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Validation of an immunochromatographic assay kit for the identification of the *Mycobacterium tuberculosis* complex

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The performance of the immunochromatographic assay, SD BIOLINE TB Ag MPT64 RAPID[®], was evaluated in Madagascar. Using mouse anti-MPT64 monoclonal antibodies for rapid discrimination between the Mycobacterium tuberculosis complex and nontuberculous mycobacteria, the kit was tested on mycobacteria and other pathogens using conventional methods as the gold standard. The results presented here indicate that this kit has excellent sensitivity (100%) and specificity (100%) compared to standard biochemical detection and can be easily used for the rapid identification of M. tuberculosis complex.

Key words: *Mycobacterium tuberculosis* - Ag MPT64 - Madagascar

Tuberculosis (TB) is a bacterial disease caused by organisms of the *Mycobacterium tuberculosis* complex (MTC). The definitive diagnosis of TB depends on the isolation and identification of the etiologic agent. Clinical identification of mycobacteria remains difficult and time-consuming. Cultures in specific media can result not only in *M. tuberculosis* (MTB) growth but also growth of nontuberculous mycobacteria (NTM). Therefore, the analysis of cultured colonies should be able to discriminate between MTB and NTM to confirm MTB specifically. Commercial diagnostic methods employ molecular biological tests to provide quick and specific tests for the identification of MTC, but false positive results cannot be excluded and these tests remain costly in terms of specialised equipment, labour and time (Wang 2006).

MPT64, a 24 kDa secretory protein, is one of the major antigens from TB bacteria. Recently, MPT64 has been shown to differentiate the MTC from other bacterial species (Tomiya et al. 1997, Abe et al. 1999, Hasegawa et al. 2002). Standard Diagnostics, Inc (SD) (Yongin, Korea) developed the SD BIOLINE TB Ag MPT64 RAPID[®] test, which is a simple and rapid assay using a mouse monoclonal anti-MPT64 antibody that is able to discriminate between MTC and NTM by immunochromatography. The purpose of this study was to evaluate the utility of the SD BIOLINE TB Ag MPT64 RAPID[®] test for the routine identification of mycobacteria in Madagascar.

A total of 171 MTB and NTM strains were used for this study (Table I). Eleven samples of the H37Rv strain were used as standard controls. Clinical strains of MTB

(88) and NTM (5) were isolated from clinical specimens and cultured on the Löwenstein Jensen (LJ) medium following decontamination and liquefaction procedures (Tison & Carbonelle 1972, Kent & Kubica 1985). Samples were inoculated in LJ medium and incubated at 37°C for growth for several weeks. For *Mycobacterium bovis* (15 isolates) and NTM (14 isolates), strains from the mycobacteria unit collection were inoculated in 8 mL of Dubos medium and incubated at 37°C for one week (for NTM) or three weeks (for *M. bovis*). Two hundred microlitres of each culture was inoculated in LJ medium and incubated at 37°C. *M. bovis* strains (15) and NTM strains (3 of *Mycobacterium chelonae*, 2 of *Mycobacterium avium*, 2 of *Mycobacterium intracellulare*, 2 of *Mycobacterium flavescens*, 1 of *Mycobacterium peregrinum*, 1 of *Mycobacterium vaccae*, 1 of *Mycobacterium aurum*, 1 of *Mycobacterium xenopi* and 1 of *Mycobacterium mucogenicum*), after cultivation in Dubos media, were applied to the immunochromatography test slide directly without any manipulation.

Mycobacteria strains were identified by their growth rate, colonial morphology, pigmentation, testing for urease, semi-quantitative catalase, heat-stable catalase, nitrate reduction and Tween 80 hydrolysis (David et al. 1989). In this study, 10 strains of *M. bovis* BCG (Pasteur) were used as a negative control for the MPT64 antigen as reported by Li et al. (1993). Other microorganism isolates (bacteria and fungi) used in this study were collected and characterised by the Clinical Biology Laboratory of the Institut Pasteur de Madagascar. Colonies were cultured on sheep's blood agar plates at 37°C for 48 h followed by incubation at room temperature for 24 h. The isolated microorganisms were identified by their biochemical characteristics using a standardised system (API[®], bioMérieux SA) combining conventional tests, assimilation tests and a database for the identification of microorganisms.

