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ORIGINAL ARTICLE

Molecular versus conventional methods: Clinical evaluation of different methods for the diagnosis of tuberculosis in Bangladesh

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Background: The diagnosis of tuberculosis (TB) in developing countries, such as Bangladesh, is based mainly on microscopic detection of acid-fast bacilli in smears from clinical specimens. On the other hand, the detection of TB by polymerase chain reaction (PCR) is quite new in Bangladesh. In this study, we compared the molecular method with the conventional diagnosis procedures, where Lowenstein—Jensen medium culture results have been used as the "gold standard."
Methods: A total of 135 sputum samples were collected from clinically suspected patients with pulmonary TB. A direct smear was made from each sputum specimen and stained by the Ziehl—Neelsen (Z—N) method. The sputum samples were then processed, and the pellet was used for both Z—N (concentration) and auramine O fluorescence staining or resuspended in phosphate buffered saline to inoculate Lowenstein—Jensen medium or processed for PCR detection of *Mycobacterium tuberculosis*.
Results: The direct smear staining yielded 44 (32.6%) sputum samples that were acid-fast positive, which increased after concentration, yielding 60 (44.4%) acid-fast-positive samples. Fluorescence microscopy using auramine O staining further increased the number of positive samples to 67 (49.6%). The biochemical tests showed 75 (55.6%) sputum samples to be culture positive, and the MB/BacT system increased the recovery up to 90 (66.7%) culture positives. On

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the other hand, PCR yielded 93 (68.9%) positive results, 20 (21.5%) of which were culture-negative sputum specimens.
Conclusion: It is suggested that the Z—N direct microscopy on its own is the best method (with high specificity) for confirming the diagnosis of acid-fast bacilli. Although the PCR diagnosis of TB appears to be a rapid and sensitive method, the results should be interpreted with care in the clinical settings.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a major cause of morbidity and mortality throughout the world. It is also one of the most serious infectious diseases and a major public health problem in Bangladesh. This is exemplified by the statistics of 2004, when the incidence (all cases) was estimated to be 229 people per 100,000 population. On the other hand, the detection rate for all TB cases was only 31% in the same year.[1]

Since 1993, a national TB control program has been implemented at the *thana* (subdistrict) level, based on the WHO recommendations, with each *thana* covering a population of about 250,000.[2] Both government and nongovernmental organizations are working under this program for the diagnosis and treatment of suspected TB patients. However, all of them practice conventional diagnostic methods, including clinical symptom observation, sputum smear examinations, chest X-ray, tuberculin test, and others. Confirmation of TB by positive culture often takes 2—8 weeks' time, the facility that is available only in a few centers in the country.

Early diagnosis followed by proper medications is essential to prevent both morbidity and mortality. Although the conventional technique of direct smear examination is cheap and easy to perform, its low sensitivity is a major drawback. On the other hand, the molecular-based diagnosis by polymerase chain reaction (PCR) technique is faster and more sensitive.[3,4]

The diagnosis of TB by molecular method is quite new in Bangladesh, and only a few diagnostic laboratories have started using PCR for TB diagnosis in recent years. There are not many reports evaluating the application of the techniques for the diagnosis of suspected TB patients in Bangladesh. We, therefore, took this prospective study to compare the molecular method with the conventional diagnostic procedures, where Lowenstein—Jensen (LJ) medium culture results have been used as the "gold standard."

Methods

Sample collection

A total of 135 sputum samples were collected from the outpatient clinic of the National Tuberculosis Program, Shayamoli, Dhaka, which is one of the biggest public TB diagnosis and treatment centers in Bangladesh. All samples were obtained from clinically suspected patients with pulmonary TB, before the start of chemotherapy.

Before collection of samples, the patients were advised to rinse the mouth with water to remove any food particle, oral drugs, remains of betel leaf, or mouth wash, which might contain growth inhibitors for acid-fast bacilli. An early morning sample, which was an exudative material after a deep and productive cough, was collected.

Acid-fast staining

A direct smear was made from each sputum specimen and stained by the Ziehl—Neelsen (Z—N) method. The sputum samples were then processed for concentration.

