

# EVALUATION OF AN IMMUNOCHROMATOGRAPHIC TEST KIT FOR DETECTING *MYCOBACTERIUM TUBERCULOSIS* COMPLEX IN SPUTUM SAMPLES AND ON SOLID AND IN LIQUID CULTURES

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**Abstract.** A rapid, cheap and effective method for diagnosing tuberculosis (TB) is essential for TB control. We evaluated the performance of an immunochromatographic assay (ICA) kit (SD Bioline TB Ag MPT64 rapid test) designed for detecting *Mycobacterium tuberculosis* complex (MTC) in liquid and solid cultures to determine its ability to detect and differentiate MTC directly in sputum samples. We attempted to optimize antigen extraction using several sputum solvents under various conditions. Adding the sputum solvent prior to using the ICA kit gave a sensitivity of 71.7% (43/60) for all acid-fast bacillus (AFB) stain positive specimens and a 100% specificity (20/20) among AFB negative specimens. Without sputum solvent, the ICA kit had 0% sensitivity for detecting MTC in sputum. We also evaluated the ICA test kit for its designed purpose of detecting MTC in 80 solid and liquid culture specimens positive for MTC using the niacin accumulation test or polymerase chain reaction (PCR) (16s-23s ITS). The ICA kit gave 100% sensitivity and specificity. We also evaluated the ICA test kit on 3 reference specimens of MTC, 15 nontuberculous mycobacteria (NTM) species, 7 bacterial species and 5 *Candida albicans* specimens. The tested ICA kit gave 100% specificity. The tested ICA kit was useful for detecting and differentiating MTC in solid and liquid cultures, but not useful for detecting MTC in sputum samples even treated with sputum solvent. The tested ICA kit should be used only for liquid and solid culture specimens. Therefore, the tested kit is inappropriate for use in evaluating sputum samples.

**Keywords:** *Mycobacterium tuberculosis* complex, immunochromatographic assay, MPT64, sputum sample

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## INTRODUCTION

Tuberculosis (TB) is a major public health problem worldwide. There are approximately 8.7 million new TB cases and 1.4 million deaths per year (WHO, 2011). TB is a devastating disease caused by *Mycobacterium tuberculosis* complex (MTC)

(El Khechine *et al*, 2009). The incidence of infections caused by non-tuberculous mycobacteria (NTM) has also increased (Lai *et al*, 2006; Polanecky *et al*, 2006). Many countries affected by TB are developing countries. An inexpensive, rapid and easy method to detect and differentiate mycobacteria is important for TB control.

Common current TB diagnostic methods include detection of acid-fast bacilli (AFB) on a smear or culture (Hanscheid *et al*, 2005). Direct microscopy is rapid but has a detection limit of around 5,000 bacilli/ml (Chakraborty *et al*, 2009). Although culture is the gold standard, it requires 6-8 weeks to obtain results (Ongut *et al*, 2006). Molecular methods, such as PCR amplification are rapid and specific for differentiating MTC, but are complicated, time consuming, and require specific equipment and highly trained technicians (Martin *et al*, 2010; Grimaldi *et al*, 2011; Perez-Osorio *et al*, 2011; Povazan *et al*, 2012).

Recently, a number of new rapid methods for diagnosing TB have been developed. Serological diagnostic methods can be inexpensive, flexible, and suitable for small laboratories and useful for developing countries (Abe and Hara, 2002; Reddy *et al*, 2002; Raja *et al*, 2008). The immunochromatographic assay (ICA) is one of the most commonly used serological tests for discriminating between MTC and NTM (Abe *et al*, 1999; Chakraborty *et al*, 2009; Park *et al*, 2009). In 2007, Standard Diagnostics (SD, Yongin, Korea) developed a rapid immunochromatographic assay (ICA) kit to differentiate between MTC and NTM. This kit is based on a specific mouse monoclonal antibody to MPT64, a specific secreted antigen found only on MTC. This test has been reported to have 100% sensitivity and specificity for detecting MTC in culture specimens (Park

*et al*, 2009). However, this test has never been evaluated for its ability to detect and differentiate MTC in the sputum.

In this study, we evaluated the performance of the ICA kit (SD Bioline TB Ag MPT64 rapid test) for detecting MTC in sputum samples. We also evaluated the sensitivity of the test kit to detect MTC in positive solid and liquid cultures and the sensitivity against MTC and NTM reference strains as well as bacterial and fungal reference strains. We also evaluated several sputum solvents to enhance antigen extraction from sputum samples.

## MATERIALS AND METHODS

### Samples and isolates

One hundred sixty acid-fast bacilli positive (AFB+) sputum samples were obtained from symptomatic pulmonary TB patients attending the TB clinic at Srinagarind Hospital and the Office of Disease Prevention and Control 6, Khon Kaen, Thailand. Eighty-four solid and 84 liquid cultures were performed on 100 AFB positive sputum samples. The ICA test kit was used to evaluate 80 AFB positive sputum samples (AFB 1+,  $n = 17$ ; AFB 2+,  $n = 23$ ; AFB 3+,  $n = 20$ ) and 20 AFB negative sputum samples.

