Evaluation of Para-Nitrobenzoic Acid to Differentiate Mycobacterium Tuberculosis from other Mycobacteria

Rajamanickam Venkata Laxmi[1]*, Seriseni Gouthami [2], Payala Vijayalakshmi[3]

1. Department of Microbiology, Government Medical College, Wanaparthy, Telangana, India.
2. Department of Pathology, Dr. Patnam Mahender Reddy Institute of Medical Sciences (PMRIMS), Chevella, Telangana, India.
3. Department of Microbiology, GITAM Institute of Medical sciences and Research, GITAM Deemed to be University, Visakhapatnam, Andhra Pradesh, India.

Abstract

For definitive diagnosis of pulmonary tuberculosis, Mycobacterium tuberculosis must be isolated and identified from patient sample.

The objective of the research is to describe the usefulness of LJ medium containing PNB to identify Mycobacterial tuberculosis in a resource-limited setting.

A prospective study was done in a Teaching hospital, Chevella District, from Dec 2017 to 2019. Overall, 468 samples were referred to central laboratory from various departments in those cases suspecting TB. For sputum, 3 to 5ml three samples were collected, first day spot, on second day and third day early morning sputum samples. In case of swollen superficial lymph node specimen, FNAC investigation was done with a sterile 21-gauge needle carrying syringe. FNA cytology by Haematoxylin and eosin stain and Ziehl-Neelsen stain were performed with aspirates. Lowenstein-Jensen (LJ) medium was used for culturing of smear positive samples.

Out of total 468 samples, 162 (34.62%) samples showed AFB on Ziehl-Neelsen staining. Among 162 AFB positive samples, culture positive were 93.21%, 3.09% culture negative and 3.70% were contaminated. The isolates which showed no growth on LJ medium without PNB doesn't show growth on LJ medium with PNB.

For the control of TB, early diagnosis, efficient treatment are required which will minimising the burden of disease.

Experimental article (J Int Dent Med Res 2022; 15(3): 1405-1409)

Keywords: AFB, LJ Medium, PNB, Mycobacterium tuberculosis, R.R district.

Received date: 23 April 2022 **Accept date:** 25 May 2022

Introduction

Tuberculosis (TB) is one of the public health issue globally, causing illness in millions of people every year, predominantly in low- and middle-income countries with the highest mortality rates. [1, 2] Nearly 1/3rd world population was effected by TB. [3] The WHO's End TB Strategy has set lofty targets for 2020–2035, including a 35% decrease in total TB mortality and a 20% drop in TB incidence by 2020 compared to 2015. [4] Mycobacterium tuberculosis is an acid-fast, non-motile, non-spore forming, obligate aerobe bacillus that primarily affects the apex of the lungs, causing pulmonary tuberculosis (PTB). A single cough or sneeze can produce a large number of infectious droplets, with as few as 10 bacilli causing infection. [5, 6, 7] Mycobacterioses are infections caused by non-tuberculosis Mycobacteria (NTM), which are becoming more common as the prevalence of immunocompromised hosts such as AIDS rises. As a result, distinguishing non-tuberculosis Mycobacteria (NTM) from Mycobacterium tuberculosis is critical. [3] Tuberculosis is a poverty-related illness, which explains why it affects different populations in different ways. Bad accommodation, nutritional insecurity, financial problems, illiteracy, and unfavourable psycho-social circumstances are among the main determinants of tuberculosis (TB) and, as a result, impair sick people's ability to access healthcare services. [4] Vulnerable groups include HIV-

