Diagnosis of cutaneous tuberculosis in biopsy specimens by PCR and Southern blotting

E Quirós, M C Maroto, A Bettinardi, I González, G Piédrola

Abstract

***Aims*—To evaluate the use of a gene amplification and hybridisation method for detecting mycobacterial nucleic acid as a possible diagnostic method for cutaneous tuberculosis infection.**

***Methods*—Biopsy specimens from 20 patients with various skin conditions of possible tuberculous aetiology were studied. Six patients had ulcerative nodules, seven lupiform lesions, two non-necrotic granulomas, one scrofulous lichen, one impetigo, one erythematosus lesions, one warty lesions, and one suspected tuberculous lipoma. Biopsy specimens were stained using Ziehl-Neelsen stain and cultured in Lowenstein-Jensen medium. DNA was extracted and then amplified by PCR using primers specific for the *Mycobacterium tuberculosis* complex. Specificity was confirmed by Southern blotting.**

***Results*—Of the specimens, 30% were positive for mycobacteria on staining with Ziehl-Neelsen stain, 60% were culture positive and 85% PCR positive. Only 35.2% of specimens were positive with all three techniques. A further 32.5% were both culture and PCR positive. All PCR negative samples were also negative when cultured or stained with Ziehl-Neelsen stain. Of the PCR positive specimens, 29.4% were negative when cultured or stained.**

***Conclusions*—PCR, using suitable primers, is an efficient and sensitive method for the diagnosis of cutaneous tuberculosis.**

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Keywords: cutaneous tuberculosis, PCR, Southern blotting.

Department of Microbiology, Medical School, University of Granada, Granada, Spain
M C Maroto
I González
G Piédrola

Combiotec, Brescia, Italy
E Quirós
A Bettinardi

Correspondence to:
Dr M C Maroto,
Department of Microbiology,
School of Medicine,
Avenida de Madrid, 11,
18012 Granada, Spain.

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Cutaneous conditions caused by *Mycobacterium tuberculosis* are relatively rare. Clinical expression may be exogenous or endogenous, the latter occurring when a tubercle lying beneath the skin releases the infection into the lymphatic system or blood stream.[1]

The clinical features are influenced by the virulence of the bacillus, the immunological state of the patient and non-specific factors such as age, race, general state of health, etc.[2,3] Given this clinical heterogeneity and the difficulties in diagnosing this condition (slow bacterial growth, ineffectiveness of antibody investigation and limited value of intradermoreaction), the need for further diagnostic markers of infection is evident.

Methods

The study population comprised 20 patients with diverse skin conditions of possible tuberculous aetiology. Six patients had ulcerative lesions, seven lupiform lesions, two non-necrotic granulomas, one scrofulous lichen, one impetigo, one erythematosus lesions, one warty lesions, and one tuberculous lipoma (table 1). Four patients were HIV positive intravenous drug users (cases 2, 10, 18, and 20). Only one patient (case 18) presented with active tuberculosis and another (case 10) cohabited with a partner who had presented with disseminated tuberculosis. Cases 2, 4, 5, 6, and 9 had painful axillary and inguinal nodules.

A skin biopsy specimen of the area affected was obtained from all patients, fixed in formalin and embedded in paraffin wax. Sections were stained with Ziehl-Neelsen stain and cultured in Lowenstein-Jensen medium (Becton Dickinson, Meylan Cedex, France), as described previously. For PCR and Southern blotting, sections (6 μm) were cut and dewaxed in xylene at 55°C for 10 minutes, centrifuged, washed with absolute alcohol, and then lyophilised. Prior to DNA amplification, the lyophilised samples were digested with a buffer containing 50 nmol Tris, 1 nmol acetic acid, 0.5% Tween 20 (pH 8.5), and 200 ng/ml proteinase K for four hours at 55°C with gentle shaking.

POLYMERASE CHAIN REACTION

After proteolytic digestion, samples were subjected to enzymatic gene amplification by PCR. The reaction mixture comprised 5–10 μl sample, 2 units Taq DNA polymerase (Promega, Madison, Wisconsin, USA), 50 pmol of each primer (positive primer: 5'-CAA-GGC-TTC-AAT-TCC-GGT-GAT-GCC- 3'; negative primer: 5'-TGG-TCC-GGT-TCA-TAC-TCG-GGC-TGG-3') and 60 nmol of each dNTP in PCR buffer (10 nmol Tris (pH 8.3), 50 nmol KCl, 4 nmol $MgCl_2$), made up to 60 μl. PCR conditions were as follows: 95°C for five minutes, 94°C for one minute and 70°C for one minute for 40 cycles.

The primers chosen amplify *M tuberculosis* complex DNA, including *M tuberculosis, M africanum,* and *M bovis* DNA, but not that of other mycobacterial species—for example, *M avium, M chelonae, M paratuberculosis, M fortuitum, M kansasii, M marinum, M terrae, M szulgai, M xenopi, M scrofulaceum,* and *M simiae.*

The specificity of PCR amplification was confirmed by running an aliquot of each PCR product on 2% agarose gels. PCR products

