e-ISSN: 0975-1556, p-ISSN:2820-2643

Available online on www.ijpcr.com

International Journal of Pharmaceutical and Clinical Research 2024; 16(8); 642-647

Original Research Article

Comparative Study on Isolation of *Mycobacterium Tuberculosis* in Lowenstein Jensen Medium and Middle Brook 7H9 Broth from Sputum Samples of Suspected Cases of Pulmonary Tuberculosis and their Molecular Characterization by CBNAAT in a Tertiary Care Hospital, Thanjavur

S. Vanathi[1], R. Hemalatha[2], Umamaheswari[3], S. Lallitha[4], S. Swarna[5]

[1]Associate Professor, Department of Microbiology, Government Pudukkottai Medical College, Tamil Nadu

[2]Tutor, Department of Microbiology, Government Stanley Medical College, Tamil Nadu

[3]Assistant professor, Department of Microbiology, Government Pudukkottai Medical College, Tamil Nadu

[4]Professor, Department of Microbiology, Government Virudhunagar Medical College, Tamil Nadu

[5]Associate Professor, Department of Microbiology, Government Thoothukudi Medical College, Tamil Nadu

Received: 25-05-2024 / Revised: 23-06-2024 / Accepted: 26-07-2024

Corresponding Author: Dr. S.Swarna

Conflict of interest: Nil

Abstract:

Tuberculosis is caused by *Mycobacterium tuberculosis*, an acid-fast bacillus. The most common form of TB is pulmonary tuberculosis and can also cause extra pulmonary Tuberculosis. Nowadays we are facing the co-infection with HIV and the emergence of MDR-TB, XDR-TB. In developing countries like India, it causes major public health problem. The delay in diagnosis and the improper treatment are the important causes of mortality. In India, in our health care setup we mainly depend upon the Sputum Microscopy. The sputum microscopy and culture both of the diagnostic methods are very important for the diagnosis of Tuberculosis. This study was conducted to compare and evaluate the efficacy of Microscopy (Zeihl Neelsen Technique) and conventional Culture methods (Solid medium-Lowenstein Jensen Medium, Liquid Medium-Middle Brook 7H9 Broth supplemented with OADC) in suspected 150 cases of pulmonary tuberculosis attending Thoracic Medicine and General Medicine OPD at Thanjavur Medical College. Sputum samples were collected and processed for acid fast staining(ZN)and culture(Solid medium-Lowenstein Jensen Medium and Liquid medium-Middle Brook 7H9 Broth).Among the 150 sputum samples, AFB positivity in concentrated sputum AFB staining by Zeihl Neelsen Technique-37(25%), Culture positivity in Lowenstein Jensen Medium(Solid egg based medium)- 48 (32%), Culture positivity in Middle Brook H9 broth (Liquid based medium)- in 54 samples (36%). All Sputum samples were processed for CBNAAT (Catridge Based Nucleic Acid Amplification Tehnique-Gene Xpert-MTB/RIF), 54 samples detected positive, among this Rifampicin Resistance (rpo B gene mutation) positivity is detected in one sample. This study confirmed that combination of Sputum microscopy by Zeihl Neelsen Staining Technique, culture by solid or liquid medium and CBNAAT is the gold standard method for the diagnosis of pulmonary tuberculosis.

Keywords: *Mycobacterium tuberculosis*, Acid fast bacilli, Zeihl Neelsen Technique, Solid medium-Lowenstein Jensen Medium and Liquid Medium-Middle Brook 7H9 Broth, CBNAAT.

Introduction

Tuberculosis is the one of the major infectious disease [1] and global public health problem since so many years. *Mycobacterium tuberculosis* most commonly causes Pulmonary Tuberculosis and also Extrapulmonary Tuberculosis. It commonly infects the lower socio-economic status, overcrowded areas, Immunosuppressive states like HIV, Diabetes[2,3] and prevalent in developing countries like India. Tuberculosis affects both adults and children. It affects the infected individual's family and leads to economic burden for the family. Nowadays we are unable to predict the Antituberculosis drug sensitivity and treatment outcome for the affected individuals. Now it emerges as MDR TB and XDR TB and create major global threat. In 2006 itself, WHO issued an alert about the threat of MDR & XDR-TB [4] and strengthening of TB control worldwide as a

strategy and necessary response. So early diagnosis and treatment of Tuberculosis is the essential one particularly in our country. One third of World population is infected with *M. tuberculosis* [5]. 3/4 of worldwide infected cases reported mainly from developing countries. One fifth of worldwide TB cases occur in India. About half a million of cases died every year due to tuberculosis.

