Original Article

Culture positivity of smear negative pulmonary and extrapulmonary tuberculosis- A study from North Kerala, India

Shabana Orvankundil[1], Beena P. Jose[1], Fahmiya L. Yacoob[2], Sreelatha Sreenivasan[3]

[1]Department of Microbiology, Government Medical College, Kozhikode, Kerala, [2]Department of Microbiology, Aspira Pathlab and Diagnostics Ltd, Mumbai, Maharashtra, [3]Department of Microbiology, Government Medical College, Thrissur, Kerala, India

Abstract

Introduction: The identification of smear negative pulmonary and extrapulmonary tuberculosis continues to remain a diagnostic challenge. This study was conducted in a tertiary care setup in north Kerala to isolate and identify mycobacteria by culture from radiologically and clinically suspected cases of smear negative pulmonary and extrapulmonary tuberculosis. **Methods:** A total of 200 samples (100 pulmonary and 100 extrapulmonary) were processed and cultured by automated (MB/BacT) and conventional methods. Heat stable catalase test, nitrate reduction test and detection of MPT 64 antigen were done to aid species identification. **Results:** Overall culture positivity was 7% (14 isolates - 8 pulmonary and 6 extrapulmonary) of which 92.9% (13) of the isolates were *Mycobacterium tuberculosis* and 7.1% (1) was *Mycobacterium fortuitum* (identified by molecular typing). Detection rate by automated method was 7% (14) and by conventional method was only 1.5% (3). **Conclusion:** Despite its shortcomings and low positivity, culture still remains the gold standard for the diagnosis of EPTB and SNPT. However, automated liquid cultures have better isolation rates than the conventional LJ culture. Subjecting these isolates to rapid diagnostic tests like antigen detection and LPA can aid in the early institution of appropriate treatment regimen.

Keywords: Culture positivity, smear negative, tuberculosis

Introduction

Tuberculosis (TB) is a major contributor of ill-health worldwide and has continued to remain a global health problem since its discovery. In 2014, the actual number of clinically diagnosed pulmonary TB and extrapulmonary tuberculosis (EPTB) cases notified in India alone comes to an astounding figure of 730600 among 1609547 total notified new and relapse TB cases.[1] Therefore, a significant 45% of the TB epidemic in India is contributed by smear negative pulmonary tuberculosis (SNPT) and EPTB. Most of the EPTB cases are smear negative and constitutes about 15-20% of all cases of TB in immunocompetent patients and in Indian studies, EPTB constitutes 45-56% of all the cases of tuberculosis in persons with AIDS.[2-4]

Though smear-negative TB is less infectious due to its paucibacillary nature, a delay in the diagnosis and treatment of smear-negative, culture-positive TB cases could contribute to increase in disease transmission and morbidity as established by various studies.[5-7] Furthermore, the dual HIV/TB epidemic has complicated the clinical case scenarios of SNPT and EPTB.[8]

Address for correspondence: Dr. Shabana Orvankundil, Department of Microbiology, Government Medical College, Kozhikode, Kerala, India.
E-mail: shabana.unais@gmail.com

Received: 27-05-2019 **Revised:** 06-06-2019 **Accepted:** 12-07-2019

Access this article online

Quick Response Code:

Website: www.jfmpc.com

DOI: 10.4103/jfmpc.jfmpc_424_19

How to cite this article: Orvankundil S, Jose BP, Yacoob FL, Sreenivasan S. Culture positivity of smear negative pulmonary and extrapulmonary tuberculosis- A study from North Kerala, India. J Family Med Prim Care 2019;8:2903-7.

Direct smear microscopy which forms the cornerstone of TB control programs in middle and low income countries has a poor track record in EPTB, paediatric TB and in patients co-infected with HIV and TB, due to reduced bacillary load in these patients.[9,10]

Though smear negative TB is primarily diagnosed by thorough history and physical examination along with radiological and laboratory evidences suggestive of TB, the misdiagnosis rates have been estimated as high as 35-52% without a standardized diagnostic protocol.[11] Thus, the definitive diagnosis of smear negative TB still depends upon culture isolation and identification of mycobacteria.

