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Colour Plate Microcolony Detection of Mycobacterium Tuberculosis in Extra Pulmonary Tuberculosis

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Abstract

Introduction: Accurate and timely tuberculosis diagnosis is the primary step for initiating effective treatment. The colour plate agar-based culture test (TB-CX test) is a low cost, simple to use, detects Mycobacterium tuberculosis faster. The study conducted to compare the diagnostic accuracy and time to detection of positive cultures using colour test and Lowenstein Jensen culture in extrapulmonary samples.

Method: Prospective, laboratory-based study, was conducted on 210 consecutive Extra pulmonary samples received for a period of 18 months in a tertiary care hospital and were directly processed for culture on LJ media and Colour TB CX test along with the microscopy.

Result: From 200 samples, 15 were found positive by both methods, 5 were positive on LJ and negative on the colour TB-CX test. The overall sensitivity and specificity of colour TB-CX test compared with conventional LJ culture were, respectively 75% and 100%. The positive predictive value and negative predictive value of colour TB-CX test were 100% and 95.45% respectively as compared to LJ culture. Among the samples Pus showed highest positivity on both LJ media and Colour plate. In addition, 35% of positive samples were detected within the first three weeks on colour TB-CX plate agar, and 5% on LJ. The minimum time for detection was 16 days for colour TB-CX test and 27 days for LJ media.

Conclusion: The colour plate test allows early detection of MTB diagnosis in a median time of 23 days. This rapid method could be used for the diagnosis of Mycobacterium tuberculosis in EPTB.

Keywords: Colour TB CX test, Extrapulmonary tuberculosis (EPTB), Mycobacterium tuberculosis, Lowenstein Jensen.

Introduction

Tuberculosis (TB) is one of the world's most important infectious causes of morbidity and mortality. The total was 10.8 million in 2023, much higher than 10.1 million in 2020.

India contributed to 26 % of TB cases globally. Although the lungs are the most typically affected site in TB (pulmonary TB), organ systems other than the lungs can also be affected. Clinical manifestations of EPTB range from nonspecific to mimicking any other disease.

Moreover, due to the paucibacillary nature of Extrapulmonary TB specimens and sample collection often requiring invasive procedures, diagnosis of EPTB is often delayed.[1] India, with its high incidence of TB, shoulders a considerable burden of EPTB. The Nikshay platform reported over 2.4 million TB cases in India in 2022, with EPTB constituting 24% of these cases.[2]

Detection by Ziehl Neelsen has a sensitivity which ranges from 20% to 60%.

Conventional detection on solid media requires 10- -100 viable organisms for a positive result but takes 4 to 6 weeks. BACTEC or MGIT using liquid culture media though rapid are expensive and requires biosafety level 3 facility. The equipment and specialized skills required to perform molecular methods make them an impractical option, especially in developing countries. There is an urgent need for simple, sensitive and rapid diagnostic tests for TB which can be introduced in a routine laboratory. Despite limited data, the (TB-CX) test Colour test has demonstrated good performance for rapid detection.

Colour Test (TB-CX) is a non-commercial, in-house thin-layer agar thin layer agar (TLA) technique in which STC redox indicator (2, 3-diphenyl-5-2-thienyl tetrazolium chloride). causes a colour change in the medium when bacteria grow, making positive results apparent by examination with the naked eye.[3] Colour Test combines the TLA technique using a selective medium to reduce contamination and colorimetric indication of bacterial growth making interpretation easy.

Rapid sensitive detection of Mycobacterium tuberculosis in sputum has been demonstrated in studies of the TB color plate test performed directly on sputum specimens in which cultures were examined to visualize coloured colonies and microscopically to detect growth of Mycobacterium tuberculosis under an ordinary light microscope [4,5,6]. The median time to detection has been shown to be 13 days as compared to solid culture 29 days.

Manipulation of a solid or liquid culture carry risk of aerosolization and spillage. Colour plate once inoculated with the specimen is sealed inside a ziploc bag as no further manipulations are required.

Evaluation of the colour plate test has been done by Mekonnen et al[6] and Shibabaw et al[7] in sputum specimens in comparison with Lowenstein Jensen (LJ) medium and the redox indicator used was STC (2,3, diphenyl 5-2- thienyl tetrazolium chloride) [7,8] Also there is paucity of data on the performance of this test on EPTB specimens.

The present proposal is designed to evaluate the performance of the colour plate test for early detection of

Mycobacterium tuberculosis in extrapulmonary TB (EPTB) specimens when used to directly test patient specimen on Middlebrook 7H11 agar with using a more easily available indicator TTC (2,3,5 triphenyl tetrazolium chloride) as the redox indicator in comparison with Lowenstein Jensen medium.

