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Multicenter study of MTT and resazurin assays for testing susceptibility to first-line anti-tuberculosis drugs

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SUMMARY

OBJECTIVE: A multicentre evaluation was performed to assess two rapid low-cost methods, MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) and resazurin assays, for testing the susceptibility of *Mycobacterium tuberculosis* to the first-line anti-tuberculosis drugs rifampicin (RMP), isoniazid (INH), ethambutol (EMB) and streptomycin (SM).

METHODS: Thirty coded *M. tuberculosis* strains were sent to seven laboratories located in Latin America, representing six countries. Each site performed the colorimetric assays, MTT and resazurin, blind for the first-line drugs RMP, INH, EMB and SM. The minimum inhibitory concentration results obtained were compared to the conventional proportion method on Löwenstein-Jensen medium.

RESULTS: After establishing the breakpoint concentrations, excellent results were obtained for RMP, INH and EMB, with levels of specificity and sensitivity of between 96% and 99%.

CONCLUSION: MTT and resazurin assays are promising, accessible new alternative methods for middle- and low-resource countries that need low-cost methods to perform rapid susceptibility testing of *M. tuberculosis* to key anti-tuberculosis drugs.

KEY WORDS: *M. tuberculosis*; MTT; resazurin; first-line drugs

THE SPREAD of *Mycobacterium tuberculosis* strains resistant to the two key anti-tuberculosis (TB) drugs, isoniazid (INH) and rifampicin (RMP), defined as multidrug-resistant TB (MDR-TB), challenges the control of the disease.[1,2] The emergence of MDR-TB highlights the need for drug susceptibility testing (DST), both for proper patient management as well as for drug resistance surveillance, a priority defined by the World Health Organization (WHO) to detect areas of emerging resistance in time and to avoid dissemination of the disease.[3]

In low-resource countries, where TB is endemic and MDR-TB is a more serious public health problem, DST is still currently based on conventional culture methods with solid media such as Löwenstein-Jensen (LJ) and Middlebrook agar, which are laborious and lengthy, requiring at least 4 weeks of incubation before an isolate can be reported as susceptible or resistant.[4–6] Newer, more rapid culture methods in liquid media are increasingly being used, as they reduce the time needed for culture and DST. Due to its reliability and speed, the BACTEC TB-460 radiometric system (Becton Dickinson, Sparks, MD) was the first to achieve worldwide recognition. However, this method requires radioisotopes and an expensive machine[7,8] that are limiting factors for its implementation in low-resource countries. The commercial Mycobacterial Growth Indicator Tube (MGIT) system (Becton Dickinson) is a good candidate to replace it;[9,10] it is rapid and reliable, but is still expensive in some low-resource countries. New molecular tools for rapid detection of drug resistance usually need skills, supplies, equipment and facilities that are not always available in endemic countries; in addition, as the molecular mechanisms of drug resistance are not completely understood, molecular methods fail to detect all those resistant strains in which no associated mutations have been identified to date.[11,12]

Among the recently described new phenotypic methods for DST, rapid colorimetric methods, based on the ability of live bacteria to reduce an indicator and produce a change of visual colour, appear promising techniques and have been described with success previously.[13–24] They are simple and require no so-

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phisticated equipment. Colorimetric methods using the oxidation-reduction indicator MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and resazurin have been used to determine the minimal inhibitory concentrations (MICs) of RMP, INH, EMB, SM and other antimicrobial drugs.[13–15,19–21,25–27]

To our knowledge, no controlled evaluation has been performed to assess the reliability and performance of colorimetric methods on a wider scale in endemic countries. In this study, we performed a multicentre evaluation of the MTT and resazurin methods in seven laboratories in Latin America, representing six countries. The objective of our study was to evaluate the reliability of these two colorimetric methods for testing *M. tuberculosis* susceptibility to the first-line drugs RMP, INH, EMB and SM, and assess its accuracy in different settings working under local conditions. The performance of these methods was determined by comparing the results with DST using the most commonly used conventional proportion method on LJ medium. This work is the first multicentre study to evaluate the reliability of colorimetric methods in different countries.

