

# *Mycobacterium tuberculosis* DNA in tissues affected by sarcoidosis

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## Abstract

**Background**—Although some studies have reported the presence of *Mycobacterium tuberculosis* (MTb) DNA in tissues affected by sarcoidosis, the data are conflicting. The aim of this study was to collect prospectively tissue from patients with sarcoidosis in whom tuberculosis had been excluded, and to use polymerase chain reaction (PCR) to search for DNA sequences specific for MTb.

**Methods**—Fresh tissue samples (node or lung biopsy) taken from 23 patients with newly diagnosed sarcoidosis, 10 with other respiratory disease, and four patients with culture positive tuberculosis were analysed using PCR to amplify a 123 bp fragment of IS6110, the insertion element present in MTb, and nested PCR to further amplify an 85 bp sequence within the 123 bp product. DNA was also extracted from formalin fixed tissue from eight additional patients with sarcoidosis.

**Results**—MTb DNA was not detected in any of the tissue samples from patients with sarcoidosis or other respiratory disease but was found in all four patients with tuberculosis.

**Conclusions**—This study has shown the absence of MTb DNA in lymph node and lung biopsy samples from patients with sarcoidosis. MTb is therefore unlikely to be a factor in the pathogenesis of this disease. (*Thorax* 1998;53:871-874)

Keywords: sarcoidosis; mycobacteria; tuberculosis

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Table 1 Characteristics of patients and controls

Patient group	No.	Mean (range) age (years)	Sex	Race	Mantoux status	MTb cultures
Sarcoidosis	31	43 (21-68)	12 F	24 E 3 M 3 PI 1 I	31 -ve	31 -ve
Control	10	46 (20-74)	3 F	9 E 1 M	5 -ve 5 ND	10 ND
Tuberculosis	4	37 (30-58)	4 F	3 I 1 PI	4 +ve	4 +ve

E = European; M = Maori; PI = Pacific Island; I = Indian; ND = not done; -ve = negative; +ve = positive.

mRNA encoding the cytokines interleukin 2 (IL-2) and granulocyte macrophage colony stimulating factor (GM-CSF) are increased at sites of disease activity, suggesting local activation of antigen specific T cells.<sup>3</sup>

*Mycobacterium tuberculosis* (MTb) has long been a likely contender as the inciting antigen in sarcoidosis, although Koch's postulates have never been fulfilled.<sup>4</sup> Where the organism is found in sarcoid-like granulomas, the diagnosis is usually considered to be tuberculosis. However, the tubercle bacillus may be present in a form without a cell wall and thus not detectable by conventional microbiological methods. Renewed interest in such organisms follows their detection in the blood of patients with sarcoidosis.<sup>5</sup>

Some investigators have found evidence of MTb in tissues using the polymerase chain reaction (PCR) to amplify mycobacterial DNA or rRNA<sup>6-8</sup> but more recent studies have failed to identify such organisms in the majority of tissue samples from patients with sarcoidosis.<sup>9-14</sup> However, the reports to date have not always provided clear clinical evidence supporting the diagnosis of sarcoidosis nor have they excluded patients with prior tuberculous disease or infection.

The aim of this study was to collect prospectively tissue from a carefully characterised cohort of patients with newly diagnosed sarcoidosis in whom tuberculous infection or prior disease had been excluded and to use PCR to search for DNA sequences specific for MTb.

## Methods

### PATIENTS AND CONTROLS

Fresh tissue samples were prospectively collected from a cohort of 23 patients with newly diagnosed sarcoidosis all seen at one outpatient clinic at one hospital. Patients were enrolled in the study if they fulfilled previously accepted clinical diagnostic criteria,<sup>15</sup> had evidence of non-caseating granulomas on biopsy specimens, a negative Mantoux test, and negative MTb cultures with no prior history of tuberculosis. In addition, formalin fixed paraffin embedded tissue was obtained from eight patients with an earlier diagnosis of sarcoidosis who were still being followed up. Ten patients with other respiratory disease undergoing lung biopsy or mediastinoscopy acted as negative controls, and four patients with tuberculosis and culture positive nodes acted as positive controls. Details of the 31 patients with sarcoidosis, the 10 negative controls, and the four positive controls are shown in table 1.

