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# Application of PCR from the Fine Needle Aspirates for the Diagnosis of Cervical Tuberculous Lymphadenitis

Sung-Sook Kim, M.D., Sung-Min Chung, M.D.*, Jong-Nam Kim, M.D.*,
Mi Ae Lee, M.D.**, Eun-Hee Ha, M.D.***

*Department of Anatomic Pathology, Division of Cell & Tissue Biology,
Department ENT*, Department of Clinical Pathology**, Department of preventive medicine***,
Ewha Womans University Hospital, Ewha Medical Research Center, Seoul, Korea*

*Tuberculosis remains a major public health problem worldwide. A definitive and accurate diagnosis of tuberculosis in cervical lymphadenopathy is important because satisfactory results can be achieved with chemotherapy alone, obviating surgery. Recently, fine needle aspiration cytology (FNAC) has provided an alternative and easy procedure for collection of material for cytomorphologic and bacteriologic examination. But the detection rate for M. tuberculosis from the aspirate material is still low with Ziehl-Neelson stain and even with culture. The authors therefore performed polymerase chain reaction (PCR) for mycobacterial DNA sequences in 31 cases of cytodiagnosis of tuberculous lymphadenitis and compared conventional bacteriologic methods. Ziehl-Neelson staining for acid-fast bacilli (AFB) was positive in 3 cases (10 %) in direct smears, and the cultures for M. tuberculosis were positive in 6 cases (19 %). In 19 (61 %) among 31 samples, mycobacterial DNA fragments were detected, using the PCR method. With combined conventional and PCR method, the rate of detection was increased to 68 percent high. In conclusion, PCR is the most sensitive technique in the demonstration of M. tuberculosis in patient with clinically suspected as tuberculosis, who have AFB stain or culture negative cytology. Combined conventional and PCR methods as well as cytologic findings are of further help in the detection and characterization of M. tuberculosis.*

Key Words: *Fine needle aspiration, M. tuberculosis, Polymerase chain reaction*

## INTRODUCTION

Tuberculosis is still rampant in developing countries such as Korea, where prevalence is reported to be as high as 1.8 % of the population and the mortality due to tuberculosis is relatively high (Hong, 1991). A definitive and accurate diagnosis of tuberculosis of the neck lymph node is important because satisfactory results can be achieved with chemotherapy alone, obviating surgery, and there are divergent etiology in cervical lymphadenopathy (Huhti et al., 1975).

Instead of excision of the lymph node for histopathologic and microbiologic examination to confirm the diagnosis of tuberculous lymphadenitis, fine needle aspiration cytology (FNAC) provides an alternative and easy procedure for collection of material for

Address for correspondence: *Sung-Sook Kim. M.D., Department of Pathology, Ewha Womans University Hospital, 70 Jongro 6 ka, Jongro-gu, Seoul, 110-126, Korea.
Tel: 82-2-760-5095, Fax: 82-2-567-5674.*

cytomorphologic and bacteriologic examination (Metro and Jayaram, 1987 ; Kadhika et al., 1989 ; Gupta et al., 1993 ; Radhika et al., 1993). The use of FNAC in the diagnosis of tuberculous lymphadenitis is being described increasingly, and cytologic criteria for its diagnosis have been well established as described in several recent papers (Dahlgren and Ekstrom, 1972 ; Betsill and Hajdu, 1980 ; Kline et al., 1984 ; Bailey et al., 1985 ; Ramsy et al., 1985 ; Rajwanshi et al., 1987 ; Kardos et al., 1989 ; Finfer et al., 1991). The diagnostic findings are epithelioid cell granuloma as with or without multinucleated giant cells and caseation necrosis(Jayaram, 1985 ; Shariff and Thomas, 1991). However, in cases of presenting with a cold abscess, well-formed epithelioid cell granulomas may not be seen ; likewise, a similar granulomatous inflammation may be seen in nontuberculous conditions as well.

