

## Original Article

# Detection of *Mycobacterium tuberculosis* in smear negative sputum by PCR.

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### Abstract

Pulmonary tuberculosis is a major health problem in Bangladesh that is responsible for about 7% of total death in a year. This study was conducted to isolate and identify *Mycobacterium tuberculosis* from sputum and to evaluate the efficacy of PCR as a modern diagnostic tool, for diagnosis of pulmonary tuberculosis, especially in the smear negative cases. One hundred and fifty suspected pulmonary TB patients (male/ female: 97/53) were included in this study. Single morning sputum was collected from each patient and diagnostic potential of PCR was compared with staining and culture. Twenty five (16.7%) sputum were positive by ZN stained smear. Among 125 smear negative samples, 13 (10.4%) yielded growth in culture in LJ media and 21 (16.8%) samples were positive by PCR. The sensitivity and specificity of PCR in smear negative cases was 100% and 92.9% respectively. Mean detection time in PCR was 24 hours. PCR detected *M. tuberculosis* in 21 smear negative and 9 culture negative samples. For diagnosis of tuberculosis in smear negative cases, PCR directly from sputum was a very sensitive and accurate method. In conclusion, PCR may be done, especially in clinically suspected pulmonary tuberculosis patients who remain negative by conventional methods.

**Key words:** *Mycobacterium tuberculosis*, PCR, Smear.

### Introduction

Tuberculosis (TB) is one of the deadliest diseases, infecting one-third of the world population, causes over 1.6 million deaths each year<sup>1,2</sup>. India, China, Indonesia, Bangladesh and Pakistan together contribute about half (48%) of the new cases diagnosed every year<sup>3</sup>. Pulmonary TB is the fourth major cause, second infectious cause of death in Bangladesh which is responsible for 7% of total deaths in the country<sup>4</sup>. Like many other TB endemic countries, many multidrug-resistant (MDR) TB and a few extensively drug-resistant (XDR) TB have been reported in Bangladesh<sup>5,6</sup>.

Diagnosis of TB is mainly based on microscopic detection of acid fast bacilli (AFB) in smear which has 40%-70% sensitivity and culture which needs up to 8 weeks time<sup>7-9</sup>. Smear is considered useful only as a screening test<sup>8</sup>. Negative sputum smear does not exclude TB in highly suspected cases (10). None of the serodiagnosis has sufficient sensitivity and

specificity<sup>11</sup>. Delay in diagnosis together with misdiagnosis spreads infection in the community and increases severity of the disease<sup>12</sup>. PCR increases the sensitivity and specificity of laboratory tests in detecting *Mycobacterium tuberculosis*<sup>13</sup>. Amplification of different DNA sequences by PCR has been done with encouraging results<sup>14,15</sup>. Most commonly used PCR target is the repetitive sequence IS6110 and is specific for it (13). This study was designed to detect *M. tuberculosis* directly from sputum by PCR.

### Material and methods

This cross sectional study was conducted from January to December 2010. All laboratory works were done in the department of Microbiology of Dhaka Medical College, Dhaka.

A total of 150 clinically suspected pulmonary TB patients, regardless of their age, sex, socio-economical status were included in this study. The patients were enrolled from the Tuberculosis Control and Training Institute, Chankharpu, Dhaka and OPD of Dhaka Medical College Hospital. Clinical suspicion was based on the presence of fever and productive cough for more than three weeks with or without result of Mantoux test and radiological evidence. Patients on anti-tubercular drugs or completing anti-tubercular drug regimen within last one year were excluded.

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The protocol was approved by the Research Review Committee and Ethical Review Committee of Dhaka Medical College. Informed written consent was taken from each patient before collection of sample.

**Sample collection:** Freshly passed single morning sputum was collected from each patient and was immediately transported to the laboratory for further processing. For Mantoux test 10 TU of PPD was used and reading was taken after 72 hours.

#### Laboratory procedures:

**Microscopic examination:** Smear was prepared directly from sputum and was stained with Ziehl-Neelsen (ZN) stain.

**Decontamination:** Sputum was treated with 4% NaOH for digestion and decontamination following Petroff's sodium hydroxide method <sup>16</sup>. Sputum sediment was used for culture and PCR.

**Culture:** Decontaminated sputum was inoculated on Lowenstein-Jensen medium and was incubated at 37°C aerobically.

**Species identification by biochemical test:** *M. tuberculosis* and atypical Mycobacteria were differentiated by biochemical tests- nitrate reduction test, niacin test, catalase test and subculture in PNB media.

