CLINICAL UTILITY OF POLYMERASE CHAIN REACTION FOR IMPROVED DIAGNOSIS OF PULMONARY TUBERCULOSIS IN CHILDREN

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Summary

Background: Conventional bacteriological methods rarely detect M. tuberculosis in clinical samples from children and, hence are of limited use in the diagnosis of active tuberculosis in them. There is need for an alternative detection method which is rapid, specific and sensitive.

Aim: The efficacy of Polymerase Chain Reaction (PCR) was evaluated in the diagnosis of pulmonary tuberculosis in children.

Methods: Sixty two (62) hospitalized children were included in the study. Thirty-one were suffering from active tuberculosis, 11 children had tuberculous infection but no active disease and 20 children had other unrelated diagnosis. Early morning gastric aspirates and sputum samples were processed using conventional techniques of mycobacterial isolation and PCR for M. tuberculosis complex specific MPB64 gene. Favorable response to anti-tubercular treatment (ATT) was taken as the gold standard.

Results: In the active tuberculosis group 12 out 31 children were positive by PCR while microscopy and culture were positive in 3 and 6 children respectively. All samples positive by microscopy and culture were positive by PCR giving a sensitivity of 100% for culture confirmed cases.

Conclusion: Benefit of rapid and reliable results with PCR offers an appreciable advantage over traditional techniques when used in conjunction with clinical profile and epidemiological factors such as age, socio-economic and nutritional status, contact history and any other intercurrent illness. [Indian J Tuberc 2006: 53:212-216]

Key Words: PCR, Mycobacterium tuberculosis, Tuberculosis, Children, Diagnosis.

INTRODUCTION

In India, the overall prevalence of infection (as judged by the standard tuberculin test) is about 30% in contrast to 2-3% in developed countries. About 20% of children become tuberculin positive by the age of 5 years and 55% by 20 years¹. As much as 43% of children under the age of 1 year, 24% of children 1-5 years of age and 15% of adolescents develop active tuberculosis sometime after primary infection. The diagnosis of active tuberculosis in children is usually epidemiological and indirect which include – exposure tracing, adequate history, tuberculin skin test, chest roentgenogram and physical examination².

Conventional diagnostic tests such as acid fast stain are frequently negative in children with active tuberculosis disease and multiple cultures give positive results in no more than 20% of samples³. A delay in diagnosis has been directly related to poor prognosis and severe disease. With PCR becoming widely available, it is important to know how to interpret its results in children. We have tried to evaluate the role of PCR for detection of M. tuberculosis in clinical samples from children with and without tuberculosis.

MATERIAL AND METHODS

Sixty-two (62) hospitalized children were taken from the Pediatrics ward, Lok Nayak Hospital, New Delhi. Thirty-one (31) cases had clinical diagnosis of tuberculosis which was made in the presence of following criteria:

(1) Abnormal chest X-ray suggestive of tuberculosis like para tracheal gland, hilar lymphadenopathy, segmental lesion, miliary lesion, unresolving pneumonia, chronic cavitary lesion.

With

(2) a) positive tuberculin test which is taken

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as 10-mm or more induration at 48-72 hrs following 1TU of PPD intradermally.

Or

b) an adult source case identified with contagious *M. tuberculosis* disease.

All the children with clinical diagnosis of pulmonary tuberculosis were given anti-tubercular treatment (ATT) and were observed regularly for a minimum period of three months for clinical improvement.

Eleven (11) children had a positive tuberculin skin test but on diagnostic and radiological evaluation had no evidence of active tuberculosis disease. These children were identified during contact investigations and presented with vague symptoms like fever/cough/anorexia/weight loss. All these children received preventive therapy. Twenty (20) children had bacterial or viral broncho-pulmonary infections with negative tuberculin skin test and who completely recovered after appropriate non-tubercular treatment (Table 1).

Early morning gastric aspirates and sputum specimens (from older children) were collected from all the children before the institution of any treatment. All the specimens were decontaminated and concentrated by using conventional techniques. Each sample was examined by Ziehl-Neelsen (ZN) stain and cultured on Lowenstein-Jensen (LJ) medium.

