THE DETECTION OF \textit{Mycobacterium tuberculosis} BY PCR IN SPITUM SAMPLES FROM LONG-TERM-TREATED LEPROSY PATIENTS: ASSOCIATIONS WITH SKIN TEST RESULTS AND IMMUNOTHERAPY WITH \textit{Mycobacterium vaccae}


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 \textit{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 \textit{Mycobacterium leprae}. Whereas immunotherapy with killed \textit{Mycobacterium vaccae} given nearly 2 years earlier to 23 of the patients strongly influenced PCR results for \textit{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.


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
PCR Detection of *M. tuberculosis* in Sputum Samples

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. 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.,
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, but two studies have suggested
that the presence of leprosy might encourage the development
of tuberculosis. 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 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 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
tubercins, and randomized to receive an injection of saline
as placebo or 10 fluid ml of *M. vaccae* plus tuberculin as
immunotherapy. The patients investigated in the present
study, and that previously reported, were selected from
among them.

**Skin-testing**

The 2 new tubercins 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. 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 intradermal 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.

**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
cases 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 (x 100) lens.

**Isolation of DNA from sputum**

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

<table>
<thead>
<tr>
<th>MB patients</th>
<th>BCG +</th>
<th>Skin-test results LA</th>
<th>IT/PE</th>
<th>First sampling TB-PCR</th>
<th>LEP-PCR</th>
<th>Second sampling TB-PCR</th>
<th>LEP-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PB patients

<table>
<thead>
<tr>
<th>MB patients</th>
<th>BCG +</th>
<th>Skin-test results LA</th>
<th>IT/PE</th>
<th>First sampling TB-PCR</th>
<th>LEP-PCR</th>
<th>Second sampling TB-PCR</th>
<th>LEP-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PB patients

<table>
<thead>
<tr>
<th>MB patients</th>
<th>BCG +</th>
<th>Skin-test results LA</th>
<th>IT/PE</th>
<th>First sampling TB-PCR</th>
<th>LEP-PCR</th>
<th>Second sampling TB-PCR</th>
<th>LEP-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MB patients</td>
<td>BCG +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Abbreviations used in the Table:**

Method of preparation of *M. tuberculosis* DNA as a positive control

Chromosomal DNA of *M. tuberculosis* was prepared and purified from a fresh culture of tubercle bacilli by a boiling method, was as used 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. from sequences repeated several times in the chromosome of *M. tuberculosis*. The sequences of the primers (synthesized by Osweol DNA Service, Edinburgh, UK) from 5' to 3' were:

CCTGCGAGCGTGGCGTCGG and CTGCGACGCGCCTTCGG which amplify a 123-bp fragment of the repetitive DNA sequence IS6110. PCR was performed on each sample using the method of Eisenach et al.

**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, 230 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
PCR Detection of *M. tuberculosis* in Sputum Samples

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 tubercle 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, 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 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 apparently shows the efficacy of the technique for the identification of paucibacillary situations for both tuberculosis 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 suggests 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* 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.

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**

6. Kumar B, Kaur S, Kataria S, Roy SN: Concomitant...


