Original Article

mRNA and DNA PCR tests in cutaneous tuberculosis

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ABSTRACT

Background: The microbiologic diagnosis of cutaneous tuberculosis is difficult because most lesions harbor only a small number of mycobacteria that cannot usually be detected by staining for the organism or by culture. Nucleic acid amplification tests based on the polymerase chain reaction (PCR) are potentially useful in this situation. **Aims:** To evaluate the utility of mRNA PCR and DNA PCR in the diagnosis of cutaneous tuberculosis. **Methods:** Biopsies from 28 cases of cutaneous tuberculosis and 19 controls with other diseases were subjected to microbiologic tests including direct smears for mycobacteria, culture and both mRNA PCR and DNA PCR. The laboratory was blinded to the clinical diagnosis. **Results:** None of the patients or controls showed a positive reaction on mRNA PCR test. Seven of 28 cases and 5 out of 19 controls showed a positive result on DNA PCR test yielding a sensitivity of 25% and a specificity of 73.7%. **Conclusion:** The results of PCR tests in cutaneous tuberculosis should be interpreted in the light of clinical and histopathological findings.

Key words: Laboratory diagnosis, nucleic acid amplification assays introduction, tuberculosis

INTRODUCTION

The diagnosis of cutaneous tuberculosis is a difficult task. Most of the time, this entity is diagnosed by its clinical presentation in combination with corroborative histopathological evidence. In association with clinical findings, histopathology is a useful diagnostic test. However, epithelioid cell granulomas may also be seen in other conditions such as sarcoidosis, leprosy, deep fungal infection, etc. which sometimes clinically also resemble cutaneous tuberculosis. The Mantoux test has high sensitivity but cannot be used in isolation as a diagnostic test as little information is available on the specificity of the test. In addition, a recent study has cast doubt on its value as a diagnostic aid in difficult cases.[1] The direct demonstration of *Mycobacterium tuberculosis* has very low sensitivity because most lesions are paucibacillary.[2-4] Culture of the organism from lesion of cutaneous tuberculosis is again highly specific but is usually tedious and unrewarding[3,5-6] though some studies have demonstrated substantially better results.[7-11] A therapeutic trial of antitubercular drugs is frequently used to confirm the diagnosis in difficult cases.[12,13]

In cutaneous tuberculosis, the sensitivity of DNA PCR technique has varied from 54% to 100%, and specificity from 80% to 100%.[14-16] In sputum samples, mRNA PCR has been used to monitor therapeutic efficacy and/or susceptibility to antibacterial agents.[17]

We report our experience with the DNA and mRNA PCR techniques in the diagnosis of cutaneous tuberculosis.

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METHODS

Patients and controls were recruited from patients presenting to the dermatology department, All India Institute of Medical Sciences, New Delhi between

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June 2006 and April 2008. All patients and controls were biopsied with a new disposable punch and 2 biopsies were taken. One biopsy was subjected to histopathological examination using standard techniques and the second biopsy was coded and transferred immediately on ice for microbiological tests. Mantoux test was done in all patients with intradermal injection of 5 TU of purified protein derivative (Span Diagnostics, Surat, India) on the volar aspect of forearm and the induration was measured after 2 days. Baseline investigations were done in all patients including haemogram with ESR, liver function tests, renal function tests and chest X-ray. Other investigations to look for underlying tuberculosis were done as indicated by the clinical presentation.

Patients were treated with short-course antitubercular regimen consisting of isoniazid 300 mg daily, rifampicin 450 mg daily, ethambutol 800 mg daily and pyrazinamide 1500 mg daily for 2 months followed by isoniazid and rifampicin in the same doses for 4 months. The dose was adjusted according to weight in case of paediatric patients. The regimen was slightly modified in 2 cases due to hepatic side effects. All patients were followed up every 4 weeks until 4 weeks after the completion of treatment.

Definition of Cases

Patients who fulfilled the following criteria were considered as cases:

1. Culture showing Mycobacterium tuberculosis.

or

2. Clinical improvement within 6 weeks of starting antitubercular treatment, and one or both of following:
   a) Biopsy showing epithelioid cell granulomas.
   b) Mantoux test showing an induration of 10 mm or greater at 48 h.

