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Comparison of PCR and antigen detection with conventional culture and acid-fast microscopy for diagnosis of pulmonary tuberculosis

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Objective : *To evaluate the methods based on DNA amplification by IS6110-PCR and antigen detection by dot immunoassay (DIA) in comparison with conventional culture and detection of acid-fast bacilli by direct microscopy for diagnosis of pulmonary tuberculosis.*

Design : *A cross-sectional analytical study*

Setting : *Central Chest Hospital, Ministry of Public Health, Nonthaburi; Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok.*

Subjects/Methods : *A total of 328 sputum samples were tested. After processing of sputum for decontamination, a part of the sputum sediments was used for culture on solid and liquid BACTEC medium, and for acid-fast staining. The remaining sediments were used in PCR amplification based on IS6110 as a DNA target, and in antigen detection by dot immunoassay (DIA). Results were compared with those obtained by conventional culture and acid-fast microscopy, and culture method was used as the "gold standard." Data were*

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analyzed statistically by the chi-square test; a P of < 0.05 was considered significant.

Results : *Of the 328 sputum specimens, 134 were positive for Mycobacterium tuberculosis by culture. In comparison with culture, PCR with Southern blot hybridization showed overall sensitivity, specificity, positive and negative predictive values, and efficiency of 96.27, 99.48, 99.23, 97.47 and 98.17%, respectively with no significant difference (P > 0.05). The same values for DIA-Ag detection were 73.13, 69.07, 62.03, 78.82 and 70.73%, respectively with a statistically significant difference (P < 0.05). For smear-positive specimens, the sensitivity and specificity of PCR were 100 and 100% equivalent to culture (P > 0.05), and those of DIA-Ag detection assay were 73.03, and 33.33%, respectively (P < 0.05). For smear-negative specimens, PCR had low sensitivity (88.89%) (P > 0.05), but high specificity (99.48%), while the DIA-Ag detection assay had a sensitivity and specificity of 73.33, and 69.63%, respectively (P < 0.05).*

Conclusion : *There was no significant difference in the performances of IS6110-PCR based method and culture (P > 0.05). In contrast, the difference between DIA-Ag detection and culture was found to be statistically significant (P < 0.05). The overall good performance of the IS6110-PCR based method for detection of M. tuberculosis makes it a very useful additional tool in establishing a rapid diagnosis of tuberculosis. Further improvements of the assay at the sample preparation step and at the detection step may lead to a more reliable, simple, and cost-effective assay to be used in routine clinical practice for rapid diagnosis of pulmonary tuberculosis.*

Key words : *IS6110-PCR, Antigen detection, Pulmonary tuberculosis, TB diagnosis.*

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วัตถุประสงค์ : *เพื่อประเมินวิธีพีซีอาร์ที่เพิ่มจำนวนดีเอ็นเอเป้าหมายในกลุ่ม IS6110 กับวิธีการตรวจหาแอนติเจนด้วยเทคนิคด็อท อิมมิวโนแอสเสย์ เปรียบเทียบกับผลการตรวจแบบมาตรฐานด้วยวิธีการเพาะเชื้อและวิธีการย้อมสีทนกรด และตรวจโดยตรงด้วยกล้องจุลทรรศน์สำหรับการวินิจฉัยวัณโรคปอด*

รูปแบบการวิจัย : *การศึกษาเชิงวิเคราะห์ภาคตัดขวาง*

สถานที่ : *โรงพยาบาลโรคทรวงอก กระทรวงสาธารณสุข นนทบุรี ภาควิชาจุลชีววิทยา คณะสาธารณสุขศาสตร์ มหาวิทยาลัยมหิดล กรุงเทพ ฯ*

ตัวอย่างและวิธีการวิจัย : *ทำการเก็บตัวอย่างสิ่งตรวจเสมหะ จำนวน 328 ตัวอย่าง ผ่านขั้นตอนการเตรียมเสมหะเพื่อขจัดการปนเปื้อน และแบ่งตะกอนบางส่วนไปเพาะเลี้ยงในอาหารชนิดแข็ง และชนิดเหลวเพื่อเพาะหาเชื้อวัณโรคตามวิธีมาตรฐาน และทำการตรวจโดยวิธีการย้อมสีทนกรด ตะกอนบางส่วนที่เหลือนำไปทำการตรวจโดยวิธีพีซีอาร์เพื่อเพิ่มจำนวนดีเอ็นเอเป้าหมายในกลุ่ม IS6110 และทำการตรวจหาแอนติเจนด้วยเทคนิคด็อท อิมมิวโนแอสเสย์ เปรียบเทียบผลของทั้ง 2 วิธี กับผลการเพาะเชื้อและการตรวจด้วยกล้องจุลทรรศน์ โดยอาศัยผลการเพาะเชื้อวัณโรคเป็นวิธีมาตรฐานในการวินิจฉัยวัณโรคปอด ใช้สถิติวิเคราะห์หาความแตกต่าง โดย chi-square test ค่า $P < 0.05$ ถือว่ามีนัยสำคัญทางสถิติ*