*Corresponding author:
Dr. R. Venkata Laxmi,
Associate Professor, Department of Microbiology,
Government Medical College, Wanaparthy,
Telangana, India.
E-mail: drlaxmi04@gmail.com,
rajamanickamvenkatalaxmi123@gmail.com

positive individuals, inmates, homeless people, migrants/refugees, and drug or heavy alcohol users. [4] Diabetes, suffocation, being overweight or obese are all risk factors for tuberculosis infection. Healthcare staff are at a high risk of contracting tuberculosis. People infected with HIV/AIDS (PLHIV) are at an extremely high risk of contracting tuberculosis (TB). Those who live in the same house are at a higher risk than those who have only casual contact. Contact tracing has gained importance in recent years, and it is now included in the Government of India's Revised National Tuberculosis Control Programme (RNTCP). [8, 9] According to recommended joint TB/HIV therapies, all newly diagnosed TB patients should be aware of their HIV status. [4, 10] To establish a conclusive diagnosis of pulmonary tuberculosis (TB), Mycobacterium tuberculosis must be isolated and identified from respiratory specimens, most often expectorated or induced sputa. [11] Despite the availability of new technology for tuberculosis diagnosis, AFB microscopy and culture remain the gold standard for tuberculosis diagnosis. [12] Culturing mycobacterium tuberculosis remains the gold standard for the laboratory diagnosis of pulmonary tuberculosis, with 9 million new cases and 1.5 million deaths mainly in developing countries. [13] Many clinical laboratories use Lowenstein-Jensen (L-J) medium, a solid medium, to detect the presence of Mycobacterium tuberculosis. [14] The most effective approaches for preventing TB transmission and reducing its occurrence are rapid case detection and early treatment. Screening high-risk groups to detect tuberculosis early has been shown to be successful in reducing the global TB epidemic. [15] Isolation of Mycobacterium tuberculosis from a sputum culture is still recommended to confirm the diagnosis of pulmonary tuberculosis, according to existing treatment recommendations. [16] In 1997, India implemented the World Health Organization-recommended Directly Observed Treatment, Short Course (DOTS) as part of its Revised National Tuberculosis Control Program (RNTCP). [17] One of the most important aspects of tuberculosis prevention is identifying infectious patients. This is due to the fact that each person with untreated tuberculosis infects 10-15 people each year. DOTS, the global tuberculosis control technique, relies solely on AFB microscopy to identify outbreaks, rather than a more precise approach such as culture determination. [18] Early diagnosis and initiation of therapy are also affected by delay of patient and health system delays. Patient and health-care system delays also hinder early diagnosis and treatment initiation. Patient delay has been linked to poverty, drug abuse, rural living, limited access to health care, and a lack of awareness about tuberculosis. [19] Tuberculosis (TB) has a well-known causative agent, pathogenesis, and treatment, and it is currently treatable. However, because of the high mortality rate associated with this disease, tuberculosis remains a major public health concern worldwide. [20] Breaking the chain of infection and preventing the spread of disease would be aided by timely and prompt laboratory results. To effectively regulate tuberculosis, the primary form of infection, pulmonary tuberculosis, must be diagnosed early and treated effectively. [21] According to recommended joint TB/HIV treatments, all newly diagnosed TB patients should be aware of their HIV status. [4] The objectives to end the global tuberculosis epidemic were mentioned in (Table-1).The aim of the present research is to describe the usefulness of LJ medium containing PNB to identify mycobacterial tuberculosis in a resource-limited setting.

GOAL	End the global tuberculosis epidemic
MILESTONES FOR 2025	–75% reduction in tuberculosis deaths (compared with 2015); –50% reduction in tuberculosis incidence rate (compared with 2015) (less than 55 tuberculosis cases per 100 000 population) –No affected families facing catastrophic costs due to tuberculosis
TARGETS FOR 2035	–95% reduction in tuberculosis deaths (compared with 2015) –90% reduction in tuberculosis incidence rate (compared with 2015) (less than 10 tuberculosis cases per 100 000 population) –No affected families facing catastrophic costs due to tuberculosis.

Table 1. Goals to end the global tuberculosis epidemic.