#### Polymerase chain reaction:

**DNA extraction:** DNA was extracted following Simple boiling method (17). One ml sterile distilled water was added to 300 µl sputum sediment. After mixing by vortexing, the content was centrifuged at 10,000 rpm for 5 minutes. Pellet was washed again and then 200 µl of distilled water was added to the pellet and was heated at 100°C for 10 minutes and was cooled down quickly. After centrifugation at 14,500 rpm for 10 minutes, 20-30 µl of supernatant was taken and used for PCR.

**Amplification of DNA:** Template DNA was amplified using IS6110 primer (F: 5'-CTC GTC CAG CGC CGC TTC GG-3' and R: 5'-CCT GCG AGC GTA GGC GTC GG-3') <sup>18</sup>. PCR was performed in a final reaction volume of 25 µl, containing 1.5 µl each primer (Ist Base, Singapore), 3 µl extracted DNA, 12.5 µl Mastermix and 6.5µl Nuclease free water. After initial denaturation at 94°C for 10 minutes, the reaction was subjected to 35 cycles (each cycle consisting of denaturation at 94°C for 1 minute, annealing at 67°C for 50 seconds and elongation at 72°C for 50 seconds) followed by a final extension at 72°C for 7 minutes.

**Gel electrophoresis:** PCR products were detected on 1.5% agarose gel that was subjected to electrophoresis for 35 minutes at 100 volts. Gel was stained with ethidium bromide and was observed under UV Transilluminator.

**Interpretation:** Samples showing presence of IS6110 specific DNA band corresponding to 123 bp were considered positive for *M. tuberculosis*. In each experiment, positive control (reference strain H37Rv), as well as a negative control (replacing DNA with distilled water during amplification) was included.

**Data processing and analysis:** All data were analyzed using 'Microsoft Office Excel 2007' program. Sensitivity and specificity was calculated following standard formula.

#### Results

Among 125 smear negative sputum samples, 13 (10.4%) samples were positive by culture in LJ media and 21 (16.8%) were positive by PCR. When either culture or PCR is considered, 23 (18.4%) samples were positive (table-I).

**Table I: Results of different tests in smear negative cases (n= 125).**

| Test                  | Samples with positive result |
|-----------------------|------------------------------|
| Culture               | 13 (10.4%)                   |
| PCR                   | 21 (16.8%)                   |
| Either culture or PCR | 23 (18.4%)                   |

Scanty' bacilli were detected in one (4%) case, '2+' bacilli were detected in 7 (28%) and '3+' bacilli were detected in 17 (68%) cases. No sample had '1+' bacilli (table-II).

**Table II: Grading of Ziehl-Neelsen staining (n=25).**

| *Grading | Number | Percentage |
|----------|--------|------------|
| Scanty   | 1      | 4          |
| 1+       | 0      | 0          |
| 2+       | 7      | 28         |
| 3+       | 17     | 68         |
| Total    | 25     | 100        |

\* WHO Grading (19).

Of the 25 smear positive samples, 23 samples were positive by both PCR and culture. One sample was positive by PCR but negative by culture and one was negative by PCR but positive by culture (table-III).

**Table III: Comparison between culture and PCR in smear positive cases (n=25).**

| PCR             | Culture    |          |
|-----------------|------------|----------|
|                 | Positive   | Negative |
| Positive (n=24) | 23 (95.83) | 1 (100)  |
| Negative (n=1)  | 1 (4.17)   | 0 (0)    |
| Total           | 24 (100)   | 1 (100)  |

Figures within parentheses indicate percentage

Of 125 smear negative samples, 13 samples were positive by both PCR and culture, whereas 104 were negative by both the methods. Remaining 8 culture negative samples were positive by PCR. The sensitivity of PCR in smear negative cases was 100% and specificity was 92.86% (table-IV).

**Table IV: Comparison between culture and PCR in smear negative cases (n=125).**

| PCR              | Culture  |             |
|------------------|----------|-------------|
|                  | Positive | Negative    |
| Positive (n=21)  | 13 (100) | 8 (7.14)    |
| Negative (n=104) | 0 (0)    | 104 (92.86) |
| Total            | 13 (100) | 112 (100)   |

Figures within parentheses indicate percentage.

Total 24 samples were positive by both ZN stain and PCR. Twenty one samples were negative by stain but positive by PCR and one sample was positive by ZN stain but negative by PCR (table-V).

**Table V: Comparison between PCR and ZN stain.**

| ZN Stain        | PCR        |             |
|-----------------|------------|-------------|
|                 | Positive   | Negative    |
| Positive (n=25) | 24 (53.33) | 1 (0.95)    |
| Negative(n=125) | 21 (46.67) | 104 (99.05) |
| Total           | 45 (100)   | 105 (100)   |

Figures within parentheses indicate percentage.