ผลการศึกษา : *จากเสมหะ จำนวน 328 ตัวอย่าง ให้ผลการเพาะเชื้อบวกบอกว่าเป็นเชื้อวัณโรค จำนวน 134 ตัวอย่าง ในการวิเคราะห์ผลโดยรวมเมื่อเปรียบเทียบกับผลการเพาะเชื้อ ค่าความไว ความจำเพาะ ค่าคาดหวังของผลบวก ค่าคาดหวังของผลลบ และประสิทธิภาพ ของวิธีพีซีอาร์ พบร้อยละ 96.27, 99.48, 99.23, 97.47, และ 98.17 ตามลำดับ; วิธีการตรวจหาแอนติเจน พบร้อยละ 73.13; 69.07, 62.03, 78.82, และ 70.73 ตามลำดับ ในกลุ่มตัวอย่างที่ให้ผลย้อมเสมหะสเมียร์บวกด้วยกันวิธีพีซีอาร์นับว่ามีความไว*

และความจำเพาะสูงถึง ร้อยละ 100 ในขณะที่วิธีตรวจหาแอนติเจน ให้ค่าความไว ร้อยละ 73.03 ความจำเพาะ ร้อยละ 33.33 ซึ่งแตกต่างจากผลของการเพาะเชื้ออย่างมีนัยสำคัญทางสถิติ (P < 0.05) ส่วนกลุ่มตัวอย่างที่ให้ผลย้อมเสมหะสเมียร์ลบด้วยกัน วิธีพีซีอาร์ ให้ค่าความไวต่ำ ร้อยละ 88.89 แต่ค่าความจำเพาะสูง ร้อยละ 99.48 (P > 0.05) ในขณะที่วิธีตรวจหาแอนติเจน ให้ค่าความไว ร้อยละ 73.33 ความจำเพาะ ร้อยละ 69.63 (P < 0.05)

สรุปผล : *วิธีพีซีอาร์ที่ใช้ IS6110 เป็นดีเอ็นเอเป้าหมาย ให้ผลการวินิจฉัยวัณโรคที่น่าเชื่อถือไม่ต่างจากวิธีเพาะเชื้อ (P > 0.05) จึงนับว่ามีประโยชน์ช่วยในการวินิจฉัยวัณโรคปอดได้รวดเร็ว แต่ควรมีการปรับปรุงวิธีการในขั้นตอนการเตรียมตัวอย่าง และการตรวจวิเคราะห์ผลิตผลของพีซีอาร์ เพื่อให้ได้วิธีการที่ง่าย ประหยัด น่าเชื่อถือ เหมาะสมสำหรับใช้ในงานประจำในห้องปฏิบัติการเพื่อการวินิจฉัยวัณโรคที่รวดเร็วต่อไป*

The recent resurgence in tuberculosis cases and in multidrug-resistance poses a serious public health problem. The global incidence of tuberculosis is predicted to increase by 36% between 1990 levels and 2000,[1] and likely to increase over the following decade, mainly due to the AIDS epidemic. There is heightened interest in rapid and reliable methods to identify people with pulmonary tuberculosis and render them noninfectious by treatment as soon as possible. This is of major importance to control the spread of *Mycobacterium tuberculosis* infection.[2] Diagnosis of tuberculosis made on the basis of patient histories and clinical and radiological findings is presumptive. The definitive diagnosis is dependent on the microscopic examination of acid-fast bacilli (AFB) stained sputum smears and then confirmation by culture. Unfortunately, both techniques have limitations. Direct microscopy by Ziehl-Neelsen staining to identify AFB is cost effective and rapid, but it lacks sufficient sensitivity and specificity. Although the sensitivity of the smear is improved by fluorescent staining,[3] the test fails to distinguish between tuberculous and nontuberculous mycobacteria. Culture on solid media is more specific and sensitive but results require up to 8 weeks. The use of radiometric BACTEC liquid medium has considerably shortened detection time, but it still takes at least 10 days.[4] As a result, cultured-based diagnosis methods are slow. The need to perform biochemical testing to identify the mycobacterial species adds to the time required to obtain a diagnosis.

