

Evaluation of the resazurin assay for the detection of multidrug-resistant *Mycobacterium tuberculosis* in Madagascar

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SUMMARY

SETTING: Multidrug-resistant (MDR) tuberculosis (TB) can jeopardise the success of national TB control programmes. Rapid, simple drug susceptibility tests applicable in developing countries would allow earlier treatment of patients with MDR infections.

OBJECTIVE: To test the feasibility and performance of the resazurin microtitre assay (REMA) as an indirect test for detecting isoniazid (INH) and rifampicin (RMP) resistance of *Mycobacterium tuberculosis* strains in Madagascar.

DESIGN: Study comparing the sensitivity and specificity of the REMA plate test with the Löwenstein-Jensen proportion method for determining the resistance of *M. tuberculosis* strains to INH and RMP.

RESULTS: The sensitivity and specificity of the resazurin test were studied in 77 strains and were respectively 95% and 97.3% for the detection of INH resistance, and 95% and 100% for the detection of RMP resistance. The sensitivity and specificity for the identification of MDR strains were respectively 89% and 100%.

CONCLUSION: The resazurin test is sensitive and specific enough for the detection of INH- and RMP-resistant strains. It is also easy to use, rapid and inexpensive, making it suitable for developing countries. Its usefulness for national drug resistance surveys should be assessed.

KEY WORDS: tuberculosis; multidrug resistance; resazurin; Madagascar

TUBERCULOSIS (TB) is a serious public health problem in many developing countries, where the problem is made even more severe by human immunodeficiency virus (HIV) co-infection. TB treatment is long, involving multidrug therapy over 6–8 months. TB with multidrug resistance (MDR), defined as resistance to at least the two major anti-tuberculosis drugs isoniazid (INH) and rifampicin (RMP), has emerged due to non-adherence to treatment. Before the 1990s MDR-TB was rare, but over the last 10 years, epidemics of MDR *Mycobacterium tuberculosis* strains have been observed around the world, with high mortality rates particularly among HIV-positive patients.^{1–3} MDR-TB is now also emerging in HIV-negative populations and in high-risk populations such as medical staff.^{4,5} MDR-TB also threatens the success of national TB control programmes (NTPs) in countries with high TB prevalence.

In Madagascar, the primary MDR rate (i.e., MDR-TB identified in new patients) is low, at less than 0.5%.^{6,7} Most of the MDR strains found were isolated from non-responsive or chronic TB patients. The drug susceptibility test currently available is the conventional proportion method on Löwenstein-Jensen (LJ) medium, which takes 3–6 weeks to yield results.

More rapid tests using liquid media, such as the BACTEC 460TB radiometric method and the Mycobacteria Growth Indicator Tube (MGIT),⁸ require specific equipment and consumables, and are still expensive. Likewise, the new rapid tests based on molecular tools are generally not easy to use and need specialised staff.^{9–11} Rapid diagnosis of MDR patients is nevertheless necessary to avoid the spread of MDR strains. The test should be specific, simple and applicable in developing countries.

Colorimetric tests such as the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method,^{12,13} the Alamar Blue method,¹⁴ the resazurin microtitre assay (REMA)¹⁵ and the nitrate reductase assay^{16,17} have been developed. These tests are less time-consuming than conventional methods and have proven to be rapid and reliable, with good sensitivity and specificity for the detection of INH and RMP resistance as compared to the proportion method as a gold standard.^{18–20} Resazurin is the oxido-reduction reagent in the Alamar Blue method and is considerably cheaper. REMA has also been described as being useful for the diagnosis of resistance to second-line drugs.²¹ This test is performed in microplates and is therefore relatively cheap.

The objective of the present study was to evaluate the feasibility of the resazurin assay as an indirect test for the detection of MDR *M. tuberculosis* strains in Madagascar using the indirect critical proportion method as a gold standard.

MATERIALS AND METHODS

Bacterial isolates

Specimen processing was performed in a level P2 biosafety TB laboratory* in the Pasteur Institute of Madagascar, Antananarivo.