MATERIALS AND METHODS

Laboratories

MIC results were generated by seven laboratories: Hospital Centrángolo, Argentina; Instituto 'Pedro Kouri', Cuba; Instituto Adolfo Lutz, Brazil; Institute Malbrán, Argentina; Instituto Nacional de Salud, Colombia; Instituto de Salud Publica de Chile; and Centro Nacional de Diagnostico y Referencia, Nicaragua. The seven laboratories were randomly numbered from 1 to 7 for the purposes of this publication.

Isolates

A panel of 30 clinical isolates of *M. tuberculosis* with a known drug susceptibility pattern was selected and coded by the Institute of Tropical Medicine, Belgium, and distributed to the participants. Twelve were resistant to INH, 15 to RMP, 9 to EMB, 12 to SM and 12 were susceptible. The reference strain H37Rv (ATCC 27294), from the American Type Culture Collection, was used as the reference susceptible strain. All strains were freshly subcultured on LJ medium in each laboratory before being tested.

Antibiotics

INH, EMB and SM stock solutions were prepared at 1 mg/ml in distilled water. RMP stock solution was prepared at 10 mg/ml in methanol. All antibiotic stock solutions were filter-sterilised and stored at −20°C until use.

Reagents

A stock solution of resazurin sodium salt (Acros Organic, Geel, Belgium) was prepared at 0.01% in distilled water, filter sterilised and kept at 4°C. A stock solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Bornem, Belgium) at 5 mg/ml was prepared in phosphate buffer saline (PBS), pH 6.8, and kept at 4°C in the dark. Formazan solubilisation buffer was prepared by mixing 1:1 (vol/vol) 20% sodium dodecyl sulfate (SDS) and a solution of 50% of N,N-dimethylformamide (DMF).

Drug susceptibility testing

The proportion method was performed on LJ medium according to the standard procedure, with the recommended critical concentrations of 40 μg/ml for RMP, 0.2 μg/ml for INH, 4.0 μg/ml for SM and 2.0 μg/ml for EMB.[4,5] The proportion method was performed blind in the 30 strains in each site. The proportion method results obtained in the reference laboratory constituted the gold standard.

Resazurin assay

The resazurin microtiter assay (REMA) plate method was carried out as previously described.[25] Briefly, the inoculum was prepared from fresh LJ medium resuspended in 7H9-S medium (7H9 broth, 0.1% casitone, 0.5% glycerol, supplemented oleic acid, albumin, dextrose, and catalase [OADC], Becton Dickinson), adjusted to a McFarland tube No. 1, and diluted 1:20; 100 μl was used as inoculum. Each drug stock solution was thawed and diluted in 7H9-S at four-fold the final highest concentration tested. Serial two-fold dilutions of each drug were prepared directly in a sterile 96-well microtiter plate (Becton Dickinson) using 100 μl 7H9-S. The range concentrations tested were: INH 0.03–1.0 μg/ml, RMP 0.06–2.0 μg/ml, EMB 0.5–16.0 μg/ml and SM 0.125–4.0 μg/ml. A growth control containing no antibiotic and a sterile control were also prepared on each plate. Sterile water was added to all perimetre wells to avoid evaporation during the incubation. The plate was covered, sealed in plastic bags and incubated at 37°C in normal atmosphere. After 7 days incubation, 30 μl of resazurin solution was added to each well, and the plate was reincubated overnight. A change in colour from blue (oxidised state) to pink (reduced) indicated the growth of bacteria, and the MIC was defined as the lowest concentration of drug that prevented this change in colour.

MTT assay

The MTT assay was carried out as described by Abate et al.[13] The inoculum was prepared as described above for the REMA plate method. The procedure to prepare the 96-well plate was identical to that used for the REMA plate assay. After 7 days incubation at 37°C, 10 μl of the MTT solution (5 mg/ml) was added to each well and the plate was reincubated overnight. If a violet precipitate (formazan) appeared in the MTT well, 50 μl of the SDS-DMF solution was added to these wells and the plate was reincubated for 3 h. A

change in colour from yellow to violet indicated the growth of bacteria, and the MIC was interpreted as in the REMA plate assay.

