Assessment of possible tuberculous lymphadenopathy by PCR compared to non-molecular methods

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Tuberculosis is a major public health problem in India and other developing countries and has formed a lethal partnership with AIDS. It often presents a diagnostic challenge especially when clinical presentation is suggestive but bacteriological proof is lacking. The objective of this study was to compare the various diagnostic techniques in clinically suspected cases of tubercular lymph nodes and to find a suitable, cost-effective but sensitive and specific method for diagnosis. A total of 100 cases were recruited for the study. Fine needle aspiration cytology was done in all cases and the smears prepared were processed for Giemsa, Ziehl–Neelsen's, Kinyoun and Papanicolaou stains. Parts of the aspirated materials were assessed by fluorescent staining, culture and PCR. Seventy-four percent of aspirates were positive by fluorescent stain while only 22 % were positive by culture. PCR could be performed in 55 cases, out of which 22 (40 %) were positive. When compared to culture, the sensitivity and specificity of PCR were found to be 89.5 % and 86.1 %, respectively. Fluorescent stain was found to be the most sensitive (81.8 %) of the conventional methods but showed poor specificity (28.2 %). Interestingly, PCR detected 80 % of smear-negative but culture-positive cases.

INTRODUCTION

Tuberculosis (TB) is on the rise globally, with an estimated one-third of the world's population being infected with Mycobacterium tuberculosis and approximately 3–4 million new cases every year. Developing countries, particularly in south-east Asia, have experienced a major increase in the burden of TB, a major public health problem that is compounded by the emergence of multi-drug-resistant tuberculosis bacilli (WHO, 2002). Extra-pulmonary TB is also much higher in Asians than in the Caucasian population (Finch et al., 1991; WHO, 2002). Tuberculous lymphadenitis (TB-L) is the most common form of extra-pulmonary TB, accounting for 30–40 % of TB cases (Hooper, 1972; Krishanaswami et al., 1972; Appling & Miller, 1981). TB-L also occurs with an increased frequency in patients with human immuno-deficiency virus (HIV) (Finfer et al., 1991; Slavik et al., 1996).

Traditionally, the diagnosis of TB-L is established by histopathology and smear microscopy or by mycobacterial culture. Over the past decade, fine-needle aspiration (FNA) 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 possible TB-L have been clearly defined as epitheloid cell granulomas with or without multinucleated giant cells and caseation necrosis (Lau et al., 1990; Finfer et al., 1991; Gupta et al., 1992, 1993).

The diagnosis of extra-pulmonary TB is difficult, especially when clinical presentation is suggestive but bacteriological proof is lacking. The diagnosis confirmed by acid-fast bacilli (AFB) using conventional microscopy is simple and rapid but lacks sensitivity, whereas culture is more sensitive and specific but takes several weeks to get the results (Grange, 1989). Fluorescent stain (FS) has been proven to be superior to the Ziehl–Neelsen's (ZN) stain, especially in paucibacillary cases (Kumar et al., 1998). In recent times, PCR has been found to be the most sensitive technique for rapid diagnosis

Abbreviations: AFB, acid-fast bacilli; FNA, fine needle aspiration; FS, fluorescent stain; TB, tuberculosis; TB-L, tuberculous lymphadenitis.
of *M. tuberculosis* (Shankar et al., 1990; Dewit et al., 1992; Schlüger et al., 1994; Banavaliker et al., 1998; Baek et al., 2000; Singh et al., 2000; Gong et al., 2002). In contrast, FNA cytology is a simple and safe out-patient technique, the efficacy of which as a diagnostic tool has already been established particularly in cases of TB-L (Gadre et al., 1991). Thus there is a need for a rapid and cost-effective technique for reliable diagnosis of TB-L particularly in low-resource settings. In the present study, we have used various diagnostic techniques including PCR to diagnose clinically suspected cases of TB-L and compared the results to see which of the techniques are more sensitive, specific and cost-effective.

**METHODS**

**Patients and specimens.** The present study was conducted prospectively in Lok Nayak Hospital, a major tertiary health-care centre in New Delhi metropolitan city over a period of 1 year. The fine-needle aspirated samples were collected from both outdoors and indoor Surgery and Medicine department of the hospital. One hundred cases of clinically and cytologically suspected TB-L were included in this study. The ages of the patients ranged between 2.5 months and 60 years and the female : male ratio was 1:0.75. The clinical symptoms suggestive of TB were fever, anorexia or weight loss, and lymphadenopathy.