Materials and methods

This is a prospective study conducted in Teaching hospital, Chevella District, from December 2017 to December 2019. In this study, a total of 468 samples were collected from patients referred to central laboratory from various departments in those cases suspecting TB. The samples were taken with the patients' permission. Sputum samples were obtained three times: on the first day, on the second day, and on the third day, early morning sputum samples. Sputum that was mucopurulent or blood stained was obtained in amounts of 3–5 ml.

Sputum samples were obtained and analysed according to World Health Organization (WHO) guidelines. [3] When the sputum specimen was mainly saliva, it was deemed "unsuitable" for microbiological analysis, and a new specimen was demanded. [4] All sputum specimens were decontaminated and concentrated prior to examination by using the *N*-acetyl-L-cysteine–sodium hydroxide procedure recommended by the Centers for Disease Control and Prevention. [1]

Centrifugation was used to concentrate body fluids, lowering the volume to around 20 ml. [21] Smears on double slides were then prepared. [22] A sterile 21-gauge needle with an attached syringe was used to conduct FNA on a swollen superficial lymph node. 70% alcohol was used to clean the overlying area. The node was then punctured using a syringe with a negative pressure. The needle made several (on average six) in and out passes without leaving the node. Drops of aspirate were put on two clean slides after the needle was removed for FNA cytology using Haematoxylin and eosin stain and ZN stain. The remainder of the sample was cultured in a falcon tube containing sterile natural saline. [23] A drop of aspirate was put on a clean slide and a smear was prepared immediately after the specimen was collected. [23] Following WHO guidelines, the smears were stained with ZN and observed under oil immersion for AFB in a traditional microscope. All smears with ≥ 1 AFB/100 high power fields (HPF) were considered positives. At least 100 microscopic fields were examined to declare a slide negative. Positive AFB stains were quantified as 1+ to 4+ (1+, 1–9 AFB/100 fields: 2+, 1–9 AFB/10 fields: 3+, 1–9 AFB/field: 4+, 9 AFB/field) [16]. Only smear positive samples were cultured on LJ media.

Lymph node Mycobacterial culture

The aspirated material was digested, decontaminated, and concentrated before being used for culture. For digestion and decontamination, the N-acetyl-l-cysteine and sodium hydroxide process (NALC/NaOH) was used. Following that, the specimens were centrifuged at 3500g for 15 minutes and suspended in 2 mL of sterile phosphate buffer saline (PBS) (pH 6.8). After that, the specimen is inoculated onto a Lowenstein–Jensen (LJ) slant. [23] After eight weeks, all specimens inoculated on LJ slants were incubated at 37°C until growth was observed or they were discarded as negative. Visual inspection of colony morphology and microscopic analysis of colonies for acid quick bacilli (AFB) were used to confirm mycobacteria growth, which was then biochemically checked [3]. Following standard procedure, samples positive for AFB by LJ culture were subjected to a para-nitrobenzoic acid (PNB) test to rule out non-tuberculosis mycobacteria (NTM) (PNB resistant). [20]

Para nitro benzoic acid (PBB) test

At a final concentration of 500 g/ml, para nitro benzoic acid was added to the LJ medium. This PNB concentration was chosen based on previous research. [24] In 0.5 mg/ml PNB containing LJ medium, one loopful of 4 mg/ml bacterial growth suspension was inoculated. Inoculating the suspension in PNB-free LJ medium served as a constructive control. All of the slants were incubated at 37°C for 4 weeks and some growth on the medium was observed. [3] Biochemical tests identified the eugonic (rough, tough, and buff) colonies as M. tuberculosis. All isolates cultured in LJ without PNB and then transferred to LJ with PNB showed no growth. [3] Quality controls were carried out in accordance with national guidelines. [15] Regardless of the sputum culture result, sputum smear positive pulmonary tuberculosis was described as having at least one sputum sample showing acid fast bacilli on direct smear microscopy. Culture-positive pulmonary tuberculosis was defined as having at least one sputum culture showing growth of M. tuberculosis, irrespective of sputum smear result. Anyone sputum sample showing acid-fast bacilli on direct smear microscopy and/or growth of M. tuberculosis considered a bacteriologically positive pulmonary tuberculosis. [18] In the presence of the following cytomorphological conditions, cytological analysis of FNA smears was considered a diagnostic method for TBLN: epithelioid cell aggregate with or without Langerhans giant cells and necrosis, epithelioid cell aggregate without necrosis, necrosis without epithelioid cell aggregate, necrosis without epithelioid cell aggregate, or polymorphocytes with necrosis. [23]