Analysis of data

MedCalc Software (MedCalc, Mariakerke, Belgium) was used to calculate the breakpoint concentration for each drug, based on receiver operating characteristic (ROC) curve analysis, which gives the corresponding cut-off point for the best resolution of resistant and susceptible strains. The ROC curve analysis is used to measure the ability of a test to distinguish diseased cases from normal cases and can also be used to compare the diagnostic performance of two or more laboratory or diagnostic tests.[28,29] MedCalc Software was also used to calculate other statistical parametres: sensitivity (ability to detect true resistance), specificity (ability to detect true susceptibility), and accuracy (the rate of correct results). The value of the area under the ROC curve can be interpreted as follows: if there is a perfect separation of the values of the two groups, resistant and susceptible strains, the area under the ROC curve is equal to 1; if the test under study can not distinguish between the two groups, resistant and susceptible, this value will be equal to or lower than 0.5.

RESULTS

By visual reading of the colorimetric methods, MIC results were obtained after 8 days and sent to the coordinating laboratory. For each site we defined a tentative breakpoint concentration for each drug using MedCalc Software (Mariakerke, Belgium) based on ROC curve analysis[28,29] and defined a tentative breakpoint of 0.25 μg/ml for INH, 0.5 μg/ml for RMP, 4 μg/ml for EMB and 1 μg/ml for SM. Table 1 shows the number of resistant and susceptible strains obtained by the MTT and resazurin assays according to the defined breakpoints compared with the results of the proportion method. Specificity and sensitivity values are shown in Table 2.

For RMP, for both the MTT and the REMA methods, results were very good. Of the seven sites, five identified all isolates correctly, giving a specificity and sensitivity of 100%. Two sites obtained a sensitivity and specificity of 93.3% due to only one discordant result. For INH, results gave a specificity between 94.4% and 100% and a sensitivity between 91.7% and 100% for all isolates. Overall specificity and sensitivity for all sites was over 97.6%. For EMB, good results were obtained and for all sites a specificity and sensitivity higher than 95% were observed. For SM, discordant results were found in all sites, and specificity and sensitivity were lower with this drug, with 82.7% and 90.5% specificity and sensitivity for the REMA test, and 85% and 90.5% specificity and sensitivity for MTT. Only sites 2 and 5 were able to correctly identify all true SM-resistant isolates by both methods.

The average area under the ROC curve (AUC) values ranged from 0.904 to 1.00 for all drugs using MTT (INH 0.994; RMP 1.00; EMB 0.982 and SM

Table 1 Susceptibility results of 30 *M. tuberculosis* strains obtained by the MTT and resazurin (REMA) assays performed in each site compared to the proportion method (PM)

	Rifampicin				Isoniazid				Ethambutol				Streptomycin			
	MTT		REMA		MTT		REMA		MTT		REMA		MTT		REMA	
PM	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Site 1																
R	15	0	15	0	12	0	12	0	8	1	8	1	10	2	10	2
S	0	15	0	15	0	18	0	18	1	20	1	20	3	15	5	13
Site 2																
R	15	0	15	0	12	0	12	0	9	0	9	0	12	0	12	0
S	0	15	0	15	0	18	0	18	0	21	0	21	3	15	3	15
Site 3																
R	15	0	14	1	12	0	12	0	9	0	9	0	9	3	11	1
S	0	15	0	15	1	17	1	17	1	20	1	20	0	18	4	14
Site 4																
R	15	0	15	0	12	0	12	0	9	0	9	0	11	1	9	3
S	0	15	0	15	0	18	1	17	0	21	1	20	3	15	0	18
Site 5																
R	12	0	12	0	10	0	10	0	7	0	8	0	9	0	9	0
S	0	11	0	11	0	13	0	13	0	16	0	15	3	11	3	11
Site 6																
R	15	0	15	0	11	1	11	1	9	0	9	0	11	1	11	1
S	0	15	0	15	0	18	0	18	0	21	0	21	3	15	3	15
Site 7																
R	14	1	14	1	11	1	11	1	8	1	8	1	11	1	11	1
S	1	14	1	14	1	17	1	17	3	18	2	18	3	15	3	15

MTT = 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide; REMA = resazurin microtiter assay; R = resistant; S = susceptible.

