The Turkish Journal of Pediatrics 1996; 38: 399-405

DIAGNOSIS OF CHILDHOOD TUBERCULOSIS BY POLYMERASE CHAIN REACTION*

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SUMMARY: Ceyhan M, Kanra G, Seçmeer G, Erdem G, Ecevit Z, Yılmaz E, Tuncer S. (Infectious Disease Unit, Department of Pediatrics, Hacettepe University Faculty of Medicine, Ankara, Turkey). Diagnosis of childhood tuberculosis by polymerase chain reaction. Turk J Pediatr 1996; 38: 399-405.

The polymerase chain reaction (PCR) using oligonucleotides based on the repetitive sequence (IS6110) of Mycobacterium tuberculosis as a primer was evaluated for detection of M.tuberculosis in clinical samples. We tested 55 clinical specimens from patients suspected of having tuberculosis and 71 specimens from control subjects. PCR was more sensitive than culture (positivity rate was 14.5% and 36.3%, respectively, in suspected patients). This approach to the diagnosis of tuberculosis is a valid diagnostic alternative to classical procedures. *Key words: polymerase chain reaction, M.tuberculosis, children.*

Tuberculosis continues to be one of the most important causes of death in developing countries and has made a dramatic comeback in developed countries, in part because of the acquired immune deficiency syndrome (AIDS) pandemic[1]. Since clinical diagnosis alone is unreliable, it must be confirmed by demonstration of Mycobacterium tuberculosis (MT)[2]. Although early diagnosis is very important in control with isolation precautions and treatment of the disease, cultures are cumbersome and time-consuming for diagnosing tuberculosis[3]. Microscopic examination of smears of acid-fast bacteria by Ziehl-Neelsen staining is a rapid but insensitive and nonspecific method for detection of MT. Immunological and serological methods, such as latex agglutination, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA) are not sensitive or specific[3]. The radiometric BACTEC system has shortened the time for detection of MT, but still requires 10 to 12 days for growth of the bacteria and completion of susceptibility tests[4,5].

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One diagnostic difficulty specific to the pediatric age-group is related to the type of infection in children. Since primary tuberculosis is the prominent type in childhood, miliary and meningeal tuberculosis are common and bronchial involvement is rare; also, the number of microorganisms in the clinical specimens, such as sputum and gastric lavage, is low[1]. Thus, a rapid, sensitive and specific diagnostic test is needed, especially in childhood tuberculosis.

Recently, several attempts have been made to diagnose MT infection specifically through biochemical or molecular biological techniques. Among them, polymerase chain reaction (PCR) has been shown to be rapid and reliable test[2, 3, 6-9]. Several sets of primers have been used in MT PCR. Of these, the IS6110 primer has been used with high sensitivity and specificity in many studies[10-12]. In this report, we describe the application of the PCR technique with the IS6110 primer for the detection of MT DNA in the clinical specimens of children with the clinical diagnosis of tuberculosis.

Material and Methods

Patients: Specimens were obtained from patients admitted to Hacettepe University İhsan Doğramacı, Dr. Sami Ulus and SSK Children's Hospitals in whom tuberculosis was suspected during the course of routine diagnostic evaluation. The age of the patients ranged from six months to 15 years. There were 55 girls and 71 boys. Among the 126 patients, 55 patients had proven or suspected tuberculosis and 71 cases were non-tuberculosis controls. Laboratory diagnosis was made with positive MT cultures in eight patients. The criteria used for the diagnosis of tuberculosis were prolonged fever, radiographic findings, cerebrospinal fluid findings (more than 0.02×10^9 cells/L with lymphocytes predominating, protein greater than 1 g/L, glucose less than 50% of blood glucose) in cases with meningitis, sterile routine bacterial and fungal cultures, a positive history for exposure to an adult who had MT in sputum, a positive tuberculin test, and a clinical response to antituberculous therapy.

The tuberculous cases were classified as being proven tuberculous (a positive culture for MT), highly probable tuberculous (met all the clinical and laboratory criteria), probable tuberculous [met at least 5 of the 6 or 7 (6 criteria plus cerebrospinal fluid findings in tuberculous meningitis) criteria]. The non-tuberculous control group consisted of 36 cases of meningitis with etiology other than tuberculosis and cases with non-tuberculous infections in whom the etiology was proven by microbiological cultures.

Clinical Specimens: The type of specimens submitted were as follows: 57 cerebrospinal fluid, 22 sputum or gastric lavage, 14 pleural fluid, seven pericardialea fluid, seven peritoneal fluid, nine blood, five skin and tissue biopsies,

two urine, two infectious aspirate, and one bone marrow aspirate. All specimens were cultured for MT and were subjected as appropriate to lysis to prepare the DNA for amplification.