Processing of samples

An equal volume of *N*-acetyl-L-cysteine-NaOH solution was added into the sputum sample, and the content was shaken for 20—30 minutes and allowed to stand for 10 minutes. The digested/decontaminated sputum sample was then diluted with sterile phosphate buffered saline (pH 6.8) and centrifuged at 4,000 $\times$ *g* for 20 minutes at room temperature. The pellet was then either (1) used to prepare smear on a glass slide for Z—N staining (concentration method) and auramine O fluorescence staining; (2) resuspended in 500 μL of phosphate buffered saline to inoculate LJ medium or MB/BacT process bottles (MB/BacT 240 Mycobacterium Detection System; Organon Teknika, Durham, NC, USA); or (3) processed for PCR detection of TB.[5]

Culture and recovery of mycobacteria from sputum samples

The inoculated LJ culture tubes were incubated for up to 8 weeks with weekly examination for evidence of growth. On the other hand, the organisms in the MB/BacT process bottles, which were designated as positive by the system in 5—7 days, were also subcultured on LJ medium. The latter cultures were incubated for up to 6 weeks with weekly examination for evidence of growth. Growth of *M tuberculosis* in all LJ culture tubes were confirmed by (1) niacin test, (2) sensitivity to thiophene-2-carboxylic acid dydrazide (TCH), and (3) sensitivity to *p*-nitrobenzoic acid (PNB).

PCR detection of TB

The primer DNA (*IS6110*) specific for *M tuberculosis* complex was obtained commercially (Genset, Paris, France) and was used as a target for amplification using PCR. The following reaction cycles were used with *IS6110*

primers: an initial 4-minute denaturation step at 94°C was followed by 35 cycles of amplification (94°C for 45 seconds, 68°C for 45 seconds, and 72°C for 1 minute). The 72°C extension step was extended for an additional 5 minutes. The reaction was stopped by cooling at 4°C, and the PCR-amplified products were analyzed by 2.5% agarose gel electrophoresis, and *M tuberculosis* complex *IS6110*-specific DNA band corresponding to 123 bp was detected by Gel Doc 1000 Trans-illuminator (Bio-Rad, Hercules, CA, USA). Positive and negative controls were run with each batch or sample analyzed.

Statistical analysis

All values were expressed as frequencies, percentages, or means, as applicable. Comparisons between groups were done by using Chi-square test and Fisher's exact test. A p value less than or equal to 0.05 was considered as significant. All data were analyzed using SPSS Program (SPSS Inc., Chicago, IL, USA). The sensitivities, specificities, and positive and negative predictive values were calculated by contrasting the results of each method.

Results

Microscopy of the sputum samples

The routine diagnostic procedure consisting of direct smear staining yielded 44 (32.6%) sputum samples that were acid-fast positive. However, after the concentration of the sputum specimen samples, the Z—N staining yielded 60 (44.4%) positive samples. On the other hand, 67 (49.6%) sputum samples were found to be positive in fluorescence microscopy using auramine O staining. Among these two latter methods, eight Z—N staining—negative sputum samples were found to be positive in fluorescence microscopy, whereas one fluorescence microscopy—negative sputum sample was found to be positive in Z—N staining microscopy (Table 1). Therefore, a significant difference ($p < 0.05$) was found between the results of these two microscopy methods.

Culture and biochemical tests

The biochemical tests revealed 75 (55.6%) sputum sample cultures on LJ medium to be positive for *M tuberculosis*. However, the MB/BacT system, which provided a suitable culture medium to recover mycobacterial species, increased the recovery to up to 90 (66.7%) positive samples in the subsequent LJ medium culture. Sixteen direct LJ culture—negative samples were found to become positive after MB/BacT processing system and subsequent LJ medium culture. On the other hand, one MB/BacT-processed negative sample was found to be positive by direct LJ medium culture (Table 2). The overall mean time periods to detect mycobacteria were 32.70 ± 4.56 days and 17.02 ± 10.96 days for direct LJ medium culture and MB/BacT processing system followed by LJ medium culture, respectively, which showed a significant difference between the two methods ($p < 0.05$).

Table 1 Comparison between bright field microscopy after Ziehl—Neelsen staining (concentration) and fluorescence microscopy after auramine O staining

Z—N staining	Fluorescence staining		Total
	Positive	Negative	
Positive	59	1	60
Negative	8	67	75
Total	67	68	135

Table 2 Comparison between MB/BacT-processed LJ medium culture and direct culture on LJ medium for recovery of mycobacteria from sputum specimen samples

MB/BacT-processed LJ culture	Direct LJ culture		Total
	Positive	Negative	
Positive	74	16	90
Negative	1	44	45
Total	75	60	135

LJ = Lowenstein—Jensen.