Definite evidence of tuberculous infection is the demonstration of mycobacteria in the smear and/or culture. There have been only a few studies on the correlation of cytomorphology with smears and culture examination of FNAC material in tuberculous lymphadenitis. Some studies demonstrated the accuracy of the conventional bacteriologic methods is less than 50 % (Gupta et al., 1993 ; Radhika et al., 1993). Recently, the amplification of specific DNA sequences by polymerase chain reaction (PCR) is a novel tool for the detection of different infectious organisms and has already been applied to detect mycobacterial DNA sequences in several materials (Pao et al., 1990 ; Plikaytis et al., 1991 ; Sritharan et al., 1991 ; Ghossein et al., 1992 ; Saboor et al., 1992 ; Popper et al., 1994). Also in Korea, several reports about the PCR of M. tuberculosis have been published (Cho et al., 1990 ; Kim et al., 1992 ; Kim et al., 1993 ; Jeon et al., 1993). However, there have been few reports which described the application to FNAC materials.

We therefore investigated FNAC samples for the presence of mycobacterial DNA in cytologically proven cases of tuberculous lymphadenitis as correlated with conventional bacteriologic studies for *M. tuberculosis.*

## MATERIALS AND METHODS

### Materials

The material for this study included all cases of lymph node aspiration over a period of one year (April 1994-March 1995) referred to the Cytology Laboratory of Ewha Womans University, Dongdaemoon Hospital, Seoul, Korea. Aspirations were performed using 22-gauge needles and disposable, 10ml plastic syringes with a detectable syringe holder. The standard technique of aspiration was used. The material thus aspirated was used for the preparation of smears that were stained with May-Gru "nwald-Giemsa and hematoxylin and eosin stain for routine cytologic diagnosis and Ziehl-Neelson stain for AFB. For further mycobacterial examination, the material was inoculated onto the slants of Ogawa medium for isolation and identification of mycobacteria using the standard technique.

The remainder of specimens were flushed into 1 ml of normal saline. The needle was not replaced between aspirates and was rinsed after the final aspirate by repeated aspiration and ejection of approximately 1ml sterile saline. These materials were used for PCR.

We studied 31 cases of cytodiagnosis of tuberculosis and randomly chosen 50 cases of non- tuberculosis as control group. Control group included mostly reactive hyperplasia, Kikuchi's lymphadenitis, and metastatic carcinoma.

### Methods

DNA preparation : The sediments of aspirated materials were mixed with 300 μℓ of proteinase K digestion buffer (100 μg proteinase K per ml, 10mM Tris, pH 7.8, 5mM EDTA, and 0.5 % SDS) and incubated for overnight at 37℃. The mixtures were transferred to 2.0 ml screwcap polypropylene tubes containing 0.5 g of 0.1mm Zirconium beads and 300 μℓ of buffer-saturated phenol. After mulling for 3 min in the Mini-beadbeater(Biospecs Products, Bartlesville, OK, USA), the 200 μℓ of supernates were mixed with 35 μℓ of 5 M NaCl and 27 μℓ of 10 % CTAB /NaCl and incubated for 10 min at 65℃. DNA was extracted with chloroform : isoamylalcohol (24 : 1) and phenol : chloroform : isoamylalcohol (25 : 24 : 1) and precipitated with 0.6 volume of isopropranol and 2 μℓ of 10 mg/ml glycogen. The pellet was washed with 1ml of 70 % ethanol and resuspended in 30 μℓ of TE buffer (10mM Tris, 1mM EDTA, pH 8.0). Cycle conditions : Two oligonucleotide primers within the *M. tuberculosis mtp40* DNA fragments squence, designated primer PT1 (5'CAACGCGCCGTCGGTGG 3') and PT2 (5'CCCCCCACGGCACCGC 3'), were used for the PCR, resulting in a 396-bp PCR fragment. All reactions were performed with a final volume of 100

μℓ containing 1X PCR buffer (Geneamp Kit ; Perkin-Elmer Cetus, CA, USA), 200 uM each dNTP (dATP, dCTP, dGTP, dTTP), 50 pmol of each primer (PT1, PT2) and 2.5 U of *Taq polymerase* (AmpliTaq ; Perkin-Elmer Cetus, CA, USA). The reaction was carried out on a DNA thermal cycler (Perkin-Elmer Cetus, CA, USA). In all PCR series, positive control (one vial of M. tuberculosis DNA) and negative control (one vial of TE buffer) for contamination with M. tuberculosis DNA during the DNA extraction and the preparation of the PCR mix were included. The PCR incubations were as follows : 5 min at 94℃, followed by 30 cycles of 1 min at 94℃, 1 min at 70℃ and 1.5 min at 72℃ and an additional 7 min in the final extension step.

