COMPARISON OF IMMUNOCHROMATOGRAPHIC ASSAY WITH BIOCHEMICAL TESTS FOR MYCOBACTERIAL ISOLATES

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ABSTRACT: BACKGROUND: Tuberculosis is considered to be a major cause of death among the infectious diseases prevalent in India. Differentiation of M. tuberculosis from Nontuberculous Mycobacteria (NTM) is very important in the management of the disease. Recently, Standard Diagnostics (SD Yongin Korea) developed a simple and rapid assay using a mouse monoclonal anti-MPT64 antibody to discriminate between M. tuberculosis and NTM by Immuno-chromatography. These tests were found to have good sensitivity and specificity. METHOD: 200 sputum samples were processed and inoculated on conventional Lowenstein Jensen culture medium. The Mycobacterial isolates were subjected to biochemical tests (Niacin test and Nitrate reduction test) and Immunochromatographic assay. The isolates were also tested by Polymerase chain reaction. H37 RV strain was used as a positive control. RESULT: The culture isolates showed positive in 47 (23.5%) cases. 44 isolates were positive and 3 were negative by both biochemical tests and immunochromatographic assay. Similar results were obtained by PCR. The sensitivity and specificity of Immunochromatography test when compared to biochemical tests and PCR was 100%. Positive predictive value and Negative predictive values were 100%. CONCLUSION: Immunochromatographic assay showed comparable results with biochemical tests and this assay can be used as an alternative to biochemical tests for the confirmation of Mycobacterium tuberculosis complex, which avoids use of hazardous chemicals (Cyanogen bromide). KEYWORDS: Mycobacterium, Immunochromatography, Biochemical tests.
Yongin, Korea) developed a simple and rapid assay using mouse monoclonal anti-MPT64 antibodies (SD Bioline TB Ag MPT64) immobilized on a nitrocellulose membrane as a capture material. Another antibody, which recognizes another epitope of MPT64 and has been conjugated with colloidal gold particles, is used for antigen capture and detection in a sandwich-type assay. In the present study, the clinical usefulness of MPT64-Immunochromatographic assay test compared with different biochemical tests was evaluated by using fresh clinical isolates and stock cultures of different mycobacterial species grown on LJ media.

**MATERIALS AND METHODS:** 47 Mycobacterial isolates from sputum samples, one H37RV culture, 12 Gram positive & Gram negative bacterial isolates from sputum & Urine were tested for the presence of MPT64 antigen by Immunochromatographic method and biochemical tests (Niacin test and Nitrate reduction test).

**Immunochromatography assay for detection of M. tuberculosis complex (MPT64 antigen)**: TB Ag MPT64 Rapid is a rapid immunochromatographic identification test for the M. tuberculosis complex that uses mouse monoclonal anti-MPT64. This test kit can be easily used for the rapid identification of M. tuberculosis complex in combination with culture systems based on liquid media without any technical complexity in clinical laboratories. This test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane, and an absorbent pad. Mouse monoclonal anti-MPT64 was immobilized on the nitrocellulose membrane as the capture material (test line). Another antibody, which recognized another epitope of MPT64, conjugated with colloidal gold particles was used for antigen capture and detection in a sandwich type assay. TB antigen MPT64 rapid test can detect MPT64 antigen specifically secreted from the tuberculosis bacteria. The MPT64 antigen is the secretory protein that is one of the major antigens secreted from tuberculosis bacteria. It is developed for differentiation of Mycobacterium tuberculosis complex from nontubercular mycobacteria (NTM). SD BIOLINE TB Ag MPT64 Rapid test device has a letter of T and C as "Test Line" and "Control Line" on the surface of the case. Both the "Test Line" and "Control Line" in result window are not visible before applying any samples. The "Control Line" is used for procedural control. Control line should always appear if the test procedure is performed properly and the test reagents of control line are working. As the test sample applied in the sample well flow laterally through the membrane, the antibody-colloidal gold conjugate binds to the MPT64 antigen in the sample liquid media. The complex then flows further and binds to the mouse monoclonal anti-MPT64 on the solid phase in the test line, producing red to purple colour band. In the absence of MPT64, there is no line in the test band region. The procedure is started by adding 200µl of buffer in a sterile test tube. To this, 3 colonies from the 3-4 week old culture is taken & emulsified and the mixture is vortexed. The whole mixture is pipette into a sample well and allowed for 15 minutes. Pink colour band formation was observed at the test region along with the control region. (Image 3)

**Niacin test**: Niacin plays a vital role in the oxidation-reduction reactions that occur during synthesis in all Mycobacteria. It functions as a precursor in the biosynthesis of coenzymes. Although all Mycobacteria produce nicotinic acid, comparative studies have shown that, because
of a blocked metabolic pathway, M. tuberculosis accumulates the largest amount, and the
detection of this accumulated niacin is useful for the definitive diagnosis of this species. The
niacin test should not be used alone to identify M. tuberculosis because, several other species (M.
simiae, M. chelonae) consistently yield positive results. This fact alone emphasizes the importance
of performing the supportive tests of nitrate reduction to confirm the identity of M. tuberculosis.
Before the niacin test was performed, cultures were (a) checked for purity by microscopy, (b) had
to be 3 to 4 weeks old on egg medium, and (c) had to have sufficient growth of 50 or more
colonies. If cultures are still niacin negative at 4 weeks and if they have been handled aseptically,
they were re incubated for retesting when 6 weeks old; otherwise fresh cultures were used.
Because Mycobacteria excrete niacin into growth medium, cultures confluent growth may give a
false-negative niacin reaction because the extracting fluid cannot contact the culture medium.
When this occurs, the underlying medium surface was exposed by either scraping away or
piercing through some of the culture growth.6