### Discussion

Tuberculosis is the first infectious disease declared as a global health emergency by the World Health Organization<sup>20</sup>. Early diagnosis and prompt treatment is necessary to limit the spread of infection. Although most of the sputum positive cases are diagnosed by observing acid fast bacilli in ZN stained smear and culture in LJ media, quite a few sputum smears become negative and some become negative in culture also. The main objective of the present study was to find out the efficacy of PCR in diagnosis of the smear negative pulmonary tuberculosis cases.

In ZN staining, scanty bacilli were detected in 4%, '2+' bacilli were detected in 28% cases, '3+' were detected in 68% cases. Similar results were reported by Iqbal (2010) in Bangladesh (5). Ninety-six percent of positive smear detected '2+' and '3+' bacilli which means in these patients bacterial load were enormous. This is probably due to negligence of patients who are very much reluctant about their health problems that led the disease progression in them. Mean duration of cough among confirmed TB cases was  $71 \pm 12.5$  days which also proves the delay in taking health care among these patients.

Thirteen (10.4%) of the smear negative cases were found positive in culture. Sensitivity of ZN stain was 64.9%. Only 1 smear positive specimen was negative in culture, thus showing 99.12% specificity of ZN stain. Higher specificity of staining was probably due to the low prevalence of Non-tuberculous Mycobacterium in this region, as they cannot be discerned from *M. tuberculosis* on smear. As also described by other authors<sup>21,22</sup>, the sensitivity of ZN stain was not as good as its specificity. However, considering the availability and technical easiness, ZN stain should still be used as the method of choice in screening of tuberculosis.

Considering culture as gold standard, in total sample the sensitivity of PCR was 97.30% and specificity was 92.04%. Findings of the present study coincide with the findings of other studies<sup>15,23</sup>. One culture positive sample which was negative by PCR was identified as Non-tuberculous Mycobacterium by biochemical tests. As the IS6110 primer was specific for *M. tuberculosis* complex this NTM specimen was negative in PCR. In 125 smear negative sputum, 13 samples were positive by both culture and PCR; whereas 8 culture negative samples were found positive by PCR. The sensitivity of PCR in smear negative cases was 100% and specificity was 92.86%, positive predictive value was 61.9%, negative predictive value was 100%.

In this study, 21 (16.8%) smear negative sputum were positive by PCR and 9 (7.96%) culture negative specimens gave positive result in PCR. These 'culture-negative, PCR-positive' cases might be due the failure of the bacteria to grow in culture, probably due to insufficient number in the samples. PCR requires as few as 10 organisms in a sample for detection whereas for ZN stain  $10^3$ - $10^4$  organisms and for culture 10-100 organisms per milliliter of sputum is required<sup>8,10</sup>.

There is provability that some of these smear negative, culture negative results may have represented actual positive cases of tuberculosis, as these samples belonged to clinically

suspected pulmonary TB patients. This is supported by the fact that among the smear negative cases that were positive by PCR total 7 cases had induration of  $10\geq\text{mm}$  in Mantoux test and 9 cases had chest X-Ray report suggestive of pulmonary TB. Besides, DNA contamination was less likely in this study, as strict procedures were employed during laboratory work. All the negative controls used were found negative, which ruled out the possibility of contamination. So these 'smear negative, culture-negative but PCR-positive' results can be considered as potential of PCR in detection of M. tuberculosis in sputum.

The aim of this study was to identify Mycobacterium directly from sputum by PCR and to compare its efficacy over routinely used methods. Radiology and smear still remain the methods of diagnosis of TB in Bangladesh<sup>7</sup>. As they have some disadvantages, PCR was studied as a possible alternative. Prompt demonstration of M. tuberculosis using species-specific primer improves the quality of diagnosis.

Among different procedures used in this study PCR has the highest sensitivity with a detection time less than 24 hours. Considering the diagnostic potential of PCR against ZN stain and culture, this newer rapid diagnostic technique can be introduced which will help in the effective management and control of TB. For the diagnosis of pulmonary tuberculosis, PCR from sputum may be recommended, especially in clinically suspected patients who remain negative by ZN stained smear.

#### Financial support:

The work was supported financially by the department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh.

#### Acknowledgement:

We acknowledge the Tuberculosis Control and Training Institute, Chankharpul, Dhaka, Bangladesh for providing the sample collection facility.

**Conflict of interest:** We do not have any potential conflicts of interest.

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