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Multicentre study to establish interpretive criteria for clofazimine drug susceptibility testing

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SUMMARY

OBJECTIVE: To conduct a multicentre study to establish the critical concentration (CC) for clofazimine (CFZ) for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* on the MGIT™960™ system using the distribution of minimum inhibitory concentrations (MIC) and genotypic analyses of *Rv0678* mutations.

DESIGN: In phase I of the study, the MIC distribution of laboratory strains (H37Rv and in vitro-selected *Rv0678* mutants) and clinical pan-susceptible isolates were determined ($n = 70$). In phase II, a tentative CC for CFZ ($n = 55$) was proposed. In phase III, the proposed CC was validated using clinical drug-resistant tuberculosis (DR-TB) isolates stratified by *Rv0678* mutation ($n = 85$).

RESULTS AND CONCLUSION: The MIC distribution of CFZ for laboratory and clinical pan-susceptible strains ranged between 0.125 μg/ml and 0.5 μg/ml. As the MIC values of DR-TB isolates used for phase II ranged between 0.25 μg/ml and 1 μg/ml, a CC of 1 μg/ml was proposed. Validation of the CC in phase III showed that probably susceptible and probably resistant *Rv0678* mutants overlapped at 1 μg/ml. We therefore recommend a CC of 1 μg/ml, with additional testing at 0.5 μg/ml to define an intermediate category. This was the first comprehensive study to establish a CC for routine phenotypic DST of CFZ using the MGIT960 system to guide therapeutic decisions.

KEY WORDS: CFZ; drug-resistant tuberculosis; MGIT™ 960™

CLOFAZIMINE (CFZ) is a riminophenazine agent that was initially developed for the treatment of tuberculosis (TB) in the 1950s.[1–3] CFZ is first-line treatment for leprosy,[4] but has now been repurposed for treatment of drug-resistant tuberculosis (DR-TB). However, due to poor in vivo results in initial studies, CFZ was thought to be ineffective for the treatment of TB.[5]

Renewed interest in the use of CFZ resulted from the findings of the 'Bangladesh regimen', which demonstrated successful outcomes in shortened DR-TB treatment regimens containing CFZ.[6] Subsequent studies confirmed those results, leading to the endorsement of a short, CFZ-containing multidrug-resistant TB (MDR-TB) regimen by the World Health Organization (WHO).

Clinical resistance to CFZ is difficult to ascertain because it is administered as part of combination therapy, and has been reported to be rare.[7] Defining resistance is thus dependent on laboratory-based criteria using the wild-type (wt) distribution of minimal inhibitory concentrations (MICs) for CFZ in *Mycobacterium tuberculosis* isolates; however, even with this approach, the literature is very limited. A cut-off point of 1 μg/ml for detecting resistance to CFZ was proposed using the MGIT™ 960™ system (BD, Sparks, MD, USA) in a local study in the Netherlands comprising only 26 MDR-TB and extensively drug-resistant TB (XDR-TB) isolates.[8] A critical concentration (CC) for CFZ testing has not been defined by the Clinical & Laboratory Standards Institute (Wayne, PA, USA) or US Food and Drug Administration (Silver Spring, MD, USA), although

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the WHO has recently determined a CC of 1 μg/ml based on small studies and unpublished data.[9]

Mutations in *Rv0678*, a regulator of the MmpS5-MmpL5 efflux pump, have been shown to lead to increased MICs of CFZ (2–4-fold) and bedaquiline (BDQ) (2–8-fold),[10–12] as well as to confer cross-resistance to both drugs.[10,13] Other genes such as *Rv1979c* or *Rv2535c* (PepQ) might be associated with increased MICs, but their resistance mechanisms have not been clearly established.[10]

With increasing use of CFZ in the treatment of MDR/XDR-TB, a reliable drug susceptibility testing (DST) method is needed. In the present multicentre study, we sought to establish the CC of CFZ for DST of *M. tuberculosis* on the MGIT960 system using wt MIC distributions and to evaluate if a *Rv0678* mutation was present.