Smear negative TB is a relatively neglected area of study compared to smear positive TB. This study, to the best of our knowledge, is the first among published articles to deal with isolation and identification of mycobacteria from SNPT and EPTB in the state of Kerala.

Methods

A total of 200 samples (100 pulmonary and 100 extrapulmonary), which were smear negative for acid fast bacilli (AFB) from patients with clinical and radiological features highly suggestive of pulmonary TB and EPTB, were included in this study. Samples from patients who were already on anti-TB drugs were excluded. Other relevant co-morbid conditions, blood investigations (total count, ESR, RBS and HIV testing) and radiological investigations were recorded where necessary. Patients with positive culture were followed up for clinical response to anti-TB drugs.

Specimen collection

Pulmonary samples and extrapulmonary samples were collected in leak proof sterile containers and under aseptic precautions, wherever necessary. The samples were processed as soon as possible. In case of delay, they were refrigerated at 4°C for not more than 24 hours and then processed accordingly.

Specimen processing

All specimen processing and inoculation was done in Biosafety cabinet class II A2. Direct smears prepared from the specimens were stained by Ziehl-Neelsen (ZN) technique. Specimens from sterile sites were inoculated directly without decontamination. Urine samples were centrifuged at 3000 g for 15 minutes and the deposits were used for further processing. Tissue biopsy specimens were ground using sterile mortar and pestle with minimum amount of sterile distilled water before decontamination. Specimens were decontaminated and concentrated using Modified Petroff's method.[12] In this study, double the volume of 4% sodium hydroxide was used for pulmonary samples, whereas, equal volume of 2% sodium hydroxide was used for decontaminating extrapulmonary samples.

Culture

Each processed sample was inoculated onto two Lowenstein – Jensen medium (LJ) slopes as well as automated MB/BacT liquid culture bottles and incubated at 37 °C. The LJ medium was checked daily for 7 days and weekly thereafter for 12 weeks. LJ media with contamination i.e. growth other than mycobacteria, were discarded after confirmation by ZN and gram staining.

Samples from MB/BacT bottles flagged positive were confirmed for growth of AFB by ZN staining and gram staining (to exclude contamination). Subculture was done on LJ medium from positive bottles. In this study, MB/BacT bottles were declared negative for AFB only after incubation for an extended period of 60 days, as the samples collected were smear negative.

Identification of the isolates

Preliminary identification of the isolates was done from LJ medium. Heat stable catalase test was done to differentiation between *Mycobacterium tuberculosis* complex (MTC) and nontuberculous mycobacteria.[12] Nitrate reduction test was done to differentiation between *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

The Detection of MPT64 antigen for MTC was done by immunochromatographic method (SD BIOLINE TB Ag MPT64 Rapid) as per the manufacturer's instructions. The nontuberculous mycobacterium was subjected to molecular typing for identification at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

Drug susceptibility testing

It was done by proportion method on drug incorporated LJ medium.[12] The concentration of the drugs used were 0.2 mg/L, 40 mg/L, 4 mg/L, and 2 mg/L for INH, RMP, EMB, and SM, respectively.

The isolates were taken to Intermediate Reference Laboratory, Thiruvananthapuram for performing LPA (GenoType MTBDR*plus*, Hain Lifescience, Nehren, Germany) to confirm sensitivity pattern to INH and RMP.

Results

A total of 200 smear negative pulmonary and extrapulmonary samples (100 each) received during the study period of 1 year. Pulmonary specimens included 88 sputum samples and 12 bronchial washings. Pleural fluid, urine and tissue biopsy samples constituted the major share of extrapulmonary specimens [Figure 1].

Culture and identification of isolates

Overall culture positivity was 7% in smear negative tuberculosis (14 isolates). Culture positivity in SNPT was 8%

and in EPTB was 6%. Of the 8 culture positive pulmonary samples, 7 were sputum and 1 was bronchial washings. The 6 culture positive extrapulmonary samples are shown in Table 1.