In India about 40% of population infected and 5% of cases having co-infection with HIV [6]. The delay in diagnosis leads to delay in treatment and increase in the transmission in community and also increase morbidity, mortality and emergence of drug-resistant tuberculosis. So, this study was conducted to compare and evaluate the efficacy of Microscopy (Zeihl Neelsen Technique) [7,8,9] and conventional culture methods (Solid medium-Lowenstein Jensen Medium and Liquid Medium-Middle Brook 7H9 Broth supplemented with OADC) and their molecular characterization by CBNAAT in suspected 150 cases of pulmonary tuberculosis attending Thoracic Medicine and General Medicine OPD at Thanjavur Medical College.

Aims & Objectives:

1. To detect and Compare the *Mycobacterium tuberculosis* infection in clinically suspected cases of Pulmonary Tuberculosis by Microscopy & Culture methods in sputum samples.
2. Molecular characterization and Detection of rpoB gene mutation of *Mycobacterium tuberculosis* by CB-NAAT.
3. To prevent the emergence of Multi Drug Resistant Tuberculosis (MDR-TB) and Extensively Drug-Resistant Tuberculosis (XDR-TB) by early diagnosis.

Materials and Methods

This study was conducted in Thanjavur Medical College and Hospital for the period of one year. Microbiology, General Medicine and Thoracic Medicine Departments were collaborated during this study. It is a Cross-Sectional Study. Institutional Ethical Committee Clearance certificate was obtained and Informed Consent was also obtained from all patients participated in this study.

Inclusion Criteria:

1. Patients those who are having cough for more than 2weeks duration.
2. History of cough for more than 2 weeks duration, associated with fever, loss of weight, loss of appetite with or without haemoptysis.
3. Adults – more than 14 years.
4. Recurrent Lower Respiratory Tract Infection not responding to routine antibiotics therapy.

Exclusion Criteria:

1. Patients those who are having cough less than 2 weeks duration.
2. Old treated and cured cases of Tuberculosis.
3. Patients on Anti Tuberculous Therapy at present.
4. Patients of Chronic Obstructive Pulmonary Diseases(COPD)

Plan of Work: Sputum samples were received from of 150 persons with history suggestive of pulmonary tuberculosis (symptoms of cough more than 2 weeks duration, fever with loss of weight and appetite with or without haemoptysis).

Methodology:

Patients were instructed to collect early morning deeply coughed out 5ml of sputum [7] samples in a sterile wide mouthed sputum collection container having 7-8ml of Trisodium Phosphate solution [10,11] at home and it was transported to Microbiology Laboratory. Proper labelling was done after receiving sputum samples which had the patient name, age, sex, IP. No, date. Under strict aseptic universal work precautions(using personal protective equipment), sample in the transport medium was to be vortexed in a vortex shaker and that then it was left over (sputum sample with transport medium) and it was stored for one overnight period under refrigeration safely [12,13].

Then the sputum sample in the transport medium was processed under strict all standard work precautions in BIOSAFETY CABINET- II-B, decant the supernatant solution using sterile pipette into the container having 5% Sodium Hypochlorite Solution. Using sterile nichrome loop of 5mm size, one loopful of inoculum from the deposit was streaked in the ready use-Himedia-LJ SLANTS(SL001-10SL) [14,15,16] Lowenstein Jensen Culture Medium (by surface plating method) and using another sterile nichrome loop, one loopful (5mm) of the same sample was inoculated in the sterile test tube containing Middle Brooks 7H9 broth Medium Himedia-Middle Brook 7H9 Broth base (M198-500G & Middle Brook OADC Growth Supplement-FD019) [15,16].

Then both the inoculated medium was incubated in BOD Incubator at 37˚C for 12 weeks duration and documentation was done. Both medium were observed for presence of any growth in both medium at every week preferably on Monday. After completion of culture processing, good quality smear was made from the deposit for Acid Fast Bacilli Staining. Then AFB staining by Zeihl Neelsen Technique was done in the smear. Then the smear was examined under oil immersion field (100X).Then all sputum samples were processed for CBNAAT. The results were documented. Standard Work Precautions and Biomedical Waste Management guidelines were followed strictly.