The patients with sarcoidosis had one or more of the following disease manifestations: hilar and mediastinal lymphadenopathy (27

patients), pulmonary (9), splenic (1), ocular (1), cervical node (1), hypercalcaemia (1). The chest radiographic appearances were as follows: stage I (22), stage II (7), stage III (2). Tissue for analysis was obtained from the following sites: mediastinal node (25), cervical node (1), open lung biopsy (2), transbronchial biopsy (3). Specimens from each patient were cultured for MTb and all were negative.

The diagnoses of the negative controls were as follows: carcinoma of the lung (4), metastatic seminoma (1), lymphoma (1), Castleman's disease (1), plasma cell granuloma (1), bronchiectasis (1), reactive lymphadenopathy (1). Tissue for analysis was collected prospectively from the following sites: mediastinal node (7), cervical node (1), open lung biopsy (2). Five patients had tissue cultured for MTb and all were negative. The four patients with tuberculosis all had lymph nodes sampled (three mediastinal, one supraclavicular) and all were culture positive. Tissue collected prospectively was stored in sealed vials at  $-70^{\circ}\text{C}$  in liquid nitrogen until analysis. Specimens were analysed in randomly selected groups, the Scientific Officer being blind to the diagnostic category of individual specimens. This study had the approval of the North Health ethics committee.

#### EXTRACTION OF DNA

When sufficient frozen specimen was available four aliquots from each specimen were processed and assayed. Each sample was suspended in 500  $\mu\text{l}$  of lysis buffer, final concentration 20 mM Tris HCl (pH 7.6), 20 mM ethylene diamine tetraacetic acid (EDTA), 20 mM sodium chloride, 1% sodium dodecyl sulphate (SDS), and 600  $\mu\text{g/ml}$  proteinase K (Boehringer Mannheim).

Samples were incubated at  $55^{\circ}\text{C}$  with shaking for 48–72 hours. DNA was purified by phenol/chloroform extraction and precipitated with ethanol. Precipitated DNA was washed once with 70% ethanol and dissolved in PCR clean double distilled water. The quantity and quality of DNA was calculated from absorbance measurements made with a Beckman spectrophotometer DU-64. Paraffin embedded samples were deparaffinised by two extractions with xylene followed by two absolute ethanol washes. Samples were then dried in a  $65^{\circ}\text{C}$  heat block and lysis buffer was added. Their subsequent treatment was identical to that for frozen samples.<sup>16</sup>

One negative extraction control (PCR clean double distilled water) for every specimen was processed to detect contamination. Positive extraction controls equivalent to 1 colony forming unit (CFU) and 10 CFU of MTb H37RV were processed with each batch of specimens. The positive controls acted as checks for adequate cell lysis, DNA purification, and activity of PCR reagents.

#### AMPLIFICATION OF DNA

PCR was performed on a Perkin Elmer Cetus DNA thermal cycler 480 according to standard methods.<sup>12</sup> Sample DNA equal to 1.0  $\mu\text{g}$  was added to each 50  $\mu\text{l}$  PCR reaction. All reactions contained 10 mM Tris HCl (pH 8.3), 50 mM

KCl, 200  $\mu\text{M}$  each of dATP, dCTP, dGTP and dTTP, 1.5 mM magnesium chloride, 1 unit *Taq* DNA polymerase (Gibco, BRL, Life Technologies) and 0.1  $\mu\text{g}$  of each primer. Primers IS1 and IS2 amplified a 123 bp fragment of IS6110, the insertion element present in mycobacteria of the MTb complex.<sup>17</sup> An aliquot of the first round amplification products (2  $\mu\text{l}$ ) was further amplified using nested primers N1 (5'-ACC ACCAGCACCTAACCG) and N2 (5'-TGAC AAAGGCCACGTAGGCG) which recognise an 85 bp sequence within the 123 bp product. As an alternative to the above primer sets TB1 (5'-CGCAAAGTGTGGCTAACCCCTGAA) and TB2 (5'-GGTCGAGTACGCCTTCTTGTTGG) which amplify a 376 bp fragment were used in conjunction with nested primers NT1 (5'-CGTGAGGGCATCGAGGTGGC) and NT2 (5'-CGCGTCGAGGACATGGAGG) which produce a 306 bp product.

