# Detection of *Mycobacterium tuberculosis* from Sputum Collected on Filter Paper and Stored at Room Temperature for 5 Days by PCR Assay and Culture

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**Abstract**

The efficacy of PCR assay and culture for direct detection of *M. tuberculosis* (MTB) from sputum specimens collected on filter paper and stored at room temperature for 5 days was evaluated in comparison with conventional culture of fresh sputum specimen. A total of 231 sputum specimens were examined. MTB was recovered from 124 samples by culture from fresh sputum samples before storage. The culture positivity rate was significantly decreased to 70 per cent after 5 day's storage on filter paper. For PCR assay, a fragment of 377-bp of the IS*6110* sequence was amplified and detected using nested PCR. Compared with culture results performed on fresh sputum samples, the sensitivity, specificity, and efficiency for the nested PCR were 96.0, 97.2 and 96.5 per cent, respectively. The nested PCR showed sensitivity and specificity with no significant difference ($p>0.05$) from culture of fresh sputum specimens.

**Conclusion :** The collection and storage of sputum on filter paper at room temperature for 5 days had no apparent effect on the performance of nested PCR. Sputum samples collected by this method could be sent by post in a minimum of space and inexpensive way and will enable a large number of samples collected in the field or from peripheral health centers to be sent to central laboratories for analysis by trained technicians and under a well-equipped and well-established quality control system. The rapid and reliable detection by PCR-based assay will be helpful for optimal patient management of therapy and effective control of tuberculosis.

**Key word :** *Mycobacterium tuberculosis,* Sputum Storage, Filter Paper, PCR, Culture

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The reemergence of tuberculosis cases and increasing multidrug resistance among *Mycobacterium tuberculosis* (MTB) strains poses a public health threat of global concern[1]. In developing countries tuberculosis is a leading cause of mortality, and the spread of the HIV epidemic contributes significantly

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to the worsening of the situation. Tuberculosis has recently become a re-emerging disease in Thailand after considerable decline during the previous decades and remains a substantial health problem in this country with a notification rate of 70/100,000 of the population noted for 1999[2]. Rapid and reliable detection of MTB has an important public health significance that may improve management of infected patients and facilitate infection control procedures.

Many diagnostic laboratories are incapable of manipulating cultures and performing antimicrobial susceptibility tests. Therefore, in many instances, sputum specimens collected in peripheral health centers must often be sent to well equipped central laboratories for *Mycobacterium* isolation, identification, and susceptibility testing. Delays in culturing of sputum specimens, because of transport time over long distances, could result in significant loss in recovery of MTB[3]. Moreover, when transportation requires more than the usual time with higher temperatures, these sputum samples usually become highly contaminated.

In a previous study we proposed a method of collecting sputum on filter paper for transportation from peripheral centers to a central laboratory and compared this method with that of collection in a plastic cup before storage and after storage for up to 5 days[4]. There was no significant difference in the rates of recovery by culture between both storage methods. However, significant loss of viability after storage for 5 days was observed. The filter paper method of sputum collection has some advantages. These include inexpensive and easy transportation by post in a minimum of space without cold storage facilities and convenience for culturing with less culture contamination. However, the transport of sputum specimens over long distances from peripheral health centers to a central laboratory may affect the culture recovery. Therefore, a PCR-based assay was considered as an alternative method to detect MTB from sputum specimens collected by the filter paper method.

In the present study, we evaluated the direct detection of MTB, from sputum collected on filter paper and stored at room temperature for 5 days, by culture and nested PCR assay based on the IS*6110* insertion sequence using a primer pair[5] that amplifies a 377-bp target of the insertion sequence IS*6110*. The results of PCR assay and culture for direct detection of MTB from sputum collected and stored on filter paper were compared with those obtained by conventional culture and microscopy performed on fresh sputum specimens.

