

# Rapid detection of multidrug-resistant *Mycobacterium tuberculosis* in Cotonou (Benin) using two low-cost colorimetric methods: resazurin and nitrate reductase assays

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We have evaluated two simple, rapid and low-cost colorimetric methods for the detection of multidrug-resistant *Mycobacterium tuberculosis*. A total of 151 *M. tuberculosis* strains were tested for resistance to rifampicin (RMP) and isoniazid by resazurin microplate assay (REMA) and nitrate reductase assay (NRA) in comparison with the conventional proportion method (PM) on Löwenstein-Jensen medium. A complete agreement was found between NRA and PM, while one false RMP-susceptible result was found by REMA. REMA and NRA tests are rapid and inexpensive, and could be good alternatives to the conventional PM in low-resource countries.

## INTRODUCTION

Tuberculosis (TB) is still a major public-health problem all over the world, particularly in developing countries. According to the latest World Health Organization (WHO) report in 2005, there were 8.8 million new TB cases and 1.6 million deaths were attributed to the disease worldwide (WHO, 2007). The situation becomes more complicated due to the rising human immunodeficiency virus/AIDS pandemic, the emergence of multidrug-resistant (MDR) TB and the recently described extensively drug-resistant TB (WHO, 2004; Aziz *et al.*, 2006; Shah *et al.*, 2007).

Current standard methods for the detection of MDR *Mycobacterium tuberculosis* include the proportion method (PM) performed on Löwenstein–Jensen (LJ) medium or agar, absolute concentration and resistance ratio methods (Canetti *et al.*, 1963, 1969; Kent & Kubica, 1985) and the radiometric method in the BACTEC-460 system (Roberts *et al.*, 1983). However, these methods either are lengthy or produce radioactive waste that is difficult to manage in low-resource countries.

Commercial methods, such as the mycobacteria growth indicator tube (MGIT) and molecular methods, have been

introduced (Rossau *et al.*, 1997; Palomino *et al.*, 1999); however, though rapid, these methods are expensive and could not be easily implemented in developing countries. Recently, several rapid and inexpensive tests to detect drug resistance in *M. tuberculosis* have been developed (Wilson *et al.*, 1997; Abate *et al.*, 2004; Caviedes *et al.*, 2000; Angeby *et al.*, 2002; Palomino *et al.*, 2002); unfortunately, such tests have been evaluated only in a few low-resource countries (Nateche *et al.*, 2006; Rivoire *et al.*, 2007) but not so far in a TB reference laboratory in West Africa.

In this study we have evaluated two rapid and low-cost colorimetric tests, the nitrate reductase assay (NRA) and the resazurin microplate assay (REMA), for the detection of resistance to rifampicin (RMP) and isoniazid (INH) in strains of *M. tuberculosis* in a TB reference laboratory in Cotonou, Benin. The results were compared to those obtained by the standard PM performed on LJ medium.

## METHODS

**Setting.** The study was performed in the National Reference Mycobacteriology Laboratory (Laboratoire de Référence des Mycobactéries) in Cotonou, Benin, a West African country. External quality control of the laboratory is performed by the Supranational Mycobacteriology Laboratory of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, and regular proficiency testing for drug susceptibility testing using the PM on LJ medium has shown excellent results.

Abbreviations: INH, isoniazid; NRA, nitrate reductase assay; PM, proportion method; REMA, resazurin microplate assay; RMP, rifampicin; TB, tuberculosis; WHO, World Health Organization.

**Bacterial strains.** All available isolates during the study period (from 1 June to 31 July 2007) were included in the study. In total, 151 clinical isolates of *M. tuberculosis* (128 susceptible, 16 MDR and 7 monoresistant to INH) from sputum specimens were included.

**PM.** The PM was performed blindly on LJ medium according to the standard procedure, with a critical concentration of 40 µg ml<sup>-1</sup> for RMP and 0.2 µg ml<sup>-1</sup> for INH, and a critical proportion of 1% (Canetti *et al.*, 1963, 1969).

**REMA.** The assay was carried out as previously described (Palomino *et al.*, 2002). Briefly, an inoculum of 1 mg ml<sup>-1</sup> was prepared by suspending fresh colonies in 7H9 S broth (consisting of Middlebrook 7H9 broth containing 0.1% casitone, 0.5% (v/v) glycerol and supplemented with 10% oleic acid dextrose catalase and diluted 1:20 in the same medium. One hundred microlitres was used as the inoculum and the drug (RMP and INH) solutions were diluted in 7H9 S medium to 8 and 4 µg ml<sup>-1</sup>, respectively (four times the highest final concentration tested). Serial twofold dilutions in 7H9 S were prepared directly in a sterile 96-well flat-bottom plate (Becton Dickinson) in 100 µl 7H9 S. The range of concentrations tested was 0.06 µg ml<sup>-1</sup> to 2 µg ml<sup>-1</sup> for RMP, and 0.03 to 1 µg ml<sup>-1</sup> for INH. A growth control containing no drug and a sterile control without bacteria were also prepared for each strain. Two hundred microlitres 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 and incubated at 37 °C under a normal atmosphere. After 7 days of incubation, 30 µl 0.02% resazurin solution was added to each well and the plate was reincubated overnight. A change in colour from blue (oxidized state) to pink (reduced state) indicated growth of the bacteria, and the MIC was defined as the lowest concentration of drug that prevented this change in colour.