PCR conditions for primers IS1 and IS2 were  $95^{\circ}\text{C}$  for three minutes followed by 50 cycles of  $95^{\circ}\text{C}$  for 40 seconds,  $68^{\circ}\text{C}$  for 40 seconds, and  $72^{\circ}\text{C}$  for 15 seconds. Alternative primers TB1 and TB2 had an initial denaturation at  $94^{\circ}\text{C}$  for three minutes followed by 50 cycles of  $94^{\circ}\text{C}$  for 50 seconds and  $70^{\circ}\text{C}$  for 50 seconds. Nested PCR with primers N1 and N2 had an initial denaturation of  $95^{\circ}\text{C}$  for three minutes followed by 30 cycles of  $95^{\circ}\text{C}$  for 40 seconds,  $61^{\circ}\text{C}$  for 40 seconds, and  $72^{\circ}\text{C}$  for 40 seconds. When nested primers NT1 and NT2 were used, the annealing temperature of the nested PCR was increased to  $68^{\circ}\text{C}$ .

A PCR reagent blank was included with each PCR assay.

#### DETECTION OF *M. TUBERCULOSIS* IN DNA

Aliquots of amplification products were electrophoresed into 2% agarose gels. Gels were stained with ethidium bromide and the results read by transillumination with UV light. The identity of bands running with the positive control were confirmed by Southern blotting and by hybridisation with  $^{32}\text{P}$ -labelled probe (oligonucleotide IS3).<sup>12</sup> Hybridised PCR products were detected by autoradiography, with overnight exposure to photographic film at  $-70^{\circ}\text{C}$  using an intensifying screen.

#### DETERMINATION OF PCR SENSITIVITY AND SPECIFICITY

To determine the sensitivity of the combined DNA extraction and PCR methods, cultures of MTb H37RV grown in Middlebrook 7M broth were diluted. Five identical aliquots of each dilution were made, three of which were cultured and two were assayed by PCR. For culture, aliquots were spread over Lowenstein-Jensen slopes, incubated at  $37^{\circ}\text{C}$  for six weeks, and the number of CFUs of MTb in each dilution was determined. For PCR, total DNA was extracted from each aliquot and assayed. DNA extracted from one CFU could be detected by first round PCR.

The sensitivity and specificity of PCR of MTb DNA in the presence of human DNA was also determined. When logarithmic dilutions of DNA purified from MTb H37RV were

each assayed in the presence of 1 µg of pooled human white blood cell (WBC) DNA, 5 fg of DNA (equivalent to one MTb bacillus)<sup>18</sup> could be detected by first round PCR. The  $A_{260}/A_{280}$  ratio of the purified MTb DNA was 1.77.

The specificity of PCR primers IS1 and IS2 was determined previously.<sup>17</sup> To determine the specificity of primers TB1 and TB2, 1 µg aliquots of DNA prepared from bacterial and mycobacterial cultures were assayed by PCR. No amplified product was detected for clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *M fortuitum*, *M simiae*, *M thermoresistible*, *M scrofulaceum*, *M chelonae* (three isolates) and *M avium* complex (three isolates). DNA from MTb H37RV and *M bovis* BCG and clinical isolates MTb (African) amplified to give a 376 base pair product. The identity of the product was confirmed by blotting and hybridisation with the oligonucleotide probe IS3.

