Comparison of in house polymerase chain reaction with conventional techniques for the detection of *Mycobacterium tuberculosis* DNA in granulomatous lymphadenopathy

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Abstract

Aims—To evaluate the usefulness of the devR based polymerase chain reaction (PCR) in the detection of *Mycobacterium tuberculosis* in lymph node aspirates and tissues of lymphadenitis and to compare PCR with conventional diagnostic techniques.

Subjects and methods—Coded specimens of fine needle aspirates and biopsies from 22 patients with tuberculous lymphadenitis, 14 patients with non-tubercular lymphadenitis, and nine patients with granulomatous lymphadenitis were processed and subjected to analysis by PCR, smear microscopy, *M tuberculosis* culture, histology, and cytology.

Results—Tuberculous lymphadenitis was correctly diagnosed by PCR in 18 patients, by culture in five patients, by histology in 13 patients, and by cytology in seven patients. PCR gave two false positive results in 14 patients with non-tubercular lymphadenitis. The sensitivity of the conventional techniques was significantly higher with biopsies (17 of 22 specimens; 77%) than with fine needle aspirates (nine of 22 specimens; 41%). However, the sensitivity of PCR was not significantly higher with biopsies (68%) in comparison with fine needle aspirates (55%). The sensitivity of either biopsy PCR or fine needle aspirate PCR was not significantly different from that of either histology combined with culture or cytology combined with culture. The overall combined specificity of PCR was 86%. *Mycobacterium tuberculosis* DNA was detected in six of nine patients with granulomatous lymphadenitis.

Conclusion—PCR is the most sensitive single technique available to date for the demonstration of *M tuberculosis* in specimens derived from patients with a clinical suspicion of tuberculous lymphadenitis. The value of PCR lies in its use as an adjunct test in the diagnosis of tuberculous lymphadenitis, particularly in those patients where conventional methods fail. Because fine needle aspiration is not an invasive procedure, it is the procedure of choice, and PCR should be performed initially on these samples. Excisional biopsy histology and PCR should be recommended only for patients in whom fine needle aspirate PCR is negative or when

there is discrepancy with the clinical impression.

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Tuberculosis (TB) of the lymph node (tuberculous lymphadenitis) is the most common form of extrapulmonary TB.¹⁻³ In developing countries where the incidence of TB is high, tuberculous lymphadenitis is one of the most frequent causes of lymphadenopathy (30-52%).⁴ In contrast, in developing countries, TB was implicated in as few as 1.6% of patients with lymphadenopathy in one series.⁴⁻⁶ Tuberculous lymphadenitis also occurs with an increased frequency in patients with human immunodeficiency virus (HIV).⁷⁻⁸ Granulomatous lymphadenopathy has an extensive differential diagnosis. Conditions including sarcoidosis, carcinoma, lymphoma or sarcoma, viral or bacterial adenitis, fungal disease, toxoplasmosis, cat scratch fever, collagen vascular diseases, and diseases of the reticuloendothelial system can present the same cytology or histopathology as tuberculous lymphadenitis. Traditionally, the diagnosis of tuberculous lymphadenitis has been established by histopathology and smear microscopy, or by mycobacterial culture on biopsy specimens. Over the past decade, fine needle aspirate cytology has assumed an important role in the evaluation of peripheral adenopathy as a possible non-invasive alternative to excisional biopsy. The cytological criteria for diagnosis of tuberculous lymphadenitis have been clearly defined as being epithelioid cell granulomas with or without multinucleate giant cells and caseation necrosis.⁴⁻⁷⁻⁹⁻¹⁰ The diagnosis is confirmed by the presence of acid fast bacilli (AFB) and by isolation of *Mycobacterium tuberculosis* on culture. The detection rate for *M tuberculosis* from fine needle aspirates is low by microbiological techniques. Therefore, there is a definite need for improving the sensitivity of tuberculous lymphadenitis diagnosis in fine needle aspirates. The AFB positive rate has ranged between 15% and 47%, depending upon the absence or presence of necrosis in patients with documented TB.¹⁰ AFB cultures were positive in 35% to 65% of patients in published studies.⁹⁻¹¹⁻¹² Moreover, culture takes six to eight weeks and causes an inordinate delay in the initiation of treatment.