Table 2 Specificity and sensitivity of the MTT and resazurin (REMA) assays against the first-line drugs obtained in the seven sites

	Rifampicin				Isoniazid				Ethambutol				Streptomycin			
	MTT		REMA		MTT		REMA		MTT		REMA		MTT		REMA	
	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec	Sens	Spec
Site 1	100	100	100	100	100	100	100	100	88.9	95.2	88.9	95.2	83.3	83.3	83.3	72.2
Site 2	100	100	100	100	100	100	100	100	100	100	100	100	100	83.3	100	83.3
Site 3	100	100	93.3	100	100	94.4	100	94.4	100	95.2	100	95.2	75.0	100	91.7	77.8
Site 4	100	100	100	100	100	100	100	94.4	100	100	100	95.2	91.7	83.3	75.0	100
Site 5	100	100	100	100	100	100	100	100	100	100	100	100	100	78.6	100	78.6
Site 6	100	100	100	100	91.7	100	91.7	100	100	100	100	100	91.7	83.3	91.7	83.3
Site 7	93.3	93.3	93.3	93.3	91.7	94.4	91.7	94.4	88.9	85.7	88.9	85.7	91.7	83.3	91.7	83.3
All sites	99.0	99.0	98.1	99.0	97.6	98.4	97.6	97.6	96.6	96.6	96.8	95.9	90.5	85.0	90.5	82.7
Overall accuracy	98.0%		97.5%		99.0%		98.5%		96.5%		96.5%		87.2%		85.7%	

MTT = 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide; REMA = resazurin microtiter assay; Sens = sensitivity; Spec = specificity.

0.904). With REMA these results ranged from 0.904 to 0.993 (INH 0.989; RMP 0.993; EMB 0.979 and SM 0.904). The difference between the average AUC results obtained by MTT and REMA was not significant for the tested drugs (INH $t = 2.490$, $P = 0.0375$; RMP $t = 0.016$, $P = 0.9879$; EMB $t = 0.201$, $P = 0.8454$; SM $t = 0.030$, $P = 0.9771$). The standard deviation for the AUC ranged from 0.0125 to 0.0341 when the strains were tested by MTT and from 0.015 to 0.0497 when tested by REMA in the seven participating laboratories.

DISCUSSION

The purpose of this multicentre study was to evaluate the reliability of two rapid low-cost methods, the MTT and resazurin assays, for DST of *M. tuberculosis* to the first-line drugs (RMP, INH, EMB, SM) in different Latin American countries working under local conditions.

It is well known that MDR-TB is a serious public health problem, and laboratories should be able to give rapid results of the susceptibility pattern of an isolate to initiate adequate patient treatment. As *M. tuberculosis* is considered to be MDR when it shows resistance to at least INH and RMP,[2] the two most effective agents for the treatment of TB, the results obtained are encouraging as no major discordances were found among the seven participating laboratories regarding detection of resistance to the two drugs. Even in underprivileged settings, the colorimetric microplate methodology can be considered a reliable tool to detect MDR-TB. EMB and SM are considered difficult drugs to test. The WHO quality assurance programme for DST of *M. tuberculosis*, created in 1994, reported that results of susceptibility to INH and RMP were highly reliable, whereas the results for SM and EMB showed substantial discordance, even when applying fully standardised methods endorsed by WHO.[30] Using such methods, highly qualified international laboratories achieved efficiencies of at least 99% for RMP, 97% for INH, and 92% for EMB and SM, according to supranational laboratory criteria.[30] In this multicentre study, using comparable methodology and evaluation criteria, both colorimetric methods yielded optimal overall efficiency for all first-line drugs, with the exception of SM. The median time for obtaining results was 8 days, which is similar to that required by the BACTEC TB-460 system but shorter than the conventional proportion method, which requires a minimum of 4 weeks to report the first results. The main disadvantage of the BACTEC TB-460 system is the radioactivity, requiring special disposal conditions and needles for inoculation. Other disadvantages are the cost of the machine and the imported culture media vials that limit the use of BACTEC TB-460 in middle- or low-income countries.