Results

Out of 468 specimens, 324 (69.23%)

were from male and 144 (30.77%) were from female patients (Table 2). Among the 468 specimens collected 71.79**%** specimens from respiratory source and 28.21% from non-respiratory sources (Table 3). Table 4 showed the AFB grading based on Ziehl-Neelsen staining method. Out of total 468 samples, 162 (34.62%) samples showed AFB on Ziehl-Neelsen staining (Table 5). Among 162 AFB positive samples, culture positive were 93.21% and 3.09% were culture negative and 3.70% were contaminated (Table 6). Among the 151 culture positive samples, 117 (77.48%) were from male and 34(22.51%) were from female. All the culture positive samples which gave growth on LJ media without PNB were further inoculated on LJ media containing PNB showed no growth (Table 7).

Gender	Number	Percentage (%)
Male	324	69.23
Female	144	30.77
Total	468	100

Table 2. Gender-wise distribution of cases.

Specimens	Number	Percentage (%)
Respiratory	336	71.79
Non-respiratory	132	28.21
Total	468	100

Table 3. Type of specimens.

AFB Grading	Number
1+	36
2+	62
3+	49
4+	15
Total	**162**

Table 4. AFB Grading based on Ziehl-Neelsen staining.

Variable		Total	Positive (number/percentage)	Negative (number/percentage)
Sex	Male	324	125 (38.58%)	199 (61.42%)
	Female	144	4 (19.05%)	107 (74.31%)
Age	<15 years	21	4 (19.05%)	17 (80.95%)
	16-25 years	184	62 (33.70%)	122 (66.30%)
	26-35 years	126	49 (38.89%)	77 (61.11%)
	36-45 years	78	26 (33.33%)	52 (66.67%)
	>45 years	59	21 (35.59%)	38 (64.41%)
	Total	468	162	306

Table 5. Sputum AFB result.

Culture result	Number	Percentage (%)
Culture positive	151	93.21
Culture negative	5	3.09
Contaminants	6	3.70
Total	**162**	**100**

Table 6. Culture results.

	Culture on LJ without PNB			Culture on LJ with PNB	
	Positive	Negative	Contamination	Positive	Negative
Males	117	3	5	0	117
Females	34	2	1	0	34
Total	151	5	6	0	151

Table 7. Categorization of cases based on Gender-wise and culture results on LJ medium.

Discussion

Identification of mycobacterium to the species level is critical because it offers a wealth of epidemiological knowledge and aids in the effective care of patients. Development characteristics (rough and cream colonies) and microscopic observation of cording formation on Ziehl-Neelsen (ZN) stain of a positive culture can be used to make a preliminary distinction between MTC and NTM. However, some NTM also manufacture cording, so this isn't a definite distinction. Conventional tests, such as the niacin test, nitrate reduction, or catalase synthesis, may provide conclusive MTC identification, but these procedures are time consuming. Urinary Lipoarabinomannan (LAM) is also used as a rapid diagnostic test for adult pulmonary Tuberculosis Annisa et al. (2021). [25] The present study is focussed on para-nitrobenzoic acid to differentiate mycobacterium tuberculosis from other mycobacteria.