**Fine-needle aspirates.** Fine-needle aspirates from the involved lymph node were divided into seven parts. One part was smeared onto a slide and fixed immediately with 95% alcohol for Papanicolaou staining (Pap stain). Another four smeared slides were prepared and air-dried for ZN stain, Giemsa, Kinyoun (cold stain) and FS. Since necrotic smears often give false-positive staining if auramine is used first, we used ZN staining followed by auramine. One portion of the material was collected in an Eppendorf tube containing sterile PBS and stored immediately at 4°C for PCR, and the last portion was stored at 37°C (for not more than 4–5 days) for culturing in Lowenstein-Jensen (LJ) medium.

**Culture.** Fine-needle aspirates were liquefied and digested by using N-acetyl l-cysteine, decontaminated by standard procedure using Petroff’s method, inoculated on to LJ slants and incubated at 37°C for 2–3 weeks. Cultures on LJ slants were subjected to biochemical testing for *M. tuberculosis*.

**DNA extraction.** Mycobacterial genomic DNA was extracted as previously described (Van Soolingen et al., 1995), with minor modifications. Briefly, 200 μl of the FNA material was incubated in a water bath at 80°C for 20 min to inactivate the bacteria and then diluted with 500 μl of Tris-EDTA buffer. The bacteria were thereafter lysed with 50 μl lysozyme (10 mg ml⁻¹; Sigma) and vortexed before incubation for 1 h at 37°C. The lysozyme-treated samples were incubated at 65°C for 10 min in the presence of 10 μg of proteinase K (Roche) and 10% sodium dodecyl sulphate (SDS). A 5 M solution of sodium chloride-cetyltrimethylammonium bromide was added to the sample, and phenol/chloroform/isooamylic alcohol (25: 24: 1) extraction was performed. The DNA precipitate was obtained by adding 1/10 volume of 3 M sodium acetate and an equal volume of 2-propanol or 2-5 vols chilled absolute ethanol. After storage for 1 h at −20°C, the DNA was collected by centrifugation at 12,000 r.p.m. for 15 min at 4°C, washed with 70% ethanol and resuspended in 30 μl of distilled water or Tris-EDTA. Finally, the sample was treated with RNase (0.2 mg ml⁻¹; final concentration; Sigma) and incubated for 1 h at 37°C before storage at −20°C until further analysis.

PCR. PCR was performed on all specimens using primers 5’-TCCGCTGCACTGTTTCC-3’ and 5’-GTCCGCGAGTCTA GGCCA-3’ to amplify a 240 bp region (460–700) from the gene encoding the MPB 64 kDa protein. Amplification of the gene was done in 25 μl reaction mixture containing 50–100 ng of genomic DNA, 10 mM Tris/HCl (pH 8.0), 1.5 mM magnesium chloride, 50 mM potassium chloride, 200 μM of each dNTP (dATP, dGTP, dCTP and dTTP), 10 pmol of each primer and 0.5 U Taq DNA polymerase. DNA was initially denatured at 94°C for 4 min, followed by 29 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 30 s. In the last cycle, extension at 72°C was allowed for 4 min. Positive (Mycobacterium tuberculosis DNA) and a negative (distilled water) controls were included for each experiment. Amplified DNA was electrophoresed on a 3% ethidium bromide-stained agarose gel along with HaeIII-digested ϕX174 DNA molecular mass markers and photographed under a UV transilluminator. Amplification identity was confirmed in each instance by Southern hybridization. The sequence of the probe was chosen from the middle part of the amplified sequence of the gene (5’-CTTCAACCCGCGGAGT-3’).

**Statistical analysis.** Taking culture positivity as standard, sensitivity and specificity of different assay methods were calculated. The results were analysed by chi-squared test and *P* values were evaluated at the 5% level of significance.

**RESULTS**

In the present study, 100 consecutive patients with a clinical diagnosis of possible TB-L were confirmed by both clinical and cytological criteria. The patients presented with a painless, discrete, solid nodule or multiple masses present for weeks to years. Most of the patients (55%) presented lymphadenopathy of variable duration of weeks to years and a few also had fever with evening rise (22%) or fever and cough (18%). A positive family history and positive past history were found in 13% and 12% of the patients, respectively. The ages of the patients ranged from 2-5 months to 60 years, with a median of 21-5 years. The female : male ratio was 1:0.75. The presenting complaint in most of the patients was swelling in the head and neck region (cervical lymph node) with fever and/or cough. Only 11% of the cases had pulmonary involvement as seen on chest X-ray (Table 1). The most frequently aspirated lymph nodes included cervical (65%), supraclavicular (13%), submandibular (11%), axillary (9%) and inguinal (2%).