Detection of amplified DNA : Aliquots (10 μℓ) from the PCR were analyzed by gel electrophoresis in 2 % NuSieve 3 : 1 agarose (FMC BioProducts, USA) gel stained with ethidium bromide and photographed on a UV transilluminator (Spectroline TVC-312A, Spectronics Cor., NY, USA). In assessing the PCR results, a PCR was "positive", showing specific amplification of 396-bp fragment on agarose gel electrophoresis.

## RESULTS

A total of 299 cervical lymph node aspirations were done during this period at Ewha University Hospital. Of these, a cytologic diagnosis of tuberculous lymphadenitis was suggested in 31 cases (10 %), with cervical lymphadenopathy. The majority of the cases were in the first four decades of life, with a peak age range of 21-30 years and male : female ratio of 1 : 1.2.

Based on the nature of the material aspirated and/or cytomorphologic findings, the cases were categorized into three types. In type 1, thick or thin necrotic, liquified material was aspirated, with the smears showing necrotic material and a sparse to moderate inflammatory reaction consisting of a mixture of degeneration polymorphs, lymphocytes and some scattered epithelioid histiocytes (Fig. 1). No well formed granulomas or giant cells were seen. In type 2, semisolid or thick necrotic material was aspirated, with the smears showing well-formed epithelioid cell granulomas, giant cells and caseation necrosis (Fig. 2). In type 3, small, solid particles of tissue were aspirated, with the smears showing well-formed epithelioid cell granulomas but no caseation necrosis (Fig. 3).

In all 31 cases, both mycobacterial culture and smear examination were done. And 50 cases of the control group (diagnosed as non-tuberculosis such as abscess, Kikuchi's necrotizing lymphadenitis, etc) were also examined. The results of bacteriologic

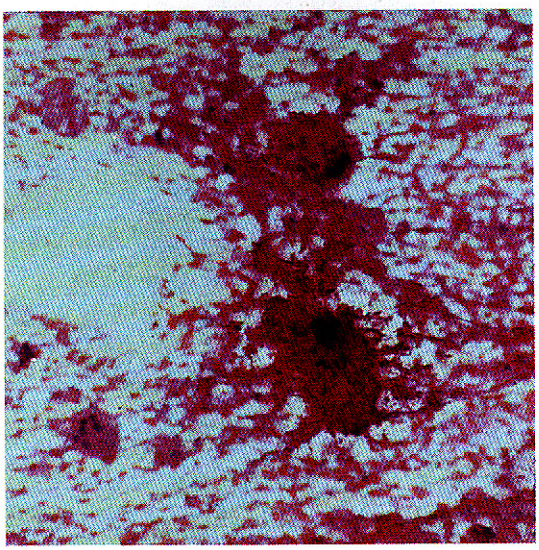

Fig. 1. Cytodiagnosis of tuberculosis of type 1 showed abundant caseation necrotic materials with a few granumomas(H & E stain, × 200).

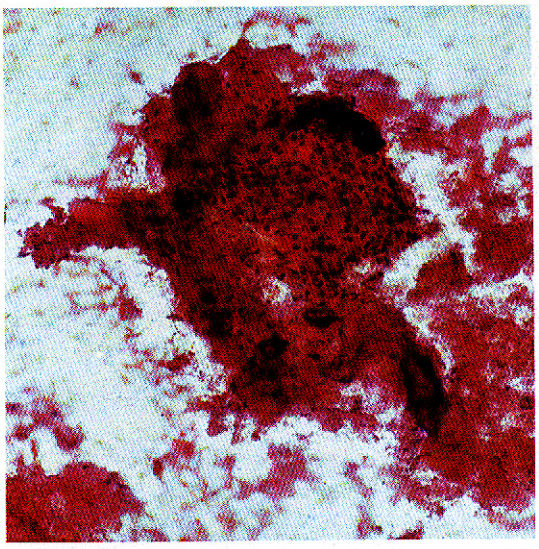

Fig. 2. Type 2 revealed typical granulomas composed of caseation necrosis and epithelioid cells with giant cells(H & E stain, × 200).

