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New Simple and Rapid Test for Culture Confirmation of *Mycobacterium tuberculosis* Complex: a Multicenter Study

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Mycobacterial antigen MPB64 has been identified as a *Mycobacterium tuberculoisis* complex-specific secretory protein since 1984. Recently, a simple culture confirmation test for *M. tuberculosis* complex has been developed by using lateral flow immunochromatographic assay (ICA) to detect MPB64 with anti-MPB64 monoclonal antibody. The current multicenter study evaluated the performance of an ICA slide test for MPB64 antigen in the clinical setting. Primary positive cultures from clinical specimens, as well as stock cultures, were tested. Approximately 100 μl of positive liquid culture medium or suspension made from colonies on solid medium was placed into the test well of the plastic slide devise, and the test was read after 15 min. No processing or instrumentation was required. A total of 304 mycobacterial isolates consisting of *M. tuberculosis* complex (171 isolates) and mycobacteria other than *M. tuberculosis* (MOTT) complex (133 isolates) belonging to 18 different species were tested. Growth in liquid media (Mycobacteria Growth Indicator Tube [MGIT] and Radiometric 12B), as well as in solid (Löwenstein-Jensen and Middlebrook 7H10 agar) media, was evaluated. Results were compared with those obtained with nucleic acid-based and/or high-pressure liquid chromatography identification. All MOTT were found to be negative on the ICA slide with no cross-reaction. All *M. tuberculosis* and *M. africanum* cultures were found to be positive, whereas the results of *M. bovis* and *M. bovis* BCG cultures were variable since some of the BCG strains are known to lack MPB64 antigen production. The results did not change with prolonged storage of cultures. This low-tech rapid test with high sensitivity and specificity could provide an alternative to currently available identification methods, particularly for recently introduced nonradiometric liquid culture systems such as MGIT.

Tuberculosis is a significant reemerging infectious disease in many parts of the world, which is of great concern. Prompt detection, isolation, identification and susceptibility testing of *Mycobacterium tuberculosis* from clinical specimens is essential for appropriate management of patients with tuberculosis. The Centers for Disease Control and Prevention has recommended the use of liquid medium for primary culture and susceptibility testing to achieve better as well as faster results (17).

Even though liquid medium allows less time for the detection of positive cultures, it is crucial for identifying isolated acid-fast cultures quickly as well as accurately. The differentiation of *M. tuberculosis* from mycobacteria other than *M. tuberculosis* (MOTT) is most important from a clinical point of view, since tuberculosis is an infectious disease. Management and treatment of a patient with tuberculosis is different from that of patients infected with other mycobacteria. The radiometric BACTEC 460 TB system (Becton Dickinson Diagnostic Systems, Sparks, Md.) offers a NAP (*p*-nitro-α-acetylamino-β-hydroxypropiophenone) test, which discriminates the *M. tuberculosis* complex from MOTT (16). The NAP test, however, requires ca. 4 to 6 days to report results. Recently, nonradiometric liquid culture media such as Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson) have been introduced and extensively evaluated (1, 3, 13). There is no rapid and simple test available to differentiate the *M. tuberculosis* complex from MOTT in these new media. Other technologies, such as the use of molecular probes, gas-liquid chromatography, and high-performance liquid chromatography, are available, but these tests are technically complex, cumbersome, and expensive, especially the ones that are based on molecular analyses (5, 7). In countries with limited resources such tests are not practical. Therefore, a rapid and simple test is greatly needed, one that is as sensitive and as specific as the probe-based tests.

Several secreted mycobacterial proteins have been reported that showed some promise in diagnostic use (4, 9, 19). Among these secreted proteins, MPB64, which was first described as MPT64, has been found in unheated culture media of *M. tuberculosis*, *M. bovis*, and some but not all substrains of *M. bovis* BCG. This is a highly specific protein for the *M. tuberculosis* complex and has shown potential for diagnostic use (4, 10, 14, 15). The MPB64 gene has been well characterized, and the antigen has been widely studied (12, 20). This immunogenic protein elicits delayed-type hypersensitivity reactions in sensitized guinea pigs and lymphoproliferative responses in patients with tuberculosis (4, 8, 14). Studies with MPB64 antigen showed promise as a skin test antigen to differentiate

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patients with active disease from BCG-vaccinated people (10, 11).