Cytomorphological analysis of 100 cases revealed acute suppurative lesion (33%), granuloma with necrosis (28%), granuloma (24%) and necrosis only (15%). Maximum AFB positivity was seen with FS (Table 2), which was found to be the most sensitive of the conventional methods, and the differences in AFB positivity by FS compared with other staining methods were statistically significant (*P* < 0.05). FS was again found to be the most sensitive in cases of granuloma without necrosis (Fig. 1), but was less specific in cases showing necrosis. FS staining improved the detection of AFB in paucibacillary smears, specifically those showing granulomas. However, the time required to screen FS smears was similar to ZN-stained smears but the bacilli had to be...
viewed under high power for confirmation. In necrotic smears, the fluorescence of necrotic material interfered with the visibility of bacilli.

Out of 100 cases, only 22% were positive for *M. tuberculosis* by culture. No other organism was found to grow in culture. This may be because of the absence of viable organisms in necrotic aspirates. Culture positivity was maximal in the presence of granulomas (61.1%); however, the difference in positivity between various cytomorphological groups was not found to be statistically significant.

A single specific PCR band of the desired size amplimer, 240 bp, was always obtained without ambiguity (Fig. 2). All PCR products were again reconfirmed by Southern blot hybridization using internal DNA sequences of the PCR-amplified genomic sequences as a probe. This excluded the possibility of picking up any atypical mycobacterial sequence since, unlike in Western countries, lymphadenopathy in India is rarely caused by atypical mycobacteria. However, we have not tested these primers against DNA from other mycobacteria that are almost absent in India.

### Table 2. AFB positivity by different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Level of positivity</th>
<th>Negative cases</th>
<th>Total no. of cases</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>KAF*</td>
<td>36 (36%)</td>
<td>4 (4%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>ZN</td>
<td>39 (39%)</td>
<td>6 (6%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>FS</td>
<td>57 (57%)</td>
<td>14 (14%)</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>PCR</td>
<td>–</td>
<td>–</td>
<td>22 (40%)</td>
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*KAF, Kinyoun acid-fast stain.*
showed necrosis only, and this difference was found to be statistically significant \((P < 0.0007)\). Of the 55 cases analysed by PCR, only 19 were culture positive. Table 3 shows a comparative picture of PCR and other conventional methods with culture positivity. The sensitivity and specificity of PCR were 89.5 and 86.1%, respectively. Although FS was the most sensitive of the conventional methods (sensitivity 81.8%), it was less specific than ZN or Kinyoun acid-fast stain and gave higher false positivity (64%). PCR was able to detect four out of five (80%) smear-negative but culture-positive cases. There were five cases (13.9%, Table 3) that were smear-positive and culture-negative but PCR positive, which is indicative of the fact that viable organisms are required for culture while genomic DNA can still be detected by PCR (Fig. 2). The other possibility could be that the culture inoculum did not contain any bacilli. Table 4 shows detectability by PCR in different morphological groups.

DISCUSSION

TB continues to be a major public health problem all over the world and especially in India. It has formed a lethal partnership with HIV and AIDS. It is therefore essential to have a reliable diagnostic method that is rapid and cost-effective for early detection and management of TB patients.

TB-L is the commonest presentation of TB. It is most frequently found in children and young adults but several reports have indicated a variable age group ranging from 18 months to 85 years (Pamra & Mathur, 1974; Arora & Arora, 1990; Gupta et al., 1992; Ersoz et al., 1998). In the present study, the ages of the patients ranged from 2.5 months to 60 years, with the majority of the cases in the third decade. A female preponderance has been seen in our study (female : male, 1:0-75), as has also been reported by several other authors (Pamra & Mathur, 1974; Gupta et al., 1992; Ersoz et al., 1998).

There is convincing evidence of increased effectiveness of using FS to demonstrate AFB as compared to the ZN method (McClure, 1953; Braunstein & Adriano, 1961; Bailey et al., 1985). In our study FS gave higher positivity (74%) especially in paucibacillary cases, hence it is the most sensitive of the conventional methods but certainly a less specific technique as the number of false positivity is quite high. The high frequency of FS-positive, culture-negative, PCR-negative samples in this study raises the possibility that the frequency of lymphadenitis due to non-tuberculous mycobacteria may be higher than previously appreciated.