PCR detection

PCR yielded 93 (68.9%) positive results, 20 (21.5%) of which were culture-negative sputum specimens (Table 3). Of the 42 PCR-negative specimens, two (4.8%) were culture positive. These two isolates, although showed typical colonial morphology similar to *M tuberculosis*, however, showed a different biochemical behavior in the TCH and PNB tests. One isolate showed resistance to PNB and the other showed sensitivity to both TCH and PNB.

Considering the direct LJ medium culture results as the "gold standard", the sensitivities of Z—N staining, both direct and concentration methods, fluorescence staining, MB/BacT processing followed by LJ medium culture, and PCR methods, were 54.6%, 72%, 81.3%, 98.6%, and 97.3%, respectively, whereas the specificities were 95%, 90%, 90%, 73.3%, and 66.7%, respectively (Table 4). The positive and negative predictive values of the methods are also shown in Table 4.

Table 3 Comparison of PCR with direct culture on LJ medium as the "gold standard"

PCR result	n (%) with the indicated PCR result	n (%) with the following result by culture	
		Positive	Negative
Positive	93 (68.9)	73 (78.5)	20 (21.5)
Negative	42 (31.1)	2 (4.8)	40 (95.2)
Total	135 (100)	75 (55.6)	60 (44.4)

PCR = polymerase chain reaction.

Discussion

A total of 135 sputum specimen samples were taken in this study, where 44 (32.6%) and 60 (44.4%) sputum samples were found to be positive in direct and concentration methods of Z—N staining, respectively. Significant differences were revealed between these two methods as 16 (12%) Z—N (direct) negative sputum samples were found to be positive in Z—N (concentration) method. This is probably because the concentration of sputum samples facilitated the smear examination in high oversetting. Similar findings were also reported previously,[6] and it was observed that, in the direct smear examination, a portion of patients with TB-positive smear may not be diagnosed as positive.

Again, in the auramine O staining fluorescence microscopy, a total of 67 (49.6%) sputum samples were found to be positive. This number also included 23 Z—N (direct)- and eight Z—N (concentration)—negative samples. On the other hand, one fluorescence microscopy—negative sputum sample was found to be positive in Z—N (concentration) microscopy. Therefore, fluorescence microscopy appears to be more sensitive than bright field microscopy for the detection of the bacillus in the sputum smear, as has been suggested by others.[7] False-positive results (specimen with positive acid-fast smears and negative direct LJ medium culture results) were revealed in this study for both direct and concentration Z—N staining methods. Only 4% false-positive results were revealed in direct Z—N smear examination, whereas 8% false-positive results were revealed in both Z—N (concentration) and fluorescent staining methods.

The direct LJ medium culture method, which was considered as the "gold standard" for the *M tuberculosis* detection, could recover 75 (55.6%) positive sputum specimens. However, the number further increased to 90 (66.7%) when LJ medium culture recovered the bacilli after the MB/BacT processing of the organisms. It is assumed that recovering this high prevalence of culture-positive samples (66.7%) could be because of the fact that the samples were obtained from clinically suspected patients attending the outpatient clinic of the National Tuberculosis Program. There is a high increase in the recovery rate of the bacilli and is close to being twice as effective as the Z—N direct microscopy method. The mean recovery time for the *Mycobacterium* was 32.70 ± 4.56 days in direct LJ medium culture method and 17.02 ± 10.96 days for LJ medium culture after MB/BacT processing system. Considering the direct LJ medium culture results as the "gold standard", the sensitivity and specificity of the MB/BacT-processed LJ medium culture were 98.6% and 73.3%, respectively. The high sensitivity was obtained because there was no false-positive result in the study. However, the 16 samples, which were negative in direct LJ medium culture but positive in MB/BacT-processed LJ medium culture, were not probably true false positives. The very low number of viable mycobacteria might have been recovered only by the MB/BacT-processed LJ medium culture system but not by the direct LJ medium culture.

PCR yielded similar number of positive results as diagnosed by MB/BacT-processed LJ medium culture system (68.9% *vs.* 66.7%). Both these methods also showed similar sensitivities (97.3% *vs.* 98.6%). However, the PCR had a lower specificity (66.7% *vs.* 73.3%), resulting in better diagnosis. Of the 42 PCR-negative sputum samples, two (4.8%) were culture positive. These two isolates, although showed typical colonial morphology similar to *M tuberculosis*, however, showed a different biochemical behavior in the TCH and PNB tests.

