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Rapid Diagnosis of Pulmonary Tuberculosis From Sputum by Polymerase Chain Reaction

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Background: Tuberculosis (TB) is a curable infectious disease yet the leading cause of death worldwide and in Bangladesh it is responsible for 7% of total deaths every year.

Objectives: This cross sectional study was conducted to isolate and identify *Mycobacterium tuberculosis* from sputum and to evaluate the efficacy of polymerase chain reaction (PCR) for the early diagnosis of pulmonary tuberculosis.

Patients and Methods: A total of 150 clinically suspected pulmonary TB patients (male/female: 97/53; age: 31.9 ±9.8 years) were enrolled in this study. Freshly passed single morning sputum was collected from each patient and diagnostic efficacy of PCR was compared with staining and culture methods.

Results: Among 150 sputum samples, 25 (16.7%) were positive by Ziehl-Neelsen (ZN) stain, 37 (24.7%) yielded growth in Lowenstein-Jensen (LJ) media and 45 (30%) were positive by PCR. Of the 37 isolated *Mycobacteria* in culture, 36 were *M. tuberculosis* and one was identified as non-tuberculous Mycobacterium by PCR and biochemical tests. The mean detection time was 26.9 days for the culture and 12 hours for the PCR method. Considering the culture method as the gold standard, the sensitivity of PCR was 97.3% and specificity was 92% with accuracy of 93.3%. Twenty-one (16.8%) smear negative cases and nine (7.9%) culture negative samples were positive by the PCR method.

Conclusions: PCR was the most sensitive, accurate and rapid method for diagnosis of TB. It was concluded that, for the diagnosis of pulmonary tuberculosis, PCR directly from sputum may be recommended in Bangladesh, especially in suspected tuberculosis patients who remain negative by conventional methods.

Keywords: Bangladesh; *Mycobacterium tuberculosis*; Polymerase Chain Reaction; Tuberculosis, Pulmonary

1. Background

Tuberculosis is the first infectious disease declared as a global health emergency by the World Health Organization (WHO) (1). The incidence of TB worldwide was 9.4 million in 2008, at a rate of 140 per 100000 individuals (2) and if current trends of TB persist, this rate will reach about 150 in 2015 (3, 4). TB, which currently holds the seventh place in the global ranking of causes of death, kills over 1.6 million people each year (5). About 90% of tuberculosis cases and deaths occur in the developing world where 75% of cases occur within income generating years (15-54 years) of people's life, with a huge social and economic disruption (5, 6). India, China, Indonesia, Bangladesh and Pakistan together contribute to about half (48%) of the new cases every year (3). Bangladesh ranked sixth among the world's high-burden TB countries in 2008 (7). Pulmonary TB is the fourth major cause and the second infectious cause of death and is responsible for 7% of total death every year (8). There is an estimated 353103 new cases with an incidence rate of 223 per 100000 individuals in Bangladesh (in 2007) (7).

The emergence of multidrug-resistant (MDR) strains of *M. tuberculosis* and the HIV epidemic has contributed globally to the resurgence of tuberculosis (1). Tuberculosis is the major opportunistic infection of AIDS which is responsible for 15% of AIDS related deaths worldwide (9).

In most developing countries, diagnosis of TB is based on microscopic detection of acid-fast bacilli (AFB) in smear samples (10). Smear examination can yield results within 24 hours but it is neither specific for *M. tuberculosis* nor very sensitive, requiring 10^3 to 10^4 organisms per milliliters of sputum (11). Smear cannot distinguish *M. tuberculosis* from other species and is considered useful only as a screening test (12). Culture of *Mycobacteria* is time consuming; it requires up to eight weeks for isolation (1). Culture also requires a high standard of technical expertise and in 10-20% of positive cases bacillus is not successfully cultured (13). This delay in diag-

Implication for health policy/practice/research/medical education:
This study will help in the early diagnosis of pulmonary tuberculosis which will be possible in 2 to 3 days. PCR may also help in the smear negative tuberculosis. The early and accurate diagnosis by this molecular method will decrease the disease burden as well as the tuberculosis related morbidity in Bangladesh.

nosis together with misdiagnosis spreads the infection within communities, increases severity of the disease and is associated with higher risk of mortality (14).