Aims and Objective

We conducted a study with the following aims and objectives:

1. To determine and compare the time to culture positivity by both colour test and LJ culture.
2. To determine the sensitivity and specificity of detection of mycobacterial growth in EPTB specimens by colour test in comparison with that conventional culture on LJ medium.
3. To assess contamination rates by both colour plate and LJ culture.

Materials and Method

Prospective, laboratory-based study, was conducted on 210 consecutive Extrapulmonary samples received in the Department of Microbiology, Tertiary care teaching hospital for a period of 18 months. Sample processing and culture was done in a Biological Safety Cabinet Class 2. EPTB specimens were directly processed for culture on LJ media and Color TB CX test along with the microscopy (ZN stain).

Colour plate inoculation[9]

Each plate was inoculated using 2 drops (100 µl) of EPTB specimen. To inoculate the Colour Test plate with the sample, the following procedure was used: Two drops of specimen were added to each quadrant using a disposable plastic pipette; one drop to each quadrant before adding the second drop to each quadrant to improve mixing and balance the inoculum. The sample was dropped onto but was not 'spread' on the Colour Test plate. This simplifies the procedure and also reduces contamination. The lid of the culture plate was sealed with parafilm or transparent tape e.g. 'Scotch' or 'Cello tape'). The plate was double-sealed by enclosing in a Ziploc bag. The plates was placed in the incubator at (37ºC). Each Petri dish was having 4 quadrants three of which was used for 3 specimens and one for H37Ra (ATCC 25177) as control

Procedure of Reading of plates

Plates were read after 24 hours and thereafter thrice weekly for 6 weeks and read double- sealed, without removal from the plastic bag. M. tuberculosis growth in the form of red color microcolony was observed by naked eye Small <3mm colony was observed then it was taken as probable mycobacterial growth. Also, a secondary smear was made and stained by ZN after opening the plates

MPT64 was performed for speciation along with inoculation on LJ with PNB.

The great majority of fungal and bacterial contamination was obviously non-mycobacterial from naked eye inspection either because no colour change occurred in the surrounding culture medium or because of the rapid appearance of a large colony that on naked-eye inspection has the obvious morphological characteristics of fungal or bacterial contamination. Plates were retained upto 42 days (6 weeks).

The detection quadrant which developed complete contamination (or extensive partial contamination covering most of the quadrant and no red colored mycobacterial growth was seen) then the result was reported as a failed, contaminated culture.

If partial contamination was present covering the only minority of the detection quadrant and no red mycobacterial growth was seen then the absence of

mycobacterial growth by 6 weeks incubation was reported as a definitively negative culture.

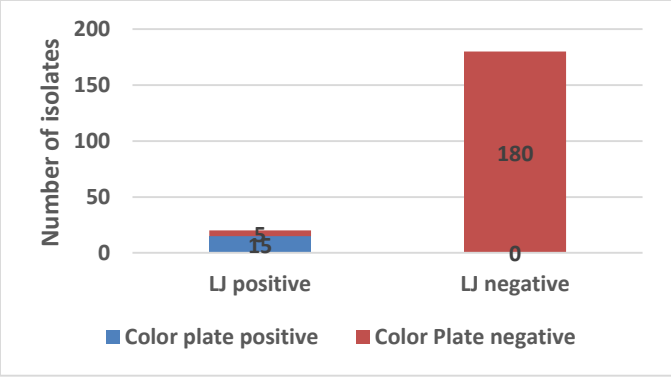
Specimen was considered to have Mycobacterium tuberculosis if culture on LJ medium was positive for M. tuberculosis. A colour plate culture with colour change was considered to be falsely positive if the companion LJ culture did not grow M. tuberculosis.

Statistical analysis: Mc Nemar's chi square test will be used to compare the sensitivities and specificities of detection of the two methods. Key proportions were reported with their 95% confidence interval (95% CI) and a p-value of <0.0001 was considered as statistically significant for sensitivity, specificity, predictive values. Sensitivity and specificity, predictive values of detection by color test was calculated against LJ culture using the Medcalc software- version 22.019 (accessed Jan 13 2024) https:// www.medcalc.org/calc/ diagnostictest. php[10]

Result

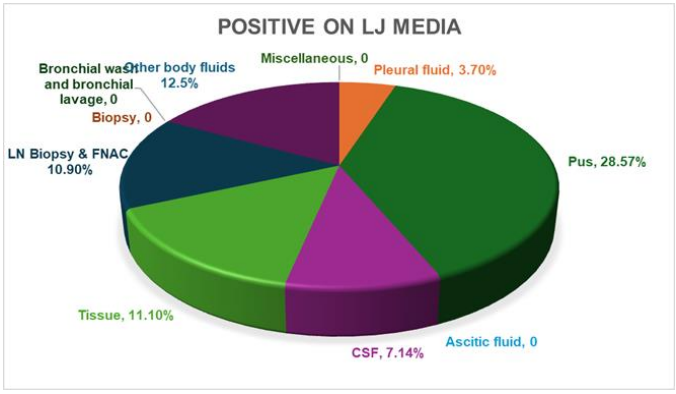
Out of 210 samples 9 samples were contaminated on both LJ media and color test and one was rapidly growing mycobacteria and after excluding 10 samples which were contaminated, we used 200 samples for statistical analysis. From 200 samples, 15 were found positive by both methods, 5 were positive on LJ and negative on the color TB-CX test. .(Figure no .1)