Of the 14 isolates, one was positive for heat stable catalase and negative for MPT 64 antigen which suggested atypical mycobacterium. It was identified to be *Mycobacterium fortuitum* after subjecting to RT-PCR and DNA sequencing in a reference laboratory. The rest of the 13 isolates (92.9%) were positive for MPT 64 antigen indicating infection by MTC species. Nitrate reduction test was positive for all 13, which excluded infection by *Mycobacterium bovis*.

Solid vs automated liquid culture

Isolation rate by automated MB/BacT liquid culture was 7% and by conventional method on LJ media was only 1.5%. The isolation of AFB by automated culture and LJ media from various specimens have been given in Table 2. The mean time for detection of AFB was 34.4 days by automated method as compared to 56.3 days by LJ culture.

Of the 200 specimens, contamination in both automated and LJ medium was seen in 5 samples. Additional 7 samples grew contaminants on LJ medium. The contamination rate by automated method was 7% (14 samples) and by solid LJ culture was 10.5% (21 samples).

Drug sensitivity testing

All the 13 *M. tuberculosis* isolates were sensitive to INH, RMP, EMB and SM. The LPA also showed concordant results with all 13 samples showing sensitive pattern to INH and RMP.

Epidemiological profile

64% of the study group comprised of males with a male: female ratio of 1.78:1. Majority (72%) of the patients were in the age range of 21-60 years [Figure 2]. HIV co-infection was present in 17 patients with SNPT and 1 patient with EPTB. Of the 18 samples from HIV infected patients (17 sputum samples and 1 bone marrow), the isolation rate was 16.7% (3 samples) and all 3 were isolated from sputum.

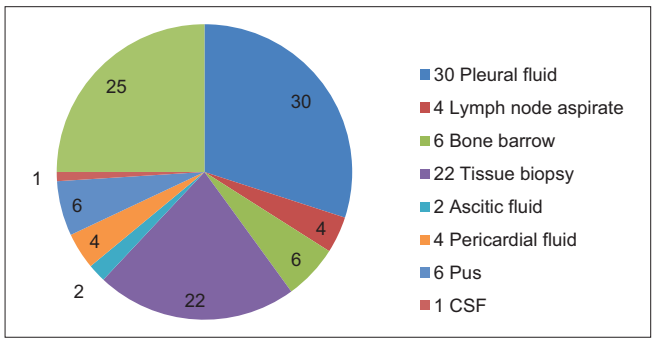

Figure 1: Description and number of the 100 extrapulmonary samples processed

At the same time, out of the 100 suspected SNPT, 97 had Chest X-ray (CXR) findings consistent with TB and 3 had non-specific findings.

Discussion

In the present study, the culture positivity of SNPT was 8%. As mentioned earlier, this subgroup of TB patients can act as a source of disease transmission. It is a well-known fact that among the MTC species, *M. tuberculosis* is the most common

Table 1: Culture positive specimens *M. fortuitum was isolated from urine sample

Specimen	Number of isolates
Sputum	7
Bronchial washings	1
Skin biopsy	1
Rectal biopsy	1
Pleural fluid	1
Pus from pelvic abscess	1
Pus from lumbar abscess	1
Urine	1
Total isolates	14

Table 2: Isolation by conventional LJ culture and automated MB/BacT liquid culture from various samples

Specimen type	Total number proceeded	Isolation by MB/BacT	Isolation by LJ media
Sputum	88	7	3
Bronchial washings	12	1	-
Bone marrow	6	-	-
Tissue biopsy	22	2	-
Ascitic fluid	2	-	-
Pericardial fluid	4	-	-
Lymph node aspirate	4	-	-
Pus	6	2	-
Pleural fluid	30	1	-
Urine	25	1	-
CSF	1	-	-
Total	200	14 (7%)	3 (1.5%)