Detection of *M. tuberculosis* by amplification of different DNA sequences by PCR has been done with encouraging results (15, 16). Many amplification targets have been reported; from these, the most commonly used is the insertion sequence IS6110, which is specific for the *M. tuberculosis* complex (17-19). Compared with the culture method, the specificity of PCR in clinical laboratories is almost 100%, having sensitivity about 95% (20), with detection limits of as few as 10 bacilli (21). PCR allows detection of *M. tuberculosis* within one day (15). In Bangladesh, pulmonary TB is mainly diagnosed by Ziehl-Neelsen (ZN) staining of sputum. Culture in Lowenstein-Jensen (LJ) media is done in a few centers.

2. Objectives

This study was designed to identify *M. tuberculosis* directly from sputum by PCR and to compare the results with ZN stain and culture methods and to evaluate their efficacy.

3. Patients and Methods

This cross sectional study was carried out from January to December 2010. One hundred and fifty clinically suspected pulmonary TB patients, who were referred to the Tuberculosis Control and Training Institute, Chankharpul, Dhaka and outpatient department of Dhaka Medical College Hospital, Dhaka were included in this study, regardless of their age, sex and socio-economical status. Clinical suspicion was based on the presence of fever and productive cough for more than three weeks with or without radiological evidence and result of Mantoux test. Patients on anti-tubercular drugs or completing anti-tubercular drug regimen within the last one year were excluded from the study. All laboratory work was done in the department of Microbiology of Dhaka Medical College, Dhaka. The protocol was approved by the Research Review Committee and Ethical Review Committee of Dhaka Medical College. Informed written consent was obtained from each patient before sample collection, according to the guidelines of the Ethical Review Committee. Freshly passed single morning sputum was collected from each patient in a sterile, disposable plastic container and was immediately transported to the laboratory for further processing. Chest X-ray and Mantoux test were performed for each patient. For the Mantoux test, 10 TU of purified protein derivative (PPD) was used and reading was taken after 72 hours.

3.1. Laboratory Procedures

3.1.1. Microscopic Examination

Smear was obtained directly from sputum and was stained with the Ziehl-Neelsen (ZN) stain.

3.1.2. Decontamination

Sputum was treated with 4% NaOH for digestion and decontamination following Petroff's sodium hydroxide method (22). Then, the sputum was concentrated by centrifugation at 3000 rpm at 4°C for 15 minutes; the supernatant was discarded and 2 mL of sediment was preserved for culture and PCR.

3.1.3. Culture

Decontaminated sputum was inoculated in Lowenstein-Jensen medium and incubated at 37°C under aerobic conditions, in the dark for 10 weeks. Culture was considered to be negative if there was no growth after 10 weeks.

3.1.4. Species Identification by Biochemical Tests

Typical and atypical *Mycobacteria* were differentiated by the niacin test, nitrate reduction test, catalase test and subculture in para-nitrobenzoicacid (PNB) media. *M. tuberculosis* was niacin positive, nitrate positive, produced heat labile catalase and did not grow on PNB media.

3.1.5. Polymerase Chain Reaction

3.1.5.1. DNA Extraction

DNA was extracted following the standard extraction process (simple boiling method) (23). One milliliters of sterile distilled water was added to 300 μL of the sputum sediment. After mixing, the content was centrifuged at 10000 rpm for 5 minutes and the supernatant was discarded. The pellet was washed again and 200 μL of distilled water was added to the pellet and the container was placed in a block heater at 100°C for 10 minutes and cooled down quickly by placement on ice. After centrifugation at 14500 rpm for 10 minutes, 20-30 μL of the supernatant was taken and used for the PCR reaction.

