PCR CAN HELP EARLY DIAGNOSIS OF PULMONARY TUBERCULOSIS

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Abstract. One hundred and fifty-one patients, clinically suspected for pulmonary tuberculosis (age: 31 ± 13 years, male/female: 112/39), were investigated to evaluate the diagnostic potential of polymerase chain reaction (PCR) based detection of the *Mycobacterium tuberculosis* complex in sputum. The diagnostic efficacy of PCR was compared with culture of *Mycobacterium tuberculosis* on egg-based Lowenstein-Jensen modified medium. PCR detected 71.5% (108/151), whereas culture detected 66.2% (100/151) of the clinically suspected patients. There was a significant association between the results of PCR and culture ($\chi^2 = 59.524$, $p<0.001$). However, 23.2% (35/151) samples were found negative in both culture and PCR. Considering culture as the gold standard, the sensitivity of the PCR was 92%, and its specificity 70%. This lower apparent specificity may be due to the higher sensitivity of PCR.

INTRODUCTION

Laboratory diagnosis of tuberculosis involves several bacteriologic, immunologic or molecular approaches. However, none of the existing methodologies is without limitations (Bennedsen *et al*, 1996). Microscopic examination of acid-fast stained smears and culture are still the methods of choice in most diagnostic laboratories for the detection *of Mycobacterium tuberculosis,* and culture is still considered the gold standard (Schirm *et al*, 1995). Although culture of mycobacteria has a high specificity and permits drug susceptibility testing, the slow growth of most pathogenic mycobacteria results in delays in definitive diagnosis. Direct staining for acid-fast bacilli (AFB) is the most rapid method, and takes less than 1 hour (Kent and Kubica, 1985). However, for sensitivity, microscopic examination requires a large number of bacteria (>10^4 /ml) in the clinical sample (Kent *et al*, 1994). Moreover, it can not distinguish *Mycobacterium tuberculosis* from other mycobacteria and considered useful only as a screening test (Morre and Curry, 1995). Although the newly introduced radiometric BACTEC, fluorescent compound embedded Mycobacteria Indicator Tube (MGIT) and biphasic Septi-Check systems (Abe *et al*, 1992) are relatively rapid compared with conventional culture, these also take 7-10 days to achieve optimal sensitivity. Studies have been carried out on the serodiagnosis of tuberculosis using different antigens; however, none of them have yet been prove to show sufficient sensitivity and specificity (Lodha *et al*, 2000; Bothamley *et al*, 1989). The tuberculin test, sometimes used for screening tuberculosis, may also indicate an immunization, earlier tuberculin test, or previous exposure. Therefore, a positive tuberculin test does not necessarily indicate infection.

Nucleic acid amplification-based diagnostic approaches may provide very sensitive, specific, and rapid detection of *Mycobacterium tuberculosis*. Various PCR-based assays for the

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detection of *Mycobacterium tuberculosis* species, such as *M. tuberculosis*, *M. leprae*, *M. avium*, *M. intracellulare* have been reported in recent years (Eisenach *et al*, 1990; Fries *et al*, 1990; Hermans *et al*, 1990; Abe *et al*, 1993; Brisson-Noel *et al*, 1998). Many target sequences have been used, but the most thoroughly evaluated assays target the *Mycobacterium tuberculosis*-specific repeat element IS*6110* / IS*986* (Insertion sequence 6110/ 986) (Thierry *et al*, 1990), which is usually present in multiple copies in *M. tuberculosis* complex (Butcher *et al*, 1996). The high copy number of IS*6110* also results in increased sensitivity. Compared with culture, the specificity of PCR in clinical laboratories is essentially 100%, having a sensitivity not less than 95% (Shinnick and Jonas, 1994), with detection limits of 1 to 100 colony-forming units (CFU) (Abe *et al*, 1992). PCR amplified DNA sequences can be identified using *M. tuberculosis* complex-specific commercial probes (Amicosaante *et al*, 1995).

Tuberculosis, especially pulmonary tuberculosis, is highly endemic in Bangladesh (Hafez *et al*, 1991). The national health services of Bangladesh have several specialized TB clinics throughout the country that offer free-of-cost diagnostic and treatment facilities. The tuberculin test, AFB smear and culture on Lowenstein-Jensen (L-J) media remain the only available diagnostic facilities in those centers. Considering the limitations of existing diagnostic facilities, the diagnostic potential of PCR has been evaluated against culture, with the intention to introduce a newer rapid diagnostic technique with improved accuracy.