The development of rapid procedures for the diagnosis of tuberculosis has been a long-standing goal for public health and therapeutic reasons. New technological diagnostic techniques such as immunological detection of mycobacterial antigens by ELISA[5,6] and DNA probe technology[7,8] have been used in medicine in tuberculosis diagnosis; however both lack the sensitivity to be useful for testing clinical specimens, especially for those specimens containing few mycobacteria. Over the past decade, diagnostic tools for the detection of *M. tuberculosis* have considerably improved. Polymerase chain reaction (PCR) is a well-developed technique used extensively for the diagnosis of numerous infectious diseases, including tuberculosis.[9-13] PCR can be specific and sensitive and may shorten clinical detection of *M. tuberculosis* from weeks to less than a day, however there are difficulties associated with the techniques. In addition, problems of contamination, the presence of amplification inhibitors, and high variations in sensitivity[14] ultimately call into question the utility of PCR in clinical laboratories on a routine basis for rapid diagnosis of pulmonary tuberculosis.

The present study was undertaken to evaluate the performance of IS*6110*-PCR based assays and immunological-based assays for detection of *M. tuberculosis* in sputum samples. In order to assess the sensitivity and specificity, the results of both tests were compared with direct acid-fast microscopy and culture, and the culture method was used as the "gold standard."

Materials and Methods

Specimen collection and processing. A total of 328 sputum samples were obtained from adult patients with respiratory symptoms at the Central Chest Hospital, Ministry of Public Health, Nonthaburi.

All samples were decontaminated for 15 min with *N*-acetyl-L-cysteine (NALC)-2% NaOH[15] and neutralized with sterile 0.067 M phosphate buffer (pH 6.8). After centrifugation at 3,000g for 15 min, the sediment was resuspended in 2 ml of phosphate buffer. The culture medium was inoculated with part of the sediment and used for AFB staining, while the remaining sediment was aliquoted and stored at - 20°C until PCR and antigen detections were performed.

Culture. The processed sediment (0.5 ml) was cultivated by using a radiometric BACTEC technique (Becton-Dickinson Diagnostic Instrument Systems).[16] In addition, Ogawa medium[17] was inoculated with 0.2 ml of the sediment. The Ogawa tubes were incubated at 37°C for 8 weeks and examined weekly for growth. BACTEC 12B vials were incubated at 37°C, and growth index readings were recorded with a BACTEC instrument twice a week for 6 weeks. Once a 12B vial attained a growth index of ≥ 100, the presence of AFB was confirmed by Ziehl-Neelsen staining.[18] Positive cultures on Ogawa media were examined for growth rate, gross and microscopic colony morphology, and pigmentation. They were subjected to conventional biochemical tests[19] for niacin, nitrate reduction, catalase, arylsulfatase, pyrazinamidase, and also to complementary tests for certain isolates.

Microscopy. The processed sediment (0.1 ml) was screened for the presence of AFB by fluorescence microscopy,[20] and positive slides were confirmed by Ziehl-Neelsen staining and scored as negative, 1+, 2+, and 3+.

DNA extraction from bacterial strains and clinical specimens. Total purified DNA was isolated from reference strains; i.e. *M. tuberculosis* H_{37} Rv and *M. flavescens* ATCC 23035 was used as a positive and a negative control DNA in PCR amplification. The extraction was performed by the conventional phenol-chloroform method as previously described.[13,21] DNA extraction from sputum samples was performed by following the protocol of Buck et al.[22] with slight modification. Briefly, the processed sediments (0.25 ml) were washed at least two times by high centrifugation and resuspension in TE buffer. After the supernatant was discarded, 100 µl of lysis buffer (100 mM Tris HCl [pH 8.3], 0.5% Tween 20; 1 mg/ml proteinase K) was added to the pellets, and the mixture was incubated at 55°C for 1 h. The tube was then placed in a boiling water bath for 10 min, and cell debris was removed by centrifugation, and 10 µl of the supernatant was used in the PCR.

PCR amplification. Two oligonucleotide primers derived from the IS*6110* insertion sequence[23] reference sequence X 17348 (GenBank), designated primer TB1 (5'-CCAACAAGAAGGCGTACTCG -3') and TB2 (5'-GGAGACTCTCTGATCTGAG-ACC -3') were designed.[24] A reaction mixture of 50µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM (each) deoxynucleoside triphosphates, 0.5 µM each of primers TB1 and TB2, 1 U of *Taq* polymerase (Promega) and 10 µl of sample DNA was prepared. The sample was denatured at 94°C for 5 min, 40 amplification cycles were performed with an automated thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of denaturation at 94°C (2 min), annealing at 60°C (2 min), and extension at 72°C (2 min). A final incubation at 72°C for 5 min was included to allow for completion of strand synthesis. To avoid possible contamination of the PCR mixture, all reactions were performed under stringent

conditions as recommended by Kwok and Higuchi.[25]

The PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μg/ml) and photographed with a UV transillumination camera. The presence of a band of the expected size (377 bp) was identified by comparison with a molecular mass marker, a PhiX 174/*Hae*III digest (Gibco; BRL). For hybridization, the DNA was transferred to a nylon membrane by Southern blotting. [26] The membrane was hybridized to internal oligonucleotide probe (TB3-5' GAAAGACGTTATCCA-CCA-3') labeled with fluorescein-11-dUTP by the ECL labeling and detection system (Amersham) according to the manufacturer's instructions. Hybridization was carried out at 48°C for 2 h in the hybridization buffer. Detection of hybridized duplex was completed by using the anti-fluorescein-peroxidase conjugate-chemiluminescent system (Amersham), according to manufacturer's protocol and exposed to Hyperfilm for 1 to 4 min.

Detection of antigen in sputum. A dot immunoassay (DIA) with immunological detection by chemiluminescence was set up by using a commercially available hyperimmune serum directed against *M. bovis*, BCG. Optimal concentrations of all reagents were determined by checkerboard titration with the use of sonicated antigens prepared from *M. tuberculosis* H_{37} Rv and *M. flavescens* ATCC 23035 as a positive and a negative control. Briefly, sonic extract was prepared with young organisms grown on LJ medium for 3 weeks. Cultures were harvested, washed with normal saline solution (NSS); then 50% (vol/vol) of pellets in NSS was frozen overnight at -70°C and thawed, and pulse-sonicated with a Vibra Cell 600-Watt Model (Sonics & Materials, Inc., Connecticut, USA) at 300 W for 15 min and centrifuged at 15,000g for 20 min. Protein concentration of the sonic extracts was measured by the method of Lowry et al. [27] Dilutions were prepared in PBS and 50 μl was used in DIA for optimization of all reagents.

A total of 328 sputum specimens were examined in the DIA in blind fashion. Briefly, the processed sediment (0.5 ml) was heat inactivated at 80°C for 30 min and sonicated with the Vibra Cell at 300 W for 15 min using a cup horn so that the sample would not come in direct contact with the metal tip, thus preventing cross-contamination among samples. To prepare dots, 50 μl of sonicated samples were spotted onto a nitrocellulose membrane (Hybond-ECL, Amersham) by using a manifold system (Hybridot Manifold, Life Technologies; BRL). Positive and negative controls were also performed on each membrane in the same manner with 100 ng of sonicated protein per well. The membrane was air dried at 37°C to stabilize antigen binding, and then blocked with 5% blocking reagent in phosphate buffered saline-Tween (PBS-T) for 1 h at room temperature. After 5 washes with PBS-T, anti-*M. bovis* BCG rabbit serum (Dakopatts) at 1:5,000 was added, and incubated for 1 h at room temperature. After 5 washes as above, sheep anti-rabbit IgG horseradish peroxidase conjugate at 1:2,000 was added and incubated for 1 h, followed by ECL detection (Amersham) according to the manufacturer's protocol and exposed to Hyperfilm as described above for the Southern procedure. The intensity of each dot was compared with those of the negative controls and of various dilutions of positive controls. Results were read visually in comparison with controls.

Patient clinical evaluation. In these cases where the results from the culture, PCR and DIA-Ag detection were discrepant, the clinical data of the patient were evaluated. Clinical assessment included the patient's history, signs, symptoms, chest X-ray, microbiological results, and follow-up observations, as well as the results obtained from additional specimens from the same patient that were sent to the mycobacteriology laboratory. For considering a truly positive result we used a criteria of Tevere et al.[28] In cases in which the culture result was positive but either PCR or DIA-Ag result was negative, the culture results were assumed to be correct.

Statistical analysis. Statistical comparison was performed by using chi-square analysis; a P of < 0.05 was considered significant.

Results

Culture and smear results. A total of 328 sputum specimens were tested. This included 222 samples from new patients who were suspected of having TB because of their clinical history and characteristic radiographs and who were not receiving anti-tuberculosis therapy; and 106 samples from patients with diseases other than tuberculosis. Growth of mycobacteria in 3% Ogawa and/or BACTEC 12B medium was detected in 140 samples; 134 of them were identified as *M. tuberculosis*, 6 were identified as mycobacteria other than *M. tuberculosis* (3 *M. fortui-tum*, 1 *M. kansasii*, and 2 *M. avium-M. intracellu-lare* complex isolates). Acid-fast bacilli (AFB) were detected in 92 samples after fluorochrome and/or Ziehl-Neelsen staining. Three of the culture-negative and 89 of the culture-positive specimens were AFB smear positive.