Both assays, MTT and REMA, showed very similar overall efficiencies; however, the interpretation of colour change seems to be easier with the MTT assay, as its colour shift was much sharper. On the other hand, resazurin is cheaper and does not precipitate as does MTT, avoiding a further step of solubilisation in the test and less manipulation of the plates. The cost of MTT and resazurin was calculated at 5 and 3 US$, respectively, for one isolate and against all drugs tested.[19,20] To perform the colorimetric assay, the laboratory needs a biosafety cabinet, which could be a limiting factor in some low-resource countries. In any case, neither method needs special instruments for reading the results, just a good eye to see the change in colour. This congruence in results obtained by eye adds evidence of the robustness of the methodology. As it is possible to conclude from the ROC AUC analyses, the general performance of the test was very high. The results obtained from the seven participating sites were homogeneously distributed, especially for INH, RMP and EMB, as indicated by the standard deviations. Both MTT and resazurin assays performed without any difference between them, suggesting that they could be implemented in clinical laboratories for the rapid detection of MDR-TB strains.

In summary, the present study is the first preliminary multicentre evaluation to assess the reliability of

the colorimetric methods MTT and resazurin in several laboratories working under local conditions and with limited facilities. The laboratories engaged in the study were located in middle- and low-income countries where TB is endemic. The work was performed under the same conditions and in facilities available for DST that these laboratories routinely provide as a service to their communities.

The results show that these colorimetric assays performed very well in detecting resistance of *M. tuberculosis* to INH, RMP and even for EMB. SM needs more standardisation to obtain acceptable levels of sensitivity and specificity. With the spread of MDR-TB there is increasing demand for new tools for rapid DST to first-line drugs. TB laboratories in countries with limited resources will need to be prepared to perform these new technologies.

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RÉSUMÉ

OBJECTIF : Une évaluation multicentrique a été réalisée dans le but d'étudier la performance de deux méthodes rapides et peu coûteuses, les tests MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) et resazurin, pour la détection de la résistance de *Mycobacterium tuberculosis* aux médicaments antituberculeux de première intention : rifampicine (RMP), isoniazide (INH), éthambutol (EMB) et streptomycine (SM).
MÉTHODES : Trente souches codées de *M. tuberculosis* ont été envoyées à sept laboratoires localisés en Amérique Latine, représentant six pays. Chaque laboratoire a réalisé de manière aveugle les méthodes colorimétriques, MTT et resazurin pour les médicaments de première ligne, RMP, INH, EMB et SM ; les résultats de la concentration minimale inhibitrice ont été comparés à la méthode des proportions sur milieu de Löwenstein-Jensen.
RÉSULTATS : Après avoir établi la concentration critique pour chaque antibiotique, d'excellents résultats ont été obtenus pour la RMP, l'INH et l'EMB, avec une spécificité et une sensibilité comprises entre 96% et 99%.
CONCLUSION : Les tests colorimétriques MTT et resazurin sont des méthodes alternatives très prometteuses et accessibles aux pays à revenus moyens ou faibles qui devraient disposer de méthodes rapides et peu coûteuses pour réaliser les tests de sensibilité de *M. tuberculosis* aux médicaments antituberculeux de première intention.

RESUMEN

OBJETIVO : Se realizó un ensayo multicéntrico con el fin de evaluar dos métodos rápidos y de bajo costo, el método del MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) y el de la resazurina, para medir la susceptibilidad de *Mycobacterium tuberculosis* a los antibióticos de primera línea rifampicina (RMP), isoniazida (INH), etambutol (EMB) y estreptomicina (SM).
MÉTODOS : Treinta cepas codificadas de *M. tuberculosis* fueron enviadas a siete laboratorios en Latinoamérica, representando seis países diferentes. Cada laboratório realizó de manera ciega los ensayos colorimétricos, MTT y resazurina, para los antibióticos de primera línea RMP, INH, EMB and SM ; las concentraciones mínimas inhibidoras obtenidas fueron comparadas con el método convencional de las proporciones en médio de Löwenstein-Jensen.
RESULTADOS : Luego de establecer los puntos de corte, se obtuvieron resultados excelentes para RMP, INH y EMB, con una especificidad y sensibilidad de entre 96% y 99%.
CONCLUSIÓN : El método del MTT y de la resazurina constituyen alternativas prometedoras y accesibles para países de bajos y medianos recursos en busca de métodos de bajo costo para realizar ensayos rápidos de susceptibilidad a antibióticos de *M. tuberculosis*.