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Multicentre laboratory validation of the colorimetric redox indicator (CRI) assay for the rapid detection of extensively drug-resistant (XDR) *Mycobacterium tuberculosis*

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Objectives: To perform a multicentre study to evaluate the performance of the colorimetric redox indicator (CRI) assay and to establish the MICs and critical concentrations of rifampicin, isoniazid, ofloxacin, kanamycin and capreomycin.

Methods: The study was carried out in two phases. Phase I determined the MIC of each drug. Phase II established critical concentrations for the five drugs tested by the CRI assay compared with the conventional proportion method.

Results: Phase I: a strain was considered resistant by the CRI assay if the MIC was ≥0.5 mg/L for rifampicin, ≥0.25 mg/L for isoniazid, ≥4.0 mg/L for ofloxacin and ≥5.0 mg/L for kanamycin and capreomycin. Sensitivity was 99.1% for isoniazid and 100% for the other drugs and specificity was 97.9% for capreomycin and 100% for the other drugs. Phase II: the critical concentration was 0.5 mg/L for rifampicin, 0.25 mg/L for isoniazid, 2.0 mg/L for ofloxacin and 2.5 mg/L for kanamycin and capreomycin giving an overall accuracy of 98.4%, 96.6%, 96.7%, 98.3% and 90%, respectively.

Conclusions: Results demonstrate that the CRI assay is an accurate method for the rapid detection of XDR *Mycobacterium tuberculosis*. The CRI assay is faster than the conventional drug susceptibility testing method using solid medium, has the same turnaround time as the BACTEC MGIT 960 system, but is less expensive, and could be an adequate method for low-income countries.

Keywords: tuberculosis, resazurin, drug resistance, second-line drugs

Introduction

Tuberculosis (TB) continues to be a global health problem due to the emergence and spread of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) worldwide.[1] TB laboratories play an important role in optimizing patient treatment by timely identification of drug-resistant cases, which is a crucial step in avoiding the spread of the disease, and initiation of adequate public health measures. Several methods of detecting drug resistance are available, but more evidence on the performance of new rapid low-cost technologies to detect MDR-TB in patients is urgently needed, especially since the Global XDR-TB Response Plan calls for wide-scale implementation of rapid methods to screen patients at risk of MDR-TB and XDR-TB.[2] Some limitations for low-income countries in the use of commercial technologies available today are the cost of equipment and delivery of consumables; therefore these settings still use the conventional methods on agar or Löwenstein–Jensen (LJ) medium that take months to obtain results.[3] Recently, the WHO Strategic and Technical Advisory Group for Tuberculosis[4] endorsed and recommended a new policy on the use of non-commercial culture and drug susceptibility testing (DST)

methods. Among them, the colorimetric redox indicator (CRI) assay has been recommended for screening patients suspected of having MDR-TB. The CRI assay is faster than the conventional proportion method (PM) performed on solid culture medium and is less expensive than commercial liquid culture methods and molecular line probe assays. There are currently only a few studies evaluating the use of the CRI assay for the detection of resistance to second-line drugs. The objective of this study was to perform a multicentre evaluation of the performance of the CRI assay using the resazurin microtitre assay (REMA) compared with the PM as the gold standard.

Materials and methods

Study sites

The study was carried out at five different sites: the State Agency 'LIC', Clinic of Tuberculosis and Lung Diseases, Riga, Latvia; the Dr Cetrángolo Hospital, Vicente López, Buenos Aires, Argentina; the Swedish Institute for Infectious Disease Control, Solna, Sweden; the Instituto Nacional de Salud, Bogota, Colombia; and the Institute of Tropical Medicine (ITM) in Antwerp, Belgium.

Study design

The study was conducted in two phases. Phase I was designed to validate the procedure and to determine the range of MICs of each drug. A total of 149 clinical isolates of *Mycobacterium tuberculosis*, consisting of 113 MDR-TB strains, 28 clinical isolates fully susceptible to first-line drugs and 8 multiresistant isolates, were included as well as the *M. tuberculosis* H37Rv strain (ATCC 27294) for quality control. Fresh subcultures of all isolates were prepared before starting the study. In Phase II, two concentrations of each drug based on the results of Phase I were chosen and a subset of 30 strains with different resistance patterns to second-line drugs [consisting of 23 MDR-TB strains (including 7 XDR-TB strains), 6 fully susceptible strains and 1 isoniazid-monoresistant strain] were sent and tested blindly by the study sites. Results were analysed and compared with the PM method performed at ITM.