Definition of Controls

Twenty one patients with adequate proof of another granulomatous disease on clinical, histopathological, microbiological and/or therapeutic grounds were taken as controls including leprosy, post kala azar dermal leishmaniasis, deep fungal infection, actinomycetoma, eumycotic mycetoma etc.

Blinding

The biopsy taken for microbiological tests was sent coded without a clinical diagnosis. The results of these tests were not available for and were not utilized in clinical evaluation or therapeutic decision making.

Microbiological Tests

The second biopsy was immediately transferred to the microbiology laboratory on ice and processed for direct microscopy by carbol fuchsin staining using Ziehl-Neelsen method, culture for mycobacteria using Lowenstein-Jensen medium and the radiometric BACTEC 460 system using Middlebrook 7H9 media, mRNA PCR and DNA PCR.

mRNA PCR

Sample processing and RNA extraction:

An aliquot of sample was frozen at -70°C as soon as it was received in the laboratory. The sample was then homogenized by vortexing with N-acetyl-l-Cysteine (NALC) solution in phosphate buffer. One ml of the homogenized sample was centrifuged at 3000 rpm for 15 min. The pellet was washed with DEPC (diethyl pyro carbonate) water. The pellet was suspended in 250 µl of DEPC water and freeze-thawed 3 times (-70°C$_{\leftrightarrow}$37°C). Five micro litres of 10 mg/ml of proteinase K was added and inoculated at 65°C for 20 min. 750 µl of trizol (3 times the initial volume = 250 µl) was added to and pipetted up and down several times to facilitate lysis. The mixture was left at room temperature for 10 min. 200 µl of chloroform was then added, mixed well, incubated at room temperature for 10 min and centrifuged at 12000 rpm. Aqueous layer was separated and added to 500 µl of chilled isopropanol, 1 µl of glycogen was added and kept for precipitation overnight at -20° C. Pellet was separated by centrifuging at 12000 rpm for 15 min. Pellet was washed with freshly prepared 70% ethanol by centrifuging at 12000 rpm for 5 min, dried and dissolved in 10 µl of DEPC water.

Reverse-transcription of mRNA:

Reverse transcription of the extracted RNA was carried out by high efficiency cDNA archive kit in a 20 µl reaction using random hexamer, reverse transcriptase and dNTPs to yield the first strand of cDNA. Primers for the 85 A antigen of M. tuberculosis were used for the assay.

Polymerase chain reaction:

cDNA was further amplified using gene specific primer, *Taq* polymerase and dNTP's. PCR was carried out in nested manner using two sets of primers (outer and inner).

DNA PCR

Details of the procedure were published previously.[18] Briefly, the sample was decontaminated using NaOH and N-acetyl-l-Cysteine (NALC). This was followed by extraction of DNA using heat lysis and chloroform. DNA obtained was amplified using gene specific primer, Taq polymerase and dNTP's. A 240 bp long region of the MPB64 gene of M.tuberculosis was amplified using primers MPB1 (5'-TCC GCT GCC AGT CGT CTT CC-3') and MPB2 (5'- GTC CTC GCG AGT CTA GGC CA -3').

Ethical clearance to conduct the study was obtained from the Ethics Committee, All India Institute of Medical Science, New Delhi.

RESULTS

Patients

Twenty nine cases of cutaneous tuberculosis were recruited. The age of the cases varied from 6 to 70 years (mean 27.8 ± 16.2 years). There were 11 men and 18 women. Thirteen patients with well defined erythematous plaques were diagnosed as lupus vulgaris. Scrofuloderma was diagnosed in 10 patients with discharging sinuses with undermining bluish to black colored margins. Verrucous hyperkeratotic erythematous plaques on dorsum of foot, thumb and fifth toe were diagnosed as tuberculosis verrucosa cutis in 3 patients.The duration of disease before diagnosis ranged from 1.5 months to 30 years with mean duration of 34 months. On histopathology epithelioid cell granulomas were seen in biopsies from 27 patients; they were absent in 2 patients.