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Nucleic acid amplification techniques, notably the polymerase chain reaction (PCR), have been used for the detection of *M tuberculosis* in spite of the considerable demands in terms of technical skills, equipment, and cost. This is largely because of the ability of PCR to increase the sensitivity and decrease the time necessary to detect *M tuberculosis* in clinical specimens.¹³ Several reports have described the application of PCR to the diagnosis of pulmonary TB from sputum specimens.^{13–15} In contrast, there is considerably less experience with the direct detection of *M tuberculosis* in extrapulmonary specimens. It is extrapulmonary TB such as tuberculous lymphadenitis, pleural effusion, or tuberculous meningitis for which a rapid, sensitive, and specific diagnosis is needed, owing to the limitations of the traditional microbiological methods (paucibacillary nature of specimens) and the extensive differential diagnosis (see above). Many reports, including those from our laboratory, have demonstrated the value of PCR in the diagnosis of extrapulmonary TB, including lymphadenitis.^{16–25}

In view of the need for a rapid, specific, and sensitive diagnosis of tuberculous lymphadenitis, our study was undertaken with the following objectives: (1) to assess the relative usefulness of cytology versus histology in the diagnosis of tuberculous lymphadenitis; (2) to evaluate the usefulness of the *devR* based PCR test and to compare it with the laboratory gold standard comprising histology/cytology combined with culture; (3) to compare the sensitivity of the PCR test on fine needle aspirates with that on biopsy tissue from the same patients (because fine needle aspirates of lymph nodes appear to be the procedure of choice); and finally (4) to evaluate the role of PCR in the detection of *M tuberculosis* DNA in granulomatous lymphadenitis.

Patients and methods

PATIENTS

Forty five (20 male and 25 female) patients with peripheral lymphadenopathy clinically suspected to be of tuberculous origin were included in our study. The clinical symptoms suggestive of tuberculosis were fever, anorexia or weight loss, and lymphadenopathy. Patients with purely mediastinal lymphadenopathy and those unwilling to undergo the fine needle aspiration or biopsy procedure were excluded. The laboratory diagnosis of tuberculous lymphadenitis was made if either tissue or aspirate revealed AFB on Ziehl-Neelsen (ZN) staining, and/or grew *M tuberculosis* on culture, and/or revealed caseating granulomas with or without AFB on histological or cytological examination. Those in the non-tubercular lymphadenitis control group were negative for TB on the basis of histopathological examination/fine needle aspirate cytology/AFB smear/*M tuberculosis* culture, or were diagnosed to have another disease. Granulomatous lymphadenitis was diagnosed if histopathology/fine needle aspirate cytology showed granulomas without AFB on ZN staining and culture did not grow *M tuberculosis*. The duration of lymphadenopathy

at presentation varied from 10 days to 20 months, the mean duration being 3.2 months. Lymph nodes from a variety of locations were biopsied. The most common site was supraclavicular, followed by posterior cervical triangle and anterior triangle/axilla. On the basis of the criteria given above, the patients were divided into three groups. All specimens were collected from the medical and surgical outpatient departments and wards of the All India Institute of Medical Sciences, New Delhi. Informed written consent for the procedures (fine needle aspiration and biopsy) was obtained from each patient.

FINE NEEDLE ASPIRATES

Fine needle aspirates from the involved lymph node were divided into five portions. One portion was smeared on a slide, fixed immediately with 95% alcohol, and subjected to Papanicolaou staining. Two other portions were made into slide smears, air dried, and subjected to ZN and May Grünwald Geimsa staining. The smears were considered positive for TB if epithelioid cell granulomas, with or without multinucleated giant cells and caseation necrosis, were found or if AFB were seen. The fourth portion was immediately frozen at -20°C for PCR. The fifth portion was stored at 4°C (maximum four days) before culture on Lowenstein-Jensen (LJ) medium.

