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Evaluation of an immunochromatographic test for discrimination between *Mycobacterium tuberculosis* complex & non tuberculous mycobacteria in clinical isolates from extra-pulmonary tuberculosis

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***Background & objectives*: Accurate diagnosis of tuberculosis (TB) is crucial to facilitate early treatment of the patients, and to reduce its spread. Clinical presentation of *Mycobacterium tuberculosis* complex (MTBC) and non tuberculous mycobacteria (NTM) may or may not be the same, but the treatment regimen is always different for both the infections. Differentiation between MTBC and NTM by routine laboratory methods is time consuming and cumbersome. This study was aimed to evaluate an immunochromatographic test (ICT), based on mouse monoclonal anti-MPT64, for simple and rapid discrimination between MTBC and NTM in clinical isolates from extra-pulmonary tuberculosis cases.**

***Methods*: A total of 800 clinical samples were collected from patients suspected to have extra-pulmonary tuberculosis. Preliminary diagnosis has been done by direct Ziehl–Neelsen (ZN) staining followed by culture in BACTEC system. A total of 150 clinical isolates, which were found positive in BD 460 TB system during September 2009 to September 2010 were selected for the screening by ICT test. p-nitro-α-acetylamino- β-hydroxy propiophenone (NAP) test was performed for differentiation of MTBC and NTM. *M. tuberculosis* complex was further confirmed by IS6110 PCR of BACTEC culture positive isolates, this served as the reference method for MTBC identification and comparative evaluation of the ICT kit.**

***Results*: Of the 150 BACTEC culture positive isolates tested by ICT kit, 101 (67.3%) were found positive for MTBC and remaining 49 (32.7%) were considered as NTM. These results were further confirmed by IS6110 PCR that served as the reference method for detection of MTBC. $H_{37}Rv$ reference strain was taken as a control for ICT test and IS6110 PCR. The reference strain showed the presence of MPT64 antigen band in the ICT test. Similar bands were formed in 101 of 102 MTBC isolates tested, proving 99.1 per cent sensitivity and no bands were detected in 48 (100%) NTM isolates tested, proving 100 per cent specificity of the ICT kit.**

***Interpretation & conclusions*: Our findings show that ICT test can be used on direct culture positive specimens. It does not require any special equipment, is simple and less time consuming. It can easily discriminate between MTBC and NTM and thus can help in appropriate management of tuberculosis.**

Key words Immunochromatographic test - *Mycobacterium tuberculosis* complex - non tuberculous mycobacteria - tuberculosis

Tuberculosis (TB) is one of the major causes of morbidity and mortality worldwide. In India, about 1.8 million new cases of TB are reported annually, that account for a fifth of new cases in the world - a greater number than in any other country[1]. The situation is further worsened by the increasing number of drug-resistant cases of TB. Thus, there is a need for rapid and correct identification of mycobacteria and rapid drug sensitivity testing for effective treatment of the disease. Strategies used for the clinical management of patients with *Mycobacterium tuberculosis* complex (MTBC) and non tuberculous mycobacteria (NTM) are different, therefore, prompt detection, isolation, and discrimination is necessary for suitable management[2,3]. Although conventional biochemical methods are able to identify mycobacterial species; but these are tedious, time-consuming and require elaborate safety precautions. The recent objective of World Health Organization (WHO) is to reduce the time for culture, identification, and drug resistance detection to as short as two days by employing Line probe assays[4]. Although molecular methods of identification are very accurate and reliable, but require a specialized set up, sophisticated and expensive equipment, trained laboratory personnel and are expensive for resource-poor countries[5]. In recent years, major advances in the understanding of the genetic structure of mycobacteria have been achieved. Various genetic probes and amplification systems for diagnosis of TB have been developed, and several of these are available as commercial kits for direct detection and identification of *M. tuberculosis* in clinical specimens[6,7]. Early and reliable identification of mycobacteria may spare patients from unnecessary treatments in cases of NTM[8]. Studies have revealed that the *M. tuberculosis* protein 64 (MPT 64) is specific for MTBC, including *M. tuberculosis*, *M. africanum*, *M. bovis,* and some, although not all, substrains of *M. bovis* BCG[5,9-12]. The MPT 64 is a *M. tuberculosis* complex specific antigen secreted during the bacterial growth, and is an excellent antigen for the identification of MTBC[9,12]. This study was carried out to evaluate clinical usefulness of immunochromatographic test (ICT) kit based on mouse monoclonal anti-MPT 64 for simple discrimination between *M. tuberculosis* complex and non tuberculous mycobacteria in clinical isolates from patients with extra-pulmonary tuberculosis.