Antimicrobial agents

Stock solutions of isoniazid and rifampicin (Sigma-Aldrich NV/SA, Bornem, Belgium) were prepared at 1 mg/mL in distilled water and 10 mg/mL in methanol, respectively. Stock solutions of ofloxacin, capreomycin sulphate (Sigma-Aldrich, St Louis, MO, USA) and kanamycin monosulphate (ICN Biomedicals Inc., Aurora, OH, USA) were prepared at 1 mg/mL (ofloxacin dissolved in 0.1 N NaOH, and capreomycin and kanamycin in sterile distilled water). Stock solutions were filter sterilized and stored at −20°C for no more than 30 days.

REMA

Resazurin sodium salt (Acros Organic NV, Geel, Belgium) was prepared at 0.02% (w/v) in distilled water, sterilized by filtration and stored at 4°C for no more than 2 weeks. The REMA plate method was carried out as previously described by Martin *et al.*[5] Briefly, the inoculum was prepared from a 3-week-old LJ culture resuspended in 7H9-S medium (Middlebrook 7H9 broth/0.1% casitone/0.5% glycerol/10% oleic acid, albumin, dextrose and catalase), adjusted to a turbidity equivalent to that of a 1 McFarland standard and diluted 1:10; 100 μL of the 1:10 dilution was used as the inoculum. Serial 2-fold dilutions of each drug were prepared directly in a sterile 96-well flat-bottomed microtitre plate (Becton-Dickinson) in a final volume of 100 μL of 7H9-S. The drug concentrations used in Phase I were: rifampicin, 2.0–0.06 mg/L; isoniazid, 1.0–0.03 mg/L; ofloxacin, 8.0–0.25 mg/L; kanamycin, 20–0.62 mg/L; and capreomycin, 10–0.31 mg/L. The drug concentrations used in Phase II were: rifampicin, 0.5 and 1.0 mg/L; isoniazid, 0.25 and 0.5 mg/L; ofloxacin, 2.0 and 4.0 mg/L; kanamycin, 2.5 and 5.0 mg/L; and capreomycin, 2.5 and 5.0 mg/L. A growth control containing no antibiotic and a control without inoculation were also prepared in each plate. The plate was covered with its lid, sealed in a plastic bag and incubated at 37°C in a normal atmosphere. After 7 days of incubation, 30 μL of 0.02% resazurin solution was added to each well and the plate was re-incubated for 24–48 h. A change in colour from blue (oxidized 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.

DST by the PM

For rifampicin and isoniazid DST, the PM was performed on LJ medium according to the standard procedure[3] with the recommended critical concentrations of 40 mg/L for rifampicin and 0.2 mg/L for isoniazid. Tubes were incubated at 37°C and read after 28 and 42 days. For ofloxacin, kanamycin and capreomycin the PM was performed on 7H11 agar according to the standard procedure[6] with the recommended critical concentrations of 2 mg/L for ofloxacin, 6 mg/L for kanamycin and 10 mg/L for capreomycin. Results were read after 21 days of incubation at 37°C at 5% CO_2.

Resolution of discrepancies

If discrepant results were obtained between the REMA and the PM in the Phase I study the test was repeated. Results obtained were used for the data analysis. If discrepant results were observed after repetition, DNA sequencing of genes involved in resistance to each specific drug and sensitivity and specificity of the REMA for each drug were recalculated and termed 'resolved discrepancies'.

Sequencing

For discordant rifampicin, isoniazid, ofloxacin and capreomycin resistance results between the REMA and the PM the genes *rpoB*, *inhA*, *katG*, *gyrA*, *rrs* and *tlyA* were sequenced.

For rifampicin, the primers OPRIF-F (5′-CGG TCG GCG AGC TGA TCC-3′) and OPRIF-R (5′-TGG ACC CGC GCG TAC ACC-3′) were used to amplify the rifampicin resistance-determining region (RRDR) of *rpoB*. PCR and sequencing of the fragment were carried out using a previously described method.[7] For isoniazid, amplification of *katG* and *inhA* was performed as previously described[8] using the following primers: *katG*, TB86 (5′-GAA ACA GCG GCG GCG CTG GAT CGT-3′) and TB87 (5′-GTT GTC CCA TTA CGT CGG GG-3′); and *inhA*, TB92 (5′-CCT CGC TGC CCA GAA AGG GA-3′) and TB93 (5′-ATC CCC CGG TTT CCT CCG GT-3′).

For ofloxacin, amplification of *gyrA* was performed using primers gyrA.PCR.F1 (5′-TTCGATTCCGGCTTCC-3′) and gyrA.PCR.R1.biotin (5′-GGGCTTC GGTGTACCTCAT-3′), the latter being biotinylated at its 5′ end. The sequencing primer gyrA.seq1 (5′-GGCAACTACCACCC-3′) was designed to detect mutations in the quinolone resistance-determining region (codons 88–94).