LYMPH NODE BIOPSIES

After fine needle aspiration, the lymph nodes were excised and divided into three portions. One part was fixed in 10% formalin, embedded in paraffin wax, sectioned, and subjected to histopathological examination and AFB staining. Haematoxylin and eosin stained sections were examined for evidence of TB or other disease. The remaining two portions were suspended in normal saline and stored at -20°C for PCR and at 4°C (maximum four days) for culture, respectively.

CULTURE

Fine needle aspirates were decontaminated, digested by standard procedures, inoculated on to LJ slants, and incubated at 37°C . Biopsy samples were homogenised, decontaminated and digested by standard procedures, cultured on LJ slants, and subjected to biochemical testing for *M tuberculosis*.

SAMPLE PREPARATION FOR PCR

A portion of 0.5 cm^3 of biopsy material was minced with a sterile blade in a sterile petri dish and homogenised in $200\text{ }\mu\text{l}$ of TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0) with the help of a pellet pestle (Kontes Glass Company, Vineland, New Jersey, USA) in a sterile 1.5 ml Eppendorf tube for five minutes at room temperature. Twofold larger aliquots were used for specimens that were reprocessed for repeat PCR. Fine needle aspirates were thawed from -20°C and $200\text{ }\mu\text{l}$ aliquots were taken for further processing. The volume was made up to $500\text{ }\mu\text{l}$ with TE buffer for both sample types. The diluted samples were heated at 80°C for 20 minutes and incubated at 37°C

for two hours after adding 50 µl of lysozyme (final concentration, 4.5 mg/ml). The lysozyme treated samples were incubated at 65°C for two hours in the presence of 1.6% sodium dodecyl sulphate (SDS) and 200 µg/ml of proteinase K. NaCl was added to the sample, to a final concentration of 700 mM, and phenol/chloroform (1/1 (vol/vol)) extraction was performed twice. To remove inhibitors, 80 µl of 0.27 M cetyltrimethylammonium bromide (CTAB)/0.7 M NaCl was added to the resulting aqueous phase, which was heated at 65°C for 10 minutes and extracted with 700 µl of chloroform/isoamylalcohol (24/1 (vol/vol)). This step was repeated twice. DNA was precipitated by adding 0.6 volumes of isopropanol to the aqueous phase. After overnight storage at -20°C, the DNA was collected by centrifugation at 15 850 ×g in an Eppendorf centrifuge, washed once with 70% ethanol, resuspended in 40 µl of water, and stored at -20°C.

POLYMERASE CHAIN REACTION

PCR was performed on all specimens using primers devRf (5'-GGTGAGGCGGGTTCG GTCGC-3') and devRr (5'-CGCGGC TTGCGTCCGACGTTTC-3') to amplify a 513 bp product of the devR gene.²⁰ Briefly, a 40 µl reaction was set up containing 0.5 µM of each of primers devRf and devRr, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 U of Taq DNA polymerase, and 5 µl of specimen DNA, added either neat or as 1/10 and 1/50 dilutions. In parallel, inhibitor check reactions were set up containing *M. tuberculosis* DNA and specimen aliquots (5 µl each of neat specimen and 1/50 dilution). The following profile was used for PCR: 10 minutes at 94°C, followed by 35 cycles each of 60 seconds at 94°C and 90 seconds at 70°C, with a final extension time of 10 minutes at 72°C. All the steps of sample processing and PCR were carried out using dedicated pipettes and filter guard tips to prevent cross contamination. PCR products were electrophoresed on a 1% agarose gel, visualised by ethidium bromide staining, transferred to a positively charged nylon membrane (Boehringer-Mannheim, Mannheim, Germany), and hybridised with γ-³²P labelled internal oligonucleotide devR1 (5'-CCGTCCAGCGCCACATCTTT-3') at 55°C in 5× saline sodium citrate (SSC), 20 mM sodium phosphate, pH 7.0, 10× Denhardt's solution, 7% SDS, and 200 µg/ml of salmon sperm DNA. The membrane was washed twice at room temperature with 2× SSC/0.1% SDS and twice at 56°C for 15 minutes with 0.2× SSC/0.2% SDS, and exposed to x ray film at -70°C for 16 hours.