Clinical specimens were processed by the Tacquet and Tison²² decontamination method, concentrated by centrifugation in an aerosol-contained centrifuge and thereafter added to standard LJ medium tubes. The tubes were incubated at 37°C until growth of colonies. *M. tuberculosis* was identified on the basis of growth rate, colony morphology on LJ medium and standard biochemical tests. All strains were stored at -20°C and freshly sub-cultured on LJ medium before use.

Drug susceptibility patterns for the first-line drugs INH, RMP, ethambutol (EMB) and streptomycin (SM) were determined by the indirect proportion method on LJ medium when required by physicians.

We studied 81 *M. tuberculosis* clinical strains selected at random among the strains with known drug susceptibility pattern: 37 isolates susceptible to INH and RMP, 21 resistant to both drugs, 21 resistant to INH and 2 resistant to RMP.

M. tuberculosis H37Rv was included as the susceptible control strain.

Anti-tuberculosis drug

Stock solutions of 1 mg/ml INH (Sigma, St Louis, MO, USA, ref. I-3377) and 10 mg/ml RMP (Sigma, ref. R-8626) were prepared in distilled water and methanol respectively, then filter-sterilised and kept at -20°C for up to one month.

Proportion method drug susceptibility test

The standard proportion method²³ for INH and RMP susceptibility testing was performed on LJ medium at the following drug concentrations: 0.2 µg/ml INH (Sigma, ref. I-3377) and 40 µg/ml RMP (Sigma, ref. R-8626). The critical proportion value was 1% for both drugs.

Resazurin solution

A stock solution of 0.01% resazurin was prepared by dissolving resazurin sodium salt powder (Sigma, ref. R7017-1G) in distilled water. The solution was filter-sterilised and stored at +4°C for up to one week.

The resazurin microtitre assay

The resazurin microtitre assay (REMA) was evaluated as an indirect test. It was carried out as described by Palomino et al.,¹⁵ with a few minor modifications. Briefly, the inoculum was prepared by dispersing 3-week-old fresh colonies in Dubos broth (consisting of 1.3 g Dubos Broth Base [Difco,™ Detroit, MI, USA, ref. 238510] in 170 ml of distilled water, supplemented with 10 ml glycerol and 20 ml Dubos medium albumin [Difco™, ref. 230910]). The inoculum concentration was adjusted to a 1 mg/ml bacille Calmette-Guérin (BCG) solution tube (around 10⁶-10⁸ colony forming units/ml;²³ Sanofi Diagnostics Pasteur, Redmond, WA, USA, ref. 53211) and diluted 1:20 in Dubos broth; 100 µl was then used as the inoculum. Two-fold serial dilutions of drug in 100 µl Dubos medium were prepared at concentrations of 2.0-0.06 µg/ml for INH and 4.0-0.12 µg/ml for RMP in a sterile 96-well microtitre plate. An aliquot of 100 µl of inoculum was then added to the wells of the microtitre plate, giving a drug concentration of 1.0-0.03 µg/ml and 2.0-0.06 µg/ml for INH and RMP, respectively. All strains were tested in duplicate for each drug concentration. Growth controls without drug and sterile controls without inoculum were included in each plate. The perimeter wells were filled with sterile distilled water to avoid evaporation. The plates were covered, placed in plastic bags and incubated at 37°C. After 7 days, incubation at 37°C, 30 µl resazurin solution was added to each well and the plates were further incubated for 24 h. A change in colour from blue to pink indicated the growth of bacteria. The minimal inhibitory concentration (MIC) is the lowest concentration of drug that prevents such a colour change. When there was no colour change in the growth control well or if a colour change was observed for the sterility controls, the test was considered invalid and the experiment was repeated.

Analysis of results

The specificity, sensitivity and predictive values of REMA for the identification of INH and RMP resistance and MDR were determined by comparison with the proportion method on LJ medium, which was used as a reference method.

RESULTS

Of 81 *M. tuberculosis* strains tested by REMA, 77 strains gave interpretable results. Four strains gave invalid results with no colour change in the control well.

Results obtained with REMA and the proportion method on LJ medium are compared in the Table. For

* Level 2 biosafety requires the following: laboratory personnel are trained in handling *M. tuberculosis* and are directed by a competent scientist; regular follow-up of staff regarding TB; access to the laboratory is limited when work is being conducted; precautions are taken with contaminated sharp items and in general with contaminated materials or products; to avoid infectious aerosols or splashes, infectious specimens are handled in a class II biological safety cabinet, and centrifugation is conducted in safety cups.