Material & Methods

This prospective study was undertaken at Mycobacteriology Laboratory, Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India. Specimens were collected from Kasturaba Chest Hospital, Department of Pulmonary Medicine, Chhatrapti Shahuji Maharaj Medical University, Lucknow, India, and from various wards of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India. A total 800 clinical specimens (2-10 ml) as lymph node aspirate and cold abscesses, pleural fluid, cerebrospinal fluid (CSF), synovial fluid, ascitic fluid, urine, gastric aspirate, pus, bone marrow aspirates, wound swabs and biopsy materials were collected from suspected cases of extra-pulmonary tuberculosis during September 2009 to September 2010. The study was approved by the institutional ethics committee of Chhatrapati Shahuji Maharaj Medical University, Lucknow, India. The age of patients varied from 12-65 yr and included both male and female population in urban and rural settlements. Written informed consent was obtained from the subjects before enrollment into the study.

Microbiological analysis for M. tuberculosis: For decontamination of specimens from normal bacterial flora, specimens were treated by N-acetyl- L-cysteine-NaOH Method[13]. Smears were stained with Ziehl-Neelsen (ZN) for detection of acid-fast bacilli (AFB). For the BACTEC method (Becton Dickinson, Cockeysville, MD, USA), 0.5 ml of processed specimen was inoculated into the BACTEC 12B vial supplemented with PANTA (a mixture of five different antibiotics: polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) and incubated at 37°C. Readings were taken twice a week for the first two wk and once a wk thereafter for culture positivity for 8 wk. After 8 wk if the growth index (GI) was zero, the specimens were considered as negative. AFB smears were made from vials with a GI of 50-100, and further identification of MTBC was done by the BACTEC NAP (p-nitro-α-acetylamino-β-hydroxy propiophenone) differentiation test (Becton Dickinson, Sparks, MD, USA)[14].

Molecular confirmation of M. tuberculosis complex by IS6110 PCR: The final confirmation of MTBC was done by IS6110 PCR of BACTEC culture positive isolates. Extraction of DNA was done by the cetyl-tri-methyl-ammonium bromide (CTAB)-phenol chloroform extraction method[15]. Identification of MTBC was done by using a specific pair of primers designed to amplify an insertion sequence IS6110 (~123-bp). The sequence of these FP1 and RP2 primers were: 5'-CCT GCG AGC GTA GGC GTC GG3' and 5' CTC GTC CAG

CGC CGC TTC GG 3', respectively[16]. PCR reaction mixture (20 µl) contained 2× Pyrostart Fast PCR Master Mix (Fermatas, India), 10 pmole of each primer (SBS Gentech Co. Ltd., India) and 5 µl of extracted DNA. Amplification was carried out in a thermal cycler (MJ Research ,PTC-100 Thermal Cycler , GMI, Inc , USA), which involved 40 cycles of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min, and primer extension at 72°C for 1 min. The amplified products were separated on 2 per cent agarose gels, visualized on a UV- light transilluminator (Bangalore Genei, Bangalore, India). The presence of 123bp fragment indicated a positive test for *M. tuberculosis* complex. Positive control included the DNA of $H_{37}Rv$ reference strain and negative control included PCR grade water (Fig. 1).

ICT tuberculosis test: A volume of 100 µl of broth from BACTEC culture was added to the well of SD TB Ag MPT 64 Rapid ICT kit cassette (Standard Diagnostics Ltd., Korea). Inoculated ICT cassettes were kept at room temperature for 20 min and were examined for the presence of control and test bands. Appearance of band in the 'C' region confirmed the validity of test. Additional appearance of Band in the 'T' region was interpreted as positive for MPT 64 antigen (Fig. 2). No band in the 'C' region was interpreted as invalid test. Standard reference strain $H_{37}Rv$ was used as positive control.