ANALYSIS

The results of culture, AFB smear, histology, cytology, and PCR were compared with the final diagnosis of TB using individual patients as the unit of analysis (table 1) and separately using individual specimens as the unit of analysis (table 2). Significance was determined by the χ^2 test with Yates's correction. The PCR results were classified as true positives (T_p),

Table 1 Results of culture, AFB smear, histology, cytology, and PCR analysis for 45 patients with lymphadenitis

Method*	Number of patients positive/number of patients tested (%)		
	TBLN (n = 22)	N TL (n = 14)	GLA (n = 9)
Culture	5/22 (23)	0/14	0/9
AFB smear	8/22 (36)	0/14	0/9
Tissue histology	13/22 (59)	0/14	7/9
FNA cytology	7/22 (32)	0/14	5/9
PCR	18/22 (82)	2/14 (14)	6/9

*Results of biopsy and FNA have been combined.

p < 0.0002 for PCR v culture; *Mycobacterium tuberculosis* was cultured from biopsy and FNA in one patient only.

p < 0.005 for PCR v AFB smear, AFB were detected in biopsy and FNA in one patient only.

AFB, acid fast bacilli; FNA, fine needle aspirate; GLA, granulomatous lymphadenitis; NTL, non-tubercular lymphadenitis; PCR, polymerase chain reaction; TBLN, tuberculous lymphadenitis.

Table 2 Results of culture, AFB smear, histology, cytology, and PCR analysis for 90 specimens of lymph node aspirate and lymph node tissue

Method	Number of positive specimens/number of specimens tested (%)		
	TBLN (n = 22)	N TL (n = 14)	GLA (n = 9)
Culture			
Overall	6/44 (15)	0/28	0/18
Tissue biopsy	4/22 (19)	0/14	0/9
FNA	2/22 (9)	0/14	0/9
AFB smear			
Overall	9/44 (20)	0/28	0/18
Tissue biopsy	5/22 (23)	0/14	0/18
FNA	4/22 (18)	0/14	0/18
Tissue histology	13/22 (59)	0/14	7/9 (78)
Histology + culture	17/22 (77)	0/14	7/9 (78)
FNA cytology	7/22 (32)	0/14	5/9 (56)
Cytology + culture	9/22 (41)	0/14	5/9 (56)
PCR			
Overall	27/44 (61)	2/28 (7)	9/18 (50)
Tissue biopsy	15/22 (68)	1/14 (7)	4/9 (44)
FNA	12/22 (55)	1/14 (7)	5/9 (56)

p < 0.00001 for overall PCR v overall culture.

p < 0.002 for overall PCR v overall AFB smear.

p < 0.05 for histology + culture v cytology + culture.

p not significant for biopsy histology + culture v biopsy PCR.

p not significant for FNA cytology + culture v FNA PCR.

Final results after repeat PCR are provided in the table; after the first PCR 10 each of FNA and biopsy specimens were positive and 13 patients with TBLN were positive.

AFB, acid fast bacilli; FNA, fine needle aspirate; GLA, granulomatous lymphadenitis; NTL, non-tubercular lymphadenitis; PCR, polymerase chain reaction; TBLN, tuberculous lymphadenitis.

true negatives (T_n), false positives (F_p), and false negatives (F_n). Sensitivity was calculated as: $T_p / (T_p + F_n) \times 100$, specificity was calculated as: $T_n / (T_n + F_p) \times 100$, and efficiency was calculated as: $(T_p + T_n) / N \times 100$, where N = 36.²¹ Positive predictive value was calculated as T_p obtained/total number of positives; negative predictive value was calculated as T_n obtained/total number of negatives.