#### DETERMINATION OF SPECIMEN DNA FRAGMENTATION AND PCR INHIBITION

DNA extracted from paraffin embedded specimens may be fragmented as a result of formalin fixation.<sup>16</sup> To confirm the integrity of DNA in these specimens, human DNA sequences of similar size to target mycobacterial DNA was amplified. Primers β2M1 (5'-TTAGCTGTGCTCGCGCTACTCTCT) and β2M2 (5'-CGCTTATCGACGCCCTAACTTGT) which amplify a 317 bp sequence in the human β<sub>2</sub>-microglobulin gene were used. Samples of DNA extracted from paraffin embedded specimens equal to 1.0 µg were added to each 50 µl PCR reaction which contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 200 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM magnesium chloride, 2 units of Taq DNA polymerase, and 0.2 µg of each primer. PCR conditions were 95°C for three minutes followed by 40 cycles of 95°C for 40 seconds, 61°C for 40 seconds, and 72°C for 40 seconds. Aliquots of amplification products were electrophoresed and compared with the product obtained by amplification of a positive control of 1 µg of pooled human WBC DNA. Decrease in signal strength of the specimen sample compared with the control sample was deemed to indicate fragmentation of specimen DNA.

To test for inhibition of the MTb PCR 5 fg and 50 fg of DNA purified from MTb H37 RV were added to two MTb PCR reactions each containing 1 µg of sample DNA and PCR assays for MTb were run as previously described. The absence of MTb amplicons was taken as evidence of inhibition.

#### Results

The results of PCR analysis are shown in table 2. Twenty six patients with sarcoidosis had intact DNA for analysis and an absence of inhibitory factors. No patients had MTb DNA detected in their tissue samples. The four tissue samples which yielded fragmented DNA were all wax embedded formalin fixed specimens. All nine negative controls with intact DNA had a negative PCR result. The four patients with tuberculosis were PCR positive.

Table 2 Results of MTb DNA PCR analysis

	No.	PCR +ve	PCR -ve	Specimen inhibitory	Fragmented DNA
Sarcoidosis	31	0	26	1	4
Control	10	0	9	0	1
Tuberculosis	4	4	0	0	0

Positive extraction controls (1 and 10 CFU) processed with each batch of specimens were always positive. All blank extraction controls were PCR negative.

Replicate tests yielded consistent results when answers of negative, inhibitory or fragmented DNA were obtained. Replicate tests for patients with tuberculosis were positive for one of four extractions (two patients), three of four extractions (one patient), and four of four extractions (one patient).

#### Discussion

In this study MTb DNA was not detected in tissue from patients with sarcoidosis. The majority of the specimens were lymph nodes sampled from patients with newly diagnosed untreated sarcoidosis which, on histological analysis, revealed abundant non-necrotising granulomas. We expected that, if MTb was involved in the pathogenesis of sarcoidosis, then these would be the samples most likely to yield positive MTb DNA. The validity of our assay was confirmed by finding MTb DNA in all samples from culture positive tuberculosis and from none of the negative controls.

The sensitivity of each assay was demonstrated by consistent extraction and detection of MTb DNA from the one CFU (extraction control) and by the ability of the PCR to detect DNA equivalent to a single MTb bacillus in the presence of 1 µg of human DNA. Sensitivity was in part dependent on the presence of multiple copies of IS6110 in the MTb genome. However, IS6110 may not be present or may be present as a single copy in 20% of MTb strains from patients of Vietnamese origin.<sup>19</sup> As far as we could ascertain, none of our patients had associations with Vietnam. Sensitivity of PCR reactions may also be affected by large amounts of DNA so we routinely restricted the total amount of DNA in the assay to 1.0 µg, equivalent to  $1.4 \times 10^5$  human cells per PCR reaction.<sup>12</sup> This PCR cannot therefore detect MTb at a concentration of less than one bacillus per  $1.4 \times 10^5$  human cells. The results for the positive control specimens indicate that MTb bacilli are not evenly distributed throughout the specimen. Thus, in our study the absence of positive results for patients with sarcoidosis could mean that sites containing bacilli have not been sampled or the organisms involved do not carry IS6110.