3.1.5.2. Amplification of DNA

The isolated DNA template was amplified using the IS6110 primer (F: 5´-CTC GTC CAG CGC CGC TTC GG-3´ and R: 5´-CCT GCG AGC GTA GGC GTC GG-3´) (24) in a thermal cycler (Eppendorf AG, Mastercycler gradient, Germany). PCR was performed in a final reaction volume of 25 μL, containing 1.5 μL of each primer (1st Base, Singapore), 3 μL of extracted DNA, 12.5 μL of master mix and 6.5 μL of nuclease free water (Promega Corporation, USA). After the 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.

3.1.5.3. Gel Electrophoresis and Interpretation

PCR products were detected on 1.5% agarose gel that was subjected to electrophoresis for 35 minutes at 100 volts. The gel was stained with ethidium bromide and observed under a UV transilluminator. Samples showing the presence of an IS6110 specific DNA band corresponding to 123

bp were considered positive for *M. tuberculosis*. In each experiment, a positive control with *M. tuberculosis* DNA extracted from reference strain H37Rv, as well as a negative control, in which DNA was replaced with distilled water during amplification, were included (Figure 1)

3.2. Data Processing and Analysis

All data were compiled and analysis was done using the Microsoft Office Excel 2007 program. The test of significance was calculated by using $\chi 2$ (Chi-square test). $P < 0.05$ was considered as the minimal level of significance. Performance of different methods was calculated by examination of sensitivity and specificity.

4. Results

A total of 150 clinically suspected pulmonary TB patients were enrolled in this study and single morning sputum sample were collected from each case. Among 150 sputum samples, 25 (16.7%) were positive for AFB by ZN staining, 37 (24.7%) samples were positive by culture in LJ media and 45 (30%) were positive by PCR. Thirty-eight (25.3%) samples were positive by either stain or culture and 46 (30.7%) samples were positive by either culture or PCR. Forty-six (30.7%) samples were positive by either ZN staining or culture or PCR.

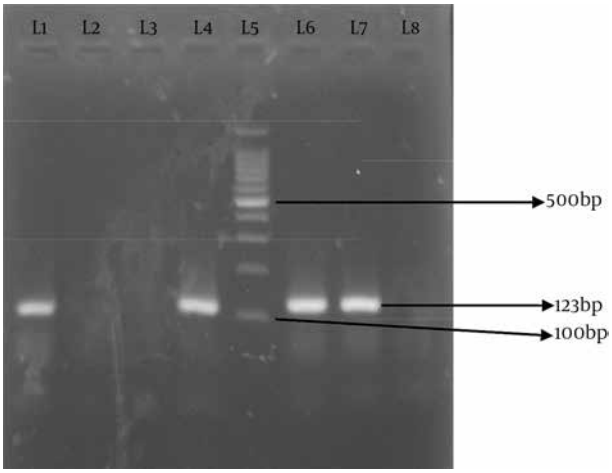
The mean age of culture positive patients was 30.7 (± 9.7) years and males were predominant than females. Most of them (67.6%) were from low socioeconomic condition and 78% had education status below primary level. Duration of productive cough was present for more than 11 weeks (78 ± 14.5 days). Previous history of tuberculosis was present in 5.4% of the positive TB patients (Table 1).

Of the 150 sputum samples, 24 samples were positive as indicated by ZN staining and culture in LJ media, whereas 112 samples were negative by both methods. One sample was positive by ZN staining but negative by the culture method and 13 samples were negative by ZN staining but positive by the culture method (Table 2). Considering culture as the gold standard, the sensitivity of ZN staining was 64% and specificity was 99%. Difference in positivity between culture and ZN staining was statistically significant ($P \leq 0.0001$).

Thirty-six samples were positive by both PCR and culture in LJ media, while 104 samples were negative by both methods. One sputum sample was negative by PCR but positive by the culture method and nine samples were negative in culture but positive by PCR (Table 3). Considering culture as the gold standard, the sensitivity of PCR was 97.3% and specificity was 92%, positive predictive value was 80% and negative predictive value was 99%. Difference in positivity between culture and PCR was statistically significant ($P \leq 0.0001$).