Table Comparison of resazurin microtitre assay results with the proportion method on LJ medium for 77 *M. tuberculosis* isolates

Drug	Proportion method [†]	Resazurin microtitre assay* [†]					
		No. of strains		Sensitivity %	Specificity %	PPV %	NPV %
		Resistant	Susceptible				
INH	Resistant (<i>n</i> = 40)	38	2	95	97.3	97.73	94.73
	Susceptible (<i>n</i> = 37)	1	36				
RMP	Resistant (<i>n</i> = 20)	19	1	95	100	100	98.3
	Susceptible (<i>n</i> = 57)	0	57				
Both drugs	MDR (<i>n</i> = 19)	17	2	89.4	100	100	96.6
	Non MDR (<i>n</i> = 58)	0	58				

* A strain was deemed resistant when MIC > 0.125 µg/ml for INH and MIC > 0.25 µg/ml for RMP.

[†] Critical proportion was 1% for both drugs.

LJ = Lowenstein-Jensen; PPV = positive predictive value; NPV = negative predictive value; INH = isoniazid; RMP = rifampicin; MDR = multidrug-resistant.

40 isolates with INH resistance $\geq 1\%$ on LJ medium, the INH MIC as determined by REMA was ≥ 0.25 µg/ml for 38 isolates and ≤ 0.125 µg/ml for the remaining two isolates. Of the 37 strains with an INH resistance proportion of $< 1\%$ on LJ medium, the INH MIC as assessed by REMA was < 0.25 µg/ml for 36 and ≥ 1 µg/ml for one. Therefore, assuming that an isolate is scored as INH-resistant when the MIC ≥ 0.25 µg/ml and INH-susceptible when the MIC < 0.25 µg/ml in the resazurin test, the sensitivity and specificity of the test were respectively 95% and 97.3%, and the positive predictive value (PPV) and negative predictive value (NPV) for INH resistance were respectively 97.4% and 94.7%.

Of the 20 isolates with a RMP resistance proportion of $\geq 1\%$ on LJ medium, the MIC for RMP was ≥ 0.5 µg/ml for 19 strains and < 0.062 µg/ml for one strain. For all the 57 strains with a RMP resistance proportion of $< 1\%$ on LJ medium, the MIC for RMP was ≤ 0.25 µg/ml. Hence, if an isolate is defined as RMP-resistant if the MIC is ≥ 0.5 µg/ml and susceptible to RMP if the MIC is < 0.5 µg/ml, as measured by REMA, only one strain was falsely identified as being RMP-susceptible (MIC for RMP < 0.062 µg/ml), and the proportion of resistance on LJ medium was 1%. Thus, REMA sensitivity and specificity for the determination of resistance to RMP were respectively 95% and 100%, and the PPV and NPV were respectively 100% and 98.3%.

One of the advantages of the REMA plate method is that it can detect MDR strains rapidly. With reference to the proportion method as the gold standard, the REMA plate method had a sensitivity of 89% and a specificity of 100%, and PPV and NPV of respectively 100% and 96.6% for the identification of MDR strains.

Predictive values according to the prevalence of resistance in a population can be calculated using Bayes' theorem.²⁴ Predictive values were therefore determined in two different study populations. The first was a population of 789 new pulmonary TB cases included in a primary resistance survey in 1999–2000, in which the prevalence of resistance was low: 2.7%, 0.1% and 0.1%, respectively, for INH resistance, RMP re-

sistance and MDR.⁷ The second population comprised 66 patients who had received TB treatment in the past (relapse, non-responsive, defaulter or chronic cases) and for whom the consulting physician had specifically requested drug susceptibility testing. The prevalence of resistance was higher in this population: 30.3%, 15.1% and 13.6%, respectively, for INH resistance, RMP resistance and MDR (unpublished data). Among new TB cases, the PPV and NPV were respectively 49.4% and 99.8% for the detection of resistance to INH, and 100% and 99.9% for the detection of resistance to RMP. For previously treated patients, these values were respectively 93.6% and 97.8% for INH resistance, and 100% and 99.1% for RMP resistance. The PPV and NPV for the identification of MDR were respectively 100% and 99.9% among new cases, and 100% and 98.3% for previously treated patients.