The SD BIOLINE TB Ag MPT64 RAPID[®] kit was used according to the manufacturer's protocol. The use of this kit was based on the detection by chromatographic diffusion of a specific MPT64 antigen of MTC.

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TABLE I
Different bacterial species tested with the SD BIOLINE TB Ag MPT64 RAPID®

| Species | Isolates (n) | Species | Isolates (n) |
|---|--------------|----------------------------------|--------------|
| <i>Mycobacterium tuberculosis</i> complex | | Bacteria and fungi | |
| <i>Mycobacterium tuberculosis</i> | 99 | <i>Proteus mirabilis</i> | 3 |
| <i>Mycobacterium bovis</i> | 15 | <i>Pseudomonas aeruginosa</i> | 4 |
| <i>M. bovis</i> BCG (Pasteur) | 10 | <i>Enterobacter cloacae</i> | 2 |
| Nontuberculosis mycobacteria | | <i>Escherichia coli</i> | 2 |
| <i>Mycobacterium chelonae</i> | 3 | <i>Streptococcus pneumoniae</i> | 3 |
| <i>Mycobacterium avium</i> | 2 | <i>Streptococcus pyogenes</i> | 2 |
| <i>Mycobacterium intracellulare</i> | 2 | <i>Staphylococcus aureus</i> | 4 |
| <i>Mycobacterium flavescens</i> | 2 | <i>Haemophilus influenzae</i> | 2 |
| <i>Mycobacterium peregrinum</i> | 1 | <i>Branchnamella catarrhalis</i> | 2 |
| <i>Mycobacterium vaccae</i> | 1 | <i>Candida albicans</i> | 2 |
| <i>Mycobacterium aurum</i> | 1 | <i>Klebsiella pneumoniae</i> | 2 |
| <i>Mycobacterium mucogenicum</i> | 1 | | |
| <i>Mycobacterium xenopi</i> | 1 | | |
| Nontuberculous mycobacteria unidentified | 5 | | |
| Total | 143 | Total | 28 |

Mouse monoclonal anti-MPT64 antibodies were immobilised on a nitrocellulose membrane as the capture material. Another antibody, which recognises a different epitope of MPT64 and has been conjugated with colloidal gold particles, was used for antigen capture and detection in a sandwich-type assay.

One hundred microlitres of a sample obtained from liquid culture was applied directly to the sample well without using the sample preparation procedure. Three or four colonies were scraped from the solid medium and suspended in 200 µL of the extraction buffer (SD); next, 100 µL of the suspension was added to the sample well. In case of condensation fluid in the egg-based medium, 100 µL of the fluid was applied directly to the sample well instead of being used as an extraction buffer. Tests were interpreted 15 min after sample application. The presence of a control band alone indicates a negative result, whereas the presence of two colour bands indicates a positive result.

The SD BIOLINE TB Ag MPT64 RAPID® assay was used to test 171 strains (143 mycobacteria and 28 other microorganisms) and was compared with conventional culture identification methods as a gold standard. The control band was strongly positive for all tests. The positive bands developed within 5-10 min. The sensitivity of the assay was calculated based on test results from MTB and *M. bovis* isolates. Strong positive bands were obtained for all MTB (n = 99) and *M. bovis* (n = 15) strains regardless of the culture medium, while no positive signal was observed for any NTM bacilli, BCG strains or other microorganisms tested (Tables II, III), which indicates a test sensitivity and specificity of 100%.