PCR was performed in three different areas physically separated from each other. The reaction mix was made in a sterile laminar flow hood. DNA extraction and PCR were carried out according to the protocol published by Dar L et al. A 240 bp region (460-700) from the gene encoding MPB 64 protein was selected for amplification using the following primers:

1. [460-479] - 5′ - TCCGCTGCCAGTCGTCTTCC- 3′
2. [700-681] - 5′ - GTTCTCGCGAGTCTAGGCCA- 3′

Amplification of the gene was done in 50µl of reaction mixture containing 10µl of genomic DNA, 10mM Tris HCl (pH-8.3), 1.5 mM MgCl₂, 50mM NaCl, 200µl of each dNTP (Boehringer Mannheim, Germany), 0.4µM of each primer and 1.25 U of Taq DNA Polymerase (Amplitaq, Perkin Elmer). The reaction mixture was overlaid with 50µl of light mineral oil to prevent evaporation. Amplification was carried out for 40 cycles in a DNA Thermal cycler (Perkin Elmer, Cetus, USA). Each cycle comprised of 3 steps:

- denaturation (94°C for 2 minutes);
- annealing (55°C for 2 minutes);
- extension (72°C for 2 minutes).

Table 1: Demographic profile of the study groups

<table>
<thead>
<tr>
<th></th>
<th>Clinically active disease</th>
<th>Infection without disease</th>
<th>No Tuberculosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of children</td>
<td>31</td>
<td>11</td>
<td>20</td>
<td>62</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>4.2</td>
<td>3.9</td>
<td>5</td>
<td>4.3</td>
</tr>
<tr>
<td>Sex ratio (male:female)</td>
<td>1:0.82</td>
<td>1:0.90</td>
<td>1:0.53</td>
<td>1:0.75</td>
</tr>
<tr>
<td>No. of children</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>vaccinated with BCG</td>
<td></td>
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The final extension step was for 5 minutes at 72°C. Each test included a positive control consisting of DNA extracted from *M. tuberculosis* 37 Ra, and a minimum of three interspersed negative controls containing distilled water in the place of sample.

**Detection and Identification of the Amplified Product:**

The amplified product was electrophoresed into 2% agarose gels. The gels were stained with ethidium bromide and visualized in an ultraviolet transilluminator. The presence of a 240 bp fragment indicated a positive PCR result. Hae III digest of FX174 DNA was used as molecular size marker.

**RESULTS**

The results are shown in Table 2. PCR was positive in all 6 culture positive samples showing 100% correlation with culture results. However PCR was positive in 6 culture negative specimens also, thus detecting 19.3% more cases when compared to culture results and 29.03% more cases when compared to microscopy results. All the 11 children who had tuberculosis infection but no disease were negative by microscopy and culture but 2(18.1%) were positive by PCR. Twenty (20) children with other unrelated broncho-pulmonary infections were negative by microscopy, culture and PCR.

**DISCUSSION**

Tuberculosis is difficult to diagnose in children because of poor yield on standard diagnostic laboratory tests and the lack of characteristic symptoms in majority of the children. A presumptive diagnosis is usually established on the basis of clinical criteria and the results of tuberculin skin test. In countries where BCG is commonly used, the decision is further complicated by difficulties in interpretation of skin test results. The problem is compounded by the fact that conventional epidemiological tools used for assessment are difficult to apply in this category because of the absence of an objective method or a ‘gold standard’ for the diagnosis of tuberculosis.

Our study confirmed the difficulty in obtaining bacterial proof of tuberculosis in children. Only 9.67% (3/31) of children with active disease had positive smears and 19.3% (6/31) had positive cultures which is comparable with other studies from India and abroad. DNA amplification by PCR identified *M. tuberculosis* in 38.7% (12/31) of children with active tuberculosis. In microscopy negative samples, the sensitivity of PCR was 53.3% (12/22) and specificity was 95.8% (18/19). The positive predictive value was 90.9% (12/13) and negative predictive value was 89.2% (18/20).