Results

There have been several investigations of the individual usefulness of excisional biopsy histology and fine needle aspirate cytology for the diagnosis of tuberculous lymphadenitis.²²⁻²⁵ However, we believe that this report is the first attempt to compare these diagnostic modalities in the same cohort of patients and to evaluate the usefulness of PCR therein. This blind study included 45 patients, of whom 22 were diagnosed as having tuberculous lymphadenitis, 14

as having non-tuberculous lymphadenopathy (including nine patients with reactive lymph node, two with non-Hodgkin's lymphoma, and one each of squamous cell carcinoma, amyloidosis, and lymphoma) and nine patients who were classified as granulomatous lymphadenitis on the basis of granulomas without AFB and the absence of *M tuberculosis* on culture. From a laboratory perspective, a total of 44 TB lymph node specimens (22 each of fine needle aspirates and biopsies), 28 non-tuberculous lymphadenitis control lymph node specimens (14 each of fine needle aspirates and biopsies), and 18 granulomatous lymphadenitis lymph node specimens (nine each of fine needle aspirates and biopsies) were analysed in our study. Because the physician integrates the results of all procedures before pronouncing a diagnosis, the PCR results were compared with the gold standard, which is the results of all tests combined.

Biopsy histology diagnosed 13 of 22 patients with tuberculous lymphadenitis, whereas fine needle aspirate cytology only diagnosed seven. Only two patients with tuberculous lymphadenitis were diagnosed by both histological and cytological criteria. *Mycobacterium tuberculosis* was cultured from five of 22 patients with tuberculous lymphadenitis, whereas smear positivity was slightly higher (eight of 22 patients with tuberculous lymphadenitis; table 1). Seventeen of 22 tuberculous lymphadenitis biopsies were diagnosed by the combined criteria of histology and culture. Application of the same criteria to fine needle aspirates (that is, cytology and culture) diagnosed tuberculous lymphadenitis in nine of 22 patients with tuberculous lymphadenitis. Thus, excisional biopsy was nearly twice as sensitive as fine needle aspirate examination ($p < 0.05$) using the conventional diagnostic criteria for tuberculous lymphadenitis (table 2).

Tables 1 and 2 show the results of the devR based PCR assay on 90 coded specimens (45 each of fine needle aspirates and biopsies) from 45 patients. The PCR assay targets a single copy gene, devR, which encodes a response regulator of a two component signal transduction system, devR-devS. This assay is specific for organisms belonging to the *M tuberculosis* complex.²⁰ The assay has shown excellent specificity (> 90% to 100%) in three phases of testing performed on 60 well characterised coded sputum specimens from tuberculous and non-tuberculous patients that were provided to JS Tyagi's laboratory (S Chakravorty and JS Tyagi, unpublished data, 1999). Further studies to evaluate the specificity and sensitivity of the assay in the diagnosis of pulmonary and extrapulmonary tuberculosis are currently in progress in the Tyagi laboratory.

In our present study, after the first round of PCR assays, the samples were decoded and the results from all the procedures were compiled. PCR was positive in 10 of 22 fine needle aspirates from patients with tuberculous lymphadenitis. The same number (10 of 22 specimens) were PCR positive in tuberculous lymphadenitis biopsies. Combining the fine needle aspirate and biopsy results, PCR was positive in 13 of 22 patients with tuberculous lymphadenitis