Statistical analysis: Data were analyzed using SPSS 15.0 (Statistical Package for the Social Sciences, Chicago, IL, USA) for Windows. Validity of ICT test

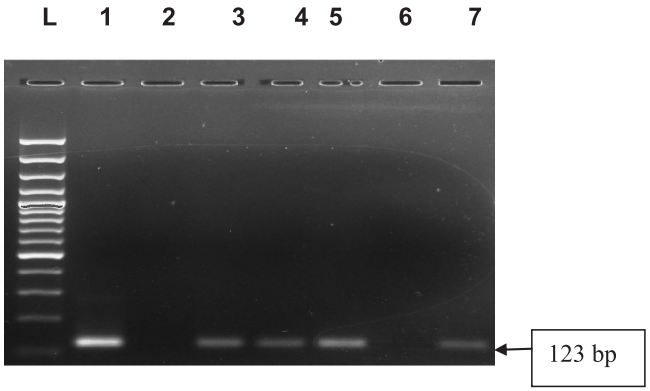

Fig. 1. IS6110PCR-based detection of *M. tuberculosis* complex. Electrophoretic separation of the amplicon into 2% agarose gel is documented across lanes 1-7. The presence of a 123 bp amplicon in lanes 3, 4, 5, 7 indicated the presence of the target while the absence of the amplicon in lane 6 showed absence of the target. Lane 2 was negative control and lane 1 was positive control ($H_{37}Rv$). L-Ladder 100 bp was well shown.

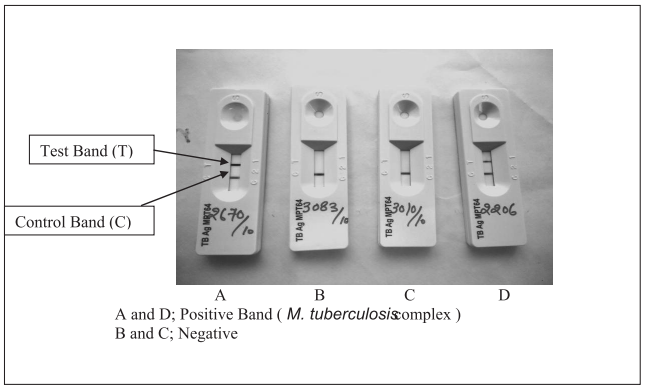

Fig. 2. Identification *M. tuberculosis* complex by MPT64 Rapid ICT kit.

was calculated by sensitivity and specificity. Sensitivity was calculated as [Tp/(Tp +Fn)] × 100; specificity was calculated as [Tn/(Tn + Fp)] × 100; Tp =total number of positives; Tn =total number of negatives; Fp= total number of false positive , Fn = total number of false negative, respectively.

Results

Of the 800 samples tested, 150 (18.7%) were found to be positive by BACTEC culture method. Of these 150 BACTEC culture positives, 20 (13.2%) were found smear positive for AFB. All BACTEC culture positive cases were examined by NAP test for differentiation between MTBC and NTM, and 102 (68%) were positive for MTBC by NAP test, remaining 48 (32%) were considered as NTM. Final confirmation of MTBC in BACTEC positive isolates was done by IS6110 PCR. All the 102 NAP positive isolates were found positive as MTBC and remaining 48 were negative by IS6110 PCR (Fig. 3). ICT test was performed on 150 BACTEC positive isolates on 1st and 5th day of BACTEC culture positive vials. In the ICT test, the 5th day BACTEC culture positive vials were able to give visibly more intense band as compared to the 1st day BACTEC culture positive vials that could be attributed to low antigen concentration on initial day of inoculation. Control band in the 'C' region of ICT test cassette was seen in all the 150 samples tested (Fig. 2) and no test was reported as invalid. $H_{37}Rv$ control strain showed the appearance of band in the test region ('T') confirming the ability of ICT test to detect the MPT64 antigen. A total of 101 of 150 (67.3%) isolates tested showed dark band in the test region ('T') confirming the presence of MPT 64 antigen and thus presence of MTBC. One sample which was found positive for MTBC by NAP test but was negative by