Ghossein *et al* also failed to detect mycobacterial DNA in formaldehyde fixed paraffin embedded tissues from 10 patients with sarcoidosis.<sup>14</sup> Thakker *et al*, using fixed tissue from 14 patients, identified mycobacterial DNA in one sarcoid lymph node but concluded that this was likely to be a contaminant because the node was preprocessed as a cryostat section and no mycobacterial DNA

was found in the remainder of the node which was immediately fixed.<sup>11</sup> Two further studies have shown that only a few patients with sarcoidosis have evidence of mycobacterial DNA in their tissues. Bocart *et al* studied tissue or cells from 16 patients with new or old sarcoidosis using similar technology to our study.<sup>12</sup> In an attempt to exclude false positives from contamination they required a sample to be positive at least three times in six PCR assays. Their analysis detected MTb DNA in samples from two patients, one of whom had past tuberculosis. The relationship of the earlier diagnosis of tuberculosis with the current diagnosis of sarcoidosis was not made clear. The second patient presented with Löfgren's syndrome but, in spite of negative tuberculous evaluation including a negative Mantoux test, all six PCR assays were positive. Richter *et al* identified one patient out of 24 with sarcoidosis in whom mycobacterial DNA could be found using PCR for amplification of DNA coding for 16Sr RNA.<sup>13</sup> The Mantoux status of this patient is not recorded and it is not clear whether tissue from this patient was cultured for mycobacteria.

Other studies provide additional conflicting data. Saboor *et al* detected MTb DNA in half of their sarcoidosis patients, but the false positive PCR rate was 9% and the authors failed to document the Mantoux status of their patients, although all had relevant MTb cultures.<sup>6</sup> Mitchell *et al* used liquid phase hybridisation to detect MTb rRNA in the spleens of patients with sarcoidosis and reported a 4.8 times greater likelihood of a positive result compared with control spleens.<sup>7</sup> The clinical details of the patients are sparse but tissue was cultured and negative for mycobacteria. Fidler *et al* also detected MTb DNA in patients with sarcoidosis but did not find the organism in two of four positive controls.<sup>8</sup> They had a problem with contamination although they addressed this by tightening the criteria for a positive result.

Graham *et al* identified mycobacteria found in skin lesions of patients with sarcoidosis as *M avium*<sup>9</sup> but the report is in letter form so patient details and numbers are not available. Likewise, Popper *et al* detected DNA from non-tuberculous mycobacteria including *M avium* in 11 of 35 patients with sarcoidosis but used only formalin fixed tissue. The insertion sequence IS6110 of the MTb complex was not detectable in any of these 11 cases.<sup>10</sup>

The debate about sarcoidosis MTb and mycobacteria is not over.<sup>20</sup> Cell wall deficient forms of mycobacteria have been postulated to be important in sarcoidosis. Such forms which were demonstrated to be similar if not identical to MTb have recently been grown from the blood of 19 out of 20 patients with sarcoidosis.<sup>5</sup> Seven of the 20 sarcoidosis patients were on systemic corticosteroids at the time of the study and were potentially immunosuppressed. These observations are nonetheless important and need to be explored by further studies including the use of molecular techniques to elucidate whether these organisms are atypical or tuberculous mycobacteria.

PCR enables tissue samples to be analysed with considerable sensitivity and a single organism can be detected. Unfortunately MTb may reside in tissue for years after infection and, unless the Mantoux response is known, prior infection status cannot be assumed. In New Zealand BCG vaccination of adolescents was routine until 1986 so the Mantoux response is less helpful in determining infection. The detection of mycobacterial DNA in lung or mediastinal nodes may simply reflect earlier infection by MTb rather than implicate the organism in the pathogenesis of sarcoidosis. Very careful clinical documentation and screening for tuberculous disease is necessary before concluding that the diagnosis is sarcoidosis and in our study we have done this.

In conclusion, our study has demonstrated the absence of MTb DNA in lymph node and lung samples from patients with sarcoidosis. We believe these findings contribute significantly to the evidence against MTb as a factor in the pathogenesis of sarcoidosis.

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