Sample Preparation: For extraction of DNA from pleural, pericardial and peritoneal paracentesis materials and cerebrospinal fluid, a 400 µl sample was incubated with 10 mM Tris-HCl pH 8.0, 0.5% SDS, 100 mM NaCl, 25 mM EDTA and 50 µl proteinase K (10 mg/ml) at 37 °C for 24 hours. Samples were bolied for 10 minutes, and phenol-chloroform extraction and ethanol precipitation were performed. The precipitates were dissolved in 30 µl distilled water, and 5 µl was used for amplification. After mincing 10-50 mg biopsy specimens, DNA was extracted as described above. For sputum and fasting gastric lavage fluids, a simplified procedure was performed[13]. Samples were incubated in 1 M NaOH for 30 minutes at room temperature on a shaker. After neutralizing with 1 M HCl, samples were centrifuged for 5 minutes at 5,000 rpm. Sediments were washed twice with TE (10 mM Tris pH 8.0, 1 mM EDTA) buffer. Suspension of the sediment in 500 µl TE was bolied for 10 minutes, and 5 µl supernatant was used for PCR.

Primers: A primer pair was selected to yield a final amplification product of 123 base pairs of the insertion element IS6110, a DNA sequence specific for the MT complex.

The sequences of the primers were:

MT1: 5' CCTGCGA GCGTAGGCGTCGG 3" and

MT2: 5' CTCGTCCAGCGCCGCTTCGG 3'[14]

Amplification: A suspension of MT isolate in TE buffer was used as positive control. PCR was carried out in 50 µl volumes. The reaction mixture contained 10 mM potassium chloride, 10 mM tris-HCl pH 8.3, 2.5 mM magnesium chloride, 100 µM of each dNTP, 50 pmol of each primer and 1.5 units of taq polymerase (Cetus stoffel fragment). After five minutes of denaturation at 94 °C for one cycle, 35 amplification cycles were performed with an automated DNA thermal cycler (Model 9600, Perkin Elmer Cetus). Each cycle consisted of 15 seconds of denaturation at 94 °C and 100 seconds of annealing and extension at 68 °C. The reaction mixture without DNA was used as a negative control. The peresence of the 123-bp amplification product was analyzed by electrophoresis of 10 µl amplified mixture on two percent agarose gel. The gel was stained by ethidium bromide and photographed on a UV transilluminater (Fig. 1).

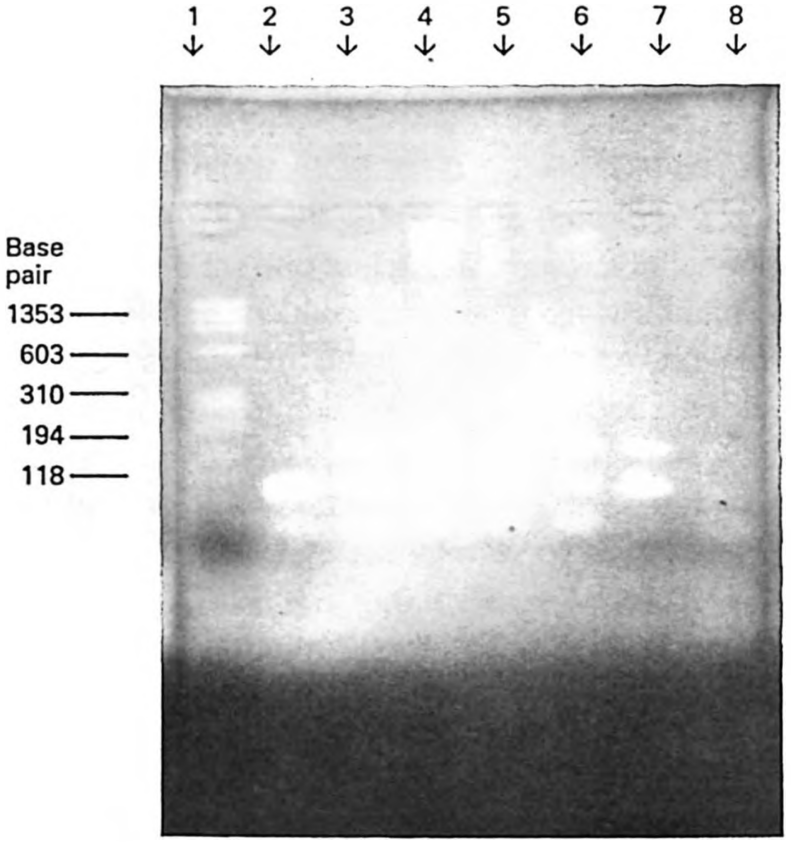

Fig. 1: Analysis of PCR products by agarose gel electrophoresis. PCR was based on the amplification of a 123-bp fragment of the insertion element IS6110 of M.tuberculosis.
Lane 1: ØX174 DNA marker. Lane 2: positive control.
Lanes 3-7: Positive cases. Lane 8: Negative control.

