

SELECTIVE INHIBITION OF *MYCOBACTERIUM TUBERCULOSIS* BY PARA-NITROBENZOIC ACID (PNB) USED IN LOWENSTEIN – JENSEN MEDIUM

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Abstract

Background: Mycobacterial growth in media to which inhibitory substances are added has been used in species identification. *Mycobacterium tuberculosis* does not grow in Lowenstein-Jensen (LJ) medium containing para-nitrobenzoic acid (PNB); which can be a basis for its identification from other mycobacteria.

Setting: National Tuberculosis Centre (NTC), Thimi, Bhaktapur, Nepal.

Objectives: To evaluate usefulness of PNB containing LJ medium in identifying mycobacterial isolates.

Methodology: This diagnostic evaluation study based at NTC was conducted from Sep 2006-Jun 2007. During the study period, a total of 857 sputum samples collected from patients attending NTC were analyzed using fluorescence microscopy. The smear positive samples were confirmed by Ziehl-Neelsen (ZN) staining. Smear positive samples were cultured on LJ media with and without PNB; followed by biochemical tests.

Results: Out of total 857 sputum samples analyzed, 246 (28.7%) were positive for AFB on fluorescence staining and 214 (87%) of smear positive samples were also positive in culture. All the isolates which grow on LJ media without PNB did not grow on LJ media containing PNB.

Conclusion: *Mycobacterium tuberculosis* can be identified and differentiated from non-tuberculous mycobacteria using PNB containing media, easily in labs where culture is being done.

Keywords: PNB, AFB, LJ medium, *Mycobacterium tuberculosis*

Introduction

Globally, two billion people, equal to one-third of the world's population, are infected with tubercle bacilli.¹ In Nepal, about 45% of the total population is infected with tuberculosis

(TB), of which 60% are adult. Every year, 40,000 people develop active TB with 20,000 infectious pulmonary diseases and 5,000 to 7,000 deaths.²

Tuberculosis is a specific infectious disease primarily affecting the apex of the lungs causing pulmonary tuberculosis (PTB). TB of man and animal is caused by a group of very closely related species forming the *Mycobacterium tuberculosis* Complex (MTC), among which *M. tuberculosis* is predominant in human.³ PTB

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can be diagnosed by sputum examination (smear and culture), chest X-ray etc.

The infections caused by non-tuberculous mycobacteria (NTM) are termed mycobacterioses which are becoming more prevalent with the increasing prevalence of immunocompromised hosts, particularly in relation to the AIDS pandemic.⁴ So, it is necessary to differentiate NTM from *M. tuberculosis*. Mycobacterial growth in media to which inhibitory substances are added has been used in species identification. Growth of *M. tuberculosis* is inhibited by PNB, whereas NTM are resistant and can grow in the culture media containing PNB.⁵

Hence this study was conducted with the objective to develop a test using PNB, and to evaluate its usefulness in the identification of *M. tuberculosis*.

Methodology

This is a diagnostic study based at NTC, Thimi, Bhaktapur, Nepal. The rationale of using NTC as study area was because it being the only National Tuberculosis Reference Laboratory in Nepal.

This study was carried out from Sep 2006-Jun 2007.

In this study, a total of 857 sputum samples were collected from patients visiting NTC. The patients were attended by the Medical Officer(s) and referred for microscopy if suspected for TB. The samples were collected with patients consent on first come first basis.

Sputum samples were collected and evaluated as per the standards given by World Health Organization (WHO).⁶ One spot sputum specimen (when the patient first present to the health service) followed by next day early morning sputum specimen and spot specimen (of that day) were collected. About 3–5 ml, mucopurulent or blood stained sputum was collected. When the sputum specimen was

mostly saliva, then it was reported as “unsuitable” for microbiological investigation and requested another specimen. Sputum smears were stained with fluorescence staining technique and examined by fluorescence microscope.⁶ Since fluorescence microscopy uses low magnification objective to scan smears, allowing a much larger area of the smear to be seen and resulting in more rapid examination, it was used as primary staining procedure. All the positive slides were confirmed by Ziehl-Neelsen (ZN) staining. Only smear positive samples were cultured on LJ media. All the tubes were incubated at 37°C until growth was observed or discarded as negative after eight weeks. The colony characteristics of the isolates were recorded and they were biochemically tested. One loopful of 4 mg/ml suspension of bacterial growth was inoculated in 0.5 mg/ml PNB containing LJ medium. The positive control was set up by inoculating the suspension in PNB free LJ medium. All the slants were incubated at 37°C upto 4 weeks and observed for any growth on the medium.⁷