Good Quality Smear Preparation: A new unscratched slide was selected for smear preparation. Yellow purulent portion of the sputum was picked with a piece of clean wooden stick and oval shaped smear of size about 2x3cm, should neither be too thick nor too thin (printed letters should be just readable through the smear) were prepared.

Preparation of Tri Sodium Phosphate Liquid Transport Medium (TSP): As per the journal of J.Jena and B.N.Panda [10] the Tri Sodium Phosphate Liquid Transport Medium (TSP) was prepared and dispersed in sterile McCartney bottle. The bottles were then stored at room temperature. Each bottle thus prepared, constituted a single unit of transport medium. Then under strict aseptic precaution, the TSP medium was transferred to sterile sputum container and supplied to patients to collect early morning deeply coughed out sputum sample.

Zeihl Neelsen Technique for Acid-Fast Staining: Sediment was taken for smear, air dry, then to be heat fixed. Smear was covered with strong carbol fuchsin (primary stain)- gentle heating for 5-7minutes without letting the stain to boil and dry. Wash the slides with distilled water.

Then decolorized with 25% Sulphuric Acid (decolorizing agent) for 1-3minutes until no more Stains come out. Wash the slide with distilled water, then counterstained with Methylene blue (counterstain) for 30seconds-1minute. Wash the slide with distilled water. Stand slides upright on paper towels to air dry. Do not blot dry. Then examine the slide in microscope at 40X and then 100X using oil immersion lens.

Biochemical Reactions [12,13]: The growth from the both culture media was processed for Niacin Accumulation Test, Nitrate Reduction Test, Thermostable Catalase Test and Urea Hydrolysis Test was documented. *M. tuberculosis* is Positive Niacin accumulation test, Positive Nitrate reduction test, Negative Thermostable Catalse test and Negative Urea Hydrolysis test. Molecular Characterisation and rpoB gene mutation detection by Catridge Based Nucleic Acid Amplification Technique (CBNAAT) [21,22,23] was done for all Sputum samples.

Results and Discussion

Zeihl Neelsen Staining in–Concentrated Sputum: The study of 150 sputum samples found that 113 samples, or 75.3%, tested negative for tuberculosis. The bulk of positive samples were classified as Positive-2+, with 20 (13.3%), followed by Positive-3+, with 11 (7.3%), and Positive-1+, with 6 (4.1%). This distribution demonstrates that a large number of the samples were negative, but a smaller subset showed varied levels of positive, indicating the presence of *Mycobacterium tuberculosis* to varying degrees (Table.1).

Table 1: Sputum Grading Distribution for Tuberculosis Diagnosis

Grading of sputum (RNTCP)	No. of samples (n=150)	Percentage (100%)
Negative	113	75.3
Positive-1+	6	4.1
Positive-2+	20	13.3
Positive-3+	11	7.3

Culture in Solid Egg Based Medium – Lowenstein Jensen medium: The results of 150 sputum samples cultivated on Lowenstein-Jensen (LJ) medium indicated a variety of growth patterns consistent with Mycobacterium TB.

The bulk of the samples (102, 68.0%) showed no growth, indicating either the absence of *Mycobacterium tuberculosis* or levels too low to be identified by the medium. Among the samples that showed bacterial growth, 14 (9.3%) showed colonies forming within 4 to 6 weeks, indicating a reasonably rapid proliferation of the bacteria.

A larger selection of 19 samples (12.7%) showed growth between 7 and 9 weeks, indicating a moderate growth rate.

Finally, 15 samples (10.0%) showed bacterial growth after a 10- to 12-week incubation period, which could indicate slower-growing strains or lower initial bacterial concentrations (Table.2).

Table 2: Growth Patterns in LJ Medium for Tuberculosis Diagnosis

Growth in LJ medium	No. of samples (n=150)	Percentage (100%)
No growth	102	68.0
Growth at 4 to 6wk	14	9.3
Growth at 7 to 9wk	19	12.7
Growth at 10 to 12wk	15	10

Among the 150 persons of screening population, 48 samples showed growth of *Mycobacterium tuberculosis* in Lowenstein Jensen medium [14] (culture positivity – 32%). On statistical analysis using One Way ANOVA test, culture in Lowenstein Jensen medium in the concentrated sputum sample significantly showed the growth (positivity) of *Mycobacterium tuberculosis*.