As per the recommendation, few colonies observed in some tubes were regarded as negative. [18] The eugonic (rough, tough and buff) colonies were confirmed as *Mycobacterium tuberculosis* by biochemical tests. All the isolates in LJ media without PNB which were further cultured on LJ with PNB showed no growth. Tuberculosis is caused by *Mycobacterium tuberculosis* as well as NTM; although clinically they produce a very similar disease. In the study done by Mahadev et al. used LJ media containing PNB for the identification of *M. tuberculosis.* [26] Tadesse et al. in his study used PNB to differentiate *M. tuberculosis*. [23] The importance of smear microscopy and conventional culture cannot be undermined despite the availability of latest diagnostic techniques.

Conclusions

The current "gold standard" for the diagnosis of tuberculosis is Mycobacterial culture. In resource-limited settings where tuberculosis is most prevalent, where facilities for molecular techniques are not readily available, simple, easy, reliable and low-cost test using growth inhibitor para nitrobenzoic acid could be incorporated in the culture media enabling identification and differentiation of *M. tuberculosis* from non-tuberculosis mycobacteria. Timely laboratory results will contribute considerably to break the chain of infection and preventing the spread of disease. For the control of TB, it is necessary that pulmonary TB, which is the primary form of infection, must be diagnosed early and treated effectively thus reducing the burden of diseases.

Acknowledgements

The authors would like to thank the faculty of Department of Microbiology, Dr. Patnam Mahender Reddy Institute of Medical Sciences (PMRIMS), Chevella road, Hyderabad, Telangana for providing me the data for publication.

Declaration of Interest

The authors report no conflict of interest.