The niacin test may be done either with chemical reagents or with commercially available
paper strips. Regardless of the method used, niacin is usually detected by its reaction with a
cyanogen halide in the presence of a primary amine. In the present study, chemical reagents
were used. The procedure is started by adding 1ml distilled water to 3–4 week old culture on
Lowenstein Jensen (LJ) medium. The tube is placed horizontally so the fluid covers the entire
surface of the medium and left for at least 15 minutes for extraction of niacin. 0.5ml of the fluid
extract is removed to a clean screwcap tube and 0.5ml of the 4% aniline solution plus 0.5ml of
10% cyanogen bromide is added. The solution is observed for the immediate formation of yellow
colour. (Image 1)

Nitr at e re d u ct ion test6: The ability of some Mycobacteria to reduce nitrate has proved valuable
in differential identification of some Mycobacteria that possess such similar characteristics as
colonial morphology, pigmentation and growth rate. Most Mycobacterial cultures to be tested for
nitrate reduction, should be examined for 4 weeks after inoculation onto the subculture medium.
Rapid growers that exhibit good growth may be tested after 2 weeks. Species that reduce nitrate
are M. tuberculosis, M. kansasii, M. flavescens and most rapid growers except M. chelonae. The
procedure is started by adding 0.2ml of sterile distilled water to a screw capped tube. To this 2
loopful of growth from a 4week old culture on LJ medium is emulsified in the distilled water and
2ml of Sodium nitrate (NaNo3) substrate was added to the tube and incubated upright for 2hrs at
37˚c. One drop of concentrated Hydrochloric acid (HCl) was added. 2 drops of 0.2%
sulphanilamide, 2 drops of 0.1% N-Naphthylethylenediamine dihydrochloride is added and
examined for pink to red colour. (Image 2)

RESULTS: The control band (C band) was seen in all the 47 samples tested validating the test.
H37 Rv control showed the appearance of a pink band in the test region (T band) confirming the
presence of MPT 64 antigen. 44 M. tuberculosis isolates showed the dark pink band in the test
region, confirming the presence of MPT64 Ag in these isolates. (Image 3) The sensitivity of the
test kit was 100%. None of the other 03 test samples including the MOTT isolates showed the
pink band formation, indicating the absence of MPT64 Ag in these isolates. This proved the
specificity of the ICT (100%). Positive predictive value and Negative predictive value was 100%. Biochemical tests also show similar results. (Image 1), (Image 2)

**DISCUSSION:** Tuberculosis is one of the leading causes of morbidity and mortality in developing country like India. Even with the implementation of several programmes like Revised National Tuberculosis control programme, the incidence and prevalence is on the rise. The main factors being related to patient himself who comes to the doctor late; by the time which he would have spread the infection to the household members and others. Even upon starting the treatment, adherence and compliance is very important for curing the disease, failing which he becomes a defaulter or the Mycobacteria might develop drug resistance.

In any case of tuberculosis, the laboratory has two important roles to fulfill. First, it plays an essential part in the diagnosis of whether it is Mycobacterium tuberculosis or atypical mycobacteria. Secondly, laboratory has an important role in performing drug sensitivity testing in fulfilling the treatment.

The recent diagnostic methods have improved the time taken for isolation, but there is a need for rapid identification of mycobacterial isolates. So at present, speciation of mycobacteria and detection of drug resistance has become the topics of interest in the diagnosis and proper treatment of the patient. Performing the biochemical tests for speciation is labor intensive, hazardous and need biosafety cabinet class two plus. Instead a new rapid Immuno-chromatographic method can be employed for the identification test in poor resource countries. With this aim, we conducted the study comparing the performance of Immunochromatographic assay with the biochemical tests to find out which is rapid and economically feasible.

The aim of this study was to find out a simple, rapid & economically feasible test which accurately identifies MTB isolates grown in culture. Our study was comparable with Hasegawa et al, which tested 63 isolates from LJ medium out of which 30 isolates were MTB complex and 33 isolates were MOTT. The difference was our study had less number of samples comparatively and didn’t perform the assay from different culture methods. Positive thing was, we compared the ICT with conventional biochemical tests. In another study, by Andrew brent et al, who also used BIOLINE SD Ag MPT64 Rapid test had sensitivity of 98.6% and 100% specificity. Our study is comparable with this study except they have done meta-Analysis of various MPT64 ICT assay and they have cultivated Mycobacterium by different methods. Our study has 100% sensitivity and specificity. In a study by Mi Young Park and Marzouk M, the sensitivity and specificity was 99% and 100% respectively. Another study by Toihir AH, sensitivity and specificity was 100 % which is the same as in our study. Similar results were also found in study by Vijay kumar et al. With these observations SD BIOLINE Ag MPT64 rapid test has got good sensitivity and specificity in identification and differentiation of Mycobacterium isolates. Prompt detection, isolation, identification and susceptibility testing of Mycobacterium tuberculosis from clinical specimens are essential for the appropriate management of patients with tuberculosis.

**CONCLUSION:** Conventional biochemical identification methods are time consuming, requiring subculture onto solid media, and cost, limited laboratory capacity preclude molecular methods in low-resource settings. Rapid immunochromatographic tests (ICT) that detect the M. tuberculosis
complex (MTBC) MPT64 protein are cheaper, simpler to use and thus may have an important role in these and other settings.

REFERENCES:

Fig. 1: Growth of M. Tuberculosis on Rapid Slide Culture
Fig. 2: Growth on Lowenstein Jensen medium

Fig. 3: Immunochromatography Assay – Positive and Negative

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