Three MTC reference specimens, 15 NTM reference species, 7 bacterial reference species and 5 *Candida albicans* reference specimens were evaluated with the tested ICA kit (Table 1).

This study was approved by the Khon Kaen University Ethics Committee for Human Research (approval number HE521199).

### The immunochromatographic assay kit

The SD Bioline TB Ag MPT64 rapid test kit (Standard Diagnostics, Yongin, Korea), was used according to the manu-

Table 1

List of reference mycobacteria, bacteria and fungi used to evaluate the ICA kit.

Species	Strain
<i>M. tuberculosis</i> (n=3)	H37Rv, H37Ra, ATCC 27294
<i>M. smegmatis</i>	16941
<i>M. xenopi</i>	ATCC 19250
<i>M. duvali</i>	MNC 442
<i>M. terrae</i>	ATCC 15755
<i>M. nonchromogenicum</i>	ATCC 19530
<i>M. austroafricanum</i>	3005
<i>M. flavescens</i>	ATCC 23035
<i>M. chelonae</i>	ATCC 23016
<i>M. fortuitum</i>	ATCC 23048
<i>M. goodii</i>	ATCC 11470
<i>M. intracellulare</i>	ATCC 13950
<i>M. szulgai</i>	JATA 3201
<i>M. avium</i>	ATCC 700898
<i>M. kansasii</i>	ATCC 12478
<i>M. scrofulaceum</i>	ATCC 19981
<i>Streptococcus pneumoniae</i>	Clinical specimen
<i>Staphylococcus aureus</i>	Clinical specimen
<i>Klebsiella pneumoniae</i>	Clinical specimen
<i>Escherichia coli</i>	Clinical specimen
<i>Neisseria meningitidis</i>	Clinical specimen
<i>Haemophilus influenzae</i>	Clinical specimen
<i>Nocardia</i> sp	Clinical specimen
<i>Candida albicans</i> (n=5)	Clinical specimen

facturer's instructions. This ICA kit is based on immunochromatographic diffusion to differentiate MTC from NTM using mouse monoclonal anti-MPT64 antibody (SD Bioline TB Ag MPT64) immobilized on a nitrocellulose membrane as the capture material.

#### Culture and identification of the clinical isolates of *M. tuberculosis* complex

Sputum specimens were cultured by standard methods (Scott *et al.*, 2002; Kaal *et al.*, 2009). Briefly, the sputum samples were decontaminated with N-acetyl-L-cysteine-NaOH and streaked on LJ

(Lowenstein-Jensen) slants (solid culture) and incubated at 37°C for 8 weeks. The LJ slants were checked weekly for growth of MTC. A standard niacin accumulation test was used to confirm the presence of MTC on LJ slants (Young *et al.*, 1970).

Zero point five milliliters each of the decontaminated samples was inoculated into the automated BACTEC Mycobacterial Growth Indicator Tube 960 (MGIT) system (liquid culture) and incubated in the MGIT 960 instrument either until they were flagged positive by the instrument or for a maximum of 6 weeks. Positive specimens on the MGIT system were evaluated by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP). In brief, the 16S-23S internal transcribed spacer (ITS) sequence was amplified by primers specific for MTC and the amplicons were digested by the restriction enzymes *Hae* III and *Msp* I, for RFLP (Telenti *et al.*, 1993; Park *et al.*, 2000).

#### Sputum solvent for antigen extraction

The efficacies of various solvents, [N-acetyl-L-cysteine (NALC), bromhexine and ambroxol solvents] to improve the efficacy of extracting MTC antigen from sputum were compared. The solvents were prepared by diluting 8 g NALC powder (Sigma, St. Louis, MO, Catalog No. A7250), bromhexine hydrochloride (Medicpharma, Bangkok, Thailand) and ambroxol hydrochloride (Boehringer Ingelheim, Berkshire, UK) in 2.0 M sodium phosphate at a pH of 8.0. Concentrations of 0.5, 2, 5, 7.5, 10, 15, 25, 30 and 40% weight/volume of each solvent were prepared. One part solvent was mixed with one part sputum before performing the test.

#### Evaluation of ICA test kit for detecting MTC from solid and liquid cultures

To determine the ability of the ICA

Table 2  
Comparison between the ICA kit and the niacin accumulation test and PCR.