Among the nine 'PCR-positive, culture-negative' cases, two cases had X-ray findings suggestive of pulmonary TB and induration of ≥ 10 mm as indicated by the Mantoux test. Four cases had X-ray suggestive of pulmonary TB but induration of < 10 mm and one case had X-ray not suggestive of pulmonary TB but induration of ≥ 10 mm. Two 'PCR-positive, culture-negative' cases had X-ray findings not suggestive of pulmonary TB and induration of < 10 mm as indicated by the Mantoux test (Table 4).

Figure 1. Photograph of Gel Electrophoresis of Amplified DNA in PCR



L represents lane; Lane 1, 4, 7: positive for DNA of *M. tuberculosis* complex; Lane 5: 100bp DNA ladder; Lane 6: positive control (DNA from reference strain); Lane 3: negative control (DNA replaced by distilled water); Lane 2, 8: negative for DNA of *M. tuberculosis* complex.

Table 1. Demographic Characters of Culture Positive Cases (n = 37)

Character	Frequency
Age, Mean ± SD, y	30.7 ± 9.1
Sex, male/female	26/11
Education, below/above primary	28/9
Socioeconomic status, low/middle/high income	25/12/0
Duration of cough, d	78 ± 14.5
Previous history of tuberculosis	2 (5.4%)

Table 2. Comparison Between Ziehl-Neelsen Stain and Culture in Lowenstein Jensen Media [a]

ZN Stain	Culture, Positive/Negative	Total
Positive	24 (64.86)/1 (0.88)	25 (16.67)
Negative	13 (35.14)/112 (99.12)	125 (83.33)
Total	37 (100)/113 (100)	150 (100)

[a] Data are presented as No. (%).

Table 3. Comparison Between PCR and Culture in Lowenstein Jensen Media[a]

PCR	Culture, Positive/Negative	Total
Positive	36 (97.3)/9 (7.96)	45 (30)
Negative	1 (2.7)/104 (92.04)	105 (70)
Total	37 (100)/113 (100)	150 (100)

[a] Data are presented as No. (%).

Table 4. Chest X-ray and Mantoux Test Findings Among 'PCR-Positive, Culture-Negative' Cases (n = 9) [a,b]

Chest X-ray	Mantoux Test, mm	
	≥ 10	< 10
Suggestive of PTB	2 (66.67)	4 (66.67)
Not suggestive of PTB	1 (33.33)	2 (33.33)
Total	3 (100)	6 (100)

[a] Abbreviations: mm, millimeter; PTB, Pulmonary tuberculosis.
[b] Data are presented as No. (%).

5. Discussion

Almost 95% of all tuberculosis cases occur in developing countries, with a great burden in South East Asia (25). A sensitive and rapid diagnostic method is required for the control and prevention of TB in Bangladesh. In the present study, among 37 culture positive cases, the mean age was 30.7 (± 9.7) years and 32 (86%) patients were between 16 and 45 years old. Males were predominant (70.3%) compared to females and the ratio between males and females was 2.4:1, in culture positive TB cases. This difference may be due to differences in the prevalence of infection between the two sexes or the referral of female patients in the studied area. Sixty-eight percent of TB cases were from a low-income class and 78% had an education status below primary level. TB is more frequent among people with little schooling and low income (26). This might be due to the fact that they are not concerned enough about their health. This is supported by the data of BDHS-2007, according to which knowledge about transmission as well as treatment of TB and rate of BCG vaccination was less among illiterate and low income individuals in Bangladesh (27).

Among 150 sputum samples, 25 were positive by ZN staining, and 37 were positive by the culture method. Considering the culture method as the gold standard, the sensitivity of ZN staining was 64.9% and the specificity was 99.1%. Various authors reported the sensitivity of ZN stainingas 53-78% (28, 29). Thirteen smear negative specimens were positive by the culture method. Despite this lower sensitivity, considering the availability and technical ease, ZN staining method should be used as a method of choice in the first line screening of pulmonary TB.