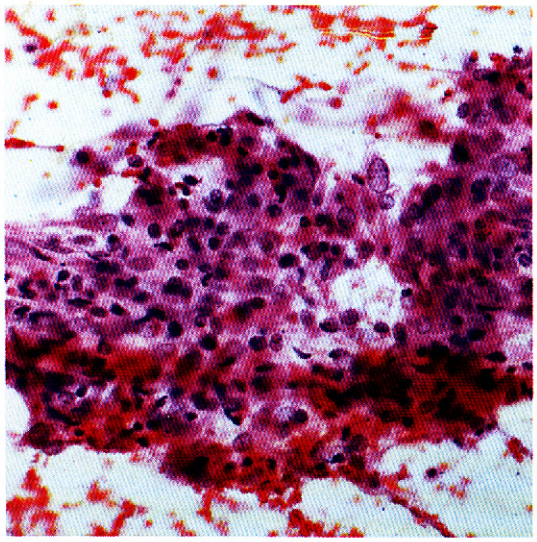

Fig. 3. Type 3 showed only a few epithelioid cells, with no evidence of typical granulomas or caseation necrosis(H & E stain, × 200).

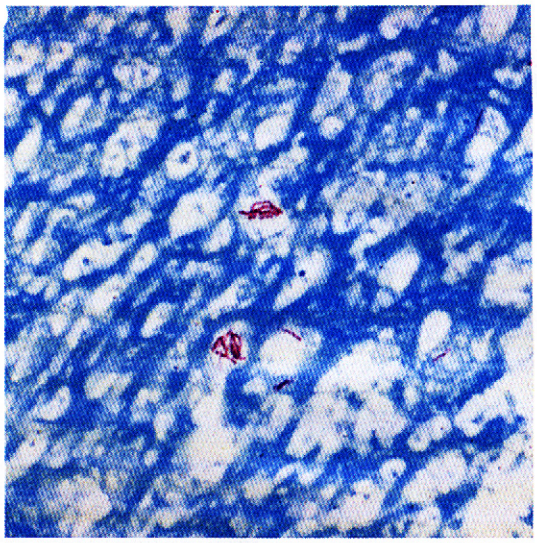

Fig. 4. AFB stain demonstrated many acid fast bacilli with type 1 smear pattern(Ziehl Neelson stain, × 1000).

examination of these cases are shown in Table 1. Direct smears for AFB was positive in 3 (10 %) of 31 (Fig. 4), and mycobacterial culture were positive in 6 cases (19 %) of 31. The combined smears and cultures were positive in 7 (23 %). In one case, the smear was positive for AFB, but the culture was negative. Table 1 showed the correlation of AFB positivity in smear and culture examination. Culture positivity was found in a higher percentage of cases with type 1 and 2 smears as compared to type 3. AFB positivity in smears was lower in type 3 and 1 compared to type 2. Of the 50 cases of the control group (non-tuberculosis with cytodiagnosis), there was no case of positive for AFB smear and culture.

PCR methods for mycobacterial DNA revealed the highest sensitivity, 19 cases (61 %) were positive (Fig. 5). In these positive cases, most cases were type 1. PCR positivity was found in a higher percentage in all

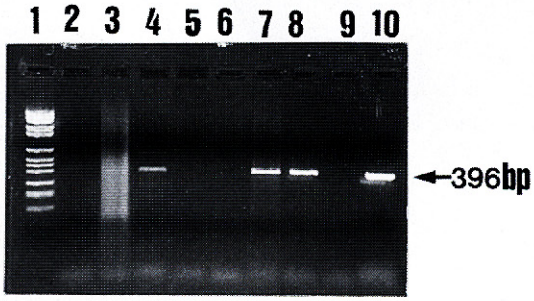


Fig. 5. The results of M. Tuberculosis PCR from the fine needle aspirates of cervical lymphadenitis patients. Lane 1, DNA molecular-weight marker VI(Boehringer-Mannheim); 2.5 and 6, Negative patients; 3,4,7 and 8, Positive patients; 9, Negative control(TE buffer); 10, Positive control(*M. tuberculosis* DNA).

Table 1. Resulsts of *M. tuberculosis* positivity in conventional and PCR methods with smear pattern

| | Smear type | Total No. | Smear$^+$ | Culture$^+$ | PCR$^+$ | Combined |
|---|---|---|---|---|---|---|
| TB | 1 | 10 | 2 | 3 | 7 | 7 |
| | 2 | 15 | 1 | 3 | 10 | 12 |
| | 3 | 6 | 0 | 0 | 2 | 2 |
| | Total | 31 | 3(9.7 %) | 6(19.3 %) | 19(61.3 %) | 21(67.7 %) |
| Non tuberculosis | | 50 | 0 | 0 | 0 | 0 |

TB : tuberculosis ; + : positive

smear types, even in type 3 which were relatively low-detectable with the conventional method. All three AFB smear positive cases were also PCR positive, but in two cases in which the culture was positive (1 case was identified into atypical mycobacterium), PCR was negative (Table 2). Table 2 shows the correlation of culture, and PCR with the smear type. With combined conventional and PCR methods, 21 cases of the cytodiagnosis of tuberculosis demonstrated the presence of *M. tuberculosis*. In the control group diagnosed as non-tuberculosis, AFB smear, culture and PCR were all negative.