If a combined BACTEC 12 B and solid-medium cultures with biochemical confirmation is regarded as the most reliable diagnostic method, then 134 of the 328 patients were diagnosed as having tuberculosis and 194 of the 328 patients as non-tuberculosis (Table 1). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency of the AFB smear method for the detection of *M. tuberculosis* were 66.42, 96.91, 93.68, 80.69, and 84.45%, respectively.

Table 1. Results of acid-fast (AF) microscopy and culture after biochemically confirmed as *M. tuberculosis* and non *M. tuberculosis* from 328 sputum specimens.

Acid fast (AF) and culture (Cul) results	No.of specimens	No. of specimens with confirmed culture result	
		TB positive	TB negative
Culture positive (N =140)			
AF-/Cul+	48	45	3[a]
AF1+/Cul+	46	44	2[a]
AF2+/Cul+	38	37	1[a]
AF3+/Cul+	8	8	0
Culture negative (N = 188)			
AF-/Cul-	188	0	188
Total	328	134	194

[a]Mycobacteria other than *M. tuberculosis* (3 *M. fortuitum*, 1 *M. kansasii*, and 2 *M. avium-M. intracellulare* complex isolates).

Comparison of PCR and DIA-Ag detection with smear and culture results. Fig. 1 shows representative results of PCR amplification from some sputum samples with different acid fast staining and culture results, i.e. AF1+/Cul+, AF2+/Cul+, AF3+/Cul+, AF-/Cul+, and AF-/Cul-. The PCR products were analyzed by agarose gel electrophoresis and confirmed by Southern blot hybridization. The hybridization step allowed specific detection of amplified *M. tuberculosis* DNA with fluorescein labeled probe, and detected by ECL detection reagent. The specificity of the hybridization reaction was illustrated by the fact that amplified *M. tuberculosis* DNA hybridized only to internal probe, TB3.

Table 2 shows the results of PCR and DIA-Ag detection in comparison with those obtained by smear and culture for detection of *M. tuberculosis* in sputum specimens. Of the 134 culture positive samples for *M. tuberculosis*, 118 (88.06%), and 129 (96.27%) were positive by PCR with amplicon detection by agarose gel electrophoresis (AGE) and Southern blot hybridization (SBH), respectively. While 98 (73.13%) were positive by DIA-Ag detection. Of the 194 culture-negative samples for *M. tuberculosis*, none was positive by PCR in AGE (specificity, 100%), while one was positive by PCR-SBH (specificity, 99.48%), and 60 were positive by DIA-Ag detection (specificity, 69.07%).

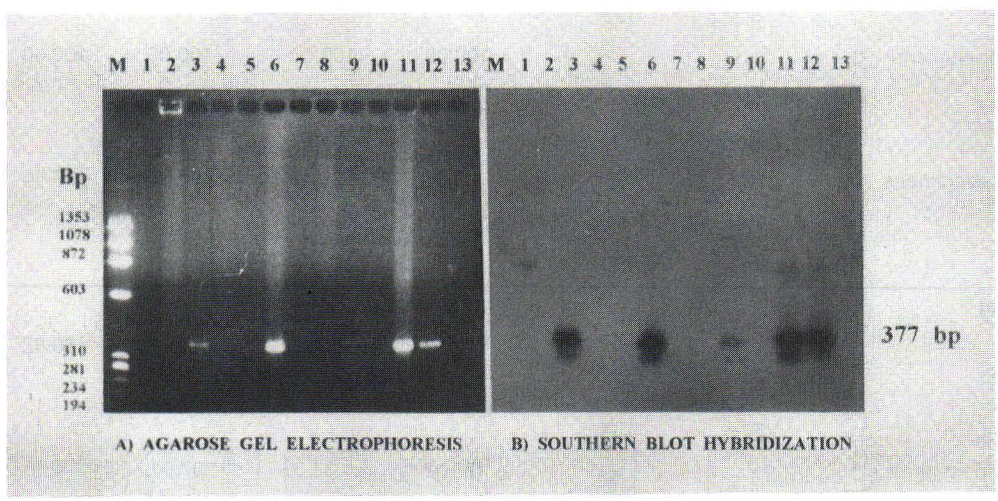

Figure 1. Representative results of PCR amplification from some clinical sputum specimens with different acid fast staining and culture results. The PCR products were analyzed on agarose gels (left), and by Southern blot (right) of agarose gel from left, hybridized with fluorescein labeled oligonucleotide probe and detected by enhanced chemiluminescence (ECL) detection reagent. Lanes : M, DNA size marker (PhiX174/*HaeIII* digest); 1-11 sputum samples; i.e. AF- /Cul- (lanes 1,2,4,5,7,8 and 10), AF1+ /Cul+ (lane 3), AF2+/Cul+ (lane 6), AF3+/Cul+ (lane 11), and AF-/Cul+ (lane 9). Lane 12 = positive control (H_{37} Rv DNA). Lane 13 = negative control (no template DNA).