Additionally contaminated growth was not noted in LJ Medium. The average mean detection time for the isolation of *M.tuberculosis* in LJ Medium was 8 Weeks in present study. The samples which showed positivity in Zeihl Neelsen staining technique in the concentrated sputum sample showed culture positivity in Lowenstein Jensen medium and it confirms the good correlation between the smear microscopy and solid egg based culture medium.

Liquid Medium-Middle Brook 7H9 Broth: The growth patterns of 150 sputum samples cultivated in MB7H9 broth were varied, demonstrating the behaviour of Mycobacterium TB in this medium. A considerable proportion of the samples, 96 (64.0%), exhibited no growth or were contaminated, indicating the absence of live bacteria, sample handling difficulties, or medium sterility.

Among the samples that did develop, 32 (21.3%) demonstrated bacterial proliferation within 3 to 4 weeks, indicating that *Mycobacterium tuberculosis* grew relatively quickly in these instances.

Another 17 samples (11.3%) showed growth between 5 and 6 weeks, indicating a moderate growth rate. A smaller selection of 5 samples (3.3%) demonstrated growth after 6 to 7 weeks, indicating slower bacterial multiplication (Table.3).

Table 3: Growth Patterns in MB7H9 Broth for Tuberculosis Diagnosis

Growth in MB7H9 BROTH	No. of samples (n=150)	Percentage (100%)
No growth and contamination	96	64.0
Growth at 3 to 4wk	32	21.3
Growth at 5 to 6wk	17	11.33
Growth at 6 to 7wk	5	3.33

Among the 150 persons of screening population, 54 samples showed growth of *Mycobacterium tuberculosis* in Liquid Culture Medium-Middle Brook 7H9 Broth [17] (culture positivity -36%).

On statistical analysis using One Way ANOVA test, culture in Middle Brook 7H9 Broth medium in the concentrated sputum sample significantly showed the growth (positive) of *Mycobacterium tuberculosis* than the solid culture medium (Lowenstein Jensen Medium) But 2 samples showed the contaminated growth in Middle Brook 7H9 Broth medium (confirmed by doing ICT & Spot inoculation in BAP [12,13]). The average mean detection time for the isolation of M.tuberculosis in Middle Brook 7H9 Broth medium was 4 weeks. The samples which showed positivity in Zeihl Neelsen staining technique in the concentrated sputum sample showed culture positivity in Middle Brook 7H9 Broth medium and it again confirms the good correlation between the smear microscopy and liquid based culture medium.

Molecular Characterisation and rpoB gene mutation detection by Catridge Based Nucleic Acid Amplification Technique (CBNAAT) [21,22,23,24] were done for all Sputum samples in which one sample showed the Rifampicin Resistance rpoB gene mutation in CBNAAT (Table.4).

Table 4: Comparison of Sputum Microscopy, Solid & Liquid Culture and CBNAAT

	ZN Method	LJ Medium	Middle Brook 7H9 BROTH	CBNAAT
Positive	37(25%)	48(32%)	54(36%)	54(36%) (rpoB gene Mutation Detected in One out of 54 samples)
Negative	113(75%)	102(68%)	96(64%)	96(64%)

Limitation of Present Study:

- Using the conventional culture methods which showed the slow growth of *M. tuberculosis*.
- Rifampicin Drug Resistance could not be detected rapidly in these conventional culture methods.

Conclusion

This study confirmed that AFB microscopy by Zeihl Neelsen technique after sputum concentration and culture methods (statistically) significantly detects the positivity in sputum samples. It also confirmed the good correlation between the smear microscopy and culture medium. Liquid medium Middle Brooks 7H9 broth gave the earlier recovery than LJ medium. In solid medium LJ medium, contamination was not observed, but in liquid medium contamination was observed.

The CBNAAT is the very useful earliest rapid diagnostic test to diagnose M.tuberculosis and detect the Rifampicin Resistance rpoB gene

mutation and to prevent the emergence of Drug Resistant Tuberculosis (MDR TB &XDR TB).

Acknowledgement: The authors are thankful to all the staffs in the Department of Microbiology, Thanjavur Medical College for their timely extraordinary help in conducting this study and writing this manuscript.

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