## MATERIAL AND METHOD

### Collection of sputum

A total of 231 sputum samples were collected from newly diagnosed patients at the TB Division, Ministry of Public Health, Thailand. These patients were suspected of having TB because of their clinical history and characteristic radiographs and were not receiving anti-tuberculosis therapy. The samples were either collection or spot sputum with the amount of at least 1-2 ml. One part (0.5 ml) was collected and stored on filter paper at room temperature (approx. 32-35°C) for 5 days. The remaining part of the fresh sputum samples was used to make smears for fluorochrome staining and then processed for culturing on the day of collection using standard procedures[6].

### Storage of sputum on filter paper

The procedure for storage of sputum specimens on filter paper was as follows : 1) Whatman no 2 filter paper (11 cm diameter) was folded in half twice to form a cone. 2) At least 0.5 ml of sputum was put down into the bottom of the cone. 3) The filter paper cone was closed using a staple, air dried and stored in a plastic bag, then placed in an aluminum box in a dark place at room temperature. 4) After 5 day's storage, each specimen was processed for culture and PCR examination.

### Processing of fresh sputum samples for culture

Sputum samples were examined first in stained smears by fluorochrome staining[7], then they were processed for culture by a modification of the method described by Petroff[8]. Briefly the sputum was treated with an equal volume of *N*-acetyl-L-cysteine-4 per cent NaOH and shaken for 15 min at room temperature and then inoculated onto 2 slants of 2 per cent Ogawa media, incubated at 37°C for 8 weeks, and examined weekly for growth. Positive cultures on Ogawa media were examined for growth rate, gross and microscopic colony morphology and pigmentation. They were subjected to conventional biochemical tests[9].

**Processing of sputum collected on filter paper for culture and PCR assay**

Individual filter paper was carefully picked up from the plastic bag, then cut 2 cm away from the cone tip using sterile scissors and placed into a centrifuge tube containing 5 ml of N-acetyl-L-cysteine-4 per cent NaOH. The sputum on the filter paper was eluted by shaking at room temperature for 15 min and 4 drops were then inoculated onto each of 2 slants of 2 per cent Ogawa. The method for culture and identification was similar to that described above. The remaining part of the processed sputum sample was then washed with 25 ml of 0.067 M phosphate buffer (pH 6.8) and centrifuged at 2,400 Xg for 15 min. The sediment was resuspended in 1 ml of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.3) and stored at -20°C for DNA extraction.

**DNA extraction from bacterial strains and clinical specimens**

Total purified DNA was isolated from reference strains; i.e. *M. tuberculosis* H37Rv and *M. flavescens* ATCC 23035 were used as a positive and a negative control DNA in PCR amplification. For extraction of DNA from sputum samples collected on filter paper, the protocol followed the method as described[10] with slight modification. Briefly, the processed sputum samples stored in TE buffer were boiled at 80°C for 15 min, vortexed and centrifuged at 12,000 X g for 15 min. After the supernatant was discarded, 100 µl of lysis buffer (100 mM Tris HCl (pH 8.3), 0.5 per cent Tween 20; 1 mg/ml proteinase K) was added to the pellets, and the mixture was incubated at 55°C for 3 h. The tube was then placed in a boiling water bath for 10 min, and cell debris was removed by centrifugation at 12,000 X g for 2 min, and 10 µl of the supernatant was used for PCR amplification.

**Polymerase chain reaction**

Two pairs of primers derived from the IS*6110* reference sequence X17348 (Gen Bank)[11] were used. The outer primers, TB1 (5'-CCAACA-AGAAGGCGTACTCG-3', position 961 to 980) and TB2 (5'-GGAGACTCTCTGATCTGAGACC-3', position 1316 to 1337); and the inner primers, TB3 (5'-TAGGGGATCTCAGTACA-3', position 1009 to 1025) and TB4 (5'-GCTCGGTCTTGTATAGGC-3', position 1128 to 1145) were designed by Tansuphasiri et al[5]. A nested PCR detection protocol was composed of the first round used primers TB1 and TB2 which generated a product of 377-bp, and the second round used primers TB3 and TB4 which were internal to the product amplified by TB1 and TB2, and generated a product of 137-bp. The first PCR reaction mixture in the final volume of 50 µl followed the optimized PCR condition as previously described[5]. The amplifications were carried out in an automated thermal cycler (Perkin-Elmer Cetus). The samples were denatured at 94°C for 5 min, and 40 amplification cycles were performed. The cycles consisted of denaturation at 94°C (1 min), annealing at 60°C (1 min), and extension at 72°C (1 min). After the final cycle, all reactions were incubated at 72°C for an additional 10 min.