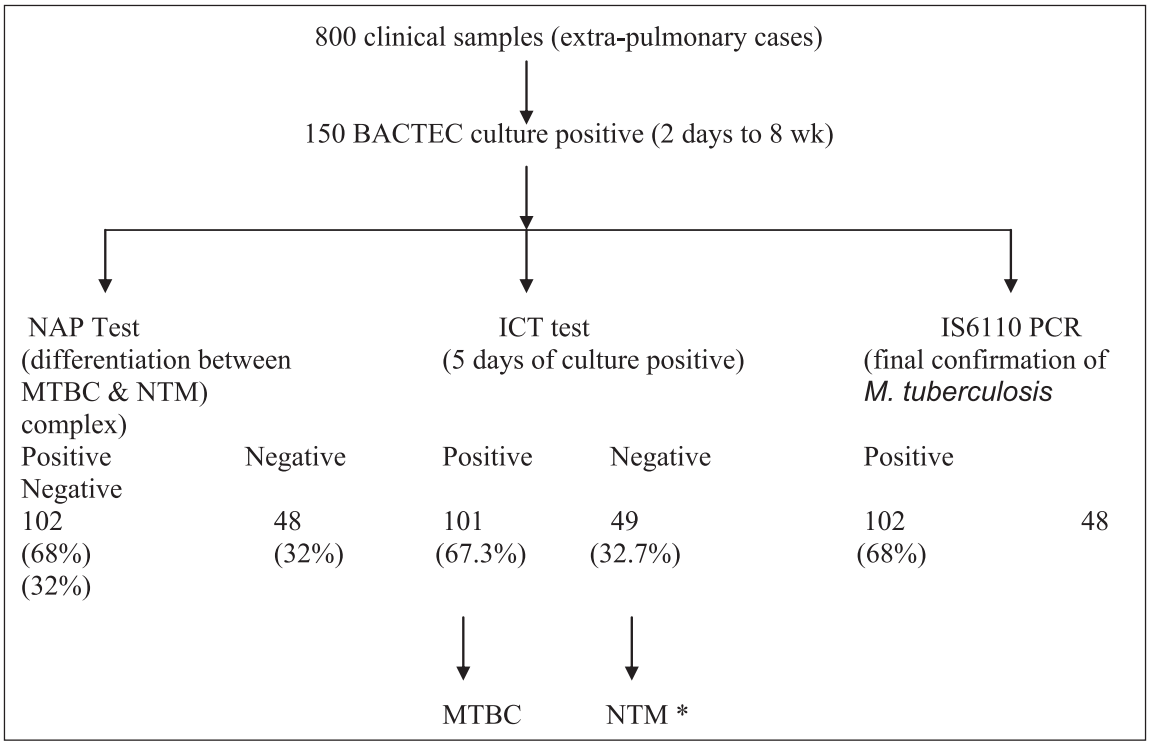

Fig. 3. Comparison of methods for detection of *M. tuberculosis* complex in extra-pulmonary tuberculosis cases. *NAP test and IS6110 PCR negative isolates were included in non tuberculosis mycobacteria. MTBC, *Mycobacterium tuberculosis* complex; NTM, non tuberculous mycobacteria.

the ICT might be related to the low-level expression of the antigen. The remaining 49 (32.7%) isolates which were identified as NTM by NAP test, showed no band formation, indicating the absence of MPT64 antigen in these isolates. The sensitivity and specificity of the ICT kit was found to be 99.1 and 100 per cent respectively (Table).