Table 2. Comparison of PCR and antigen detection with acid-fast microscopy and culture results[a] for direct detection of *M. tuberculosis* in 328 clinical sputum specimens.

Specimen type and test	No. of specimens with the following culture result :		Sensitivity (%)	Specificity (%)	Predictive value (%)		Efficiency (%)
	Positive[b]	Negative			Positive	Negative	
PCR-AGE[c]							
All specimens			88.06	100.00	100.00	92.38	95.12
Positive	118	0					
Negative	16	194					
Smear-positive specimens			100.00	100.00	100.00	100.00	100.00
Positive	89	0					
Negative	0	3					
Smear-negative specimens			64.44	100.00	100.00	92.27	93.22
Positive	29	0					
Negative	16	191					
PCR-SBH[d]							
All specimens			96.27	99.48	99.23	97.47	98.17
Positive	129	1					
Negative	5	193					
Smear-positive specimens			100.00	100.00	100.00	100.00	100.00
Positive	89	0					
Negative	0	3					
Smear-negative specimens			88.89	99.48	97.56	97.44	97.46
Positive	40	1					
Negative	5	190					
DIA - Ag detection[e]							
All specimens			73.13	69.07	62.03	78.82	70.73
Positive	98	60					
Negative	36	134					
Smear-positive specimens			73.03	33.33	97.01	4.00	71.74
Positive	65	2					
Negative	24	1					
Smear-negative specimens			73.33	69.63	36.26	91.72	70.34
Positive	33	58					
Negative	12	133					

[a] Using culture as the "gold standard."

[b] Culture positive for *M. tuberculosis* in 134 specimens (89 smear-positive and 45 smear-negative).

[c] PCR with amplicon detection by agarose gel electrophoresis (AGE).

[d] PCR with amplicon detection by Southern blot hybridization (SBH).

[e] Antigen detection by dot immunoassay (DIA).

The results of both PCRs and DIA-Ag detection assays were also analyzed according to the smear results. Of the 89 specimens that were smear-positive and culture positive, all (100%) were positive by PCR in both AGE and SBH while 65 (73.03%) were positive by DIA-Ag detection. Of the 45 specimens that were smear-negative culture positive, 29 (64.44%) were positive by PCR-AGE and 40 (88.89%) by PCR-SBH, while 33 (73.33%) were positive by DIA-Ag detection.

If combined liquid- and solid-medium cultures is considered to be "gold standard" procedure, PCR exhibited an overall sensitivity, specificity, PPV, NPV and efficiency of 88.06, 100, 100, 92.38 and 95.12%, respectively for AGE, and 96.27, 99.48, 99.23, 97.47 and 98.17%, respectively for SBH with no significant difference ($P > 0.05$). The same values for DIA-Ag detection were 73.13, 69.07, 62.03, 78.82, and 70.73%, respectively with a statistically significant difference ($P < 0.05$).

In comparative evaluation of PCR detection by AGE and SBH with all clinical specimens, SBH showed the outstanding of sensitivity and specificity (96.27% and 99.48% by SBH compared to 88.06% and 100% by AGE). Discrepant results between AGE and SBH were found for 12 specimens (1 AGE-/SBH+ and 11 AGE-/SBH+). The overall results of culture and PCR-SBH were concordant for 322 (98.17%), culture and PCR-AGE for 312 (95.12%), and culture and DIA-Ag detection for 232 (70.73%) of the 328 samples.

Discrepant results between PCR-SBH and culture were found for 6 specimens (1 PCR+/Cul- and 5 PCR-/Cul+). One PCR positive but smear and culture negative sample (AF-/Cul-) came from one patient suspected of having tuberculosis by chest X-ray and symptom, not on treatment at time of study. Unfortunately, no sample was available for culture confirmation, but for PCR testing, it could be repeated and again it was positive when retested by PCR. Other five samples that were missed by PCR assay, despite repeat testing, were from samples with negative AFB but culture positive for *M. tuberculosis* (AF-/Cul+), indicated low numbers of organisms in these sputum samples. These five samples were considered to be "true" positive for *M. tuberculosis* based on culture positive.