Data analysis: Data were analyzed using SPSS version 11.5 systems.

Results

Of 857 specimens, 585 (68.3%) were from male and 272 (31.7%) were from female patients. Out of total 857 samples, 246 (28.7%) sputum samples showed AFB on fluorescence staining; of which 193 (78.5%) were from male and 53 (21.5%) were from female. Among 246 sputum smear positive samples, 214 (87.0%) samples were culture positive, 20 (8.1%) were culture negative and 12 (4.9%) were contaminated. In 214 culture positive samples, 168 (78.5%) were from male and 46 (21.5%) were from female. All the culture positive patients were registered in the laboratory register to report them to the Medical Officer(s) for treatment.

The eugonic (rough, tough and buff) colonies were confirmed as *M. tuberculosis* by

biochemical tests. All the isolates in LJ media without PNB which were further cultured on LJ with PNB showed no growth. The pattern of culture result with respect to fluorescence

staining is given in table 1 and the result of culture positive samples on LJ medium containing PNB is given in table 2.

Table 1 Pattern of culture results with respect to fluorescence staining

		Fluorescence staining			Total
		1+	2+	3+	
Culture Results	1+	37	7	7	51
	2+	30	10	12	52
	3+	37	43	21	101
	4+	4	0	6	10
Total		108	60	46	214

Table 2 Result of culture positive samples on LJ medium containing PNB

Gender of the patients	Culture on LJ medium without PNB			Culture on LJ medium with PNB	
	Positive	Negative	Contaminated	Positive	Negative
Male	168	15	10	0	168
Female	46	5	2	0	46
Total	214	20	12	0	214

Discussion

Out of 857 sputum samples, 246 (28.7%) samples were positive for AFB by fluorescence microscopy. As per the recommendation of WHO⁶, all the smear positive slides were confirmed by ZN staining. Of 246 sputum samples, 214 (87.0%) samples were positive for culture. Among 214 culture positive samples 168 (78.5%) were from male and 46 (21.5%) were from female. All the culture positive samples which gave growth on LJ media without PNB gave no growth on inoculation and incubation in LJ media containing PNB. As per the recommendation, few colonies observed in some tubes were regarded as negative.⁷

Lowenstein Jensen (LJ) medium containing PNB has been successfully used by many researchers. In a similar study done by Giampaglia *et al*⁵ showed that PNB can be used successfully in the identification of mycobacteria isolates. Mahadev *et al*⁸ had also used LJ media containing PNB for the identification of isolates as *M. tuberculosis*. PNB can also be used with other agar medium to identify *M. tuberculosis*⁹ and can also be

incorporated in rapid techniques like in the rapid Mycobacterium Growth Indicator Tube (MGIT)/PNB method. WHO has also recommended the use of PNB along with other simple biochemical tests for the identification of *M. tuberculosis*.⁶ This study would be helpful in our setting to identify *M. tuberculosis* where culture is regularly done. Finally, this work has also shown that LJ medium containing PNB can be effectively used to identify *M. tuberculosis*-the method is easy, cheaper and reliable.

Conclusion

In conclusion, this study revealed that PNB added to culture media could be used for the identification of *M. tuberculosis*. All the isolates failed to grow when inoculated and incubated in LJ media containing PNB. So, a simple and low-cost test using growth inhibitor may be incorporated in the culture media enabling identification and differentiation of *M. tuberculosis* from non - tuberculous mycobacteria.

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