The reaction mixture of the second round in a final volume of 25 µl contained the same chemicals and concentrations as the first round and 5-µl volume of the first-round reaction mixture was added to each reaction mixture as target DNA. Reaction conditions for the second round were 94°C for 5 min then 40 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 1 min with a final extension phase of 72°C for 10 min. For detection, 10 µl of PCR product was electrophoresed on 2 per cent agarose gel, stained with ethidium bromide and viewed under UV light. To avoid possible contamination of the PCR mixture, all reactions were performed under stringent conditions[12]. All available specimens that were culture positive for MTB but negative by PCR were retested. Inhibition of the PCR was checked by spiking duplicates of the previously tested DNA extracts with 100 fg of *M. tuberculosis* H37Rv DNA. Samples showing inhibition were retested at two different dilutions: 1/5, and 1/10.

**Statistical analysis**

The McNemar chi square test was used to estimate the statistical significance of the differences in detection of MTB from sputum collected on filter paper by culture and PCR. The results of the AFB smear and culture on fresh sputum specimens were used as the "gold" standard for diagnosis of MTB. All tests were considered statistically significant when P value was less than 0.05.

## RESULTS

**Analysis of fresh sputum samples by direct microscopy and culture**

Of the 231 sputum samples examined, they were divided into four groups according to AFB

**Table 1. Comparison of culture results between sputum collected on filter paper and stored for 5 days at room temperature with conventional culture of fresh sputum samples for detection of *M. tuberculosis*.**

| Culture of sputum collected on filter paper and stored for 5 days* | Culture of fresh sputum | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 87 | 0 | 87 |
| Negative | 20 | 107 | 127 |
| Total | 107 | 107 | 214 |

* Contamination in 17 of 231 sputum specimens.

**Table 2. Comparison of nested PCR on 231 sputum specimens collected on filter paper and stored at room temperature for 5 days with conventional culture of fresh sputum specimens for detection of *M. tuberculosis*.**

| Nested PCR results of sputum collected and stored on filter paper for 5 days | Culture results of fresh sputum | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 119 | 3 | 122 |
| Negative | 5 | 104 | 109 |
| Total | 124 | 107 | 231 |

Sensitivity, 96.0%; specificity, 97.2%; efficiency, 96.5%

staining and culture results : smear negative and culture positive (2.2%), smear positive and culture positive (51.5%), smear positive and culture negative (0.9%), and smear negative and culture negative (45.4%). Conventional culture of fresh sputum samples yielded positive results for MTB in 124 of 231 samples (53.7%).

**Analysis of sputum collected on filter paper for 5 days by culture**

Of the 231 sputum specimens collected on filter paper and stored for up to 5 days, MTB were recovered from a total of 87 samples, while culture was negative in 127 samples, and contamination in 17 samples. In comparison with culture results of fresh sputum samples, the positivity rate by culture of sputum collected on filter paper for 5 days was 70.2 per cent (87 of 124). When the culture positivity rates of MTB were classified by smear results, it was revealed that in group smear negative and 1+, the culture positivity rates were nearly 40 per cent and 56 per cent, respectively. Corresponding, in-groups with grading smear 2+ and 3+ were substantially increased with the culture positivity rates of 71 per cent and 77 per cent, respectively. Comparison of MTB detection between the conventional culture of fresh sputum samples and the culture results of sputum collected on filter paper for 5 days is presented in Table 1. Statistical comparison was performed by using Mc Nemar $\chi^2$ test and there was significant difference in the positivity rates between the two periods of culture detection ($p<0.05$). The sensitivity and specificity were 81.3 and 100 per cent.