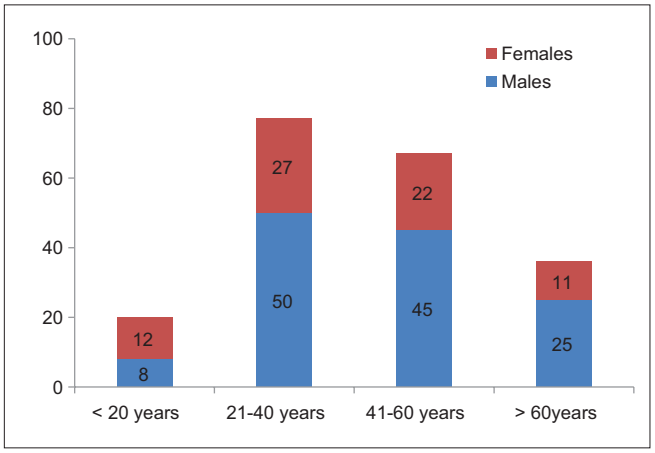

Figure 2: Age and Gender distribution of the study population

cause of human infection worldwide. In the Indian setup, *M. bovis* has been implicated in human infection too.[13,14] Apart from these two, there have been no reports of cases from India due to other species of the *M. tuberculosis* complex, except for a single case report from New Zealand of transmission of *M. orygis* from a woman of Indian origin to a cow.[15] Hence, with the exclusion of *M. bovis* by nitrate reduction test in all the 13 isolates positive for MPT 64 TB antigen, we can conclude with high possibility that all the 13 isolates are *M. tuberculosis* species. Immunochromatographic assay kit for MPT 64 TB antigen is a simple, reliable, rapid identification kit with 97-100% sensitivity and specificity, as demonstrated by various studies, which can markedly reduce the turn-around time in MTC identification and aid in the proper management of atypical mycobacterial infections.[16-18]

Out of the 6 EPTB isolates in this study, one urine sample isolate, negative for MTC by immunochromatographic test, was identified as *Mycobacterium fortuitum* after molecular typing from Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. Urinary tract infection can be rarely caused by *M. fortuitum* and a case of nephritis mimicking renal tuberculosis have also been reported.[19,20] *M. fortuitum* is a common saprophyte found in environmental and nosocomial sources.

Solid vs automated liquid culture comparison

Various studies have already demonstrated earlier time of detection, better isolation rates and lower contamination rates with automated liquid culture as compared to conventional culture for SNPT and EPTB similar to the present study.[21-24] A multicentric study from India demonstrated that automated liquid culture system had significantly higher sensitivity than solid LJ culture in pulmonary TB patients, independent of their HIV status. It also demonstrated significant reduction in the time to detect mycobacteria compared to LJ culture, thus speeding up the final diagnosis of both pulmonary and EPTB.[25]

Drug susceptibility testing

All the 13 *M. tuberculosis* isolates were sensitive to the 4 first line anti-TB drugs by proportion method on LJ medium. In addition, LPA was also performed on the 13 culture isolates which showed sensitive pattern to INH and RMP, thus demonstrating good concordance with conventional proportion method. All 13 patients clinically improved on first line anti-TB drugs (INH, RMP, EMB, SM and pyrazinamide) under the 6 month regime of DOTS (directly observed treatment short course). Studies have shown primary drug resistant tuberculosis to be low in Kerala and in this study, no drug resistance was found in the 14 smear negative TB isolates to INH, RMP, EMB and SM.[26,27] A major limitation of this study was that drug sensitivity testing to pyrazinamide could not be performed.

Epidemiological profile

As seen from Figure 2, this study population comprised of 128 males and 72 females. Other Indian studies have also shown similar male predominant pattern in tuberculosis which can be attributed to the gender dependent sociocultural factors, affecting the risk of exposure to TB and the ease of access to healthcare facility.[26,28] In this study, 16.7% of HIV co-infected patients gave positive cultures from smear negative sputum samples. In India, the magnitude of smear negative TB in HIV infected patients is underestimated and there is a substantial diagnostic delay which increases the morbidity and mortality.[25]