This study evaluated the performance of the SD BIOLINE TB Ag MPT64 RAPID® test for the identification of MTB from positive culture samples. The results presented above reveal that SD BIOLINE TB Ag MPT64 RAPID® identification had 100% sensitivity and specificity for the strains, compared with conventional methods, demonstrating excellent agreement. These results are similar to those observed by other studies (Abe et al. 1999, Fabre et al. 2010, Gaillard et al. 2011, Marzouk et al. 2011). Likewise, in an earlier multicenter study, Hasegawa et al. (2002) reported a notably high specificity for MPB64 using the ICA slide test kit. Park et al. (2009) also reported excellent sensitivity (99%) and specificity (100%) of the test along with an appropriate detection limit (10⁵ CFU/mL). In 2009, Ismail et al. (2009) used this test to demonstrate sensitivity, specificity and positive and negative predictive values of 97%, 100%, 100% and 92%, respectively. Several cases of MTB have been reported with negative test results. A possible explanation for the false negative results is that the strain had mutations within the *mpt64* gene, which may have led to the production of an incomplete protein (Hirano et al. 2004). In this case, conventional techniques for the diagnosis of MTB should be recommended if clinical symptoms and microbiological criteria are suggestive for TB. No positive signal was observed for any NTM bacilli or *M. bovis* BCG in this study, which agrees with most previous studies (Hasegawa et al. 2002, Park et al. 2009, Fabre et al. 2010, Gaillard et al. 2011). In terms of the specificity, a few false positives were reported with some strains of *Mycobacterium marinum* and *M. flavescens* (Abe et al. 1999), though Park and Lee (2003) and Gaillard et al.

TABLE II
Sensitivity of SD BIOLINE TB Ag MPT64 RAPID® test

| Test method | Culture method | | |
|-------------------------------|--|-----------------------------------|----|
| | <i>Mycobacterium tuberculosis</i> (n) | <i>Mycobacterium bovis</i> (n) | |
| SD BIOLINE TB Ag MPT64 RAPID® | Positive | 99 | 15 |
| | Negative | 0 | 0 |
| | Total | 99 | 15 |
| Sensitivity (%) | | 100 (114/114) | |

TABLE III
Specificity of SD BIOLINE TB Ag MPT64 RAPID® test

| Test method | Bacterial strains | | | |
|-------------------------------|--------------------------|-----------------------|------------------------------------|--------------|
| | Bacterial strains (n) | Fungal strains (n) | Nontuberculous mycobacteria (n) | Total (n) |
| SD BIOLINE TB Ag MPT64 RAPID® | Positive | 0 | 0 | 0 |
| | Negative | 26 | 2 | 19 |
| | Total | 26 | 2 | 19 |
| Specificity (%) | | 100 (47/47) | | |

(2011) did not report any false positives for these species when evaluating the SD MPT64. These two species may be easily differentiated from the *MTB complex* by the following properties: *M. marinum* has photochromogenic and smooth-type colonies on solid media and is isolated from skin lesions in most cases; *M. flavescens* has pigmented, smooth-type colonies; both species grow rapidly (Tison & Carbonelle 1972, Wayne & Kubica 1986, David et al. 1989).

In this study, all strains of *M. bovis* BCG were observed to be nonreactive by the SD MPT64 test. It has been reported that some BCG strains used in this study, e.g., BCG Glaxo or BCG Pasteur, do not produce the MPT64 antigen, whereas others, such as BCG Japan, are robust secretors of the antigen (Abe et al. 1999). No indeterminate results were found in this study and there were no discrepancies in the interpretation of the results. However, a negative result does not always exclude the possibility of isolating a *Mycobacterium* belonging to the *MTB complex*, especially if there is a mutation in the *mpt64* gene. Other techniques must be used if an equivocal result (e.g., genotype) is obtained. Moreover, the test is not able to distinguish between species of the *MTC*. The test is only applicable for culture specimens, requiring the manipulation of an important bacterial inoculum and thus requires a level 3 biosafety laboratory with ap-

propriate equipment to ensure personnel safety. To our knowledge, this study is the first to be carried out in a national reference centre of mycobacteria in a country with endemic TB. The SD BIOLINE TB Ag MPT64 RAPID® kit is affordable for low-income countries at a cost of \$25 (USD) per box of 25 tests or \$1 per specimen.

In conclusion, the data presented in this study have demonstrated the value of this assay for the clinical identification of mycobacteria. However, when negative test results are obtained despite clinical signs for *MTB* or *BCG*, other identification techniques should be used. No cross-reactivity was observed with the most frequently isolated *NTM* in Madagascar.

This test is extremely simple and does not require any sample preparation or instrumentation. It is rapid and can be performed by growth in either liquid or solid media. These features make the kit suit for any laboratory performing culture and could replace biochemical tests for the identification of the *MTC*.

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