Out of the 37 isolated Mycobacterium in culture, 36 (97.3%) were detected as *M. tuberculosis* complex by biochemical tests and PCR and 1 (2.7%) was a non-*tuberculous Mycobacterium*. The mean time for detection of *Mycobacterial* growth in LJ media was 26.9 days (27 ±4.2 days). For smear positive cases the detection time was 25.3 days (25 ± 2.9 days) and for smear negative specimens, this was 29.9 days (29 ± 4.8 days). On the other hand, PCR detected *M. tuberculosis* within 12 working hours after collection of sputum.

PCR had positive results for 45 sputum samples. Considering the culture method as the gold standard, the sensitivity of PCR was 97.3% and specificity was 92%, positive predictive value was 80%, negative predictive value was 99% and accuracy was 93%. This higher sensitivity of PCR, ranging between 92 and 97%, has been reported by various authors (16, 30, 31). Comparatively lower sensitivity of 83%-84% was also found by some studies (29, 32). Most authors reported specificity of PCR to be almost 100% (30, 32, 33). However, 70% specificity was reported by one study (16), which is lower than that reported by the present study.

Nine (20%) PCR positive specimens were negative by the culture method. These 'PCR-positive, culture-negative' cases, which were 20% of the entire sample, suggested the failure of growth of bacilli in culture, probably due to insufficient number or non-cultivable bacteria in sputum. PCR requires as few as 10 organisms in a sample, whereas for culture, the detection limit is 10-100 organisms per milliliter of sputum (21, 34). PCR detects DNA of both live and dead or damaged organisms (35). If decontamination process is too harsh, it is possible that some bacilli might lose viability and thus cannot grow on culture. Also there is a possibility of hiding the history of recent treatment for the symptoms, which the patient was receiving, due to social stigma. Different studies (10, 16, 29) have reported 'PCR-positive, culture-negative' cases to range from 16 to 20%, which is in agreement with the present study. Among these nine 'PCR-positive, culture-negative' cases, three cases had induration of ≥10 mm in the Mantoux test and six cases had chest X-ray findings suggestive of pulmonary TB. Besides, DNA contamination was less likely, as strict procedures were employed during the laboratory work. Also, the negative controls or DNA-free controls used during amplification, all showed negative results. Thus, it is possible that these 'PCR-positive, culture-negative' results indicate better effectiveness of PCR in detection of *M. tuberculosis*.

Since this study used culture results as the only criteria for confirmation of tuberculosis, it was not possible to totally exclude the possibility of 'culture negative' cases as TB cases. The smear and culture negative but PCR positive cases may have actually been positive cases of tuberculosis, as these samples belonged to clinically suspected TB patients living in a TB prevalent country. If they are not further evaluated and treated accordingly they will spread the infection in the community and may later come back with more serious disease complications. The recent advancements in some immunodiagnostic tests like Quanti FERON-TB test and antibodies in lymphocyte supernatant (ALS) assay may have a role in diagnosis of smear negative pulmonary TB as well as extrapulmonary TB, especially cryptic tuberculosis. Therefore, further studies with combined clinical, microbiological, immunological, serological and molecular approaches are necessary to evaluate the value of each diagnostic tool.

This study aimed at identification of *Mycobacterium* directly from sputum by the PCR method and compared its effectiveness with conventional methods. Radiological examination, ZN staining and culture still remain the methods of choice for the diagnosis of TB but as they have

disadvantages such as low sensitivity, specificity and time consumption, PCR was studied as a possible alternative. Early diagnosis, effective treatment and cessation of transmission are major strategies for the control of TB. Using species-specific DNA targets, PCR has supplemented clinical diagnosis by prompt demonstration of the presence of *M. tuberculosis*. Therefore, in Bangladesh, PCR directly from sputum should be included in laboratory procedures for the early diagnosis of pulmonary tuberculosis.

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Authors'Contributions

Mohammad Jobayer designed the study, wrote the protocol, performed the statistical analysis, managed literature search and wrote the manuscript. SM Shamsuzzaman and Kazi Zulfiquer Mamun managed analyses of the study and literature searches. All the authors have read and approved the final manuscript.

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