(table 2). Because lymph node specimens are characterised by a low bacillary load, we investigated whether using a twofold larger aliquot of the stored specimens would alter the outcome of the PCR test. Accordingly, DNA extractions and PCR were repeated for 25 specimens (13 biopsies and 12 fine needle aspirates, including the false negative and false positive specimens). After repeat PCR, *M tuberculosis* DNA was detected in seven additional tuberculous lymphadenitis specimens (five biopsies and two fine needle aspirates), confirming that the use of larger amounts of clinical material enhanced the PCR results. After repeat PCR, the test was positive in 12 of 22 fine needle aspirates and 15 of 22 biopsies from patients with tuberculous lymphadenitis (table 2). Nine patients with tuberculous lymphadenitis were PCR positive with both types of specimens (fine needle aspirates and biopsies). Combining the PCR results of fine needle aspirates and biopsies, 18 of 22 patients with tuberculous lymphadenitis were diagnosed positive. PCR had a sensitivity of 82% and was the most sensitive test in comparison with the others (biopsy histology 59%, fine needle aspirate cytology 32%, culture 23%, and smear microscopy 36%; table 1). PCR was false negative for four patients with tuberculous lymphadenitis (table 1). In two of these patients, AFB smear and *M tuberculosis* cultures were negative and TB was diagnosed histologically.

The data from the biopsy and fine needle aspirates were also analysed using individual specimens as the unit of analysis (table 2). *Mycobacterium tuberculosis* was cultured from four of 22 biopsy specimens and two of 22 fine needle aspirates (table 2). Overall, only 14% (six of 44) of the specimens grew *M tuberculosis* on culture. Smear positivity was nearly equivalent in both kinds of specimens, being four of 22 in fine needle aspirates and five of 22 in biopsies (table 2). The rates of smear and culture positivity were slightly higher for biopsies when compared with fine needle aspirates, although both were extremely low, and consequently of limited usefulness in diagnosis. All five smear positive biopsies were PCR positive and three of four smear positive fine needle aspirates were PCR positive. A PCR negative fine needle aspirate remained negative, although the biopsy specimen from the same patient was positive upon repeat PCR. Both culture positive fine needle aspirates were PCR positive; however, one culture positive biopsy specimen stayed negative even after repeat PCR. The PCR test was also negative in the fine needle aspirate from this patient. Obtaining a PCR negative result with this culture positive biopsy specimen is puzzling; an extremely low bacterial load might be responsible for the discrepant results. In the control group, PCR was positive in one of 14 fine needle aspirates and one of 14 biopsies (table 2). Both false positive specimens remained positive after repeat PCR. The false positive fine needle aspirate specimen was diagnosed as non-Hodgkin's lymphoma and the false positive biopsy specimen as reactive lymphadenitis.

Because the *devR* gene target is specific for organisms of the *M. tuberculosis* complex, the positive PCR results of the non-tuberculous lymphadenitis controls were considered as false positive. Combining the results obtained with biopsy and fine needle aspiration, the PCR test had a specificity of 86% and an efficiency of 83%. PCR efficiency was the highest when compared with the AFB smear (61%), culture (53%), biopsy histology (75%), and fine needle aspirate cytology (58%).

The patients with granulomatous lymphadenitis ($n = 9$) showed interesting results. PCR was positive in four of nine biopsies and five of nine fine needle aspirates (table 2). Combining the PCR results of biopsy and fine needle aspirate specimens, *M. tuberculosis* DNA was detected in six of nine patients with granulomatous lymphadenitis (table 1). One patient showed a Mantoux positive reaction and was PCR positive in both biopsy and fine needle aspirate specimens. Another patient had a history of TB (see below). The rest of the patients were lost to follow up.

Discussion

Nucleic acid amplification techniques including PCR have had a considerable impact on disease diagnosis on account of their speed, specificity, and enhanced sensitivity. The application of PCR to the diagnosis of tuberculous lymphadenitis has the potential to resolve one of the foremost challenges facing a diagnostic laboratory. In our study, an in house PCR test was compared with conventional techniques for the detection of *M. tuberculosis* in 45 patients with lymphadenitis. Lymph node aspirates, in

particular, pose a constraint and a challenge for the diagnostic laboratory because of the small volumes (a few microlitres to < 2 ml) of specimens available for analysis and because of the low positivity with smear and culture techniques. Biopsies do not suffer from this limitation, but the surgical procedure is generally used only when fine needle aspirate cytology is inconclusive. The efficiency of biopsy PCR (78%) was somewhat lower than that of biopsy histology combined with culture (86%), whereas the efficiency of fine needle aspirate PCR (69%) was roughly equal to that of fine needle aspirate cytology combined with culture (64%). Biopsy PCR was more sensitive than fine needle aspirate PCR (68% *v* 55%, respectively; table 2). However, these differences were not significant using χ^2 test analysis.

