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Short Communication

Rapid low-cost identification of *Mycobacterium tuberculosis* complex using p-nitro-benzoic acid (PNB) as inhibitor and the resazurin microplate assay (REMA): A preliminary study

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A rapid test for the differentiation of *Mycobacterium tuberculosis* complex from non-tuberculous mycobacteria (NTM) was developed using p-nitro-benzoic acid (PNB) as inhibitor and the resazurin microplate assay (REMA) for detection of growth. One hundred and fifty-one *M. tuberculosis* strains and 36 NTM were evaluated. Using a cut-off of 250 µg/ml of PNB, all *M. tuberculosis* strains were correctly differentiated from NTM; only two NTM strains failed to be correctly identified with this procedure. The time to obtain results was 8 days compared to 28 days or more with the conventional method. The use of REMA and PNB represents a rapid and inexpensive procedure that could be used in laboratories in low-income settings for the rapid differentiation between *M. tuberculosis* complex and NTM.

Key words: *Mycobacterium tuberculosis*, identification, resazurin.

INTRODUCTION

Tuberculosis (TB) is a highly communicable disease whose treatment differs from that of patients infected by non-tuberculous mycobacteria (NTM) (Frieden et al., 2003; Wagner and Young, 2004); accurate identification of *Mycobacterium tuberculosis* is thus, essential for TB control. However, procedures for the identification of mycobacteria are cumbersome and laborious requiring either a battery of biochemical tests or expensive molecular assays (Mokaddas and Ahmad, 2007; Ortu et al., 2006; Rieder et al., 2007; Wu et al., 2007). Accordingly, the International Union against Tuberculosis and Lung Disease (IUATLD) has recently proposed a simple identification algorithm in which p-nitrobenzoic acid (PNB) susceptibility testing is used as a key step in the differentiation between *M. tuberculosis* and NTM (Rieder et al., 2007). In this algorithm, PNB susceptibility testing is based on conventional methods and requires several weeks to produce results (Canetti et al., 1969;

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Abbreviations: REMA, Resazurin microplate assay;**TB,** tuberculosis; **NTM,** non-tuberculous mycobacteria; **PNB,** p-nitrobenzoic acid; **MGIT,** mycobacterial growth indicator tube; **MIC,** minimal inhibitory concentration; **MTT,** 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Table 1. MIC (µg/ml) of PNB by REMA compared to PNB susceptibility testing on LJ.

PNB susceptibility testing on LJ	MIC of PNB by REMA					
	≤ 31.25	62.5	125	250	500	≥1000
PNB resistant (n = 36)			1	1	5	29
PNB susceptible (n = 151)	35	21	51	44		

Rieder et al., 2007). There is, therefore, a great need for faster PNB susceptibility testing methods for rapid differentiation of *M. tuberculosis* complex from other mycobacteria. Using PNB, researchers recently developed a rapid procedure that can be used to differentiate *M. tuberculosis* complex from other mycobacteria with the commercial automated mycobacterial growth indicator tube (MGIT) (Giampaglia et al., 2007; Giampaglia et al., 2005). However, MGIT is costly and could not be easily implemented in low-resource countries.

Resazurin microplate assay (REMA) has been used as a low-cost as well as a rapid technique for drug susceptibility testing on *M. tuberculosis* isolates and have been implemented in several low resource countries (Palomino et al., 2002; Nateche et al., 2006; Rivoire et al., 2007). However, to our knowledge, this test has not so far been used for identification of mycobacteria.

In this study, we have used PNB as a marker to develop a rapid and inexpensive test for identification of *M. tuberculosis* complex based on REMA for detection of growth.

MATERIALS AND METHODS

Bacterial strains

One hundred and fifty-one clinical strains of *M. tuberculosis* (including both multidrug resistant (MDR) and non-MDR strains), from the Mycobacteriology Reference Laboratory (Laboratoire de Référence des Mycobactéries: LRM, Cotonou, Benin) and thirty-six NTM strains (including 6 *Mycobacterium fortuitum*, 6 *Mycobacterium kansasii,* 5 *Mycobacterium intracellulare,* 4 *Mycobacterium chelonae*, 3 *Mycobacterium peregrinum*, 3 *Mycobacterium gordonae,* 2 *Mycobacterium parascrofulaceum*, 2 *Mycobacterium xenopi*, *2 Mycobacterium avium,* 1 *Mycobacterium smegmatis*, 1 *Mycobacterium terrae*) from the collection of the Institute of Tropical Medicine (Antwerp, Belgium) were tested. All strains were freshly sub-cultured on Löwenstein Jensen (LJ) medium before use. PNB was obtained from Acros Organics (Geel, Belgium) and dissolved in *N,N*-dimethylformamide at a concentration of 40 mg/ml and stored at -20°C.

Conventional method

PNB susceptibility testing was performed on LJ medium as previously described (Rieder et al., 2007). Briefly, LJ medium without PNB or with PNB at a final concentration of 500 µg/ml were inoculated with a 10^{-1} dilution of 1 mg/ml bacterial suspension, and incubated at 37°C for 28 days and finally for 42 days. A strain was considered as NTM if there was growth on the PNB-containing tube and as *M. tuberculosis* complex if there was no growth on the PNB-containing tube despite a growth on the PNB-free tube.