For capreomycin, the *tlyA* gene plus ∼200 bp upstream and 50 bp downstream was amplified using primers tlyA.F2 (5′-CCATCGTTCGGG CTGTG-3′) and tlyA.R2 (5′-CAGAGTCAAGCGGTCTTCCA-3′). The entire *rrs* gene was amplified using primers F133 (5′-TGGCCGTTTGTTTTGTC AGGATA-3′) and R585 (5′-AAGTCCGAGTGTTGCCTCAGG-3′). All PCR products were purified using the GFX PCR Purification Kit (GE Healthcare, Little Chalfont, UK) and sequenced using the Big Dye v3.1 Kit (Applied Biosystems, Foster City, CA, USA).

Analysis of data

We have calculated the sensitivity, the true positive rate expressed as a percentage [true positive (TP)/TP+false negative (FN)], and the specificity, the true negative rate expressed as a percentage [true negative (TN)/ TN+false positive (FP)], for each drug compared with the PM.

Results

The MICs for 149 isolates of *M. tuberculosis* were determined by visual reading of the REMA plate for the five drugs (rifampicin, isoniazid, ofloxacin, kanamycin and capreomycin), with results obtained after 8 days of incubation. Results of the PM were available after 3–6 weeks. Isolates found susceptible by the REMA and resistant by the PM or vice versa were considered as discordant and were re-evaluated by both methods, and additionally by sequencing if discordant twice.

Phase I

Table 1 summarizes the results of the Phase I study. Most strains tested gave a high concordance between the MIC obtained with the REMA and the PM. A strain was considered resistant by the

Table 1. Susceptibility testing results of the Phase I study of 149 isolates of *M. tuberculosis* by the PM and the REMA plate method for rifampicin, isoniazid, ofloxacin, kanamycin and capreomycin

		PM		Initial results		Resolved discrepancies[a]	
Drugs	REMA MICs (mg/L)	resistant (no. of isolates)	susceptible (no. of isolates)	sensitivity (%)	specificity (%)	sensitivity (%)	specificity (%)
RIF	≥2	113		100	94	100	100
	1						
	0.5[b]		2				
	0.25		22				
	0.125		3				
	≤0.062		9				
INH	≥1	113		99.1	100	99.1	100
	0.5	6					
	0.25[b]	1					
	0.125	1					
	0.062		1				
	≤0.031		27				
OFX	≥8	17		97.5	100	100	100
	4[b]	23					
	2	1	1				
	1		17				
	0.5		55				
	≤0.25		35				
KAN	≥20	51		100	100	—	—
	10	7					
	5[b]	4					
	2.5		20				
	1.25		53				
	≤0.62		14				
CAP	≥10	18		100	97.9	100	97.9
	5[b]	29					
	2.5	1	3				
	1.25		38				
	0.62		50				
	≤0.31		10				

[a]Sensitivity and specificity of the REMA taking into account the results of sequencing.
[b]Rifampicin (RIF), MIC ≥0.5=resistant; isoniazid (INH), MIC ≥0.25=resistant; ofloxacin (OFX), MIC ≥4=resistant; kanamycin (KAN), MIC ≥5=resistant; capreomycin (CAP), MIC ≥5=resistant.

Table 2. DST results of the Phase II study for the subset of 30 *M. tuberculosis* strains; DST was performed at each site and tested at two critical concentrations by the REMA and compared with the PM

		REMA																	
		Rifampicin (mg/L)				isoniazid (mg/L)				ofloxacin (mg/L)				kanamycin (mg/L)				capreomycin (mg/L)			
		0.5		1.0		0.25		0.5		2.0		4.0		2.5		5.0		2.5		5.0	
Site	PM	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Site A	R	23	0	23	0	24	0	24	0	12	0	9	3	11	0	11	0	9	1	3	7
	S	0	7	0	7	0	6	0	6	0	18	0	18	0	19	0	19	0	20	0	20
Site B	R	22	1	20	3	24	0	24	0	9	3	4	8	10	1	10	1	4	6	1	9
	S	0	7	0	7	0	6	0	6	0	18	0	18	0	19	0	19	0	20	0	20
Site C	R	23	0	23	0	24	0	24	0	12	0	9	3	11	0	11	0	10	0	5	5
	S	0	7	0	7	2	4	2	4	1	17	1	17	4	15	0	19	3	17	2	18
Site D	R	23	0	23	0	24	0	24	0	12	0	11	1	11	0	11	0	8	2	4	6
	S	1	6	1	6	2	4	2	4	0	18	0	18	0	19	0	19	0	20	0	20

R, resistant; S, susceptible.