The combined sensitivity of PCR for biopsies and fine needle aspirates (82%) was the highest among all the investigations in comparison with the combined sensitivity of AFB smear and culture (36% and 23%, respectively). Perhaps the use of more sensitive microbiological techniques, such as a liquid culture system, would result in higher culture positivity. However, because of equipment constraints this could not be investigated in our study.

Our results are comparable with those of other laboratories performed either on lymph node aspirates or tissue. In a recent study on lymph node aspirates from 23 patients in whom the cytological diagnosis was consistent with tuberculosis, a PCR positivity of 83% was reported based on the amplification of the IS6110 insertion sequence, which is present at a copy number of between one and 24 in *M. tuberculosis*.²⁶ The authors concluded that the diagnosis of granulomatous lymphadenitis consistent with tuberculosis can be given even though the AFB smears were negative.²² In another study on 38 specimens of paraffin wax embedded lymph nodes, a sensitivity of 50% was reported in contrast to a smear positive rate of 0.8%.²⁵ Likewise, a study on fine needle aspirates from 31 patients with tuberculous lymphadenitis reported a PCR sensitivity of 61% in comparison with AFB smear and culture positivity of 10% and 19%, respectively.²³ A study compared two PCR assays in lymph node tissue sections and concluded that the primers that targeted the IS6110 element had higher sensitivity (89%) than those amplifying the 65 kDa antigen coding gene (6%), highlighting the value of using multicopy targets.²⁷ However, caution needs to be exercised when interpreting PCR data based on IS6110 because some isolates of *M. tuberculosis* from south India have been reported to lack this element.²⁸

Using PCR, *M. tuberculosis* was detected in fine needle aspirate but not in biopsy specimens of two patients with tuberculous lymphadenitis, whereas in six patients *M. tuberculosis* was detected in biopsy but not in fine needle aspirate specimens. The latter could be the result of aspiration from an area not containing bacilli, and might justify performing multiple aspirations from different sites of the enlarged

Figure 1 Polymerase chain reaction (PCR) test and inhibitor check reactions on fine needle aspirates and biopsies. PCR assays were set up with DNA from fine needle aspirate and biopsy specimens of a patient with tuberculous lymphadenitis (tuberculous panel) and a patient with non-tuberculous lymphadenitis (control panel). The products of the amplification reactions were electrophoresed, transferred to a nylon membrane, and detected by hybridisation to a γ^{32} P ATP end labelled oligonucleotide, *devR1*,²⁹ followed by autoradiography. Lanes 1 and 6, neat test DNA sample (5 μ l); lanes 2 and 7, 1/10 dilution of test DNA (5 μ l); lanes 3 and 8, 1/50 dilution of test DNA (5 μ l); lanes 4 and 9, inhibitor check reactions containing neat test DNA (5 μ l) spiked with *Mycobacterium tuberculosis* DNA (10 ng); lanes 5 and 10, inhibitor check reactions containing 1/50 dilution of test DNA (5 μ l) spiked with *M. tuberculosis* DNA (10 ng). The arrows indicate the amplification product of the *devR* gene.