MATERIALS AND METHODS

Study subjects

This study included 151 patients clinically suspected for pulmonary tuberculosis, regardless of age, sex or occupation. They were recruited from the Tuberculosis Control and Training Center, Chankharpul, Dhaka. Clinical suspicion was based on signs and symptoms with or without evidence from chest X-ray or sputum smear for AFB. A single early morning sputum sample was collected from each patient in a sterile, disposable plastic container and immediately transported to the laboratory. Samples were collected before starting chemotherapy. Subjects were included in this study only after their consent and approval of the protocol by the Ethical Review Committee of BIRDEM (Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine and Metabolic Disorders).

Sample processing

Immediately after collection, sputum samples were treated with N-acetyl-L-cysteine-NaOH (NALC-NaOH) following the standard procedure (Gullans, 1992) and concentrated by centrifugation. From the pellet, half was inoculated on to egg-based Lowenstein-Jensen (L-J) modified medium (Lambi, 1992) and incubated at 37ºC for 10 weeks, with weekly examination for growth. For identification of the *M. tuberculosis* complex, the combined niacin-nitrate test and the thiophene-2-carboxylic acid hydrazid (TCH) susceptibility test were performed according to Kent and Kubica (1985). The remaining half of the sputum pellet was washed with cold PBS and further processed for DNA extraction.

DNA extraction

DNA was extracted from the processed sputum pellet, using the commercial reagent, InstaGene Matrix (Bio-Rad, California, USA). 200 µl of InstaGene Matrix was added onto the sputum pellet in the 1.5 ml microcentrifuge tube. The contents of the tube were mixed by vortexing at high speed for 10 seconds and incubated at 56ºC for 30 minutes. Then the tube was placed in a boiling waterbath for 10 minutes after vortexing at high speed for 10 seconds. The content was again mixed by vortexing at high speed for 10 seconds and centrifuged at 10,000 rpm for 3 minutes; the supernatant was used for the PCR.

Amplification of *M. tuberculosis* DNA

A 123 bp DNA fragment was amplified

using IS*6110* specific primers P1 5′CCT GCG AGC GTA GGC GTC GG 3′ and P2 5′ CTC GTC CAG CGC CGC TTC GG 3′ (Eisenach *et al*, 1990). The PCR was performed in 25 μl of reaction volume, containing 10 μl of the extracted DNA, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP, 10 pmol of each primer and 1 unit Taq DNA polymerase (Promega, Medison, USA). After initial denaturation at 94ºC for 10 minutes, the reaction was subjected to 35 cycles (94ºC for 1 minute, 67ºC for 50 seconds and 72ºC for 50 seconds) followed by a final extension at 72ºC for 7 minutes on a Techne genecycler (Duxford Cambridge, UK). Ten microliters of the amplified products were analyzed by electrophoresis on 2.5% agarose gel containing ethidium bromide, and IS*6110* specific DNA band corresponding to 123 bp was detected by a Gel Doc 1000 Transilluminator (Bio-Rad). PCR products were further confirmed by DNA-Enzyme Immunoassay (DEIA) using a biotinylated IS*6110* specific probe (Gen-ETI-K, Diasorin, Saluggia, Italy).

Statistical analysis

Data was expressed in frequencies or percentages**.** Statistical analysis was done by chi-square test to determine the association between the PCR and culture results. P values ≤ 0.05 were considered statistically significant.

RESULTS

Of the 151 cases of clinically suspected pulmonary tuberculosis, 112 (74%) were male. The mean (±SD) age was 31 ± 13 years, ranging from 14 to 80 years. Eight percent of the patients had a history of pulmonary tuberculosis and 28% had a family history of tuberculosis. The mean duration of disease before the patients reported to the clinic was 120 ± 20 days (Table 1).

Of the 151 sputum samples, 100 (66.2%) were found to yield growth of *Mycobacterium* within 3 to 4 weeks and no more subsequent isolate could be detected during the next 6 weeks' observation. Primary isolates were subcultured and then biochemical tests (niacin production, nitrate reduction and TCH susceptibility test) were performed. Among the 100 isolates, 92 forming rough and raised colonies on L-J medium, positive for niacin production, nitrate reduction and resistant to TCH, were identified as *Mycobacterium tuberculosis* (Table-2). The other 8 isolates were negative for niacin production and nitrate reduction, but

Table1
Demographic profile of the studied subjects (N=151).