REMA if the MIC value was ≥0.5 mg/L rifampicin, ≥0.25 mg/L isoniazid, ≥4.0 mg/L ofloxacin and ≥5 mg/L capreomycin and kanamycin. For rifampicin we had two discordant strains, classified as resistant by the REMA with an MIC of 0.5 mg/L, but susceptible by the PM. The RRDR for *rpoB* was sequenced and one of the strains had a double mutation P505V/L511P and the other had a single mutation H526L. P505V has, to our knowledge, never been reported before. L511P and H526L are associated with rifampicin resistance; therefore these strains were classified as resistant. As seen in Table 1, we recalculated the specificity and the sensitivity of the REMA according to the sequencing results (resolved discrepancies), which gave a specificity and sensitivity of 100%. For isoniazid, we found one discordant strain, for which the REMA MIC was 0.125 mg/L and which was classified as resistant by the PM. Subsequent sequencing of *katG* showed a wild-type sequence and a −15C/T mutation was found in the promoter region of *inhA*; −15C/T is associated with low-level isoniazid resistance.[8] Accordingly, the sensitivity and specificity of the REMA for isoniazid were 99.1% and 100%, respectively.

For ofloxacin, one discordant strain was classified as susceptible by the REMA, with an MIC of 2 mg/L, but was found resistant by the PM. After sequencing, we found a Ser95Thr mutation in the *gyrA* gene; however, Ser95Thr is a polymorphism not correlated with fluoroquinolone resistance.[9] We have classified this strain to be most probably susceptible to ofloxacin, giving a sensitivity and specificity of 100%. For kanamycin we found 100% concordance with the PM.

For capreomycin, one discordant strain showed an MIC by the REMA of 2.5 mg/L (susceptible), but was classified as resistant by the PM. Resistance to capreomycin is associated with mutations in the 16S rRNA gene (*rrs*) and also with mutations in the *tlyA* gene.[10] Sequencing results revealed a mixed population—a resistant population with a mutation in the *rrs* gene (A1401G) and a susceptible population. This was a difficult strain to classify and in this case we elected not to take into account the result of the sequencing, giving 100% sensitivity and 97.9% specificity.

Phase II

Results of the Phase II study are summarized in Table 2. Critical concentrations used in the REMA were 0.5 mg/L and 1.0 mg/L for rifampicin, 0.25 mg/L and 0.5 mg/L for isoniazid, 2.0 mg/L and 4.0 mg/L for ofloxacin and 2.5 mg/L and 5.0 mg/L for kanamycin and capreomycin. Table 2 shows the number of resistant and susceptible strains for each site obtained by the REMA according to the two critical concentrations defined compared with the results of the PM. Specificity and sensitivity values are shown in Table 3 for rifampicin and isoniazid and in Table 4 for ofloxacin, kanamycin and capreomycin. For rifampicin, the critical concentration of 0.5 mg/L yielded the most concordant results with an overall accuracy of 98.4%, for isoniazid a critical concentration of 0.25 mg/L gave an overall accuracy of 96.6%. For second-line drugs, we chose a critical concentration of 2 mg/L for ofloxacin, 2.5 mg/L for kanamycin and 2.5 mg/L for capreomycin giving an overall accuracy of 96.7%, 98.3% and 90%, respectively.

Discussion

The aims of this study were to determine the critical concentrations of second-line drugs to be used in a CRI assay using resazurin as a dye indicator and to evaluate the performance of the REMA in a multicentre study. The PM was considered the gold standard for comparison purposes and DNA sequencing was performed for the discordant results between the two methods. The critical concentration (also termed breakpoint) is not identical to the MIC. The MIC is the lowest concentration of the drug that inhibits visible growth of the bacterium.[3,11] The MIC is usually considered as reference for critical concentration

Table 3. Specificity and sensitivity of the REMA for rifampicin and isoniazid obtained at each site

	Rifampicin (mg/L)						Isoniazid (mg/L)					
	0.5			1.0			0.25			0.5		
Site	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)
Site A	100	100	100	100	100	100	100	100	100	100	100	100
Site B	95.6	100	96.7	86.9	100	90	100	100	100	100	100	100
Site C	100	100	100	100	100	100	100	66.6	93.3	100	66.6	93.3
Site D	100	85.7	96.6	100	85.7	96.6	100	66.6	93.3	100	66.6	93.3
Overall	98.9	96.4	98.4	96.8	96.4	96.6	100	83.3	96.6	100	83.3	96.65