Results

Cultures for MT were not as sensitive as PCR. Cultures were positive in only eight of 55 (14.5%) cases of suspected tuberculosis. We were able to detect eight of eight (100%) proven cases eight of 18 (44.4%) highly probable cases, and four of 29 (13.7%) probable cases (Table I). In the non-tuberculous control group, the PCR was positive in one cerebrospinal fluid sample. In this case, a second DNA preparation was made from the remaining sample, which had been stored elsewhere. This case was PCR-negative on retesting. The first positive result in PCR was evaluated as cross-contamination. The positivity rate was highest in culture-proven cases.

Table I: Results of PCR in Tuberculous and Control Children

	No (%) of Patients with Positive Results					
	Tuberculosis					
Sample	Proven	Highly Probable	Probable	Total	Control	Total
Cerebrospinal fluid	2/2 (100)	2/8 (25.0)	2/11 (18.2)	6/21 (28.5)	1/36 (2.7)	7/57 (12.2)
Sputum-gastric lavage	1/1 (100)	1/3 (33.3)	1/10 (10.0)	3/14 (21.4)	0/8 (0)	3/22 (13.6)
Peritoneal fluid	1/1 (100)	2/2 (100)	–	3/3 (100)	0/4 (0)	3/7 (42.8)
Pleural fluid	1/1 (100)	2/2 (100)	1/3 (33.3)	4/6 (66.6)	0/8 (0)	4/14 (28.5)
Pericardial fluid	–	1/2 (50.0)	0/2 (0)	1/4 (25.0)	0/3 (0)	1/7 (14.2)
Urine	–	0/1 (0)	0/1 (0)	0/2 (0)	–	0/2 (0)
Biopsy specimen	3/3 (100)	–	0/1 (0)	3/4 (75.0)	0/1 (0)	3/5 (60.0)
Blood	–	–	–	–	0/9 (0)	0/9 (0)
Bone marrow	–	–	0/1 (0)	0/1 (0)	–	0/1 (0)
Infectious aspirate	–	–	–	–	0/2 (0)	0/2 (0)
Total	8/8 (100)	8/18 (44.4)	4/29 (13.7)	20/55 (36.3)	1/71 (1.4)	21/126 (16.6)

Discussion

The diagnosis of pediatric tuberculosis is dependent on a high index of suspicion based on history, physical examination and initial laboratory examination of the samples and culture. However, smears are usually negative and cultures take too long and frequently are negative in pediatric cases; generally the clinical findings alone lead to initiation of antituberculous therapy. Thus, diagnosis of tuberculosis in childhood requires a reliable and fast technique. However, many tests developed for this purpose have been found to be lacking in either specificity or sensitivity. PCR is a promising test in the diagnosis of tuberculosis with its high sensitivity and specificity. In this technique, following amplification, mycobacterial DNA can be detected in clinical samples from many children with primary tuberculosis, despite the fact that the same samples are negative by culture. The sensitivity of PCR is related to the type of mycobacteria. M.tuberculosis and M.microti harbor 10 to 20 copies of IS6110, which was the primer used in our study, while M.bovis and M.bovis BCG appear to contain a single copy[15].

We found that 21 of the 126 samples were positive in the PCR (Table I). Only eight of the samples were also culture-positive.

We performed PCR on cerebrospinal fluid, sputum or gastric lavage, and eight other samples from 126 children and found a significant number of PCR-positive

(13 of 118), culture-negative and smear-negative samples from different sites. Other investigators have reported similar high positive rates[16-18].

The specificity of the PCR was excellent. There was only one sample with an amplicon of 123 bp in the control group. It is not possible to exclude the possibility that this case had a subclinical tuberculous infection. If it was a false-positive result, it could have been caused by true contamination or carry-over (contamination by the products of the amplification which can serve as a substrate for the generation of more product)[19]. The proportion of false-positive results observed here (1.4%) is comparable to that seen in other series[20].

Although the sensitivity of PCR in this study was 100%, with eight positive results in eight culture-positive samples, false-negative results have been reported previously[21, 22]. These false-negative results could have resulted from 1) The presence of inhibitors not detected by the control amplification; 2) non-homogeneous distribution of bacteria in the specimen such that the fraction tested did not contain mycobacteria; or 3) low numbers of bacilli in the specimen, which decreases the probability of mycobacteria being found in the fraction analyzed by PCR[17].

Detection of M.tuberculosis by PCR has previously been shown to be more sensitive than microscopy and much faster than culture[16, 17, 18, 23]. We believe that PCR is a definitive diagnostic alternative for tuberculosis.

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