Character	Frequency/Mean
Age (Mean±SD) (years)	31 ± 13
Sex (male/ female)	112/39
Previous history of tuberculosis	12 (8%)
Family history of tuberculosis	42 (28%)
Duration of fever (Mean±SEM) days	120±20 days

The percentages or ranges are shown in parentheses. SD= Standard deviation, SEM= Standard error mean.

Table 2
Culture and PCR results among clinically suspected tuberculosis subjects (N = 151).

Methods	Positive N (%)	Negative N (%)
Culture and biochemical tests (N = 151)	100 (66.22)	51 (33.77)
M. tuberculosis	92 (92.0)	
M. bovis	05 (5.0)	
Other species of *Mycobacterium*	03 (3.0)	
PCR and DEIA	108 (71.5)	42 (28.47)

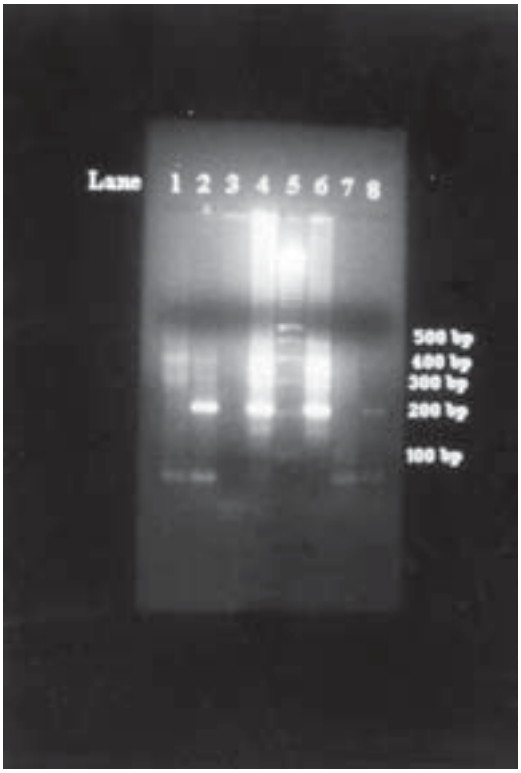

Fig 1–Analysis of PCR products by agarose gel electrophoresis. PCR was based on the amplification of a 123-bp fragment of the IS*6110* of *M. tuberculosis* complex. Lanes 1-5 : sputum from 5 patients suspected for tuberculosis. Lanes 1 and 4 were found negative and lanes 2, 3 and 5 were positive for MTB-DNA. Lane 6- Negative control, Lane 7- *M. tuberculosis* strain H37Rv and Lane 8- Molecular weight (MW) markers.

sensitive to TCH. However, 5 of these 8 isolates formed smooth, transparent colonies on L-J medium and were identified as *Mycobacterium bovis* (Table 2). The remaining 3 isolates, which formed pigmented, rough colonies on L-J medium, could not be identified by the biochemical tests and were designated as other mycobacteria.

Mycobacterium tuberculosis complex specific (IS*6110*) DNA was detected in 71.5% (108/151) sputum samples by PCR and confirmed by DEIA. Only 28.5% (43/151) sputum samples from the clinically diagnosed patients were negative by PCR and DEIA (Table 2). A representative example of agarose gel analysis is shown in Fig 1.

Of the 151 clinically suspected patients, 92 were positive by both PCR and culture, whereas 35 were negative by both methods. Of the remaining 24 patients, 16 were positive by PCR but negative by culture and 8 negative by PCR but positive by culture (Table 3). There was a significant association between the results of culture and PCR (χ^2 = 59.524, $p<0.001$). Considering culture results as the gold standard, the sensitivity of PCR was 92%, with 70% specificity (Table 3).

DISCUSSION

The early and accurate identification of the causative organism of tuberculosis (*ie M. tuberculosis*) facilitates the treatment, preven-

Table 3
Comparison of culture and PCR.

	PCR	Culture		
		Positive	Negative	Total
	Positive	92	16	108
	Negative	08	35	43
	Total	100	51	151
χ^2			59.524	
p			0.000	

tion and control of this chronic infectious disease. PCR, with its rapidity and higher sensitivity, may revolutionize the early detection of *M. tuberculosis.* However, the feasibility of PCR in light of its diagnostic efficacy needs to be evaluated before introducing such molecular techniques in Bangladesh due to its high cost.