Discussion

Although presumptive diagnosis of pulmonary tuberculosis can be made on the basis of patient histories and clinical and radiological findings, the definitive diagnosis of tuberculosis continues to depend on the microscopic examination of AFB smears for initial screening and then cultural confirmation. X-rays can be helpful in the diagnosis of a tuberculosis case but the results depend on the quality of the X-rays and require very experienced doctors looking at chest X-rays. Even very experienced doctors looking at chest X-rays find it extremely difficult as the findings may not show clear signs of tuberculosis, and it may be difficult to differentiate between active tuberculosis, inactive tuberculosis and other diseases. However, many radiologists and chest physicians still widely believe that tuberculosis of the lung can be diagnosed by chest radiography alone.[29] But there are many disadvantages such as the expense of chest radiography, availability is usually only in hospitals, and the difficulty in reading. These may lead to both over- and under-diagnosis of tuberculosis. In our study, if the diagnosis of

tuberculosis is based on clinical and radiological findings alone, it might lead to over-diagnosis, because of 222 samples from new suspected cases of tuberculosis by chest X-ray, only 134 samples (60.36%) were confirmed as true *M. tuberculosis* by positive sputum culture.

Today, the "gold standard" and most extensively used method for TB diagnosis is still detection of *M. tuberculosis* by a combination of microscopy and culture. However, neither of these methods is really satisfactory. Direct staining for AFB takes less than an hour but lacks sensitivity and specificity. In our study, the sensitivity, specificity, PPV, NPV and efficiency of the AFB smear method for the detection of *M. tuberculosis* were 66.42, 96.91, 93.68, 80.69, and 84.45%, respectively. Culturing of *M. tuberculosis* is sensitive and specific but may take 6 to 10 weeks. Since the routine diagnostic methods are insensitive and time-consuming, faster and more reliable methods with increased sensitivities and specificities would be useful adjuncts to the conventional direct microscopy and culture methods.

In this study, we evaluated the performance of a PCR based assay and a DIA-antigen detection assay for direct detection of *M. tuberculosis* in sputum samples, in comparison with conventional culture and direct acid-fast microscopy. We used combined culture results from both liquid- and solid-medium cultures since BACTEC broth allows rapid growth, and Ogawa medium provides an opportunity to examine the colonial morphology and is useful as a backup to detect mixed cultures. The sensitivity of combined cultures was quite satisfactory in this study. For AFB staining, we used fluorescence microscopy and confirmation by the Ziehl-Neelsen method, however low levels of sensitivity (66.42%) but high specificity (96.91%) were expected.

For amplification, the IS*6110*-PCR based method with our newly designed primer pair developed in previous study [24] permitted the amplification of 377-bp DNA fragments located at the end region (position +961 to +1337) of the IS*6110* sequence.[23] This method has been demonstrated quite good sensitivity and specificity for *M. tuberculosis* complex.[24] For detection of amplified product, conventional gel analysis and Southern blot hybridization with enhanced chemiluminescence (ECL) detection allowed high sensitivity and specificity of amplicon detection, however the method is more laborious, complicated and is highly expensive. We also applied a dot immunoassay (DIA) with ECL detection for detecting *M. tuberculosis* antigen by using commonly available reagents.

Overall agreement between culture and PCR was rather high (K = 0.962 by kappa analysis). In contrast, agreement between culture and DIA-Ag detection was low (K = 0.411). Discrepant results between culture and PCR were found for 6 specimens; all were from smear-negative specimens and no inhibitor was detected in the internal control of PCR. Review of clinical charts and radiographs indicated that all samples came from patients suspected of having tuberculosis by positive chest X-ray. In 5 cases (PCR-/Cul+), the culture results were assumed to be correct and were considered to be "true" positive for *M. tuberculosis.* In one case (PCR+/Cul-), the PCR result was assumed to be false positive. The reason for the positive PCR result for this patient is unclear. However, it is possible that this patient may have been coinfected with *M. tuberculosis* which was undetected

by culture, or the problem of cross-contamination among sputum samples during processing or extraction of specimens for PCR amplification. Unfortunately, no sample was available for culture confirmation. The difference between culture and PCR was not statistically significant ($P > 0.05$). The overall good performance of the IS*6110*-PCR based method for detection of *M. tuberculosis* makes it a very useful additional tool in *M. tuberculosis* diagnostics, as was also reported earlier by other investigators.[28,30] In contrast, the overall sensitivity (73.13%) and specificity (69.07%) of DIA-Ag detection was much less than those of culture and PCR. The difference between PCR and DIA-Ag detection sensitivities and specificities was found to be statistically significant ($P < 0.05$). The DIA-Ag assay was considerably less specific due to the use of anti-*M. bovis* BCG rabbit serum since BCG has been shown to be antigenically very similar not only to *M. tuberculosis* but also to many mycobacteria other than *M. tuberculosis* and also other bacterial species which are commonly found in sputum specimens.[31] Therefore, antigen detection in sputum specimens by using anti-BCG is not useful in the diagnosis of pulmonary tuberculosis, unless a monoclonal antibody directed against highly specific *M. tuberculosis* antigen is commercially available. A possible explanation for the apparent lack of sensitivity even using sensitive ECL detection was some denaturation of antigens after exposure to agents used in sputum decontamination process, or antigens in sputum may have been coated with secretions and thus unavailable to the antibody.[32] It is wise to admit that as long as specific antigens of *M. tuberculosis* are not available to the clinical laboratory, there is no hope for serodiagnosis of tuberculosis because of lack of specificity. Sensitivity also is required for a useful serodiagnostic test. Unfortunately, both are not yet available.