Recently, MPB64 has been studied to differentiate the *M. tuberculosis* complex from MOTT in isolated cultures (2, 18). Utilizing specificity of MPB64 Tomiyana et al. recently developed a simple immunochromatographic assay (ICA) with anti-MPB64 monoclonal antibody (18). Abe et al. demonstrated that the MPB64-ICA on a slide format could be easily used for rapid identification of the *M. tuberculosis* complex as a liquid culture confirmation test by using the MGIT and MB-REDOX (2). In the present multicenter study, the clinical usefulness of MPB64-ICA test and its different parameters were evaluated by using fresh clinical isolates and stock cultures of different mycobacterial species grown in several liquid and solid media.

MATERIALS AND METHODS

The study was conducted at four different sites: the Clinical Microbiology Laboratory of the National Minami Yokohama Hospital, Yokohama City, Kanagawa Prefect, Japan (site 1); the Missouri State Tuberculosis Laboratory, Mount Vernon, Mo. (site 2); the North Carolina State Lab of Public Health, Raleigh, N.C. (site 3); and the Research and Development Department of Becton Dickinson, Sparks, Md. (site 4).

Clinical samples were mostly sputa but also included extrapulmonary specimens (i.e., 15 bronchial-washing, 8 pleural-effusion, 15 gastric-lavage, 3 urine, 8 stool, 3 pus, and 1 knee joint fluid specimens) and were processed according to the routine *N*-acetyl-L-cysteine-NaOH (NALC-NaOH) method for specimens suspected to have contaminating bacteria (6). The digested, decontaminated, and concentrated specimens were inoculated into BACTEC MGIT 960 culture tubes containing 7 ml of Middlebrook 7H9 broth base supplemented with enrichment and PANTA (Becton Dickinson). The tubes were placed in the BACTEC MGIT 960 instrument (Becton Dickinson), where they were incubated and monitored continuously for fluorescence. The time to detection of positive growth in the MGIT was defined as the interval between the day of specimen inoculation and the day of positive fluorescence detected by the BACTEC 960 instrument. At site 4, growth in radiometric BACTEC 12B medium and Middlebrook 7H10 medium was tested. At two sites (sites 2 and 4) Löwenstein-Jensen (LJ) medium was also evaluated. After the detection of a positive culture the presence of acid-fast bacilli (AFB) was confirmed by Ziehl-Neelsen staining.

As a reference identification method, all of the cultures tested in this study were identified using either with the AccuProbe culture confirmation kits (GenProbe, San Diego, Calif.), by the DNA-DNA hybridization method (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) (7) (site 1), by high-pressure liquid chromatography, or by biochemical testing. In some instances more than one method was used for the final identification.

In order to have additional isolates and a wider range of different mycobacterial species, stock cultures were also tested. The frozen cultures were first inoculated into liquid and/or solid media. Once liquid culture was instrument positive or colonies on solid media were observed, the growth was tested by the ICA method.

Identification by ICA slide test. The ICA slide test kit for MPB64 (MPB64-ICA, CapiliaTB; TAUNS, Numazu, Japan) consists of a plastic slide with a sample pad, a reagent pad, a nitrocellulose membrane, and an absorbent pad. On the slide there is a sample well where the sample is applied and two test reaction zones where the results of the test and control are observed as pink to reddish purple bands. An ~100-μl sample is applied to the test well, and it flows through the nitrocellulose membrane laterally. The monoclonal antibody against MPB64, which is conjugated with colloidal gold and is immobilized at the membrane, is rehydrated by the liquid from the sample and reacts with the MPB64 antigen if present in the sample. The MPB64-antibody complex then flows further laterally and is captured by the test band, where anti-MPB64 antibody is present, producing a color band. A second anti-mouse immunoglobulin G antibody immobilized at the control band reacts with the free gold conjugate antibody when the sample continues to migrate through the control band area and gives another reddish purple color band. The test is read after 15 min. If the test band is positive it indicates the presence of the *M. tuberculosis* complex, but if there is no band then it indicates the presence of MOTT bacilli. The control band color indicates that all of the test reactions performed satisfactorily and should be positive in every testing. The test was performed according to the method developed by Tomiyama et al. (18). All of the testing was carried out inside a biological safety cabinet in a properly equipped tuberculosis laboratory.

