi Y, Madlener F, Rees RJW: Vaccination and skin test studies on the children of leprosy patients. Int J Lepr 57: 38-44, 1989.
9. Stanford JL, Bahr GM, Rook GAW, Shaaban MA, Chugh TD, Gabriel M, Al-Shimali B, Siddiqui Z, Ghartdani F, Shahin A, Behbahani K: Immunotherapy with *Mycobacterium vaccae* as an adjunct to chemotherapy in the treatment of pulmonary tuberculosis. Tubercle 71: 87-93, 1990.
10. Rolfs A, Schuller I, Finckh U, Weber-Rolfs I: PCR: clinical diagnostics and research. Berlin: Springer-Verlag, p. 75, 1992.
11. Victor T, Du Toit R, Van Halden PD: Purification of sputum samples through sucrose improves detection of *Mycobacterium tuberculosis* by polymerase chain reaction. J Clin Microbiol 30: 1514-1517, 1992.
12. 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 28: 495-503, 1990.
13. Eisenach KD, Cave MD, Bates JH, Crawford JT: Polymerase chain reaction amplification of repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J Infect Dis 161: 977-981, 1990.
14. 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 144: 1160-1163, 1991.
15. McFadden J: Molecular biology of mycobacteria. UK: Surrey University Press, pp. 139-172, 1990.
16. Skinsnes OK, Matsuo E, Chang PHC, Andersson B: *In vitro* cultivation of leprosy bacilli on hyaluronic acid based medium. 1. preliminary report. Int J Lepr 43: 193-203, 1975.

The contents appearing in this publication are indexed by PERIODICA ISLAMICA

For further information, please contact:
Dr. Munawar A. Anees, Editor-in-Chief, *Periodica Islamica*
31 Jalan Riong, Kuala Lumpur-59100, Malaysia
Tel (+60-3)282-5286 • Fax (+60-3)282-8489
eMail: America Online: *dranees* • CompuServe: *dranees*
Delphi: *drmanees* • InterNet: *dranees@klcyber.pc.my*
URL: *http://www.ummah.org.uk/dranees/periodica/*