Discussion

M. tuberculosis poses diagnostic and therapeutic problems due to low sensitivity of the diagnostic tools available for its identification and discrimination with NTM. Conventional culture is time consuming and lacks sensitivity; smear for AFB is rapid but the sensitivity has not been yet evaluated and does not differentiate between MTBC and NTM[17]. The diagnostic delay can affect treatment[18]. Rapid identification of mycobacteria is important and a simple, sensitive, and specific identification method is required. Conventional methods like direct staining of a colony is simple and fast but does not discriminate between *M. tuberculosis* and NTM, whereas traditional biochemical tests take a long time. Newer techniques like chemiluminescent DNA probes, nucleic acid amplification, high-performance liquid chromatography and sequencing of 16S rRNA genes are more sophisticated methods but are not cost-effective and require expensive equipment[19-21]. Attorri *et al*[22] showed that *M. tuberculosis* often exhibits serpentine cording when grown in liquid medium, NTM can form true cords in liquid culture but do so rarely, despite the fact that many species contain the cell wall glycolipid that mediates cord formation. Previous studies[23-25] utilized cord formation for the cost-effective employment of DNA probes for the identification of *Mycobacterium* species. Chihota *et al*[5] reported the cost of organism identification per positive culture on BACTEC as US$35.94 using standard biochemical tests, US$15.49 for anti-MPB64 assay and US$2.28 for cording. Cost-effective analytical studies[26] of SD MPT64 TB Ag ICT test, other rapid molecular methods and culture combined with conventional biochemical tests have shown SD MPT 64 TB Ag ICT as more economical than the other two methods. Further, ICT requires only 20 min of analysis as compared to five days for NAP test. Ismail *et al*[27] reported sensitivity, specificity, positive and negative predictive values of the SD AgMPT64 kit to be 97, 100, 100 and 92 per cent, respectively. Using the same kit, Chihota *et al*[5] reported sensitivity and specificity of 100 per cent in a total of 108 broth cultures. In the present study, the

Table. Sensitivity and specificity of MPT64 test results as compared to IS6110 PCR

Samples	No. of test	MPT64 test positive	MPT64 test negative	IS6110 PCR test positive	IS6110 PCR test negative
H_{37}Rv reference strain (Control)	1	1 (100)	0	1 (100)	0
M. tuberculosis complex	102	101 (99.1)	1 (0.9)	102 (100)	0 (100)
Non tuberculosis mycobacteria	48	0 (100)	48 (100)	0 (100)	48 (100)

Values in parentheses are percentages

sensitivity and specificity of ICT kit were 99.1 and 100 per cent, respectively. MTBC isolates showed same band similar to H_{37}Rv control strain.

In conclusion, ICT test can be used on direct culture positive specimens, it does not require any special equipment. It can discriminate between MTBC and NTM. The low cost, simplicity, rapidity, high sensitivity and high specificity for the MPT 64 antigen detection make the ICT as a useful diagnostic tool for diffentiation between MTBC and NTM diagnosis and can help in appropriate management of tuberculosis.

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***Conflict of interest*:** The authors declare that they have no conflict of interest.