**Table 2: Bacteriological and PCR results in the three study groups**

<table>
<thead>
<tr>
<th>Tuberculosis status</th>
<th>Children with a positive result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smear</td>
</tr>
<tr>
<td>Active disease (n=31)</td>
<td>5(9.67%)</td>
</tr>
<tr>
<td>Infection without disease (n=11)</td>
<td>0</td>
</tr>
<tr>
<td>No Tuberculosis (n=20)</td>
<td>0</td>
</tr>
</tbody>
</table>

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specimens and culture negative specimens PCR was able to detect 29.03% and 19.3% more positive cases respectively. It was unlikely that these were false positive samples because all the positive patients by PCR showed improvement on institution of ATT.

Most of the American and European studies\textsuperscript{7,8,10} have shown PCR positivity on specimens from children with pulmonary tuberculosis to be between 24% and 83%.

There were 19 cases of active tuberculosis in whom PCR was negative. Of these, 11 children had hilar shadows but no pulmonary infiltrates on chest X-ray which is consistent with the common observation that positive results are obtained more often in specimens from children with pulmonary infiltrates than with hilar adenopathy alone\textsuperscript{1}. Other negatives could have resulted from various reasons. Because samples were collected in the course of routine diagnostic evaluation, determined by the child’s primary physician, we had limited control over the number of specimens collected from each child. Although it is possible that a higher number of specimens may have increased the yield of PCR\textsuperscript{3,8}, our study is representative of clinical practice. Moreover, our hospital is a referral centre and rarely receives patients who have not received some treatment (including ATT) prior to admission. The previous treatment status often proves difficult to establish, as many of our patients are illiterate.

\emph{M. tuberculosis} was also detected in 2 children (18.1%) with tuberculosis infection without disease. It was unlikely that this was a false positive reaction because a rigorous protocol was followed to prevent carry over contamination and negative controls yielded consistently negative results. Both these children had a recent history of exposure to active pulmonary tuberculosis. Our results signify that \emph{M. tuberculosis} was present in sufficient density to be detected in samples by PCR and also highlight the artificial character of the clinical distinction between infection and disease. Recognition of infected patients without symptoms is difficult in pediatric practice. In children, the development of tuberculosis disease is usually a continuum from infection to lympho-hematogenous spread to pulmonary disease. It can be difficult to determine clinically at what point tuberculosis infection progresses to disease\textsuperscript{10}. More than half of these children have enlarged lymphnodes as assessed on CT scan but with no detectable disease on chest radiograph.

Positive PCR results have been reported in children with tuberculosis infection but no apparent disease\textsuperscript{3,8,10}. American Thoracic Society\textsuperscript{11} gives guidelines for treatment of infected children based on clinical and radiographic criteria and recommends a one-drug regimen with isoniazid. Thus according to these established criteria, PCR is not a good test to distinguish between tuberculous infection from disease in children who have been infected recently. We therefore, consider it unjustified to collect gastric aspirates or sputum samples for PCR routinely in children with suspected tuberculosis infection and rather it should be reserved for clinically suspected cases of tuberculosis disease with abnormal pulmonary findings.

We conclude that though, the sensitivity of PCR is certainly higher than that of culture for detecting \emph{M. tuberculosis} in children, PCR alone is insufficient as a single diagnostic test. Technical considerations, including the use of suitable controls and the retesting of doubtful positive samples considerably influence the sensitivity and specificity. But a well standardized PCR when taken in conjunction with epidemiological and clinical factors, offers the benefit of rapid and reliable results within 48 hrs thereby removing a significant amount of clinical diagnostic uncertainty and influences the decision regarding ATT. Specific early diagnosis will lead to evidence based management of children with tuberculosis in comparison to empirical therapy adopted in most cases.

REFERENCES


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**57th TB SEAL CAMPAIGN**

His Excellency Dr. A.P.J. Abdul Kalam, President of India, who is also the Patron of the Association, inaugurated the 57th TB Seal Campaign – Gandhi Jayanti Day - at a solemn function held at Rashtrapati Bhawan on 2nd October, 2006, when TB Seals on ‘Cultural Heritage’ were presented to Rashtrapati by Dr. Anbumani Ramadoss, Minister for Health and Family Welfare. The special souvenir brought out on the occasion was presented by Smt. P. Lakshmi, Minister of State for Health & Family Welfare, to Rashtrapati.