DISCUSSION

In Madagascar, previously treated patients are currently treated using the Category II regimen recommended by the World Health Organization (WHO).²⁵ The spread of MDR strains could seriously jeopardise the fight against TB. The diagnosis of MDR-TB in Madagascar is not easy because the only available method to determine drug resistance is the standard proportion method on LJ medium, which requires about 4 weeks to yield results. Liquid culture methods such as the BACTEC TB-640 system and molecular methods such as INNO LiPA are very much quicker, but these technologies cannot be used in low-income countries because they are expensive and require specific equipment. Thus, even though the primary MDR rate is still low in Madagascar ($< 0.25\%$ ^{6,7}), a rapid, simple test for the identification of MDR would be useful, as earlier detection of these strains would help prevent their transmission.

A comparison of the quantitative results obtained by the proportion method on LJ medium and the MIC results for INH and RMP by REMA led to the conclusion that the breakpoint concentrations determined by Palomino et al.¹⁵ are appropriate. Using these breakpoints,

detection of resistance to INH and RMP by REMA showed good sensitivity (95% for both drugs) and specificity (97.3% and 100%, respectively) with reference to the gold standard method, with INH and RMP cut-off values of respectively 0.25 µg/ml and 0.5 µg/ml. The very discordant INH results for two strains (one susceptible according to the proportion method but with an MIC of ≥ 1 µg/ml according to the REMA test, and one with 100% resistance using the proportion method but with an MIC of < 0.031 µg/ml according to the REMA test) were surprising. These results were confirmed in repeat tests by REMA and the proportion method; we have no explanation, as no mutations were found in the *katG* and *inhA* genes in these two strains (unpublished results). Another isolate was found to be susceptible by the resazurin assay but had a low resistance rate (1%) with the proportion method, which may explain the discordant result. Four strains gave no colour change in the control well, thus implying the limit of the REMA test and explaining the lower sensitivity of this test.

Palomino et al. found very good predictive values for susceptibility and resistance to INH and RMP: respectively 100% and 98.2% for INH and both 100% for RMP.¹⁵ Although we found lower values (predictive values for susceptibility and resistance of 94.7% and 97.7% for INH and 100% and 98.2% for RMP), the REMA test gave acceptable predictive values for non-MDR and MDR (96.6% and 100%, respectively).

Used as an indirect test, REMA was rapid (8 days after culture on solid medium vs. 3–6 weeks for the indirect testing on LJ medium) and technically straightforward. The total turnaround time was 4–5 weeks for REMA vs. 7–10 weeks for LJ. It was also cheaper (including the cost of reagents and consumables) than the proportion method using LJ medium prepared in-house. The REMA method could therefore prove useful for both the diagnosis of MDR and primary resistance surveys in low-income countries. The predictive values of the test were also calculated in both cases. Drug susceptibility tests are often requested by clinicians in cases of treatment failure and relapse, or for chronic TB patients at high risk of developing MDR. The predictive values for MDR and non-MDR calculated using the Bayes' theorem were acceptable (respectively 100% and 98.3%); the REMA test can therefore be used for rapid diagnosis of MDR. Moreover, in primary resistance surveys with a low rate of drug resistance, the predictive values for MDR and non-MDR were 100% and 99.9%, respectively.^{6,7} Hence, even if the predictive value for resistance to INH is low in primary INH resistance surveys (49.9%), the test should still be useful for MDR surveys.

One disadvantage of the REMA test is the risk of contamination, as it is carried out in microplates using liquid medium. The contamination rate was low: a colour change in the sterility control well was observed for only 0.06% of the isolates. However, with

the use of microplates, other wells may be contaminated without significant contamination of the control wells. It could therefore have been more appropriate to confirm that there was no bacterial contamination with a Ziehl-Neelsen stain. This process was not performed, however, as it may have made the test significantly less practical.