**Analysis of sputum collected on filter paper for 5 days by nested PCR**

Of those 124 specimens that were culture positive for MTB, 119 samples were positive by nested PCR (sensitivity; 96.0%). Of the 107 culture-negative samples for MTB, 3 samples were positive by nested PCR (specificity; 97.2%). All 5 false-nega-

tive samples by nested PCR were from smear positive (AFB 1+), culture positive samples. Likewise, 3 false-positive samples were from smear negative, culture negative samples. Statistical comparison was performed by using Mc Nemar $\chi^2$ test, and there was no statistically significant difference ($p > 0.05$) in sensitivity and specificity for nested PCR compared with conventional culture of fresh sputum samples.

## DISCUSSION

In Thailand, tuberculosis laboratory services are organized down to the district hospital level providing smear microscopy at all levels and culture facilities at the central, regional and provincial levels. However, reliable results can hardly be expected unless all the laboratory procedures are performed by trained technicians and under a well-established quality control system. Therefore, in some district hospitals and primary health care centers that lack facilities for culture examination, the sputum specimens must be sent to a well equipped reference central laboratory for processing and culture. For transporting sputum specimens from peripheral centers to the central laboratory, it is generally recommended to transport the sputum in plastic containers, tightly sealed and leak-proof, placed in special boxes which are as cool as possible and protected from the light. However, the transport of sputum specimens over long distances from peripheral health centers to a central laboratory generally takes about 3 - 5 days by post which may affect the culture recovery.

The transport of non-fixed sputum smears rather than sputum specimens has previously been proposed by Kantor and Isola[13] who studied the efficiency of this method by culturing of mycobacteria from non-fixed smears of sputum after storage at 20-25°C or 4°C for 4, 8 and 15 days. Mycobacterial growth from smears after storage at 4°C was better than those stored at 20-25°C, and also growth was better from smears stored for 4 days than those stored for 8 or 15 days. In this study, we used the filter paper collection method as previously described [4] for nonprocessed sputum specimens and stored at room temperature for 5 days, then processed for analysis by culture and PCR. The culture positivity rate for MTB from sputum storage on filter paper by this study was higher than previously described (70% compared to 50%). This may be explained by differences in the number of specimens with various smear grading results analyzed. In this study, sputum samples with a grading smear 2+ and 3+ were found in higher proportion than smear 1+ and negative. In the group smear negative and 1+, the culture positivity rates were 40 per cent and 56 per cent, respectively. Corresponding, in-groups with grading smear 2+ and 3+ were substantially increased with culture positivity rates of 71 per cent and 77 per cent, respectively. In general, the culture positivity rate decreased to 70 per cent when sputum specimens on filter paper were kept at room temperature for 5 days but the advantage of this method was low contamination (7%). While another study[14] showed the contamination rates of 5, 7, 12 and 18 per cent after storage for 0, 3, 5 and 7 days, respectively.

As time of storage before culture was an important factor of MTB growing, in addition slow growth rate and the limited sensitivity and specificity of direct microscopy made rapid diagnosis difficult. Therefore, a rapid PCR-based method was considered since it can detect both viable and non-viable organisms. In this study, we used nested PCR which gave high sensitivity and specificity (96 and 97%), and there was no significant difference between the results obtained with nested PCR and conventional culture of fresh sputum ($p > 0.05$). However, three false-positive samples (2.8%) were from smear-negative and culture-negative samples. This technique increased sensitivity, but there is also a greater risk of cross-contamination. As a result, single one-tube nested PCR should be used for solving this risk of cross-contamination. However, contamination with DNA is necessary to strictly control the procedure in PCR assay. Five false-negative samples (4%) were detected by nested PCR. The factors may be traced to the low number of organisms, the presence of inhibitors, the lack of IS*6110* in the genome and degradation. All 5 samples were AFB smear 1+ and also after DNA extraction, they were stored at –20°C for a month before amplification which might cause DNA degradation. The presence of inhibitors of PCR in these specimens was another diagnostic problem that led to false-negative results.