CXR consistent with pulmonary TB were observed in 97 patients and 3 patients had non-specific findings but the latter group had clinical features highly suggestive of TB (evening rise of temperature, night sweats, weight loss, fatigue) with raised ESR. Culture was positive in one of these 3 patients with non-specific findings who was also HIV positive. Radiological findings may be altered in the presence of HIV co-infection proportional to the degree of immunosupression.[29]

Conclusion

Smear negative TB which are paucibacillary forms pose a challenging issue in the diagnosis and management of TB. Culture, despite its shortcomings and low positivity, still remains the gold standard for the diagnosis of EPTB and SNPT. However, automated liquid cultures have better isolation rates than the conventional LJ culture. Subjecting these isolates to rapid diagnostic tests like antigen detection and LPA can aid in the early institution of appropriate treatment regimen. For a wholesome effort to stop TB transmission, primary care physicians should be sensitized to the role of culture in the diagnosis of EPTB and SNPT and more research initiatives in this direction is a necessary step in the combat against TB.

Acknowledgements

Dr. Shabana is thankful to Dr. Praveen Shankar, IRL, Thiruvananthapuram, and Dr. Sathish Mundayoor, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, for their unreserved help in molecular studies. Dr. Shabana is also grateful to Dr. Rema Devi S, Dr. Junais, Dr. Aquil, and Ms. Lolitha for their co-operation and suggestions during the study. Dr. Shabana and Dr. Fahmiya would also like to thank Dr. Saradadevi K.L., Dr. Biju George, Dr. Muhammed Unais, Dr. Jaishid Ahdal and Dr. N.A. Uvais for their sincere support.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. World Health Organization. Global Tuberculosis Report 2015. Geneva, Switzerland: World Health Organization; 2015.
2. Sharma SK, Mohan A. Extrapulmonary tuberculosis. Indian

J Med Res 2004;120:316-53.