Table 2. Correlation of *M. tuberculosis* positivity between conventional and PCR methods

| | PCR+ | − |
|---|---|---|
| Smear+ | 3 | 0 |
| Smear− | 16 | 12 |
| Culture+ | 4 | 2 |
| Culture− | 15 | 10 |
| Total | 19 | 12 |

+ : positive ; − : negative

## DISCUSSION

In the present study, cytodiagnosis supplemented with the PCR method as well as the smear and culture examination helped in establishing a definitive diagnosis of tuberculous cervical lymphadenitis in 68 % of cases. That a negative mycobacterial examination does not exclude the possibility of tuberculosis is evident from our cases in which the conventional method were negative for AFB but the PCR was positive. Whether the cases with negative bacteriologic examination should still be regarded as tuberculosis is made easier by performing PCR because of its high sensitivity.

Some previous report demonstrated AFB detection was relatively low, 25 to 45 % on AFB smear (Gupta et al., 1993 ; Radhika et al., 1993). Combined smear and culture positivity of this study was also low, found in only 7 cases (23 %). But the detection rate of our PCR results was much higher than theirs. Because for bacilli to be demonstrated in smears, their number should be 10,000-100,000/ml of material. If the number is less than this, the bacilli may not be detected in the smears. Theoretically only two organisms of AFB are enough to detect successfully with PCR amplification (Pao et al., 1990 ; Plikaytis et al., 1991). So, the culture showed a little more sensitivity, and the PCR revealed far more sensitivity.

False-positive reactions are a major problem with PCR methods for the detection of the *M. tuberculosis* (Eisenach et al., 1990 ; Friess et al., 1990 ; DelPortillo et al., 1991 ; Eisenach et al., 1991). In our study, laboratory-associated contamination is excluded since we performed a negative as well as a positive control during the DNA preparation, amplification and detection procedures. And prevention of amplicon contamination was accomplished by physical separation of different steps in the PCR procedure, using different pipettes, wearing separate gloves in each laboratory, and by using positive displacement pipette tips for preparing the PCR mixes and agarose gel electrophoresis step. Thus, the higher number of positive PCR results in the culture negative (15/31) is not due to contamination, but indicates a more sensitive method than the culture in FNAC materials because PCR detects DNA of nonviable or quantitatively irrelevant *M. tuberculosis* pathogens. The PCR method using primers PT1 and PT2 within the *mtp40* DNA fragment is specific for *M. tuberculosis* in which it confirms the presence of this fragment exclusively within the *M. tuberculosis* genome and excludes other mycobacterial species, including those belonging to the *M. tuberculosis* complex. Thus, it is possible to obtain, with the PCR method, differential diagnosis of infections due to *M. tuberculosis*, to atypical mycobacteria. Fifty cases of cytodiagnosis of nontuberculosis showed negative with AFB stain, culture and PCR.

In this study, the material showing typical granuloma with caseation necrosis or the necrotic caseation were over 60 % PCR positive, and even the material with a few granuloma (smear type 3) were 33 % positive. But the fact that two cases of the culture positive were PCR negative, informed us that PCR negative can not completely exclude the possibility of tuberculosis, and combined bacteriologic study should be indicated. Those cases included an atypical mycobacterial infection, so in case of suspicious atypical mycobacteria, culture should be recommended. Also culture provides a more information such as sensitivity and identification rather than the PCR method.

In summary, PCR is, as expected, more sensitive in the detection of *M. tuberculosis* in FNAC materials than either AFB smear or culture. But the culture positive PCR negative were two cases in especially atypical mycobacterial cases, demonstrating that a

negative PCR still does not exclude the possibility of tuberculosis. Conclusively, FNAC supplemented by bacteriologic studies, PCR methods as well as conventional examination could confirm the diagnosis of tuberculosis.

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