References

1. *RNTCP. Status TB Report, 2010*. New Delhi: Central TB Division. Directorate General of Health Services, Ministry of Health and Family Welfare. Available from: *www.tbcindia.org*, accessed on February 7, 2011.
2. Hasegawa N, Miura T, Ishii K, Yamaguchi K, Lindner TH, Merritt S, *et al*. New simple and rapid test for culture confirmation of *Mycobacterium tuberculosis* complex: a multicenter study. *J Clin Microbiol* 2002; *40* : 908-12.
3. Park MY, Kim YJ, Hwang SH, Kim HH, Lee EY, Jeong SH, *et al*. Evaluation of an immunochromatographic assay kit for rapid identification of *Mycobacterium tuberculosis* complex in clinical isolates. *J Clin Microbiol* 2009; *47* : 481-4.
4. World Health Organization. Use of liquid TB culture and drug susceptibility testing (DST) in low and medium income settings. Summary report of the expert group meeting on the use of liquid culture media. Geneva, March 26, 2007.
5. Chihota VN, Grant AD, Fielding K, Ndibongo B, van Zyl A, Muirhead D, *et al*. Liquid vs. solid culture for tuberculosis: performance and cost in a resource-constrained setting. *Int J Tuberc Lung Dis* 2010; *14* : 1024-31.
6. Wu TS, Lu CC, Lai HC. Current situations on identification of nontuberculous mycobacteria. *J Biomed Lab Sci* 2009; *21* : 1-6.
7. Lee JC, Yu FL, Lin MH, Huang GS, Chang CY, Cheng CL, *et al*. Utility of immunochromatographic assay for detecting *Mycobacterium tuberculosis* from positive BACTEC MGIT 960 cultures. *J Biomed Lab Sci* 2010; *22* : 64-8.
8. Abe C, Hirano K, Tomiyama T. Simple and rapid identification of the *Mycobacterium tuberculosis* complex by immunochromatographic assay using anti-MPB64 monoclonal antibodies. *J Clin Microbiol* 1999; *37* : 3693-7.
9. Harboe M, Nagai S, Patarroyo ME, Torres ML, Ramirez C, Cruz N. Properties of proteins MPB64, MPB70, and MPB80 of *Mycobacterium bovis* BCG. *Infect Immun* 1986; *52* : 293-302.
10. Li H, Ulstrup JC, Jonassen TO, Melby K, Nagai S, Harboe M. Evidence for absence of the MPB64 gene in some substrains of *Mycobacterium bovis* BCG. *Infect Immun* 1993; *61* : 1730-4.
11. Nagai S, Wiker HG, Harboe M, Kinomoto M. Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. *Infect Immun* 1991; *59* : 372-82.
12. Wagner D, Young LS. Nontuberculous mycobacterial infections: a clinical review. *Infection* 2004; *32* : 257-70.
13. Scott CP, Dos Anjos Filho L, De Queiroz Mello FC, Thornton CG, Bishai WR, Fonseca LS, *et al*. Comparison of C(18)-carboxypropylbetaine and standard N-acetyl-L-cysteine-NaOH processing of respiratory specimens for increasing tuberculosis smear sensitivity in Brazil. *J Clin Microbiol* 2002; *40* : 3219-22.
14. Siddiqi SH. *BACTEC 460 TB system. Products and procedure manual*. Becton Dickinson Microbiology System, Sparks, MD, USA; 1996.
15. Somerville W, Thibert L, Schwartzman K, Behr MA. Extraction of *Mycobacterium tuberculosis* DNA: a question of containment. *J Clin Microbiol* 2005; *43* : 2996-7.
16. Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis* 1990; *161* : 977-81.
17. Sharma SK, Mohan A. Extrapulmonary tuberculosis. *Indian J Med Res* 2004; *120* : 316-53.
18. Montenegro SH, Gilman RH, Sheen P, Cama R, Caviedes L, Hopper T, *et al*. Improved detection of *Mycobacterium tuberculosis* in Peruvian children by use of a heminested IS6110 polymerase chain reaction assay. *Clin Infect Dis* 2003; *36* : 16-23.
19. French AL, Benator DA, Gordin FM. Nontuberculous mycobacterial infections. *Med Clin North Am* 1997; *81* : 361-79.

20. Ichiyama S, Iinuma Y, Yamori S, Hasegawa Y, Shimokata K, Nakashima N. *Mycobacterium* growth indicator tube testing in conjunction with the AccuProbe or the AMPLICOR-PCR assay for detecting and identifying mycobacteria from sputum samples. *J Clin Microbiol* 1997; *35* : 2022-5.

21. Laughon BE. New tuberculosis drugs in development. *Curr Top Med Chem* 2007; *7* : 463-73.

22. Attorri S, Dunbar S, Clarridge JE 3rd. Assessment of morphology for rapid presumptive identification of *Mycobacterium tuberculosis* and *Mycobacterium kansasii*. *J Clin Microbiol* 2000; *38* : 1426-9.

23. Kadam M, Govekar A, Shenai S, Sadani M, Salvi A, Shetty A, *et al*. Can cord formation in BACTEC MGIT 960 medium be used as a presumptive method for identification of *M. tuberculosis* complex? *Indian J Tuberc* 2010; *57* : 75-9.

24. Yagupsky PV, Kaminski DA, Palmer KM, Nolte FS. Cord formation in BACTEC 7H12 medium for rapid, presumptive identification of *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 1990; *28* : 1451-3.

25. Morris AJ, Reller LB. Reliability of cord formation in BACTEC media for presumptive identification of mycobacteria. *J Clin Microbiol* 1993; *31* : 2533-4.

26. Kumar VG, Urs TA, Ranganath RR. MPT 64 antigen detection for rapid confirmation of *M. tuberculosis* isolates. *BMC Res Notes* 2011; *4* : 79.

27. Ismail NA, Baba K, Pombo D, Hoosen AA. Use of an immunochromatographic kit for the rapid detection of *Mycobacterium tuberculosis* from broth cultures. *Int J Tuberc Lung Dis* 2009; *13* : 1045-7.

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