Isolates	Results <sup>a</sup>	Test methods			Sensitivity compared with		Specificity compared with	
		ICA kit	Niacin	PCR	Niacin	PCR	Niacin	PCR
LJ culture (n=84)	Positive	80	80	ND	100% (80/80)	NA	100% (4/4)	NA
	Negative	4	4	ND				
MGIT culture (n=84)	Positive	82	ND	82	NA	100% (82/82)	NA	100% (2/2)
	Negative	2	ND	2				
Reference and clinical isolates (n=30)	Positive (MTC)	3	3	3	100% (3/3)	100% (3/3)	100% (27/27)	100% (27/27)
	Negative (Others)	27	27	27				
	Total	198	198	198				

<sup>a</sup>Positive and negative results refer to the finding of MTC. ND, not done; NA, not available.

test kit to detect MTC isolates on solid culture, 3 to 4 colonies were scraped from the solid media and suspended in 100  $\mu$ l of extraction buffer from the test kit (SD Bioline TB Ag MPT64 rapid test), then added to the test window. To determine the ability of the ICA test kit to detect MTC in liquid culture, 100  $\mu$ l of culture fluid was applied directly to the test window. *M. tuberculosis* H37Rv and sterile water were used as positive and negative controls, respectively. The results of the tests were interpreted 15 minutes after applying the sample. The presence of only a control band was considered negative and two bands (control and test) were considered positive. If the control band was not visible by 15 minutes, the result was considered invalid and the sample was retested.

#### Sputum solvent evaluation protocol

One hundred microliters of each solvent was added to an equal volume of sputum sample, vortexed for 5 min-

utes and then applied to the ICA kit. *M. tuberculosis* H37Rv and sterile water, both mixed with extraction buffer, were used as positive and negative controls, respectively.

Another solvent tested for extracting antigen (MPT64) was modified from Pereira Arias-Bouda *et al* (2000). Two hundred fifty microliters of sputum was added to an equal volume of 2 M sodium phosphate (pH 8) containing 7.5% NALC. The samples were then added to 5, 10, 15, 20 or 30  $\mu$ l of proteinase K (10 mg/ml) and vortexed for 5 minutes. Each mixture was then placed in a hybridization oven at 50°C for 30 minutes, 1, 2, 4 or 6 hours. The mixtures were then placed in boiling water for 10 minutes to inactivate the proteinase K and centrifuged for 1 minute at 12,000g at room temperature. One hundred microliters of the supernatant from each sample was placed in the ICA kit. Two negative controls were used for the test: 1) an equal mixture of sterile water



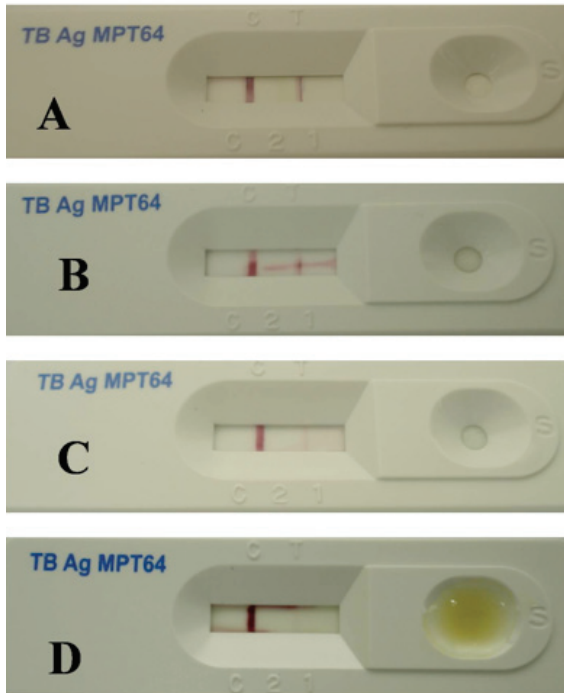


Fig 1–Test results with the ICA kit for detecting *Mycobacterium tuberculosis* reference strain MPT64 antigen directly from sputum samples. A) Sputum with 7.5% NALC; B) sputum with 7.5% ambroxol; C) sputum with 10% bromhexine HCl; D) sputum only.

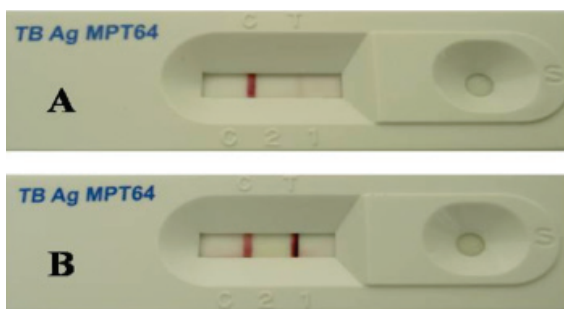


Fig 2–Evaluating the enhancing effect of 10% proteinase K in addition to 7.5% NALC for detecting MPT64 *Mycobacterium tuberculosis* antigen with the ICA kit. A) Sputum sample with 7.5% NALC only; B) sputum sample with 7.5% NALC and 10% proteinase K incubated for 10 minutes.

and extraction buffer and 2) an equal mixture of sterile water and sputum obtained from healthy subjects.