lymph node, as reported previously.²² In the former, it is possible that inhibitors might not have been removed completely. The presence of PCR inhibitors was a notable feature of the lymph node specimens. Fifteen per cent of the analysed specimens (five fine needle aspirates and nine biopsies) were inhibitory at the standard 5 µl aliquot. Inhibition was judged by the inability to amplify spiked *M tuberculosis* DNA (fig 1). In four samples, amplification of *M tuberculosis* DNA was detected only with the 1/10 dilution, clearly proving the presence of inhibitors. The usefulness of setting up assays with various dilutions is shown in fig 1; PCR was positive only with the 1/10 dilution of the biopsy sample, but not with either the neat specimen or with a 1/50 dilution. The interpretation is that by diluting the sample 10-fold (but not 50-fold), PCR inhibitors are also diluted out and *M tuberculosis* DNA is still of sufficient concentration to be amplified. Two additional cycles of CTAB/NaCl extraction were effective in the removal of inhibitors from those samples that showed inhibition. The presence of inhibitors has also been reported by other laboratories; pus samples, tissue biopsies, and sputum were found to be up to 20% inhibitory in PCR based systems.^{13 19}

The occurrence of false positives and false negatives in PCR remains a matter of concern. The two false positive results remained positive after repeat PCR, suggesting a possible contamination during sample collection. In both these patients, either biopsy or fine needle aspiration alone were consistently PCR positive. Analysis of the false negative samples revealed that one smear positive fine needle aspirate and one culture positive biopsy were missed by PCR, which is surprising in view of its theoretically high sensitivity. False negatives can theoretically be ascribed either to sampling errors (non-uniform distribution of micro-organisms), low bacterial load, inefficient extraction of DNA, or to the presence of PCR inhibitors. The paucibacillary nature of lymph node specimens was highlighted in our study. Only 16 of 38 (42%) biopsy and fine needle aspirate specimens were detected by ethidium bromide; the others were detected only by radioactive hybridisation.

Our study and those of others^{22 23 26} suggest that PCR could make a considerable impact in the diagnosis of those cases of tuberculous lymphadenitis (such as granulomatous lymphadenitis and sarcoidosis) that are missed by conventional procedures. Despite an extensive differential diagnosis, granulomatous lymphadenitis cases are tentatively diagnosed as tuberculous lymphadenitis because it continues to be the most frequent cause of granulomatous lymphadenitis in India.⁴ Granulomatous lymphadenitis can also be caused by other conditions, including atypical mycobacterial lymphadenitis, fungal lymphadenitis, sarcoidosis, toxoplasmosis, and cat scratch fever, making it important to arrive at a definitive diagnosis. In our study, of the nine patients who were diagnosed as having granulomatous lymphadenitis, six were PCR positive. One patient had a history of TB and had undergone

antitubercular treatment. *Mycobacterium tuberculosis* DNA was demonstrated in this patient, probably because PCR does not require the presence of viable bacteria. A patient with the provisional diagnosis of sarcoidosis was PCR positive in our study. Interestingly, the biopsy specimen of this patient grew *M tuberculosis* and accordingly was included in the tuberculous lymphadenitis group of patients. Earlier reports have suggested that the presence of mycobacteria in the sarcoid lesion might be capable of inducing the pathological changes of sarcoidosis, or that in some patients tuberculous lymphadenitis might present as sarcoidosis like lesions.²⁹⁻³¹

In our experience, a combination of conventional techniques and PCR must be applied for the rapid and early diagnosis of TB in paucibacillary specimens to achieve maximum sensitivity. PCR might be particularly useful for the diagnosis of tuberculous lymphadenitis in patients where conventional diagnosis fails—for example, in granulomatous lymphadenitis. The positive and negative predictive values of PCR were 0.9 and 0.75, respectively. However, false positive and false negative results are practical issues that must be dealt with to improve the efficiency and predictive value of PCR. In conclusion, PCR has a potentially important role in improving the diagnostic accuracy of tuberculous lymphadenitis. Because fine needle aspiration is not invasive, it is the procedure of choice, and we suggest that PCR is performed initially on these specimens. Excisional biopsy histology and PCR should be recommended only in patients who are negative on fine needle aspirate PCR, or if there is discrepancy with the clinical impression.

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