Testing growth from liquid media. All cultures, positive in liquid media, were applied to the ICA test slide directly without any manipulation. Growth in the MGIT was tested within 1 to 3 days of positive signal in the MGIT 960 instrument. At site 1, with 10 cultures, growth in MGIT was also monitored daily to determine the time (in days) that a culture turns positive by the ICA slide test compared to the time to detection by the instrument. At site 4, growth in radiometric BACTEC 12B medium was tested at different growth index (GI) values. The test results and the intensity of the color was recorded after 15 min for both the test and the control bands.

Testing growth from solid media. A few colonies from the surface of the medium were scraped by using a loop (1 μl) and suspended in 0.2 ml of Extraction Buffer (TAUNS, Numazu, Japan). The suspension was vortexed to homogenize it. Approximately 100 μl of the suspension was applied into the sample well of the ICA slide test. The presence or absence of red to pink bands both for control and the test was observed after 15 min. The intensity of the color was also recorded.

Stability of MPB64 in MGIT. The effect of age of MGIT and LJ cultures was studied at two sites (sites 1 and 4) to evaluate stability of MPB64 in the media stored at room temperature without any treatment. Cultures were tested at different intervals up to 1 year. As a control, MOTT cultures were also tested.

Evaluation of the effect of chemotherapy. At site 1, 25 fresh patients with pulmonary tuberculosis were followed up by culturing sputum specimen monthly with BACTEC MGIT 960 to evaluate effect of chemotherapy on the ICA test. During the follow-up, samples determined to be culture positive in the MGIT and the first time culture-negative sample in MGIT medium were tested by ICA slide test. The patients were monitored every 4 weeks for up to 12 weeks.

RESULTS

In all, 304 isolated cultures were tested at the four sites. Among 171 cultures belonging to the *M. tuberculosis* complex, 26 were stock cultures, while 145 were fresh clinical isolates. Among 131 isolates belonging to 18 different species of MOTT bacilli, 43 were from stock cultures and 90 were fresh clinical isolates. All *M. tuberculosis* and *M. africanum* isolates were determined to be positive by ICA slide test, while 3 of 11 *M. bovis* or *M. bovis* BCG were determined to be positive. One mixed culture of *M. tuberculosis* and *M. avium* was also determined to be negative in the initial testing but positive on repeat testing after 3 days (Table 1).

All of these cultures were tested in MGIT medium, whereas only selected cultures were tested in LJ ($n = 63$), 7H10 Middlebrook agar ($n = 9$), and BACTEC 12B ($n = 25$). The ICA slide test was positive in the case of *M. tuberculosis* complex in all of the different media tested (Table 2). The positive band developed within ca. 5 to 10 min, but the intensity of the color was maximum at 15 min. The intensity of a positive test color band varied from a 4+ (dark reddish purple) to 1+ (easily visible pink band). The control band was strongly positive in all of the testing. There was no difference in the outcome of the results when 235 fresh primary isolated cultures were tested compared to the 69 stock cultures. Sequential evaluation of MGIT on a daily basis by ICA slide test demonstrated that the day of conversion from negative to positive by this test was concurrent or sometimes 1 to 2 days earlier than the day when the BACTEC MGIT 960 instrument detected culture positive (Table 3). In the case of the *M. tuberculosis* complex, there was no sample for which the ICA slide test was negative when the BACTEC MGIT 960 identified it as culture positive. In our earlier experiments the CFU count at the time the MGIT medium flagged it as positive was ca. 2×10^5.