Most other studies (Gadre et al., 1991; Claridge et al., 1993) carried out on FNA samples from TB-L have revealed a culture positivity rate of 17–55.5%. We, however, observed a low culture-positive rate of 22%. Several reasons could explain this, including partially treated status (Dewit et al., 1992), presence of bacteriostatic substance, scanty bacilli in glands (Pamra & Mathur, 1974), harsh decontamination procedures (Pamra & Mathur, 1974), unrepresentative/

| Table 3. Comparison of PCR and conventional methods with culture positivity |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Method          | Positive (n = 22)* | Negative (n = 78)* | Sensitivity (%) | Specificity (%) | Positive predictive value (%) | Negative predictive value (%) |
| PCR+ (n = 22)   | 17              | 5               | 89.5            | 86.1            | 77.3            | 93.9            |
| ZN+ (n = 47)    | 17              | 30              | 77.3            | 61.5            | 56.7            | 96.0            |
| FS+ (n = 74)    | 18              | 56              | 81.8            | 28.2            | 24.3            | 95.6            |
| KAF+ (n = 42)†  | 16              | 26              | 72.7            | 66.7            | 38.1            | 94.6            |

*For PCR comparison was done with culture positive n = 19 and culture negative n = 36.
†KAF, Kinyoun acid-fast stain.

| Table 4. Sensitivity and specificity of PCR method in detecting TB infection in lymphadenopathy |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Morphological group | Sensitivity (%) | Specificity (%) | Positive predictive value (%) | Negative predictive value (%) | False negative (%) | PCR-positive, culture-negative (%) |
| ASL* (n = 33)    | 100             | 93.7            | 75              | 100             | 0               | 6.2             |
| Granuloma and necrosis (n = 28) | 100             | 83.3            | 87.5            | 100             | 0               | 16.6            |
| Granuloma only (n = 24) | 85.7             | 40              | 66.6            | 66.6            | 14.2            | 60              |
| Necrosis (n = 15) | 50              | 100             | 100             | 90              | 50              | 0               |
| Overall          | 89.5            | 86.1            | 77.2            | 93.9            | 10.5            | 13.8            |

*ASL, acute suppurative lesion.
inadequate sampling and lesser cases of TB abscesses (Lau et al., 1990). PCR positivity of several cases that were culture-negative indicates that PCR is very sensitive and useful in picking up cases that may not harbour live bacilli.

Thus, PCR is a powerful and reliable technique for rapid diagnosis of *M. tuberculosis*, with a reported sensitivity of 55–95% in culture positives but 100% in both smear- and culture-positive clinical specimens (Lau et al., 1990). The usefulness of PCR in the diagnosis of TB by using a variety of unselected clinical specimens is not clear as studies have differed in techniques including lysing method and target nucleic acid to detect products as well as the number and type of samples used, making the reported sensitivities and specificities difficult to compare (Holodniy et al., 1991; Schluger et al., 1994). A recent study comparing four conventional techniques, FNA cytology, ZN staining, culture and lymph node biopsies, and TB PCR indicated 94-8% diagnosis but PCR was found to be highly sensitive (94-4%) though less specific (38-2%) (Singh et al., 2000; Goel et al., 2001). In the present study, PCR was found to be the most sensitive (89.5%) as well as specific (86.1%) method for early diagnosis of TB.

The limiting factor in the detection of these micro-organisms appears to be the processing of samples by lysis of mycobacteria and DNA purification (Clarridge et al., 1993) as the complex cell wall of the mycobacteria may require several enzymic steps for lysis (Holodniy et al., 1991). In our study the overall frequency of false negatives was low (10-5%) and that of the PCR-positive, culture-negative cases was 13-8%, which is indicative of mycobacterial genomic DNA being present although the live organisms were absent.

The ability of PCR to detect smear-negative but culture-positive cases is remarkable as it can reduce the need for more invasive diagnostic approaches. However, the cost and labour-intensiveness preclude its use as an initial diagnostic test (Schluger et al., 1994). The low PCR positivity (40%) in this study could be explained by the small sample size (Table 3). But, PCR was able to detect four out of five cases (80%) that were smear-negative and culture-positive, thus helping in rapid and reliable diagnosis.

Studies on PCR in FNA cytology of TB-L are limited. The positivity rate varies from 61 to 94% but it is found to be the most sensitive technique (Kim et al., 1996; Ersoz et al., 1998; Manitchotipisit et al., 1999; Baek et al., 2000; Goel et al., 2001; Kidane et al., 2002). The PCR positivities in cases of granuloma only and granuloma with necrosis are reported to be 42% each, and 15-7% for necrosis only (Ersoz et al., 1998). We also observed a higher positivity in cases with only granulomas (81-8%) followed by granuloma with necrosis only (57-1%) and the least in necrosis only (10%).

Although conventional diagnostic techniques remain the method of choice in regions with low-resource settings, PCR may be employed in cases with strong clinical suspicion and equivocal results, especially at an early stage of the disease, for better diagnosis, management and treatment.

REFERENCES