Resazurin microplate assay (REMA)

The assay was carried out as previously described (Palomino et al., 2002). Briefly, an inoculum of 1 mg/ml was prepared in distilled water and diluted 1:20 in 7H9-S medium (consisting of Middlebrook 7H9 broth containing 0.1% casitone and 0.5% glycerol, supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase), (Becton Dickinson, Erembodegem, Belgium)); one hundred microliters was used as the inoculum. Different concentrations of PNB were tested to establish the optimal PNB concentration for the test. The range of PNB concentration used was 31.25 to 1000 µg/ml. The PNB solution was diluted in 7H9-S medium to four times the highest final concentration tested (4000 µg/ml). Serial two-fold dilutions of PNB in 7H9-S were prepared directly in a sterile 96-well flat-bottom microtiter plate in 100 µl of 7H9-S. A growth control well without PNB and a sterile control well without inoculation were also prepared for each strain. Two hundred microliters of sterile water was added to all outer perimeter wells to avoid evaporation during incubation. The plate was covered with its lid, replaced in the original plastic bag, sealed and incubated at 37°C. After 7 days of incubation, 30 µl of 0.02% resazurin was added to each well and the plate was re-incubated overnight. A change in colour from blue (oxidized state) to pink (reduced state) indicated growth of the bacteria, and the minimal inhibitory concentration (MIC) was defined as the lowest concentration of PNB that prevented this change in colour. The same bacterial suspension was used for the two tests (conventional method and REMA) performed in parallel.

RESULTS AND DISCUSSION

With REMA, all results were available in eight days compared to at least 28 days for the conventional method. All 151 strains of *M. tuberculosis* susceptible to PNB by LJ had an MIC ≤ 250 µg/ml by REMA and were correctly identified with a cut-off of 250 µg/ml. Out of the 36 NTM strains resistant to PNB by LJ, the majority (29 strains) had an MIC ≥ 1000 µg/ml, 5 had an MIC of 500 µg/ml and 2 had an MIC ≤ 250 µg/ml (Table 1). Using the cut-off of 250 µg/ml of PNB, all NTM strains, except two were correctly differentiated from *M. tuberculosis* complex. The two discordant results obtained came from two *M. parascrofulaceum* strains which gave MIC values of 125 and 250 µg/ml, respectively. Using this cut-off, the sensitivity and specificity of the REMA were 100 and 94%, respectively and the kappa value was 0.96 (Table 1).

Conventional procedures for identification of *M. tuberculosis* are laborious and cumbersome and include biochemical methods such as the niacin test and heat-labile catalase test (Rieder et al., 2007). Niacin testing is becoming difficult to carry out in many low-resource countries due to recent international transport restrictions of the toxic reagents used. Alternative niacin strips are expensive and could yield doubtful results. Heat labile

catalase testing, on the other hand, may fail in the identification of mycobacteria and was placed as second option after PNB susceptibility testing by the IUATLD algorithm for the identification of mycobacteria in low-resource countries. Other alternatives for rapid identification of *M. tuberculosis* include molecular tests but these are not affordable in low-resource countries as routine procedures.

Susceptibility testing to PNB by the conventional method is a lengthy procedure, thereby emphasizing the necessity of a rapid test for PNB susceptibility testing (Rieder et al., 2007). Using PNB on the commercial automated MGIT, Giampaglia et al. (2007) and Giampaglia et al. (2005) were able to rapidly differentiate *M. tuberculosis* complex from other mycobacteria. However, MGIT is 2 to 3 times more expensive than REMA (Affolabi, 2010) and could not be easily implemented in low-resource countries.

In this study, using a cut-off concentration of 250 µg/ml of PNB and the REMA plate for detection of growth, we were able to rapidly and correctly identify all 151 *M. tuberculosis* strains tested. We could also correctly differentiate all tested NTM except two strains of *M.* parascrofulaceum. Thus, TB diagnosis can be definitely and rapidly dismissed using a cut-off of 500 µg/ml but cannot be definitely confirmed with a MIC of ≤ 250 µg/ml. Further studies are needed to assess the actual prevalence of *M. parascrofulaceum* in clinical samples. NTM can be fairly well differentiated from *M. tuberculosis* complex by colony morphology when grown on solid media (Lévy-Frébault Vincent and Portaels, 1992). On the contrary, if primary culture is performed in liquid medium, which is faster than solid medium, colony morphology cannot be assessed emphasizing the need for a rapid identification test, such as the one here described that could then be applied to positive liquid cultures. Lateral flow immunochromatographic assays for rapid and simple differentiation of M. tuberculosis from NTM can be used but are costly. On the contrary, REMA is a rapid and inexpensive method, which has so far been successfully used for drug susceptibility testing of *M. tuberculosis* and implemented in several low income countries (Palomino et al., 2002; Nateche et al., 2006; Rivoire et al., 2007). By combining the identification procedure described in this study with drug susceptibility testing, all results could be obtained at the same time.

In this study we used REMA to assess the PNB concentration needed for the correct differentiation between *M. tuberculosis* complex and NTM. Taking into consideration biosafety concerns due to the use of liquid medium in a microtiter plate format, the test could also be performed in closed screw-capped tubes as has already been shown for drug susceptibility studies with resazurin with good results (Coban et al., 2006). REMA has not yet been applied directly on sputum samples for TB diagnosis; however, this could be an interesting possibility since two studies using liquid-based colorimetric methods with 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and malachite green directly in sputum samples have already shown promising results (Abate et al., 2004; Farnia et al., 2008) thus, dramatically reducing the time to obtain results.

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