Besides the problems of favorable sensitivity and specificity, diagnostic laboratories also need acceptable predictive values of the test results. The overall PPVs were very good for both PCR, culture and microscopy, but were lower for DIA-Ag assay. A high predictive value of AFB smears for *M. tuberculosis* in our study was close to the 92% reported by Yajko et al,[33] despite the high prevalence of *M. avium* complex in respiratory specimens.

When the results were evaluated according to smear status, PCR and culture were found to give equivalent results in smear-positive specimens ($P > 0.05$). In smear-negative specimens, PCR had far lower sensitivity than that of culture, but specificity remained high. Again, the difference between culture and PCR was not statistically significant ($P > 0.05$). These results are in accordance with other findings[12,28,30,34] in that PCR was less sensitive for smear-negative culture-positive samples. Overall sensitivities were affected by the number of smear-negative, culture-positive samples included in the studies. In this study, all 5 false-negative samples were smear-negative specimens which indicating low organism loads in these specimens. Uniform dispersion of organisms in sputum specimens is also difficult to obtain due to a common clumping problem of mycobacteria. Another reason for low sensitivity was likely due to the low sample utilization and nonhomogeneity of the aliquots because the NALC sediment was divided into several aliquots, each with a relatively small volume, and only 10 μl was used per PCR,

whereas 200 to 500 μl was used in the cultures. Use of the whole specimen or larger volume and subjecting the specimen to subsequent centrifugation should allow greater sensitivity.

However, the presence of inhibitors of PCR in clinical specimens is a well-known diagnostic problem that frequently leads to false-negative results. To deal with this problem, PCR inhibitors must be diluted, inactivated or removed from the samples. We used two successive high-speed washes to remove inhibitors from the NALC sediments; and to monitor PCR inhibition, we spiked duplicates of the previously tested DNA extracts with a small amount of H_{37} Rv DNA. No inhibitors were found by using this control in all samples tested. Duplicate samples from these same five negative PCR specimens were retested in PCR assay, and again none became positive by retesting. Thus, these specimens were considered false negative. In part, the cause for such false negativity may be due to a combination of low sample utilization, non - uniform distribution of microorganisms in the test suspension, and/or the presence of small amounts of inhibitors in the sputum specimens that could not be detected by internal control DNA. Thus for the analysis of patients with smear-negative specimens, we recommend testing several specimens from these patients before reporting a negative PCR result.

In general, PCR is a valuable adjunct to the laboratory diagnosis of tuberculosis but it cannot be expected to replace cultures because of the need for isolation of organisms for susceptibility testing. A practical use for PCR assays would be for patients who are at high risk for *M. tuberculosis* infection and whose sputum smears are negative. In our study, 41 of 45 patients with negative smears could be rapidly diagnosed by PCR. In cases of smear-negative specimens, diagnosis of tuberculosis by culture may take up to 8 weeks and the species identification procedures extend the diagnosis time even further, while the time for detection of *M. tuberculosis* by PCR-hybridization can be within one day. Optimal patient management requires early initiation of specific drug therapy and isolation of infectious individuals as soon as possible. Therefore, for a number of these patients, if reliable results had been reported prospectively, *M. tuberculosis* would have been detected earlier, which would have significantly altered their management. Since detection of AFB by smears is an inexpensive and rapid method for patients clinically suspected of tuberculosis, it might be rational to use PCR for smear-negative specimens.

In conclusion, we have demonstrated that there is no significant difference in the performances of IS*6110*-PCR based methods and culture. In contrast, the difference between DIA-Ag detection and culture was found to be statistically significant. The good overall performance of the IS*6110*-PCR based method for detection of *M. tuberculosis* makes it a very useful additional tool in providing a rapid diagnosis of tuberculosis. However, in a routine clinical laboratory, it is desirable to use detection methods that have low complexity and that are relatively simple and cost-effective. Thus further improvements of the assay at the sample preparation step and at the detection step may lead to a more reliable, simple and cost-effective assay useful in routine clinical practice for the rapid diagnosis of tuberculosis.

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