Radiometric BACTEC 12B medium was not positive at the threshold of instrument positive (GI = 10 to 50). Even at a

TABLE 1. Results of different species of mycobacteria tested with the MPB64-ICA test

Species	Total no. of isolates tested	Results of MPB64-ICA test (no. of isolates)	
		Positive	Negative
M. tuberculosis	158	158	0
M. bovis	4	2	2
M. bovis BCG	7	1	6
M. africanum	2	2	0
M. avium-M. intracellulare	62	0	62
M. abscessus	4	0	4
M. chelonae	5	0	5
M. celatum	1	0	1
M. flavescens	1	0	1
M. fortuitum	9	0	9
M. gastri	1	0	1
M. gordonae	8	0	8
M. kansasii	15	0	15
M. marinum	6	0	6
M. mucogenicum	1	0	1
M. nonchromogenicum	1	0	1
M. scrofulaceum	1	0	1
M. simiae	2	0	2
M. szulgai	4	0	4
M. terrae	5	0	5
M. xenopi	1	0	1
Unidentified MOTT	6	0	6
Mixed *M. tuberculosis* and *M. avium*[a]	1	1	1

[a] Negative in the initial testing but positive after 3 days.

higher GI value of 500 the ICA test was not always positive. The CFU counts at GI values of 50, 100, and 500 were ca. 4×10^4, 1×10^5, and 5×10^5, respectively. Once a 12B vial with *M. tuberculosis* growth achieved GI of ca. 500 and was incubated for one additional day it become positive for the ICA slide test. At this GI, the CFU count is usually 10^6. Once the test was positive in a BACTEC 12B vial, it gave a positive reaction even if the medium was diluted 1:2 to 1:4, whereas with MGIT medium a positive reaction was observed even at a 1:8 dilution.

It was also observed that MPB64 antigen, once secreted in the medium, is stable, since the test remained positive even if performed 1 year after the detection of growth both in solid and in liquid media. On the other hand *M. kansasii* and other MOTT bacilli remained negative upon prolonged incubation and storage (Table 4).

The data in Table 5 demonstrates that there is no effect of antituberculosis treatment on the test results when sequentially obtained sputum samples were tested up to 4 months after the initiation of chemotherapy. All culture-positive MGIT media were also ICA slide test positive, except for one sample (Table 5). This patient, who was initially culture positive for *M. tuberculosis* and positive for the ICA slide test, became MGIT culture positive but ICA slide test negative. On further evaluation it was found that at this point of therapy the patient's sample did not yield *M. tuberculosis* growth, but actually *M. gordonae* was identified by the DNA-DNA hybridization method. During the chemotherapy none of the first-time culture-negative samples in MGIT medium showed a positive ICA reaction, even though some of them were initially smear positive but remained culture negative.

TABLE 2. Growth on different culture media

Medium	No. of isolates tested (no. positive for MPB64-ICA test)[a]		Total no. of tests
	M. tuberculosis complex	MOTT	
LJ	30 (30)	33 (0)	63
7H10	9 (9)	0	9
BACTEC MGIT 960	171 (171)	133 (0)	304
BACTEC 12B	9 (9)	16 (0)	25

[a] All *M. tuberculosis* cultures were positive, while all MOTT bacilli were negative, by the MPB64-ICA test.

TABLE 3. Daily evaluation of growth in BACTEC MGIT 960 and MPB64-ICA test

Case no.	AFB smear result[a]	Day of positivity[b] by:	
		BACTEC 960	MPB64-ICA
1	Positive	10	10
2	Positive	12	11
3	Positive	6	6
4	Positive	9	8
5	Positive	12	11
6	Positive	13	13
7	Positive	4	3
8	Negative	18	17
9	Positive	8	7
10	Positive	12	12

[a] Smears were prepared with decontaminated and concentrated sputum specimens by the NALC-NaOH method.

[b] That is, the number of days required to detect first positivity by the BACTEC MGIT 960 instrument or the MPB64-ICA test.

DISCUSSION

We demonstrate here the usefulness of the ICA slide test for routine laboratory testing for differentiation of the *M. tuberculosis* complex from MOTT bacilli. In addition, this test is extremely simple and does not require any sample preparation or instrumentation. It is very rapid and can be performed using growth in both liquid and solid media.

Among the different media tested we concentrated on the BACTEC MGIT 960 system, as this is the most recent nonradiometric method, the performance of which is comparable to the radiometric BACTEC method (3, 13). All of the *M. tuberculosis* cultures once determined to be positive by the BACTEC MGIT 960 instrument were also determined to be positive by the ICA slide test, and the test results were available within 15 min, which makes the ICA slide test more rapid and attractive than any other available technique.