In summary, the REMA test was found to be reliable, inexpensive and simple to perform. In addition, it required less cumbersome incubators than those needed for the proportion method involving LJ medium. Furthermore, unlike the molecular methods, it does not require specific equipment. Like the proportion method, however, it cannot be used in peripheral laboratories and must be carried out in laboratories able to perform *M. tuberculosis* culture under appropriate conditions to avoid the risk of contamination. Thus the minimum major equipment required for performing this test includes a level P2 biosafety cabinet, an aerosol-contained centrifuge and a 37°C incubator. We conclude that the REMA test could easily be implemented and might even replace the proportion method in central laboratories in low-resource countries such as Madagascar. The next step will be to implement and evaluate REMA for determining resistance to other first-line drugs, particularly SM and EMB.^{18,19} It will also be very useful for testing susceptibility to second-line drugs, including ofloxacin (OFX),²⁶ as OFX-containing regimens have been used for treating MDR-TB and have proved effective in some developing countries.²⁷ Although this drug is not yet available in Madagascar, a rapid test to detect OFX-resistant strains will be needed when DOTS-Plus is implemented.

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R É S U M É

CONTEXTE : La tuberculose (TB) multirésistante (MDR) peut être un obstacle à la réussite des programmes de lutte contre la TB. Des tests de sensibilité simples, rapides et utilisables dans les pays à faibles ressources devraient permettre le traitement précoce des patients infectés par des souches MDR de *Mycobacterium tuberculosis*.

OBJECTIF : Évaluer la faisabilité et la performance du test à la résazurine sur microplaque (REMA) comme test indirect de détection des souches *M. tuberculosis* résistantes à l'isoniazide (INH) et à la rifampicine (RMP) à Madagascar.

SCHÉMA : Comparaison des sensibilité et spécificité du REMA à celles de la méthode indirecte des proportions sur milieu Löwenstein-Jensen pour la détermination des souches résistantes à l'INH et à la RMP.

RÉSULTATS : La sensibilité et la spécificité du REMA, déterminées à partir de 77 souches, étaient respectivement de 95% et 97,3% pour la détection de la résistance à l'INH, respectivement de 95% et 100% pour la détection de la résistance à la RMP, et respectivement de 89% et 100% pour la détection des souches MDR.

CONCLUSION : Le test REMA est suffisamment sensible et spécifique pour la détection des souches résistantes à l'INH et la RMP. Il est, par ailleurs, facile à réaliser, rapide, bon marché, donc facilement utilisable dans les pays en développement. Ses performances devront être évaluées pour des enquêtes nationales de surveillance de la résistance.

MARCO DE REFERENCIA: La tuberculosis (TB) multi-drogas resistente (MDR) puede constituir un obstáculo al éxito de los programas nacionales de control de la TB. Las pruebas rápidas y sencillas de sensibilidad a los medicamentos, utilizables en los países en vía de desarrollo, facilitarían un tratamiento más oportuno de los pacientes con infecciones MDR.

OBJETIVO: Verificar la viabilidad y el rendimiento del ensayo de microvaloración con resazurina (REMA) como prueba indirecta para detectar la resistencia de cepas de *Mycobacterium tuberculosis* a isoniazida (INH) y a rifampicina (RMP) en Madagascar.

MÉTODOS: En el estudio se comparó la sensibilidad y especificidad de la prueba de REMA con el método proporcional en medio Löwenstein-Jensen en la determi-

nación de la resistencia de cepas de *M. tuberculosis* a INH y a RMP.

RESULTADOS: En el estudio de 77 cepas, la prueba REMA demostró una sensibilidad del 95% y una especificidad del 97,3% para la detección de resistencia a INH y una sensibilidad del 95% y especificidad del 100% en la detección de resistencia a RMP. Con respecto a la identificación de cepas MDR, la sensibilidad fue del 89% y la especificidad del 100%.

CONCLUSIÓN: La prueba REMA es suficientemente sensible y específica en la detección de cepas resistentes a INH y RMP. Además, su ejecución es sencilla, rápida y de bajo costo, por lo cual podría ser adecuado emplearla en países en vías de desarrollo. Es preciso evaluar la utilidad de la prueba en las encuestas nacionales de farmacoresistencia.