### Data analysis

The sensitivity and specificity were calculated by comparing the performance of the ICA kit with the niacin accumulation test and PCR (16s-23s ITS). The sensitivity and specificity for these tests were calculated for the organisms isolated from solid (LJ) and liquid (MGIT) cultures and in sputum samples.

## RESULTS

### The ICA test kit for detecting MTC in sputum samples (Fig 1)

The sensitivity of the ICA kit for detecting MTC among AFB positive sputum samples was 71.7% (43/60) and the specificity was 100 % (20/20 negative AFB samples). Without the sputum solvent the ICA kit had 0% sensitivity for detecting MTC in sputum. The specificity without the sputum solvent for AFB negative smears was 100 %.

### Sputum solvents

The sputum solvents (NALC, bromhexine HCl and ambroxol HCl) were used at various concentrations (0.5-40% w/v) and compared for their ability to extract MTC antigen. The optimal concentrations of sputum solvent for enhancing the sensitivity of the test were: 7.5% NALC, 7.5% ambroxol HCl and 10% bromhexine. Ten microliters of proteinase K at an incubation time of 30 minutes gave the best sensitivity (Fig 2).

### Evaluation of the ICA test kit on solid and liquid culture isolates

Eighty-four solid culture (LJ) and 84 liquid culture (MGIT) isolates were examined with the ICA test kit and compared with the results of the niacin and

PCR tests, respectively. The ICA test kit had 100% sensitivity and 100% specificity compared to both the niacin accumulation test and the PCR test (Table 2) when evaluating culture isolates. In 2 isolates MTC was found in liquid but not solid cultures. The liquid culture is more sensitive than the solid culture (Lu *et al*, 2002; Mohamed *et al*, 2009). The 3 MTC reference culture isolates were all positive with the ICA kit and the 15 reference NTM species were all negative with the ICA kit. The 7 reference bacteria species and 5 *Candida albicans* isolates were all negative with the ICA kit (Table 2).

## DISCUSSION

The tested ICA kit for detecting MTC in sputum was 0% without solvent and only 71.7% with solvent making it unsuitable for use in sputum samples. A previous study demonstrated the detection limit of this ICA kit at  $10^5$  CFU/ml with *M. tuberculosis* H37Rv culture isolate but was not tested in sputum samples (Park *et al*, 2009). A different ICA kit (rapid immunochromatographic assay, RICA) which uses an antigen-cocktail-based assay has been developed to detect mycobacterial antigen in sputum specimens (Chakraborty *et al*, 2009). When examining 200 sputum samples in HIV positive patients, the RICA gave a 97.9% sensitivity and a 99% specificity compared to sputum culture on solid medium (Chakraborty *et al*, 2009).

To enhance the ability of the tested ICA kit to detect MTC, proteinase K was added to the solvent as suggested by Pereira Arias-Bouda *et al* (2000). Adding 10 l of proteinase K and incubating for 30 minutes improved the clarity of the result but did not improve the sensitivity. The AFB positive sputum samples were stored at 4°C for 1-3 days prior testing; this could

have caused degradation of the antigen in the sputum giving a false negative result, decreasing the sensitivity of the test.

The tested ICA kit gave 100% sensitivity and 100% specificity for detecting MTC in both solid and liquid cultures. The performance of the tested ICA kit on specimens from solid and liquid culture has been evaluated in many studies. The ICA kit gave 99.4% sensitivity for detecting MTC reference isolates cultured in 3% Ogawa medium (158/159) and 98.3% in BACTEC MGIT 960 culture tubes (59/60); it also gave 100% specificity for differentiating it from 137 bacterial isolates, 20 fungal isolates and 51 NTM isolates (Park *et al*, 2009). In another study, the ICA kit gave 99% sensitivity for detecting MTB (190/192) and 100% for detecting *M. bovis* (18/18) and 100% specificity for differentiating it from 28 isolates of NTM and 22 non-mycobacterial organisms (Marzouk *et al*, 2011). In still another study the ICA kit gave 100% sensitivity for detecting 99 isolates of *M. tuberculosis*, 15 isolates of *M. bovis* and 10 isolates of *M. bovis* BCG Pasteur strain and 100% specificity for differentiating it from 14 isolates of NTM (9 species), 5 isolates of NTM unidentified and 28 isolates of other microorganisms (11 species), (Toihir *et al*, 2011).

The ICA kit had poor sensitivity for detecting MTC in the sputum but good sensitivity for detecting MTC in solid and liquid cultures. The addition of a sputum solvent improved the sensitivity in sputum samples but not to the point where the ICA kit may be used to detect MTC in sputum.

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