

RESEARCH NOTE

A SIMPLE METHOD TO DIFFERENTIATE BETWEEN *MYCOBACTERIUM TUBERCULOSIS* AND NON-TUBERCULOUS MYCOBACTERIA DIRECTLY ON CLINICAL SPECIMENS

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Abstract. We report the applicability of testing susceptibility to paranitrobenzoic acid (PNB) directly on clinical samples as a rapid screening assay, to detect *M. tuberculosis* and differentiate it from non-tuberculous mycobacteria (NTM). One hundred smear positive sputum samples from patients with pulmonary tuberculosis attending the Department of Respiratory Medicine at VP Chest Institute, Delhi, were cultured on Löwenstein Jensen medium with and without 0.5 mg/ml paranitrobenzoic acid. Serial concentrations of known cultures of H37Rv, *M. fortuitum*, *M. scrofulaceum* and *M. avium* were used as controls in the study. After 3 weeks of incubation, growth was observed on all the drug free Löwenstein Jensen slants but none of the slants containing PNB, which inhibited the growth of *M. tuberculosis*. The cultures were further confirmed to be *M. tuberculosis* by niacin, nitrate and catalase tests. Direct susceptibility to PNB was thus found to be a simple, cheap and technically feasible method of preliminary identification of *M. tuberculosis* and its effective differentiation from NTM, which may be adapted for use at Level II laboratories, especially in developing countries.

INTRODUCTION

With the dramatic increase in tuberculosis world-wide, laboratory services for diagnosing mycobacterial infection face new challenges (Mathew *et al*, 1999). Although *Mycobacterium tuberculosis* infection is the most common mycobacterial infection, infections due to non-tuberculous mycobacteria (NTM), are on the rise in many countries (Falkinham, 1996). It is important to differentiate between infection due to *M. tuberculosis* and NTM at an early stage in the disease for adequate therapy. Clinical microbiologists require simple, reliable tests to identify *Mycobacterium* sp, and laboratory results need to be accurate and rapid. There is no universally ac-

cepted scheme. Technical practices vary from laboratory to laboratory. Identification of mycobacteria is time consuming; *M. tuberculosis* complex requires 2-3 weeks for growth and additional time for identification using biochemical reactions. Although some new methods, including BACTEC, have successfully reduced the time for culture and sensitivity tests to 2-3 weeks, the high cost of instruments and reagents involved in these methods puts them beyond the reach of peripheral laboratories in developing countries (Venkataraman *et al*, 1998; Mathew *et al*, 1999).

The ability of mycobacteria to grow in the presence of inhibitory substances in a suitable medium has been widely used in the identification of different species (Tsukamura and Tsukamura, 1964; Laszlo and Siddiqi, 1984). It has been reported that growth of *M. tuberculosis* is inhibited by 0.5 mg/ml of paranitro-

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benzoic acid (PNB) while NTM is not (Rastogi *et al*, 1989). Mycobacterial growth in PNB containing medium has been used as a presumptive test for NTM in several laboratories (Martins *et al*, 1997). However, all previous reports are regarding culture isolates of *Mycobacterium* sp. Here we report adaptation of the method for detection and presumptive identification of *M. tuberculosis* complex directly from sputum samples in a single test system.

MATERIALS AND METHODS

Sputum samples from patients with pulmonary tuberculosis attending the Department of Respiratory Medicine at VP Chest Institute, Delhi are routinely examined by Ziehl-Neelsen staining and cultured on Löwenstein Jensen medium, after Petroff's method of decontamination. One hundred randomly selected smear positive sputum samples were cultured on Löwenstein Jensen medium containing p-nitrobenzoic acid at a concentration of 0.5mg/ml and incubated at 37°C. The AFB smears were graded according to WHO recommendations (Salfinger and Pfyffer, 1994). Serial concentrations of known cultures of *M. fortuitum*, *M. scrofulaceum* and *M. avium* were used as positive controls and inoculated on Löwenstein Jensen medium with and without p-nitrobenzoic acid (PNB). Serial concentrations (10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} organisms/ml) of the standard laboratory strain of *M. tuberculosis* H37Rv were used as negative controls. The culture slants were tested for growth at 3, 7, 14 and 21 days. Slants with no growth at 21 days were further incubated for 3 weeks. Positive cultures were subjected to a battery of tests including niacin accumulation, nitrate reduction and catalase tests. Of these, 50 cultures were confirmed to be *M. tuberculosis* complex by BACTEC NAP test (Becton Dickinson Microbiology Systems, Sparks, Md, USA) in addition to conventional biochemical reactions. The cultures were checked every second day and the degree of growth was ex-

pressed in terms of growth index. The vials were reincubated till a growth index of 50 or more was reached and then 1 ml of the culture was inoculated into vials with discs of p-nitro-alpha-acetylamino-propiofenone (NAP). A daily decrease in growth index or an initial increase followed by no further increase was taken as susceptibility to NAP.