However, the nested PCR showed outstanding sensitivity and specificity when compared with conventional culture of fresh sputum. PCR cannot be expected to replace cultures because of the need for isolation of organisms for susceptibility testing. In general, PCR is a valuable adjunct to laboratory diagnosis of tuberculosis. PCR results

were positive, whereas, culture and AFB were negative in patients with anti-tuberculosis medication (15). A practical use for PCR assay would be for patients who are at high risk for TB infection and whose sputum smears are negative, whereas, the specimens positive by AFB, PCR permit distinction between MTB and other mycobacteria rapidly and, thus, may be helpful for optimal patient management of specific drug therapy and isolation of infectious individuals as soon as possible. Since diagnosis of tuberculosis by culture may take up to 8 weeks and the species identification extends the diagnosis time even further, while the average time for detection of MTB by nested PCR is 4-6 h. In cases of a false-negative PCR result from a clinical specimen, analysis of multiple sputum specimens should maximize detection of MTB.

In conclusion, this study has shown that MTB could be rapidly detected from sputum collected on filter paper and stored at room temperature for 5 days by using the IS*6110*-PCR based method. We recommend using both culture and PCR assay for detection of MTB from sputum collected by this method. In cases of low culture positivity rate when transportation requires more time than usual, it may prove useful if specimens are processed and then neutralized with acid before shipment of multiple specimens from one patient. In cases where sputum is identified by DNA-based diagnostics only, ethanol fixation(16) of the sputum on filter paper may provide a simple and inexpensive way to store and transport the sputum sample without refrigeration and render the clinical sample noninfectious while preserving the integrity of genomic DNA suitable for testing by PCR. However, PCR is unreliable if outside the research laboratory, thus the assay should be performed by a trained technician and under a well-equipped and well-established quality control system. This filter paper collection method will enable large numbers of specimens collected in the field or from peripheral health centers to be sent by post without refrigeration to a well-established central laboratory for analysis. The rapid and reliable detection by PCR-based assay will be helpful for optimal patient management of specific drug therapy and effective control of tuberculosis.

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## REFERENCES

1. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Global burden of tuberculosis : Estimated incidence, prevalence, and mortality by country. JAMA 1999; 282: 677-86.
2. Payanandana V, Kladpaung B, Somsong W, Jittimanee S. Battle against TB, National Tuberculosis Programme, Thailand 1999. Tuberculosis Division, Department of Communicable Disease Control. 1999.
3. Babakhani FK, Warren NG, Henderson DP, Dalton HP. Effect of transportation and acid neutralization on recovery of mycobacteria from processed specimens. Amer J Clin Pathol 1995; 104: 65-8.
4. Tansuphasiri U, Patoomkaew O, Rienthong S, Boonshuyar C. Comparison of storage sputum impregnated on filter paper and in plastic cup at room temperature for 5 days before culture of *Mycobacterium tuberculosis*. Thai J Tuberc Chest Dis 1997; 18: 269-83.
5. Tansuphasiri U, Vathanophas K, Pariyanonda A, Chanyasanha CH. Detection of *Mycobacterium tuberculosis* in respiratory specimens using IS*6110*-PCR based method with new designed primers. Mahidol J 1998; 6: 71-6.
6. Bates JH. Diagnosis of tuberculosis. Chest 1979; 76 (Suppl): 757-63.
7. Bennedsen J, Larsen SO. Examination of tubercle bacilli by fluorescence microscopy. Scan J Resp Dis 1966; 47: 114-20.
8. Petroff SA. A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and fecae. J Exp Med 1915; 21:

38-42.
9. Kent PT, Kubica GP. Public Health Mycobacteriology : A guide for the level III laboratory. Atlanta: Centers for Disease Control. 1985.
10. Buck GE, O'Hara LC, Summersgill JT. Rapid, simple method for treating clinical specimens containing *Mycobacterium tuberculosis* to remove DNA for polymerase chain reaction. J Clin Microbiol 1992; 30: 1331-4.
11. Thierry D, Cave MD, Eisenach KD, et al. IS*6110*, an IS-like element of *Mycobacterium tuberculosis* complex. Nucleic Acids Res 1990; 18: 188.
12. Kwok S, Higuchi R. Avoiding false positive with PCR. Nature (London) 1989; 339: 237-8.
13. Kantor IN, Isola NC. Culture of *Mycobacterium tuberculosis* from non-fixed sputum smears. Tubercle 1985; 66: 137-9.
14. Paramasivan CN, Narayana ASL, Prabhakar R, Rajagopal MS, Somasundaram PR, Tripathy SP. Effect of storage of sputum specimens at room temperature on smear and culture results. Tubercle 1983; 64: 119-24.
15. Tan MF, Chan SH, Tan WC. Comparative usefulness of PCR in the detection of *Mycobacterium tuberculosis* in different clinical specimens. J Med Microbiol 1997; 46: 164-9.
16. Williams DL, Gillis TP, Dupree WG. Ethanol fixation of sputum sediments for DNA-based detection of *Mycobacterium tuberculosis*. J Clin Microbiol 1995; 33: 1558-61.

# การตรวจหาเชื้อวัณโรคจากเสมหะที่เก็บในกระดาษกรอง นาน 5 วัน ที่อุณหภูมิห้อง ด้วยเทคนิคพีซีอาร์และการเพาะเชื้อ

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การศึกษาประสิทธิภาพของเทคนิคพีซีอาร์และการเพาะเชื้อเพื่อหาเชื้อวัณโรคจากเสมหะที่เก็บในกระดาษกรองเป็นเวลา 5 วัน ที่อุณหภูมิห้อง โดยอาศัยผลของการเพาะเชื้อจากเสมหะก่อนการเก็บเป็นมาตรฐาน จากตัวอย่างเสมหะของผู้ป่วยจำนวน 231 ตัวอย่าง ตรวจพบเชื้อวัณโรคโดยวิธีเพาะเชื้อแบบมาตรฐาน 124 ตัวอย่าง หลังจากเก็บเสมหะไว้นาน 5 วัน อัตราการตรวจพบเชื้อโดยการเพาะเชื้อเหลือร้อยละ 70 ส่วนวิธีพีซีอาร์ที่เพิ่มจำนวนดีเอ็นเอขนาด 377 คู่เบส จากดีเอ็นเอเป้าหมายในกลุ่ม IS*6110* และวิเคราะห์โดยวิธี nested PCR ให้ค่าความไว ความจำเพาะ และประสิทธิภาพ ร้อยละ 96.0, 97.2 และ 96.5 ตามลำดับ ซึ่งไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติกับการเพาะเชื้อแบบมาตรฐาน ($p > 0.05$)

**บทสรุป** : การเก็บเสมหะในกระดาษกรองตามวิธีดังกล่าวไม่มีผลกระทบต่อการตรวจหาเชื้อวัณโรคโดยวิธี nested PCR จึงอาจใช้ร่วมกับการเพาะเชื้อในการตรวจหาเชื้อวัณโรคจากเสมหะที่ส่งมาในกระดาษกรองได้ การเก็บเสมหะโดยวิธีนี้ทำให้การขนส่งตัวอย่างจากต่างจังหวัดหรือจากภาคสนามจำนวนมากและขนส่งมาทางไปรษณีย์นั้นทำได้สะดวก ไม่กินเนื้อที่และราคาถูก เพื่อส่งมาตรวจวิเคราะห์ที่ห้องปฏิบัติการส่วนกลางที่มีความพร้อมทั้งเครื่องมือและผู้เชี่ยวชาญ ซึ่งผลการตรวจที่รวดเร็วและน่าเชื่อถือของวิธีพีซีอาร์นี้นับว่ามีประโยชน์ส่งผลให้การดูแลรักษาผู้ป่วยทำได้ถูกต้องและการควบคุมวัณโรคมีประสิทธิภาพขึ้น

**คำสำคัญ** : เชื้อวัณโรค, การเก็บเสมหะ, กระดาษกรอง, พีซีอาร์, การเพาะเชื้อ

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