References

1. Peierdun M, Li Y, Xue F et al. Prevalence of pulmonary tuberculosis in western China in 2010–11: a population-based, cross-sectional survey. The Lancet Global Health. 2016; 4(7): e485-e494.
2. Afsar I, Gunes M, Er H, Gamze Sener A. Comparison of culture, microscopic smear and molecular methods in diagnosis of tuberculosis. Rev Esp Quimioter. 2018;31(5):435-438.
3. Nepali S, Ghimire P, Khadka DK, Acharya S. Selective inhibition of Mycobacterium tuberculosis by Para-Nitrobenzoic Acid (PNB) used in Lowenstein –Jensen Medium SAARC J. Tuber. Lung Dis. HIV/AIDS. 2008; 5(1): 25-28.
4. Giorgia S, Roggi A, Matteelli A, Mario CR. Tuberculosis: Epidemiology and Control. Mediterr J Hematol Infect Dis. 2014; 6(1): e2014070.
5. MacNeil A, Glaziou P, Sismanidis C, Maloney S, Floyd K. Global Epidemiology of Tuberculosis and Progress Toward Achieving Global Targets-2017. MMWR Morb. Mortal. Wkly. Rep. 2019; 68: 263–266
6. Dunn JJ, Starke JR, Revell PA. Laboratory diagnosis of Mycobacterium tuberculosis infection and disease in children. J Clin Microbiol. 2016; 54:1434 –1441
7. Monika A, Ashish B, Vinay B, Sarjana D. Comparative Study of GeneXpert with ZN Stain and Culture in Samples of Suspected Pulmonary Tuberculosis. Journal of Clinical and Diagnostic Research. 2016; 10 (5): DC09-DC12
8. Singer-Leshinsky S. MSED, RPAC; Pulmonary tuberculosis: Improving diagnosis and management. Journal of the American Academy of Physician Assistants. 2016; 29(2):20-25.
9. Erawati M, Andriany M. The Prevalence and Demographic Risk Factors for Latent Tuberculosis Infection (LTBI) Among Healthcare Workers in Semarang, Indonesia. Journal of Multidisciplinary Healthcare. 2020; 13: 197–206.
10. Singh J, Sankar MM, Kumar S, Gopinath K, Singh N. Incidence and Prevalence of Tuberculosis among Household Contacts of Pulmonary Tuberculosis Patients in a Peri-Urban Population of South Delhi, India. PLoS ONE. 2013; 8(7): e69730.
11. Susan M Nelson, Marcia A Deike, Charles P Cartwright. Value of Examining Multiple Sputum Specimens in the Diagnosis of Pulmonary Tuberculosis. Journal of clinical microbiology. 1998; 36 (2): 467–469.
12. Odubanjo MO, Dada-Adegbola HO. The microbiological diagnosis of tuberculosis in a resource limited setting: Is acid-fast bacilli microscopy alone sufficient? Ann Ib Postgrad Med. 2011;9(1): 24–29.
13. Asmar S, Drancourt M. Rapid culture-based diagnosis of pulmonary tuberculosis in developed and developing countries. Front. Microbiol. 2015;6:1184.
14. Cui Z, Wang J, Zhu C, Huang X, Lu J, et al. Evaluation of a Novel Biphasic Culture Medium for Recovery of Mycobacteria: A Multi-Center Study. PLoS ONE. 2012; 7(4): e36331.
15. Zhang CY, Zhao F, Xia YY et al. Prevalence and risk factors of active pulmonary tuberculosis among elderly people in China: a population based cross sectional Study. Infectious Diseases of Poverty. 2019; 8(1):7.
16. Chang CY, Jen-Yee H, Mei-Kang Y et al. Risk factors in patients with AFB smear-positive sputum who receive inappropriate antituberculous treatment. Drug Design, Development and Therapy. 2013; 7: 53–58.
17. Sathiyamoorthy R, Kalaivani M, Aggarwal P, Gupta SK. Prevalence of pulmonary tuberculosis in India: A systematic review and meta-analysis. Lung India 2020; 37:45-52.
18. Cudahy P, Shenoi S. Diagnostics for pulmonary tuberculosis. Postgraduate Medical Journal. 2016; 92(1086):187–193.
19. Kadioglu EE, Yilmaze E, Araz UO, Aktas E , Saglam L. A Comparison of Two Different Culture Methods for Use in the Diagnosis of Pulmonary Tuberculosis. Eurasian J Med. 2014; 46:74-7
20. Thakur R, Goyal R, Sarma S. Laboratory diagnosis of tuberculous meningitis - is there a scope for further improvement? J Lab Physicians. 2010; 2(1):21-24
21. Martin RS, Sumarah RK, Robart EM. Comparison of Four Culture Media for the Isolation of Mycobacterium Tuberculosis: A 2-Year Study. Journal of clinical microbiology. 1975; 2 (5): 438-440.
22. Ayele B. The Yield of First Spot Double Slide Smears for the Diagnosis of Pulmonary Tuberculosis. Mycobact Dis. 2016; 6 (1): 1000205.
23. Tadesse M, Abebe G, Abdissa K, Bekele A, Bezabih M et al. Concentration of Lymph Node Aspirate Improves the Sensitivity of Acid Fast Smear Microscopy for the Diagnosis of Tuberculous Lymphadenitis in Jimma, Southwest Ethiopia. PLoS ONE. 2014; 9(9): e106726.
24. Babita S , Nita P, Bharti M, Leela V. Evaluation of a Rapid Differentiation Test for Mycobacterium Tuberculosis from other Mycobacteria by Selective Inhibition with p-nitrobenzoic Acid using MGIT 960. Journal of Laboratory Physicians. 2010; 2 (2): 89-92.
25. Annisa K, Atika Mahira Yanfaunnas, Dandy Permana Supandi, RA e al. The use of Urinary Lipoarabinomannan (LAM) as a Rapid Diagnostic Test for Adult Pulmonary Tuberculosis in HIV-Positive Patients: an Evidence-based Case Report. Journal of International Dental and Medical Research. 2021;14 (1):461-466
26. Mahadev B, Srikantaramu N, James P, Mathew PG and Bhagirathi R. Comparrision between rapid colorimetric mycobacterial isolation and susceptibility testing method and conventional method using LJ medium. Indian J. of Tuberc. 2001; 48(3):129.