Table 4. Specificity and sensitivity of the REMA for ofloxacin, kanamycin and capreomycin obtained in each site

	Ofloxacin (mg/L)						Kanamycin (mg/L)						Capreomycin (mg/L)					
	2.0			4.0			2.5			5.0			2.5			5.0		
Site	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)	sensitivity (%)	specificity (%)	accuracy (%)
Site A	100	100	100	75	100	90	100	100	100	100	100	100	90	100	96.7	30	100	76.7
Site B	75	100	90	33	100	73.3	90.9	100	96.6	90.9	100	96.6	40	100	80	10	100	70
Site C	100	94.4	96.6	75	94.4	96.6	100	95	96.6	100	95	96.6	100	85	90	50	90	76.6
Site D	100	100	100	91.6	100	99.6	100	100	100	100	100	100	80	100	93.3	40	100	80
Overall	93.7	98.6	96.7	68.7	98.6	89.8	97.7	98.8	98.3	97.7	98.7	98.3	77.5	96.3	90	32.5	97.5	75.8

Table 5. MIC and critical concentration recommended to be used in the REMA compared with BACTEC MGIT 960

Drug	MIC to define resistance in the REMA (mg/L)	Critical concentration recommended (mg/L)	
		REMA	MGIT 960
Rifampicin	≥0.50	0.50	1.0
Isoniazid	≥0.25	0.25	0.1
Ofloxacin	≥4.0	2.0	2.0
Kanamycin	≥5.0	2.5	2.5
Capreomycin	≥5.0	2.5	2.5

assessment. To interpret DST results and avoid testing several different concentrations, the critical concentration has been used to define isolates as susceptible or resistant. Only a few studies in the literature have reported the MICs of second-line drugs using the CRI method.[5,12] This study confirmed the tentative breakpoints of a previous study[5] as 2.0 mg/L for ofloxacin and 2.5 mg/L for kanamycin and capreomycin. A study by Morcillo *et al.*[12] in 2004 using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) as indicator proposed breakpoints for kanamycin and capreomycin of 8.0 mg/L and 4.0 mg/L, respectively.

Phase I of this study was carried out in order to establish the REMA MIC of each drug and for this purpose we selected a high number of MDR-TB strains (76%) to be representative. Phase II led us to propose breakpoints. Further evaluations (Phase III) need to validate the critical concentrations established in Phase II. In a Phase III evaluation, clinical isolates will have to be tested in the field with a high number of susceptible and resistant strains. For rifampicin, only the REMA could correctly identify the resistant strains showing mutations P505V/L511P and H526L in *rpoB*. These strains were found to be borderline by the REMA (MIC 0.5 mg/L).

A summary of the MICs and critical concentrations chosen for the REMA are shown in Table 5. These are compared with the critical concentrations recommended for the BACTEC MGIT 960 system.[13,14] Data from the present study suggest that the more appropriate critical concentration for capreomycin is 2.5 mg/L as it differentiates resistance more correctly according to the PM and the DNA sequencing results. For the REMA we proposed critical concentrations of 2.0 mg/L for ofloxacin and 2.5 mg/L for kanamycin and capreomycin. These concentrations are the same as those that have been proposed for the BACTEC MGIT 960 system. This study showed that susceptibility testing of second-line drugs with the REMA yielded reliable results and a Phase III study is ongoing. The CRI assay requires a minimal training period during which the technician can become familiar with the method before implementing it in the field. We recommend in a first step to do a comparison of the CRI assay and the PM and/or BACTEC MGIT 960 before using the CRI assay in routine work. Quality control is necessary for any DST method. On the other hand, protocols should be strictly followed without any unjustified modifications to avoid unsatisfactory results as has been recently reported[15] showing a low sensitivity probably due to the modification of the inoculum in the plate. For quality control one fully susceptible and one MDR strain should be run in each new lot of drugs and reagents to control whether the medium was prepared correctly and the test is performing as expected. The CRI assay is faster than the conventional DST methods using solid medium giving results after only 1 week of incubation compared with 3–6 weeks for solid medium, and has the same turnaround time as the BACTEC MGIT 960 system, but is less expensive.

The use of the CRI assay for DST has recently been recommended by the WHO.[4] This study promotes a wider use of the REMA as a new and rapid phenotypic diagnostic test for the detection of MDR-TB and XDR-TB, especially in developing countries where rapid and inexpensive methods are urgently needed. It is important to ensure that the CRI assay can be properly integrated within the national TB control programmes that can be used within appropriate TB laboratory services and biosafety requirements.

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Transparency declarations

None to declare.

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