The specificity of PCR is the lowest (66.7%) in this study, when compared with those of all the detection methods (Table 4), where direct culture on LJ medium was used as the "gold standard" for the presence of TB. It is possible that PCR is more sensitive than the LJ culture method used here, effectively resulting in an underestimation of the specificity of the PCR, when direct LJ culture only is used as the "gold standard." It should be mentioned that the specificity of the PCR, as calculated in this study (66.7%), is lower than that reported in many other studies, where automated liquid culture systems were used as the "gold standard."[8—10] However, the MB/BacT or the liquid culture system, which is operated in only a few private diagnostic facilities in Bangladesh, has not been sufficiently evaluated, which compelled us to use the direct LJ medium culture system as the reference "gold standard."

The diagnosis of TB in a developing country, such as Bangladesh, is based on mainly the microscopic detection of acid-fast bacilli in smears from clinical specimens, a method with a variable sensitivity ranging from 22% to 78%.[11] Considering the culture results as the "gold standard", sensitivities of bright field microscopy after Z—N staining, both direct and concentration methods, and fluorescence microscopy after auramine O staining were 54.6%, 72%, and 81.3%, respectively, whereas the specificities were 95%, 90%, and 90%, respectively (Table 4). In this study, the specificity of the direct smear examination was very high and specificities of Z—N staining and fluorescent staining

Table 4 Sensitivities, specificities, and positive and negative predictive values (direct LJ culture as the "gold standard") of Z—N (direct), Z—N (concentration), fluorescence staining, MB/BacT-processed LJ culture, and PCR methods

Tests	Sensitivity (%)	Specificity (%)	Predictive value (%)	
			Positive	Negative
Z—N staining (direct)	54.6	95.0	93.2	62.6
Z—N staining (concentration)	72.0	90.0	90.0	72.0
Fluorescence staining	81.3	90.0	91.0	79.4
MB/BacT-processed LJ culture	98.6	73.3	82.2	97.8
PCR	97.3	66.7	78.5	95.2

LJ = Lowenstein—Jensen; PCR = polymerase chain reaction; Z—N = Ziehl—Neelsen.

were same. The concentration method was followed between the two techniques for smear examination. Moreover, the magnifications used by the two methods were similar, which may have given the equal specificities of the two methods. It may be likely that the use of higher magnification in fluorescence microscopy would further increase the specificity of the test.

Considering the culture results as the "gold standard", sensitivity of MB/BacT was 98.6%, whereas specificity was 73.3% (Table 4). As the sensitivity was high, the specificity was revealed as low. The high sensitivity was obtained because of 0% false-positive rate in this study. However, it was shown that 16 samples, which were negative for LJ medium culture but positive for MB/BacT, were not true false positives. All these results were sent to the respective diagnostic centers for necessary actions.

The positive predictive values of the methods were found to be 93.2%, 90%, 91.0%, and 82.2%, respectively, whereas the negative predictive values of the methods were 62.6%, 72% 79.4%, and 97.8%, respectively. Positive and negative predictive values follow Bay's theorem and depend on the prevalence of positive sputum samples examined. As the prevalence of positive sputum samples was high in this study, the positive predictive value for Z—N direct microscopy was higher (93.2%) than those of the other methods (Table 4). On the other hand, the negative predictive value of MB/BacT-processed LJ medium culture (97.8%) was higher than that of any other method. Thus, any new test to replace Z—N direct microscopy in screening suspects should perform as well as (or better than) the present diagnostic tests, when used under the identical conditions.

When we compare the performance of PCR and MB/BacT-processed LJ medium culture system with that of the other routine diagnostic methods, it is suggested that Z—N direct microscopy on its own is the best method (with high specificity) for confirming a diagnosis of acid-fast bacilli, although a negative result should not rule out TB. However, other advantages of the rapid method should also be considered for the diagnosis of TB. An advantage of PCR is that it can be performed on a single specimen and yields result within a day. This makes the diagnostic process shorter and more patient friendly, which may reduce dropout levels and, altogether, contribute to reduced transmission of TB.[4]

Although molecular diagnosis of TB appears to be a rapid and sensitive method, the results should be interpreted with care in the clinical setting. PCR also has great potential for diagnosis of smear-negative and extrapulmonary TB patients, which is another major challenge of "implementing the stop TB strategy" of Bangladesh.[12] In this preliminary study, we only compared different TB diagnosis methods but could not compare the economic aspects of the methods. However, practicality, cost-effectiveness, or operational studies are required to support an evidence-based decision to introduce the molecular-based or other rapid methods for TB control in this low-resource setting.

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