RESULTS

Of the 100 smear positive sputum samples tested, growth was observed on the drug free LJ medium in 86%. No growth was observed on the LJ slants containing PNB in any of the above samples irrespective of the bacterial load, determined by the grading of the AFB smear. Identification of the isolates as *M. tuberculosis* was confirmed by a positive niacin test and nitrate reduction assay and a negative result on the catalase test. Fifty samples were randomly selected and cultured on BACTEC 12B with NAP (p-nitroacetylamino- β -hydroxy-propiofenone). No growth was detected in any of these samples in the presence of NAP. Since direct patient specimens can have varied numbers of organisms, we wanted to evaluate the efficiency of PNB incorporated into LJ medium in supporting the growth of NTM. Serial concentrations of known cultures of H37Rv, *M. fortuitum*, *M. scrofulaceum* and *M. avium* at concentrations of 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} organisms/ml were used as controls and cultured on Löwenstein Jensen medium with and without p-nitrobenzoic acid (PNB) (Table 1). PNB incorporated LJ medium did not support the growth of H37Rv, whereas *M. fortuitum*, *M. scrofulaceum* and *M. avium* were isolated from the medium at all the culture concentrates studied.

DISCUSSION

Re-emergence of tuberculosis is a common public health concern in different regions of the world. Although *Mycobacterium tuberculosis* infection is the most common, infec-

tion due to nontuberculous mycobacteria (NTM) is on the increase in many countries (Falkinham, 1996). It is important to establish *M. tuberculosis* infection at an early stage for the initiation of adequate treatment of tuberculosis patients who follow treatment regimens different from patients infected with other mycobacteria. The aim of the present study was to reduce the time taken for identification of *M. tuberculosis*. We used the characteristic of the *M. tuberculosis* which is inhibited by the presence of PNB in Löwenstein Jensen (LJ) medium. LJ slants with and without PNB were inoculated with processed clinical samples directly for primary isolation of *M. tuberculosis*. None of the samples with *M. tuberculosis* had evidence of growth on LJ with PNB. The test described in the present study is a relatively rapid, low cost method that eliminates the need for molecular methods of preliminary identification, which are costly and technically demanding. We shortened the time for identification of *M. tuberculosis* by two weeks, as we

were able to identify *M. tuberculosis* as soon as the first growth appeared on LJ medium. Our results were confirmed by biochemical assays. We did not get any NTM in the samples studied. However, we inoculated LJ medium impregnated with PNB, with serial dilutions (10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} organisms/ml) of known isolates of *M. fortuitum*, *M. avium*, and *M. scrofulaceum* and a standard laboratory strain of *M. tuberculosis*, H37Rv. No growth on PNB slant was observed when H37Rv was subcultured on LJ medium. We were able to isolate various concentrations of all the NTM studied from the PNB slants. Similar results have been reported by others (Giampaglia *et al*, 2005) who have been able to differentiate between *M. tuberculosis* and NTM using MGIT with PNB. Our assay reduces the cost of identification by using inexpensive LJ medium, which is easily available in all laboratories handling mycobacteria. Inoculation of a PNB slant for highly suspect tuberculous patients or smear positive samples can be used for early

Table 1
Growth of serial concentrations of different species of *Mycobacterium* on LJ medium incorporated with PNB.

Organism	Concentration (org/ml)	Growth on LJ	Growth on LJ with PNB	Growth on BACTEC 12B with NAP
H37Rv	10^{-3}	+	-	-
	10^{-4}	+	-	-
	10^{-5}	+	-	-
	10^{-6}	+	-	-
<i>M. fortuitum</i>	10^{-3}	+	+	+
	10^{-4}	+	+	+
	10^{-5}	+	+	+
	10^{-6}	+	+	+
<i>M. scrofulaceum</i>	10^{-3}	+	+	+
	10^{-4}	+	+	+
	10^{-5}	+	+	+
	10^{-6}	+	+	+
<i>M. avium</i>	10^{-3}	+	+	+
	10^{-4}	+	+	+
	10^{-5}	+	+	+
	10^{-6}	+	+	+

presumptive identification of *M. tuberculosis* in peripheral laboratories. For definitive diagnosis, culture isolates need to be evaluated further by standard biochemical assays or molecular tests.

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