Figure 1:



The overall sensitivity and specificity of color TB-CX test compared with conventional LJ culture were, respectively 75% (95% CI 50.90% - 91.34%) and 100% (95% CI: 97.7%– 100%) .The positive predictive value and negative predictive value of color TB-CX test were 100% and 95.45% respectively as compared to LJ culture. Among the samples Pus showed highest positivity on both LJ media and Color plate. (Figure no .2)

Figure 2:



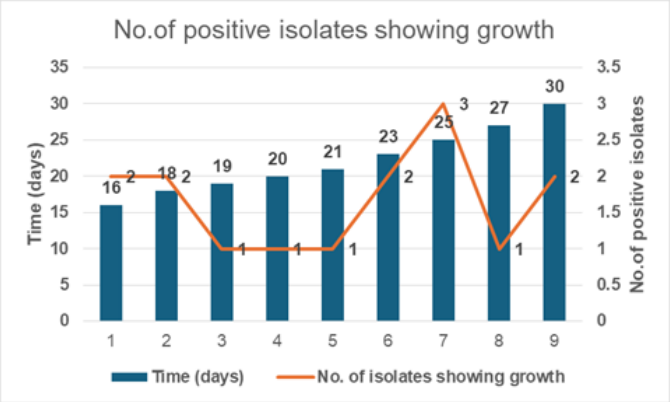
The Positivity percentage of ZN smear ,Color Plate and LJ media were 2%, 7.5% and 10% respectively (N=200)**.**The sensitivity of color plate in ZN positive smear and negative smear ~~is~~ was 100% (95% CI 39.76% to 100%).

The minimum time for detection was 1 6 days for color TB-CX test and 27 days for LJ (Table 1) (Figure No.3) and the maximum was 30 days for color TB-CX test and 48days for LJ.

Table 1: Time to detection of MTB growth by Color Plate medium, for isolates positive on both LJ medium and Color Plate (N=15)

Time (days)	No. of isolates showing growth	Cumulative isolates showing growth
16	2	2
18	2	2
19	1	1
20	1	1
21	1	1
23	2	2
25	3	3
27	1	1
30	2	2

Figure 3:



All smear-positive samples yielded mycobacterial growth on both the color TB-CX test and LJ. The median time in days from sample processing to detection of M. tuberculosis growth was shorter for the color TB-CX test (Median 23 days, IQR 18–25) than LJ (Median 34 days, IQR 30-39) ($p<0.05$).(Table 2).

In addition, 35% of positive samples were detected within the first three weeks on color TB-CX plate agar, and 5% on LJ.

Table 2: Comparison of Median time to detection by Color Plate and LJ medium for isolates positive on both Color Plate and LJ medium. (N=20)

Method	Time for detection of growth in days	
	Median	Interquartile Range
LJ medium	34	30-39
Color Plate	23	18-25

For the color TB-CX plate, the median time to detection was significantly shorter for smear-positive samples (Median, 17 days) than for smear-negative ones (Median, 23 days) ($p<0.05$). (Table 3 &4)

Table 3: Median time to detection in ZN positive samples. (N=4)

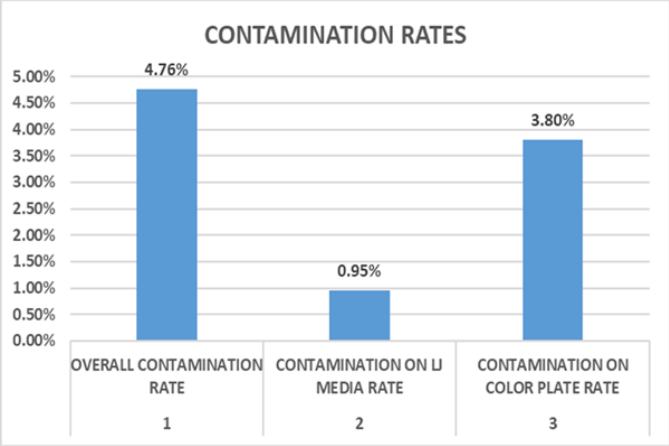
Method	Median Time for detection of growth in days	
	Median	Interquartile Range
LJ medium	28	23.5-31.5
Color Plate	17	16-22.5

Table 4: Median time to detection in ZN negative samples (N=196)

Method	Median Time for detection of growth in days	
	Median	Interquartile Range
LJ medium	39	33-41
Color Plate	23	20-25

The overall contamination was 4.76%. The contamination rate was (3.8%) on the color TB- CX test and (0.95%) on LJ medium. (Figure No.4)

Figure 4:



Discussion

Microbiological diagnosis of TB constitutes a fundamental step towards disease control. The present study was conducted on Middlebrook7H11 agar after adding a redox indicator TTC which gives red coloured colonies of M.tuberculosis.