Table 1 Clinicopathological details of the patients studied

Case number	Lesion	Case history	AFB	Culture	PCR
1	Recurrent ulcerative nodule on leg	Leg fracture one year previously	−	+	+
2	Non-necrtotic granuloma on forearm	Painful axillary nodules; HIV positive	−	−	−
3	Recurrent ulcerative nodule on knee	Meniscus operation two years previously	−	−	+
4	Non-necrtotic granuloma on shoulder	Painful neck and axillary nodules	−	+	+
5	Ulcerative nodule on arm	Painful axillary nodules	+	+	+
6	Ulcerative nodules on arm	Painful axillary nodules	−	−	−
7	Scrofulous lichen on thigh		−	−	−
8	Lupus vulgaris on abdomen	Previous pulmonary tuberculosis	+	+	+
9	Impetigo on elbow	Painful axillary nodules	−	+	+
10	Lupus-like lesions on legs	Intravenous drug user; HIV positive; partner with disseminated tuberculosis	−	−	+
11	Lupus vulgaris on nose		−	−	+
12	Lupus vulgaris on earlobe		+	+	+
13	Ulcerative nodule on arm		−	+	+
14	Lupus vulgaris on leg		−	−	+
15	Lupus vulgaris on back		−	+	+
16	Tuberculous lipoma on face		−	+	+
17	Erythematosus patches on hand	History of inoculation with infected needle 15 days previously;	−	−	+
18	Ulcerative nodule	HIV positive; active pulmonary tuberculosis	+	+	+
19	Lupus vulgaris on ear		+	+	+
20	Warty lesion	HIV positive; history of pulmonary tuberculosis; diagnosed subsequently with Leishmaniasis	−	−	−

AFB = acid-fast bacillus.

Table 2 Results of staining, culture and PCR in skin samples studied. Results are expressed as n (%)

Method	Positive
AFB smear	6 (20)
Culture	12 (60)
PCR	17 (85)

AFB = acid-fast bacillus.

(285 base pair bands) were visualised by staining with ethidium bromide and viewing under ultraviolet light.

The usual precautions were taken to prevent cross contamination, and positive and negative controls were included in each PCR run. The positive control comprised DNA from a patient with known *M tuberculosis* infection (confirmed by culture) and was run at 10, 5 and 1 μg/ml. The negative controls comprised DNA from *M avium*, DNA from *M paratuberculosis*, a lymph node biopsy specimen (positive culture) from a patient infected with *M scrofulaceum*, a biopsy specimen of a *M leprae* lesion, tissue samples from patients with sarcoidosis, and tissue from a patient with lupus erythematosus.

SOUTHERN BLOTTING

Amplified samples were run on agarose gels and transferred to Hyband N+ nylon filters (Amersham, Little Chalfont, UK), according to the manufacturer's instructions. Filters were incubated for one hour at 58°C in prehybridisation solution containing 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 ng/ml denatured salmon sperm DNA, then hybridised overnight at 58°C with a ^{32}P 5' end labelled (about 1 × 10^6 cpm/ml) internal oligonucleotide probe (5'-TCA-GCG-GAG-AGC-CGG-TGA-ACT-TGT-C-3').

Filters were washed twice for 15 minutes each at room temperature in 2× SSC and 2% SDS, and then for 60 minutes at 58°C in 0.2% SSC and 0.2% SDS and autoradiographed overnight at 70°C. All chemical reagents were purchased from Sigma Diagnostics, St Louis, Missouri, USA.

Table 3 Comparison of results obtained on staining, culture and PCR

	AFB smear positive (n = 6)		AFB smear negative (n = 14)	
	Culture positive n (%)	Culture negative n (%)	Culture positive n (%)	Culture negative n (%)
PCR positive (n = 17)	6 (35.2)		6 (35.2)	5 (29.4)
PCR negative (n = 3)				3 (100)
Total	6		6	8

AFB = acid-fast bacillus.

Results

The results are presented in tables 2 and 3. As can be seen from table 3, the number of mycobacteria positive results increased from 30% on staining to 60% on culture and rose substantially to 85% on PCR and Southern blotting.

Of the 17 PCR positive specimens, six (35%) were culture positive only, and more importantly, five were negative when cultured or stained. All PCR negative samples were also negative when cultured or stained. No false negative results were observed and all controls gave the expected results.

Discussion

Concurrent with the AIDS epidemic, there has been a notable increase in the incidence of tuberculosis.[5–7] This increase in incidence results partly from the emergence of multiresistant strains and the evolution of diagnostic techniques, PCR being one example. PCR has been used repeatedly for detecting mycobacteria in sputum,[8] cerebrospinal fluid[9] and stool samples.[10] It has been used to increase diagnostic sensitivity,[11] to improve treatment by controlling resistance phenomena[12–14] and to facilitate characterisation of different *M tuberculosis* strains.[15]

Mycobacterial infection is notoriously difficult to diagnose on staining. In the present study the percentage of positive results on staining was very low compared with conventional culture in Lowenstein-Jensen medium, which in turn was not as sensitive as the PCR method. We were unable to compare our

results on skin samples with those of other authors, but we could compare results on other sample types—for example, pleural, bronchial, sputum. Schijman *et al*[16] detected mycobacterial DNA in 34% of samples; in the study by Kiehlbauch *et al*[17] 14% of samples were PCR positive but not all patients included in that study were thought to have mycobacterial infection. In spite of this, PCR was more sensitive than the conventional methods. Of the 20 skin lesions studied here, 29% were PCR positive but negative when stained or cultured. We consider these PCR positive results to be of great importance. With the advent of the AIDS epidemic the number of patients presenting with extrapulmonary tuberculosis is increasing. If a diagnosis of tuberculosis is suspected, but staining and culture are inconclusive, it is advisable either to increase the length of time specimens spend in culture or to search for mycobacterial DNA using PCR. In the PCR method described here, the specificity of the amplification reaction was confirmed by subjecting an aliquot of the PCR product to agarose gel electrophoresis followed by Southern blotting. The latter can also be used to characterise the strain present.[18 19] This method facilitates diagnosis of extrapulmonary tuberculosis and can be used readily in the routine diagnostic laboratory.[20]

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