Mantoux test showed an induration greater than 10 mm in 28 out of 29 patients. One patient showed no induration. Nineteen patients completed the treatment and there was complete resolution. Ten patients could not be followed up till the completion of the treatment; however all 10 had shown satisfactory improvement during antitubercular therapy. We recorded the time after onset of treatment at which clinically evident response was first noted. This was recorded in 28 patients; one patient did not follow-up after starting treatment. In 14 out of 28, a clinically evident response was first noted between 21-30 days (mean 30.71 days). Time to complete healing was recorded in 18 patients and ranged from 60 to 270 days (mean 128 days). In 25 patients, there were no side effects. Mild dyspepsia controlled with antacids was observed in 2 patients. Transaminitis was seen in 2 patients and required modification of the regimen. In one patient rifampicin was stopped and isoniazid, ethambutol and levofloxacin were given for 9 months; this patient completed the treatment with complete healing. The other patient had pyrazinamide induced hepatitis; the drug was replaced with ofloxacin while remaining drugs, i.e. isoniazid, rifampicin and ethambutol were continued. This patient had received treatment for 5 months to date and showed marked improvement. The serum transaminases in both patients returned to normal after 58 and 18 days.

Thus, all the patients fulfilled the criteria for inclusion as cases. Twenty six patients showed clinical features, prompt treatment response, Mantoux test ≥10 mm and epithelioid cell granulomas on biopsy. In two patients clinically suggestive of scrofuloderma and lupus vulgaris, biopsy did not reveal epithelioid cell granulomas but the Mantoux reading was more than 10 mm and both responded promptly to treatment. In one patient with a clinical diagnosis of tuberculosis verrucosa cutis, Mantoux test did not show any induration but there were epithelioid cell granulomas in the biopsy and there was a prompt response to anti-tubercular therapy.

Twenty one controls were recruited. Seven out of 21 were diagnosed as leprosy, 5 as post kala azar dermal leishmaniasis, 2 as actinomycetoma. Other patients in the control group had diagnoses of chromoblastomycosis, lupus miliaris disseminatus faciei, eumycetoma, subcutaneous phycomycosis and cutaneous Rosai-Dorfman disease.

Microbiological Tests

Results of microbiological tests were decoded in 2 installments, for 21 cases and 9 controls on 14th September 2007 and for 7 cases and 10 controls on 8th and 15th April 2008. Samples for microbiological test were sent in 29 cases and 21 controls. However, samples were misplaced in one case and 2 controls. None of the cases and controls showed acid fast bacilli on the smear from skin biopsy. None of the cases and controls showed any growth on the L-J medium or in the BACTEC system.

Nucleic Acid Amplification Tests

DNA-PCR was positive for *Mycobacterium tuberculosis* in 7 out of 28 cases. DNA-PCR for *Mycobacterium tuberculosis* was positive in 5 out of 19 controls. The diagnoses in the controls who were positive were borderline lepromatous leprosy, borderline tuberculoid leprosy, cutaneous Rosai-Dorfman

disease, subcutaneous phycomycosis and post kala azar dermal leishmaniasis.

mRNA-PCR was negative in all cases and controls.

DISCUSSION

Conventional microbiological techniques for the diagnosis of cutaneous tuberculosis have limitations. This prompted the use of molecular techniques for diagnosis. DNA-PCR has been evaluated as a tool in the diagnosis of various forms of cutaneous tuberculosis, but it has variable sensitivity.[14-16,19-27] It is also a relatively expensive test.