The majority of the studied population comprised adult males (31 ± 13 years). It has been reported that 80% of tuberculosis patients in developing countries are under the age of 50, whereas in developed countries, most patients are of older age (Murray *et al*, 1990). Among the patients, 8% (12/151) had histories of previous pulmonary tuberculosis. Incomplete and inadequate treatment may be responsible for such relapse (Chowdhury *et al*, 1997). Twenty-eight percent (42/151) of the tuberculosis patients also had previous histories of contact with infected family members, reflecting intra-familial transmission as one important mode of tuberculosis transmission in Bangladesh. This is particlarly important in Bangladesh, where overcrowding is common, and may facilitate disease transmission. The study also revealed that most of the patients were diagnosed too late (duration: mean ± SEM: 120 ± 16 days), which may be due to the insidious onset and slow progression of the disease, poor economic conditions, lack of health consciousness and limited health care facilities (Mollah *et al*, 1992).

Bacterial culture is considered the gold standard for the diagnosis of tuberculosis worldwide (Schirm *et al*, 1995). In this study, the culture of sputum samples showed mycobacterial growth in 100 (66.2%) cases and all the isolates were detected in 3 to 4 weeks after inoculation. Although the average culture period was considered to be up to 10 weeks, no further isolates could be detected in the subsequent 6 to 7 weeks' observation. Among the 100 isolates, 92 were biochemically identified as *Mycobacterium tuberculosis*, 5 as *Mycobacterium bovis* and the other 3 isolates were other species of *Mycobacterium*. There have been several reports describing human tuberculosis in developed countries caused by *Mycobacterium bovis* (Griffith, 1973; Schiesser, 1992). However, there is very little information on the prevalence of such occurrence in the developing countries (Kleeberg, 1984).

In the present study, 71.5% (108/151) of the sputum samples were found positive for MTB complex specific DNA by PCR. Hybridization with IS*6110*-specific probes in DEIA confirmed the exclusion of non-specific amplification in all those positive cases. PCR could not detect MTB-DNA in 8 out of 100 (8%) culture-positive cases. Such PCR-negative results might be due to the presence of *Mycobacterium* species other than *M. tuberculosis* complex, or the presence of PCR inhibitors in the sputum samples. Of these 8 cases, 3 were characterized as other *Mycobacterium* species, which do not contain the IS*6110* sequence and thus PCR failed to detect. There are some reports regarding the presence of inhibitors in sputum in 5% to 13% of specimens (Nolte *et al*, 1993; Wobeser *et al*, 1996). In our preliminary observation, sputum samples with betel nut stain were found to be negative even for primer dimers. In such cases, a repeat sample was collected 1 week after starting betel nut chewing. However, further studies are required to identify and characterize such inhibitors.

There were 16 PCR-positive cases found to be negative by culture method. This apparent false-positivity in PCR might have resulted from a failure of growth in culture, due to insufficient or non-cultivable bacteria in the samples and carry-over contamination in PCR. Different studies reported false-positive results ranging from 6-12% (Pierre *et al*, 1991; Shankar *et al*, 1991). Carry-over contamination is a major problem in PCR-based detection. However, in this study, all necessary precautions were maintained to avoid contamination. Hence, 16 culture-negative but PCR-positive samples, showing low specificity of PCR, might not be true false-positives, but rather represent the apparent low sensitivity of the culture. Therefore, in the absence of an alternate gold standard, it is not possible to clarify whether samples

with false negative culture actually contain non-cultivable bacteria.

At present, there are no adequate data to evaluate a new method for *M. tuberculosis* diagnosis other than clinical assessment or culture results. Nevertheless, clinical assessment is not always reliable and culture is not without limitations. The slow growth of the *M. tuberculosis* complex delays their detection by culture (Wayne and Sramek, 1992). When clinical suspicion is high but a smear is negative, laboratory diagnosis eventually depends on culture, which takes several weeks (Abe *et al*, 1992). In such cases, the presence of *M. tuberculosis* in clinical specimens can be detected rapidly by PCR-based DNA amplification. Moreover, when smears are positive, PCR allows confirmation as well as identification of *Mycobacterium* and rapid distinction between MTB complex and other mycobacterial infections. Considering these potentials, it may be prudent to hypothesize that PCR assays may not only prove to be more sensitive than culture, but may also provide an insight into the prevalence of tuberculosis.

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