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Evaluation of p-nitrobenzoic acid using Lowenstein–Jensen medium and rapid immunochromatographic test for early differentiation between *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria

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ABSTRACT

Background: *Mycobacterium tuberculosis* (MTB), *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, and *Mycobacterium microti* are all members of the MTB complex [MTBC], which cause tuberculosis. Mycobacteria other than TB also known as non-tuberculous mycobacteria [NTM]. **Aims and Objectives**: The purpose of this study was to see how accurate and efficient the MPT64 Ag fast test and Lowenstein–Jensen (LJ) medium with p-Nitrobenzoic acid (PNB) was in differentiating MTB and NTM. **Materials and Methods:** This Prospective cross-sectional, diagnostic study was conducted in the department of Microbiology, Chalamadaanandarao Medical college, Karimnagar, Telangana. India. The study was conducted from September 2020 to September 2021. The study was preapproved by the Institutional ethical and research committee. **Results**: PNB inhibits the growth of MTBC, whereas NTM species were resistant. MPT64 is a protein antigen secreted specifically by the members of MTBC. This study was carried out to establish the accuracy and efficiency of LJ medium with PNB and MPT64 immunochromatography test (ICT) in differentiation between MTB and NTM. Of the 426 culture positive isolates tested, 382 (89.67%) were recovered from pulmonary specimens and 44 (10.32) from extrapulmonary sources. Of the 426 isolates tested for the presence of MPT64 antigen, 407 (95.53%) were found positive for ICT test. Whereas, 18 (4.22%) isolate and were considered as NTM and 408 as MTBC (95.77%) by selective inhibition by PNB in LJ medium. **Conclusion:** According to our findings, the SDbioline kit is a fast, accurate, and reliable approach for separating MTB from NTM for TB management. MPT64ICT results were compared to culture smear results, which was particularly informative in the situation of MPT64ICT negative isolates with liquid cultures.

Key words: MPT 64 antigen; *Mycobacterium tuberculosis* complex; Non-tuberculous mycobacteria; Para-nitrobenzoic acid

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INTRODUCTION

India has the greatest tuberculosis (TB) load, accounting for one-fifth of global cases. *Mycobacterium TB* (MTB), *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, and *Mycobacterium microti* are all members of the MTB complex [MTBC], which cause TB. Mycobacteria other than TB, also known as non-tuberculous

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mycobacteria [NTM], have been on the rise in recent years.

The clinical presentation of pulmonary disease caused by NTM mimics MTB as a result, NTM were often under-diagnosed. It is necessary to accurately characterize mycobacteria, because NTM were inherently resistant to first line anti-TB drugs and need modified treatment regimens and NTM were being misdiagnosed as multidrug-resistant TB.[1]

To promote early and successful treatment of patients, rapid distinction of MTBC and NTM is critical.[2] There are several ways for identifying mycobacteria, although traditional biochemical tests are often time-consuming.[3] Moreover, molecular techniques like as the accu-probe assay necessitate specialized laboratory equipment and people. These tests are costly, and they are not recommended in low-resource situations.[4]

MPT64, a secretory protein of 24 kDa, is one of the most important antigens found in TB germs. The MPT64 gene has been found to distinguish the MTBC from other bacteria, including the Bacille Calmette-Guérin strain.[3,5] Antigen for TB standard diagnostics, South Korea, manufactured MPT 64 Ag rapid immunochromatography test (ICT) kits, which were used according to the manufacturer's instructions. For MTB isolate confirmation, mouse monoclonal antibodies against MPT64 antigen were immobilized on nitrocellulose membranes. Under level two biosafety protocols, the test should be conducted inside a biosafety class II cabinet.

In this investigation, growth on suspect findings was detected due to faint bands produced by MTBC, false positives to mycobacterium marinum and Staphylococcus aureus, and false negatives in the case of MPB64 mutations, all of which were verified positive by the accuprobe test.[6,7]

The selective inhibition test can be used to distinguish MTBC from NTM. MTB is inhibited by p-Nitrobenzoic acid (PNB), while NTM are resistant. To distinguish between MTB and NTM, a PNB inhibitory test was used. PNB was added to a final concentration of 500 g/ml in the Lowenstein–Jensen (LJ) medium.[8]

Aims and objectives

The aim of this study was to see how accurate and efficient the MPT4Tb Ag fast test and Lj medium with PNB were in differentiating M.tb and NTM.

The goal of this work is to distinguish between Mycobacterium TB complex and Nontuberculous mycobacteria species isolated from various clinical specimens of human tuberculosis patients.

MATERIALS AND METHODS

This prospective cross-sectional, diagnostic study was conducted in the Department of Microbiology, Chalamada Anandarao Medical College, Karimnagar, Telangana, India. The study was conducted from September 2020 to September 2021. Permission was taken from the Institutional Ethical and Research Committee. The goal of this study was to see how many non-TB mycobacteria species were present among the patients at our tertiary care hospital. The goal of this work is to distinguish between MTBC and NTM species isolated from various clinical specimens of human TB patients. The purpose of this study was to see how accurate and efficient the MPT4Tb Ag fast test and LJ medium with PNB were in differentiating MTB and NTM.