3. Sharma SK, Mohan A, Gupta R, Kumar A, Gupta AK, Singhal VK, *et al.* Clinical presentation of tuberculosis in patients with AIDS: An Indian experience. Indian J Chest Dis Allied Sci 1997;39:213-20.
4. Kumar P, Sharma N, Sharma NC, Patnaik S. Clinical profile of tuberculosis in patients with HIV infection/AIDS. Indian J Chest Dis Allied Sci 2002;44:159-63.
5. Tostmann A, Kik SV, Kalisvaart NA, Sebek MM, Verver S, Boeree MJ, *et al.* Tuberculosis transmission by patients with smear-negative pulmonary tuberculosis in a large cohort in the Netherlands. Clin Infect Dis 2008;47:1135-42.
6. Hernandez-Garduno E, Cook V, Kunimoto D, Elwood RK, Black WA, FitzGerald JM. Transmission of tuberculosis from smear negative patients: A molecular epidemiology study. Thorax 2004;59:286-90.
7. Pollett S, Banner P, O'Sullivan MV, Ralph AP. Epidemiology, diagnosis and management of extra-pulmonary tuberculosis in a low-prevalence country: A four year retrospective study in an Australian tertiary infectious diseases unit. PLoS One 2016;11:e0149372.
8. World Health Organization. Improving the diagnosis and treatment of smear-negative pulmonary and extrapulmonary tuberculosis among adults and adolescents. Recommendations for HIV prevalent and resource-constrained settings; 2007.
9. Desikan P. Sputum smear microscopy in tuberculosis: Is it still relevant? Indian J Med Res 2013;137:442-4.
10. Parsons LM, Somosköví Á, Gutierrez C, Lee E, Paramasivan CN, Abimiku AL, *et al.* Laboratory diagnosis of tuberculosis in resource-poor countries: Challenges and opportunities. Clin Microbiol Rev 2011;24:314-50.
11. Mello CD, do Valle Bastos LG, Soares SL, Rezende VM, Conde MB, Chaisson RE, *et al.* Predicting smear negative pulmonary tuberculosis with classification trees and logistic regression: A cross-sectional study. BMC Public Health 2006;6:1.
12. Revised National TB Control Programme, Training Manual for Mycobacterium tuberculosis Culture and Drug susceptibility testing, Central TB Division, Ministry of Health and Family Welfare, New Delhi, India, 2009April.
13. Mishra A, Singhal A, Chauhan DS, Katoch VM, Srivastava K, Thakral SS, *et al.* Direct detection and identification of Mycobacterium tuberculosis and Mycobacterium bovis in bovine samples by a novel nested PCR assay: Correlation with conventional technique. J Clin Microbiol 2005;43:5670-8.
14. Shah NP, Singhal A, Jain A, Kumar P, Uppal SS, Srivastava MVP, *et al.* Occurrence of overlooked zoonotic tuberculosis: Detection of Mycobacterium bovis in human cerebrospinal fluid. J Clin Microbiol 2006;44:1352-8.
15. Dawson KL, Bell A, Kawakami RP, Coley K, Yates G, Collins DM. Transmission of Mycobacterium orygis (M. tuberculosis Complex Species) from a tuberculosis patient to a dairy cow in New Zealand. J Clin Microbiol 2012;50:3136-8.
16. Park MY. Evaluation of an immunochromatographic assay kit for rapid identification of Mycobacterium tuberculosis complex in clinical isolates. J Clin Microbiol 2009;481-4.
17. 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.
18. Kumar VGS, Urs TA, Ranganath RR. MPT 64 antigen detection for rapid confirmation of M. tuberculosis isolates. BMC 2011;4:79.
19. Serra C, Loi G, Saddi B, Pautasso M, Manzin A. Unusual clinical presentation of Mycobacterium fortuitum infection in an immunocompetent woman. J Clin Microbiol 2007;45:1663-5.
20. Oren B, Raz R, Hass H. Urinary Mycobacterium fortuitum infection. Infect 1990;18:105-6.
21. Garcia FG, Angulo GP, Garcia FG, Quero JH, Maroto Vela MC, Ureña JR. Evaluation of the MB/BACT automated mycobacteria culture system versus culture on Lowenstein medium. Clin Microbiol Infect 2008;4:339-43.
22. Concepcion F, Ang R, Myrna T, Mendoza M, Bulatao W, Cajucom M. Culture isolation of mycobacteria by MB/BacT system compared to Lowenstein Jensen egg medium culture method. Philipp J Microbiol Infect Dis 2001;30.
23. Mahadev B, Srikantaramu N, James P, Mathew PG, Bhagirathi R. Comparison between rapid colorimetric mycobacterial isolation and susceptibility testing method and conventional method using LJ medium. Ind J Tub 2001;48:129-34.
24. Mirovic V, Lepsanovic Z. Evaluation of the MB/BacT system for recovery of mycobacteria from clinical specimens in comparison to LJ medium. Clin Microbiol Infect 2002;8:709-14.
25. Lagrange PH, Thangaraj SK, Dayal R, Despande A, Ganguly NK, Girardi E, *et al.* A toolbox for tuberculosis diagnosis: An Indian multicentric study (2006-2008): Microbiological results. PLoS One 2012;7:e43739.
26. Yacoob FL, Philomina Jose B, Karunakaran Lelitha SD, Sreenivasan S. Primary multidrug resistant tuberculosis and utility of line probe assay for its detection in smear-positive sputum samples in a tertiary care hospital in South India. J Pathog 2016;Article ID 6235618:8 pages. doi: 10.1155/2016/6235618.
27. Joseph MR, Shoby CT, Amma GR, Chauhan LS, Paramasivan CN. Surveillance of anti-tuberculosis drug resistance in Ernakulam District, Kerala State, South India. Int J Tuberc Lung Dis 2007;11:443-9.
28. Mukherjee A, Saha I, Sarkar A, Chowdhury R. Gender differences in notification rates, clinical forms and treatment outcome of tuberculosis patients under the RNTCP. Lung India 2012;29:120-2.
29. van Cleeff MR, Kivihya-Ndugga LE, Meme H, Odhiambo JA, Klatser PR. The role and performance of chest X-ray for the diagnosis of tuberculosis: A cost-effectiveness analysis in Nairobi, Kenya. BMC Infect Dis 2005;5:111.