The production of MPB64 antigen in 12B medium is rather slow and could not always be detected even at a GI of 500. However, when the 12B vial is incubated one more day there is enough MPB64 antigen produced and the ICA slide test is positive. The delay in the production of MPB64 antigen in 12B medium could be due to the fact that this medium is not very rich and so mycobacteria may not be able to produce this antigen at an early stage of growth. On the solid media LJ and 7H10 the ICA slide test is positive once there is visible growth on the surface of medium. The positive color bands on the ICA slide were easily visible with a dark red-purple to a light pink color. There was no color reaction observed in the case of

TABLE 4. Effect of age of culture on the MPB64-ICA test

Time after positive growth	MPB64-ICA test result[a] (no. of cultures tested)				
	M. tuberculosis		*M. kansasii*		Other MOTT bacilli (MGIT)
	MGIT[b]	LJ	MGIT	LJ	
5 days	Pos (1)	Pos (1)	Neg (1)	Neg (1)	ND
10 days	Pos (1)	Pos (1)	Neg (1)	Neg (1)	ND
15 days	Pos (1)	Pos (1)	Neg (1)	Neg (1)	ND
1 mo	Pos (1)	Pos (1)	Neg (1)	Neg (1)	ND
1 yr	Pos (20)	Pos (20)	Neg (2)	ND	Neg (20)

[a] Pos, positive; Neg, negative; ND, no data.
[b] Tested in duplicate with or without PANTA.

MOTT bacilli cultures. This test, in terms of its sensitivity and specificity, compares very well with the probe-based tests since in our analyses all *M. tuberculosis* cultures and *M. africanum* cultures were positive (100%). Among *M. bovis* strains, two of four tested strains were determined to be negative by this test. The isolation of *M. bovis* is rare from human samples. Occasionally, the BCG strain is isolated from patients who receive BCG immunotherapy. All of the four *M. bovis* strains were from stock cultures frozen for some time. Many laboratories do not differentiate *M. bovis* from *M. bovis* BCG. It is possible that the two of the four strains that tested negative are actually BCG strains and were not further identified. Further study of these *M. bovis* isolates is needed in order to establish whether these were indeed non-BCG *M. bovis* strains. It has been reported that some BCG strains, such as BCG Glaxo, do not produce MPB64 antigen whereas others, such as BCG Japan, are good secretors of the antigen (2, 8). In our analyses, one of seven *M. bovis* BCG isolates was determined to be positive. We were not able to obtain the history of these BCG strains. On the other hand, we did not observe any cross-reaction of this antigen with MOTT bacilli. This indicates excellent specificity of the test. Abe et al. in their earlier studies reported a very high specificity for this test with only one *M. marinum* strain (one of three) and one *M. flavescens* strain (one of one) giving a very weak reaction (2). In our study six strains of *M. marinum* and one strain of *M. flavescens* were tested with no positive reaction. Besides confirming the high sensitivity and specificity of the test that Abe et al. had reported, our multicenter study evaluated some other parameters of the test.

Although we did not perform an in-depth study on the sta-

TABLE 5. Sequential evaluation of sputum cultures prior to and after antituberculosis treatment[a]

Case no.	Result prior to treatment			Result 4 wk after treatment			Result 8 wk after treatment			Result 12 wk after treatment		
	Day of MGIT positive	AccuProbe	MPB64-ICA test	Day of MGIT positive	AccuProbe	MPB64-ICA test	Day of MGIT positive	AccuProbe	MPB64-ICA test	Day of MGIT positive	AccuProbe	MPB64-ICA test
1	8	+	+	15	+	+	CN	ND	−			
2	7	+	+	CN[c]	ND[d]	−						
3	20	+	+	CN	ND	−						
4	8	+	+	19	+	+	CN	ND	−			
5	5	+	+	21	+	+	32	+	+	42	+	+
6	14	+	+	28	+	+	CN	ND	−			
7	8	+	+	23	+	+	CN	ND	−			
8	5	+	+	16	+	+	CN	ND	−			
9	6	+	+	15	+	+	30	+	+	CN	ND	−
10	5	+	+	10	+	+	21	+	+	CN	ND	−
11	8	+	+	22	+	+	14	+	+	56	+	+
12	6	+	+	20	+	+	19	+	+	CN	ND	−
13	7	+	+	24	+	+	CN	ND	−			
14	5	+	+	28	+	+	36	+	+	CN	ND	−
15	8	+	+	21	+	+	27	+	+	37	+	+
16	18	+	+	CN	ND	−						
17	7	+	+	12	+	+	40	+	+	CN	ND	−
18	8	+	+	22	+	+	30	+	+	8[b]	−	−
19	16	+	+	29	+	+	CN	ND	−			
20	9	+	+	35	+	+	CN	ND	−			
21	5	+	+	15	+	+	28	+	+	CN	ND	−
22	6	+	+	CN	ND	−						
23	8	+	+	37	+	+	CN	ND	−			
24	11	+	+	18	+	+	CN	ND	−			
25	10	+	+	24	+	+	18	+	+	CN	ND	−