SD bioline MPT64 Ag detection rapid test

SD bioline MPT64 rapid antigen detection is a straightforward rapid test that requires no extra equipment or sample preparation and may be completed in about 30 minutes. SD bioline MPT64 rapid antigen detection test kits come in individual packs of 25 strips. These test kits are suitable for any basic laboratory that performs mycobacterial culture and replaces traditional biochemical tests that are time consuming and difficult to handle, as well as having other limitations such as the use of positive and negative controls for each of the phenotypic tests.

Procedure

Sputum sample inoculated in LJ Medium for MTB, after incubation period pickup 2–4 colonies using inoculation loop were suspended in 200 l of extraction buffer (supplied in the kit), which was then applied to the sample well.

- Let it sit for 15 min before interpreting the results, which took place after 15 min of sample application at room temperature
- The presence of both control and test pink colored bands indicates a positive test, while the presence of only the control band suggests a negative MTBC result
- Any culture positive and negative ICT findings were deemed to the species of the NTM isolates if there was no control band following the test.

PNB test

At a final concentration of 500 g/ml, PNB was added into the LJ medium.[9] Decontamination and concentration using the NALC-NaOH procedure (Microxpress Lyfectol) were used to prepare the inoculum, which was then inoculated into LJ medium vials.

RESULTS

Of the 426 culture positive isolates tested, 382 (89.67%) were recovered from pulmonary specimens and 44 (10.32) from extrapulmonary sources. Of the 426 isolates tested for the presence of MPT64 antigen, 407 (95.53%) were found positive for ICT test representing, MTBC. The reference strain H37Rv was taken as a control for ICT. Selective inhibition by PNB in LJ medium exhibited growth of 18 (4.22%) isolates and were considered as NTM and 408 as MTBC (95.77%). One strain neither exhibited growth on either LJ medium with PNBA nor positive for MPT64 antigen and it was confirmed by Niacin and nitrate reduction test, which identified it as MTB. Table 1 shows the Results of SD MPT 64 Ag rapid test and growth inhibition by para-nitrobenzoic acid in LJ medium.

DISCUSSION

MTB protein 64 antigen (MPT-64) also called Rv1980c antigen is a 24kDa secretary/excretory protein produced by viable and actively dividing cells of MTBC. Once secreted, they will be stable within the culture medium, up to 1 year. This rapid ICT (MPT64 TB Ag Kit) was developed by SD Bioline, South Korea, using anti-MPB64 monoclonal antibodies, which is easier in rapid detection and differentiation of MPT 64 antigen within the isolates of MTB and NTM. In our study, we report 95.53% sensitivity with MPT 64 TB Ag rapid ICT. Almost similar findings were seen with many studies, Kumar et al.,[10] from Mysore reported 100% sensitivity and Kanade et al.,[11] evaluated phenotypic and commercial method for differentiation of MTBC and NTM, he found 99.19% sensitivity and negative predictive value was 97.3% with rapid ICT (MPT64 TB Ag Kit). A study from Mumbai by Nerurkar et al.,[12] found 94.2% sensitivity with MPT 64 antigen ICT. A study by Martin et al.,[13] from Belgium, studied on 131 isolates with TB Ag MPT64 rapid test and PCR and found 96.5% sensitivity with ICT, as four MTBC isolates were failed to be detected by TB Ag MPT64 rapid test. Hirano et al.,[7] from Japan an immunochromatographic assay, Capilia TB, found 99.2% sensitivity (381 of 384 samples) and reported that Capilia TB-test negative isolates had mutations within their mpb 64 gene, leading to production of incomplete protein antigen. Similarly, Park et al.,[14] found 99% sensitivity and reported 10^5 CFU/ml is required for the detection of antigen.

Table 1: Results of SD MPT 64 Ag rapid test and growth inhibition by para-nitrobenzoic acid in LJ medium

Results	MPT64 ICT result	Growth on LJ Medium with PNBA/Niacin test/Nitrate test
Positive	407	408
Negative	19	18
Total	426	426

ICT: Immunochromatography test, LJ: Lowenstein–Jensen,

Inhibition of MTBC growth was carried out to distinguish between MTB and NTM by adding PNB to the LJ medium at 500 μg/ml concentration. We observed 100% sensitivity and 100% specificity in our study. Similar findings were seen with, Kandhakumari and Stephen.[15] from Pondicherry observed, 100% sensitivity with PNB, 33 MTB and 4 NTM were grown in MGIT with PNB. Nepali et al.,[16] from Khatmandu, identified 100% sensitive for MTBC to grow in medium with PNB. In a study conducted by Sharma et al.,[8] to assess the reliability and efficiency of PNB in identifying MTB from NTM, they discovered 99.05% reliability between PNB inside MGIT 960 and PNB within LJ medium in distinguishing MTB from NTM. Giampaglia et al.,[17] observed 99.4% accuracy between MGIT with PNB and LJ medium with PNB in another investigation.

CONCLUSION

According to our findings, the SD bioline kit is a fast, accurate, and reliable approach for separating MTB from NTM for TB management. MPT64 ICT results were compared to culture smear results, which was particularly informative in the situation of MPT64 ICT negative isolates with liquid cultures. We show that LJ medium combined with the PNB test is a very sensitive and specific inhibitor of MTB.

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OG- Concept and design of the study, prepared first draft of manuscript; **PKV-** Reviewed the literature and manuscript preparation; **RM-** Preparation of manuscript and revision of the manuscript; **SMR-** Concept, coordination, statistical analysis and interpretation.

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