mRNA-PCR was originally developed for the monitoring of drug sensitivity of *M. tuberculosis*. Using this technique Desjardin *et al.,* reported 100% sensitivity in baseline pulmonary samples and monitored the disappearance of mRNA with treatment.[17] This prompted our evaluation of the test for the diagnosis of the tuberculosis. In our study, mRNA was not detected in any of 28 cases of cutaneous tuberculosis. Cutaneous tuberculosis is a paucibacillary form of tuberculosis and this could be one of the reasons for negative test results. Jou *et al.*, evaluated the limit of detection of RT–PCR using the same target as we did, i.e., 85B antigen of mycobacteria. They found that the technique was able to detect mRNA in *M. tuberculosis* culture aliquots that contained as few as 38 CFU (colony forming units). The technique was able to detect *M.tuberculosis* in clinical samples that contained as few as one to nine bacilli per high power field on flourochrome staining and 12 CFU on the culture.[28] In smear-negative samples (1 to 1,000 bacilli/ml), the assay showed a sensitivity of 44.83%. In extrapulmonary tuberculosis including skin, synovial fluid and tissue, pus, CSF, urine, menstrual blood and bone marrow aspirate, 64 samples out of 88 were positive for mRNA-PCR.[29] It is possible that the submitted skin samples in our study contained fewer than the detection threshold of the test. Non viability of the M. tuberculosis in our samples could be another reason for RT PCR to be negative. This is borne out by the fact that none of our samples were positive for culture on LJ medium, as well as with the BACTEC system. The quantity of the sample we sent for the microbiological test could be a possible reason for low and negligible sensitivity since one 4 mm biopsy was used for the all microbiological tests including Z-N staining, culture on L J medium, BACTEC culture, DNA-PCR and mRNA-PCR. Another possible explanation for RNA PCR to be negative could be the labile nature of mRNA.[28]

The utility of DNA-PCR based on the MBP64 gene is well established in both pulmonary and extrapulmonary tuberculosis from our laboratory.[18,30] The sensitivity of DNA-PCR for *M.tuberculosis* in the current study was found to be 25%. Specificity of DNA-PCR for *M.tuberculosis* was found to be 73.68%. Reasons for low sensitivity could be the predominantly paucibacillary nature of cutaneous tuberculosis. False positivity of DNA-PCR in the controls is difficult to explain. It is possible that cases of cutaneous tuberculosis were mis-diagnosed and labeled as controls. However, this appears unlikely as the evidence for an alternate diagnosis was based on multiple criteria: clinical, histopathological and response to therapy. It is possible that the controls had, in addition to the diagnosed skin condition, a co-existing infection with tuberculosis at another site. Mycobacteria from the distant focus may have been seeded to the skin and picked up on the molecular test. We did not rigorously exclude tuberculosis at all sites, but this appears to be a distant possibility. False positivity could also be attributed to contamination. We used new disposable punches for each patient, aseptic precautions were taken during the biopsy procedure and the sample was transferred immediately in a sterile vial on ice to the laboratory. Another possible explanation for low specificity could be the contamination at the laboratory level. But once again, standard laboratory protocol was stringently implemented and this too appears unlikely. Another limitation of our study is the relatively small sample size.

Other workers have reported their results with DNA PCR in cutaneous tuberculosis. Most studies used an assay based on the IS6110; there are 4 to 20 copies of this element scattered throughout the genome of M. tuberculosis. Margall *et al.,* found a sensitivity of 77.1% and specificity of 100% with the assay.[15] Tan *et al*, showed that the PCR technique was 100% sensitive and specific in multibacillary cutaneous tuberculosis but in paucibacillary tuberculosis, DNA-PCR positivity rates were 55% for tuberculosis verrucosa cutis (38 cases) and 60% for lupus vulgaris (5 cases). The overall sensitivity was 73%, when confirmed cases of tuberculosis were considered.[16] In other studies the sensitivity was found to be less. Sentrunk *et al*, found that 1 out of 23 cases and 1 out of 22 controls were positive yielding a sensitivity of4.5% and specificity of 96.5%.[25] In this context, it should be noted that the PCR technique has shown variability of results when it was compared among different laboratories.[31,32]

Our study indicates that the sensitivity of DNA PCR (25%) is greater than that of mRNA PCR (0%) in the diagnosis of cutaneous tuberculosis. However, since the specificity of the former test is 73.7%, it is advisable to interpret test results in the context of the clinical and histopathological findings.

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