Adding a redox indicator TTC leads to appearance of red colored colonies which can be seen by naked eye.

Overall positivity of extrapulmonary tuberculosis (EPTB) on LJ media was 10% and 7.5% on (Color plate test) which was similar to findings of Robeldo et al[11], Satti L et al [12] and Meija GI et al[13] .The sample wise highest positivity was found in pus specimen in present study which was 28.57% which was similar to Yousef et al of[14] 33% and higher than positivity in Satti et al [12] study which was 12.5%. Sensitivity of 75% of colour plate was similar to studies of Martin A, Dai Guangming et al [15] and Meijia G.I. et al[13]. The sensitivity in the present study was lower when compared to the Mekonnen B et al 2019[6].They evaluated sputum samples on TLA with redox compared to LJ media, whereas we tested consecutive extrapulmonary samples received in mycobacteriology laboratory from patients.

Specificity of detecting MTB in extrapulmonary samples by color plate in the present study shows similar finding with the study of Shibabaw A et al[7] and Mekonen B et al.[6] Present study showed positive predictive value of 100% which is similar to the study of Shibabaw A et al [8] but higher than findings of Mejia GI.[16]

In the present study 46 % of positive samples were detected within the first three weeks on color TB-CX plate agar, and 5% on LJ while in the study of Meijia GI et al[13] they observed that more than 80% of the positive samples were detected on TL7H11 during the first 2 weeks compared with less than 20% observed on L-J ,while in another study of Martin A et al[17] conducted on pulmonary samples observed that 70 % of cultures were found to be positive in 2 weeks on TLA and majority of cultures were positive after 3 weeks on LJ media.

In a study by Shibabaw A et al[7] the majority of 70.8% were detected within the first two weeks on color TB - CX test which shows the advantage over convectional LJ medium for the early detection of mycobacteria. This study included pulmonary samples which is multibacillary samples might be the cause of early positive culture growth. Secondly, they used microscope

to observe the TLA plate while in the present study we examined the plate of color TB CX test with naked eye for the color of red color colonies so it may be the cause of late detection of samples on plates.

Our study median time to detection by Color Plate and LJ medium for isolates positive on both Color Plate and LJ medium were 23 days (IQR 18-25 days) and 34 days (IQR 30-39 days)(p <0.0001) respectively, which shows median time for growth detection on color TB -CX test was shorter than LJ which resembles with the study done by Tovar M et al[18], Shibabaw A et al[7], Martin A et al[17], Robledo J et al[11] and Guangming D et al[15].

M.tuberculosis colonies grows faster and easy to visualize by the naked eye as the red color colonies due to addition of TTC indicator in the medium.

Present study revealed that Color TB-CX t est detection time was shorter for smear positive than smear negative samples which showed concordance with the study of Shibabaw A et al[8] and Robledo J et al[12].

The contamination rate of color TB-CX test and LJ in the present study, was 3.8095% and 0.95% respectively and overall contamination was 4.76%. The contamination of Color TB- CX test was comparable with study conducted by Shibabaw A et al[7] and Tovar M et al[18]. Study by Guangming D et al[15], Battaglioli et al[19] and Robledo et al[11] contamination rate was found to be higher than the present study for color TB -CX test. The reason for low contamination rate observed might be explained by using PANTA in order to prevent the growth of bacteria and fungi.

A disadvantage of the TBCX plate as far as staff requirements is that the rapid time to growth requires reading of the plate for culture detection three times a week opposed to once a week for LJ. However, the less frequent interval of once per week for LJ culture reading may result in limited detection of M. tuberculosis growth in between reads. Advantage of the test is low biohazard risk is plates are sealed after inoculation. Reading and disposal of the plates are performed without removing the seal, decreasing handling risk. Color plate TB -CX test does not require sophisticated laboratory. M.tuberculosis colonies grows faster and easy to visualize by the naked eye as the red color colonies due to addition of TTC indicator in the medium.

Conclusion

In summary the Color TB-CX test is a rapid, simple to use TB diagnostic method and could be alternative option in resource limited settings as it is easy to perform, faster and cost sensitive technique which allows the presumptive identification of Mycobacterium tuberculosis.

As it is rapid alternative method for the detection of mycobacterial growth, its use in routine diagnosis should be considered in laboratories with limited resources.

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