[a] "Day of MGIT positive" means the number of days required to detect first positivity by the BACTEC MGIT 960 instrument.
[b] Only *M. gordonae* was isolated.
[c] CN, culture negative.
[d] ND, no data.

bility of MPB64 antigen in the medium, we did look at the effect of age of growth in a culture medium on the performance of the test. It is quite clear from the findings that the antigen is stable in both liquid and solid media. On the other hand, MOTT bacilli did not produce detectable antigen with an extended incubation or when growth in a medium was left for a long period. We also observed that a positive reaction band on the slide was very stable and could be stored for months without any loss of color intensity.

We also evaluated the effect of chemotherapy on this test. The results indicate that as long as the culture is positive for *M. tuberculosis* from the sputum of a patient on chemotherapy, the test would be positive, indicating that the antituberculosis treatment has no effect on the production of this antigen. Once sputum becomes culture negative, the test also becomes negative even if the smear made from the specimen shows AFB, indicating that tubercle bacilli, if present in the specimen, are not viable.

In our experience there have been some instances in which a tuberculosis patient, after beoming culture negative can become culture positive again, though at this time not for *M. tuberculosis* but for MOTT bacilli (usually *M. avium* or *M. gordonae*). This could represent environmental contamination, colonization, or secondary infection. In such situations the ICA slide test offers a very simple and quick way to monitor the presence or persistence of *M. tuberculosis* in follow-up specimen cultures of a patient receiving antituberculosis treatment.

Molecular methods can rapidly identify mycobacteria within a few hours with great accuracy, but these methods, compared to the ICA slide test, are complex and cumbersome, require instrumentation, and are labor-intensive. We found that the ICA slide test was extremely simple, requiring very little tech time, and that the results are ready within 15 min.

One of the disadvantages of a culture system with liquid medium is the high potential for contamination or for the coexistence of *M. tuberculosis* and MOTT bacilli, which may go undetected or may lead to erroneous reporting of results. Even though the ICA slide test could be an excellent identification tool and could serve as an alternative to molecular-analysis-based approaches, the positive result of this test does not necessarily rule out the coexistence of *M. tuberculosis* and MOTT bacilli. We experienced one case in the present study supporting this concern. In this case a PCR test with sputum sample for primary cultures detected *M. tuberculosis*; therefore, PCR for *M. avium-M. intracellulare* was not performed. Initially, BACTEC MGIT 960 gave a positive result and the ICA slide test was negative, indicating the absence of *M. tuberculosis*. Since this MGIT tube turned positive within a short time, the test was repeated after 3 days of further incubation, and the ICA test was then clearly positive. Both *M. tuberculosis* and *M. avium* were present in the culture, and the additional incubation allowed *M. tuberculosis* to produce sufficient antigen detectable by this test. This is an inherent problem of molecular tests as well. However, in a probe test, such as GenProbe, there is a provision of testing for *M. avium* and a few other species if there is any suspicion of having MOTT in the culture. Development of a similar kind of lateral flow immunochromatography test for *M. avium* and a few commonly isolated mycobacteria would be desirable.

In conclusion, this immunochromatography test with monoclonal antibody against MPB64 is a low-tech and rapid method for the differentiation of *M. tuberculosis* from other mycobacteria. Considering its accuracy as well as its simplicity, the method should offer significant savings of both labor and time and could be a good alternative to probe-based tests, especially in countries with limited resources.

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