**NRA.** The NRA was performed as described by Angeby *et al.* (2002) with a few modifications. Briefly, standard LJ medium was used with 1000 µg potassium nitrate ml<sup>-1</sup>, without or with drugs (40 µg RMP ml<sup>-1</sup> or 0.2 µg INH ml<sup>-1</sup>). For each strain, 0.2 ml 1 mg ml<sup>-1</sup> of the bacterial suspension was inoculated in the drug-containing tube and 0.2 ml 1:10 dilution of the bacterial suspension into four drug-free tubes. After 8 days of incubation at 37 °C, 0.5 ml freshly prepared reagent mixture (one part 50% concentrated hydrochloric acid, 2 parts 0.2% sulphanilamide and 2 parts 0.1% *N*-1-naphthylethylenediamine dihydrochloride) was added to one drug-free tube. The results were classified as negative (no colour change) or positive and depending on the colour change, from 1+ (pink) to 4+ (deep red to violet). If the result in the drug-free tube was at least 2+ positive, the drug-containing tube was developed. Otherwise, the other tubes were reincubated and the procedure was repeated at day 10, day 14 and

finally at day 18. A strain was considered resistant if the drug-containing tube produced a colour change that was similar to or more intense than that in the drug-free tube. A strain was considered susceptible if there was no change in colour or the colour change in the drug-containing tube was less than that in the drug-free tube.

The same bacterial suspension was used for the three tests performed on the same day. Internal quality control was done using the fully susceptible *M. tuberculosis* H37Rv and a known MDR *M. tuberculosis* isolate. No discordant results were found. The technician who performed the NRA and the REMA was not aware of the PM results.

**Data analysis.** The performance of the NRA and the REMA in comparison with the PM was evaluated in terms of sensitivity (ability to detect a true resistance) and specificity (ability to detect a true susceptibility) (Altman, 1999).

## RESULTS AND DISCUSSION

With the REMA, results for all strains were available in 8 days, while with the PM on LJ medium results were available in 4–6 weeks. The majority of resistant strains had an MIC  $\geq 2$  µg ml<sup>-1</sup> for RMP and  $\geq 1$  µg ml<sup>-1</sup> for INH, while most of susceptible strains had an MIC  $\leq 0.06$  µg ml<sup>-1</sup> for RMP and  $\leq 0.03$  µg ml<sup>-1</sup> for INH (Table 1). Using a cut-off of 0.5 µg ml<sup>-1</sup> for RMP and 0.25 µg ml<sup>-1</sup> for INH as previously described (Palomino *et al.*, 2002), there was complete agreement between REMA and PM for INH. One discordant result was obtained for RMP, determined to be susceptible by REMA (MIC of 0.25 µg ml<sup>-1</sup>) but resistant by PM. This strain, which was also found to be resistant by the NRA, was retested by both methods (REMA and PM) and showed the same results. Thus, the sensitivity and specificity of the REMA was 94 and 100%, respectively.

With the NRA the results were available for 24 strains (16%) at day 8, 75 strains (51%) at day 10, 128 strains (85%) at day 14 and all strains at day 18. There was a complete agreement between NRA and PM both for RMP and INH. Thus, both the sensitivity and specificity of the NRA was 100%. Overall there was a complete agreement of the three tests for 150 strains.

MDR-TB is an increasing threat for TB control, particularly in developing countries. In Benin, a country located in

**Table 1.** MIC (µg ml<sup>-1</sup>) of RMP and INH by REMA compared to PM

PM result (no. of strains)	MIC of RMP with REMA (no. of strains)					
	$\leq 0.06$	0.125	0.250	0.500	1	$\geq 2$
RMP resistant ( <i>n</i> =16)	–	–	1	–	1	14
RMP susceptible ( <i>n</i> =135)	74	42	19	–	–	–
	MIC of INH with REMA (no. of strains)					
	$\leq 0.03$	0.06	0.125	0.250	0.500	$\geq 1$
INH resistant ( <i>n</i> =23)	–	–	–	2	2	19
INH susceptible ( <i>n</i> =128)	123	4	1	–	–	–

West Africa, though the MDR rate is relatively low in patients living in the country, many patients from neighbouring countries, where the MDR prevalence is higher, come to Benin seeking treatment (Affolabi *et al.*, 2007a). It is therefore important to rapidly detect patients with MDR-TB so that appropriate treatment, now available in Benin, can be started. As in other studies with REMA in some African countries (Nateche *et al.*, 2006; Rivoire *et al.*, 2007), the two tests performed in our laboratory gave an excellent agreement as compared to the conventional PM on LJ medium.

Of the two tests, REMA was the most rapid with all results available in 8 days, while only 85% of the results were available with NRA after 14 days. REMA has also the advantage that it allows us to easily determine the MIC; however, one concern related to REMA is the use of a liquid medium in a microplate format, which might represent a biohazard due to the generation of aerosols. The use of closed screw-cap tubes would avoid this risk as has been recently reported with good results (Coban *et al.*, 2006).

The NRA was easier to implement since it needs no additional material or training for laboratories that already perform PM on LJ medium. For most of the strains, results were available in 14 days with NRA, while PM gave results in 4–6 weeks. One limitation of the NRA, however, could be that some strains of *M. tuberculosis* lack nitrate reductase, rendering the test invalid. However, these strains are rare in our setting (Affolabi *et al.*, 2007b) and could be tested by REMA. NRA has recently been applied directly in sputum samples with good results (Musa *et al.*, 2005; Solis *et al.*, 2005; Affolabi *et al.*, 2007b). Application of REMA directly on sputa is not described so far; however, it could also represent a good option for the rapid detection of MDR-TB.

This study evaluated the feasibility of the implementation of these rapid tests in our setting. A cost analysis study is actually on-going to assess the cost-effectiveness of the tests. The decision on the use of one or the other test will depend on the results of this study.

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