MATERIALS AND METHODS

Study design and setting

The present study was carried out in three phases at four mycobacteriology laboratories. The participating sites were the Forschungszentrum Borstel, National Reference Centre for Mycobacteria, Borstel, Germany (site 1); the P D Hinduja National Hospital and Medical Centre, Mumbai, India (site 2); the Centre for Tuberculosis, National Institute for Communicable Diseases, Johannesburg, South Africa (site 3); and the Mycobacteriology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium (site 4).

In phase I, we determined the MIC range and a tentative CC for CFZ using laboratory isolates (pan-susceptible H37Rv [American Type Culture Collection 27294] and in vitro-selected *Rv0678* mutants), as well as pan-susceptible clinical isolates. In Phase II, we determined the MIC distribution and proposed a tentative CC using clinically resistant isolates. In phase III, we validated the proposed CC.

Ethics approval was not required for this laboratory-based study, as anonymised stored clinical isolates were used.

Minimum inhibitory concentration testing and drug preparation

CFZ MIC testing was performed using the MGIT960 system following the standard protocol for DST for first-line drugs (Becton Dickinson, Franklin Lakes, NJ, USA). CFZ powder was obtained from Sigma-Aldrich (Saint Louis, MO, USA). A 40 μg/ml stock solution was prepared by dissolving CFZ in dimethylsulfoxide (DMSO), and stored in small aliquots at –20°C until further use. On the day of testing, a 1:10 dilution was prepared from the thawed stock solution. Test concentrations were then made by two-fold serial dilutions ranging between 0.06 and 4.0 μg/ml (working solutions). All the dilutions were made in DMSO, and leftovers of working solutions were discarded. *M. tuberculosis* H37Rv was included for each batch as a control at all sites. MIC was defined as the lowest drug concentration to inhibit a strain.

Phase I: Determining the MIC distribution of CFZ for reference strains, in vitro-selected resistant strains and pan-susceptible clinical isolates

Pan-susceptible H37Rv was tested at each site in triplicate using the stock available onsite. In addition, nine local pan-susceptible clinical isolates and eight in vitro-selected *Rv0678* mutants provided by site 4 were tested. The pan-susceptible and in vitro-selected *Rv0678* mutant strains were tested at respectively 0.06 to 1 μg/ml and 0.25 to 4 μg/ml. The MIC distribution for H37Rv, pan-susceptible, clinical and in vitro-selected *Rv0678* mutant strains was plotted. The tentative CC was defined as the concentration at which 95% of the susceptible isolates were inhibited. A graph of this tentative CC for CFZ was visually compared to that of the in vitro-selected *Rv0678* mutants for additional confirmation.

Phase II: Determining the MIC distribution and evaluating the tentative CC for CFZ among clinical MDR/XDR M. tuberculosis *isolates*

The MIC distribution for CFZ was determined by each site using local clinical isolates with known drug resistance to first-line (MDR-TB) and/or to second-line anti-tuberculosis (XDR-TB) drugs. Five concentrations from 0.25 to 4.0 μg/ml were tested.

Phase III: Validation of critical concentration

Each site independently tested local MDR/XDR-TB isolates not included in phases I and II. In addition, all isolates underwent sequencing to detect mutations in *Rv0678*. Isolates with wt *Rv0678* were classified as 'probably susceptible' (PS), while isolates with resistance-associated *Rv0678* mutations were classified as 'probably resistant' (PR). Isolates were classified as PS if a mutation with MICs in the wt range which had not been previously described was found; if the MICs were in the non-wt range, they were considered PR. However, all singleton mutants were classified as PR.

Five concentrations from 0.25 to 4.0 μg/ml were tested. The final CC was established based on phase III results and defined as the concentration at which 95% of the PS isolates were inhibited. The number and proportion of isolates classified as susceptible using the final CC was evaluated against PR isolates harbouring the *Rv0678* mutation. If the MICs of >20% of the PR *Rv0678* mutants and the CC overlapped, an intermediate category was proposed to minimise reporting errors.

Table 1 Minimum inhibitory concentration of clofazimine for H37Rv using the MGIT™960™ system

	H37RV		
Laboratory	Sample 1	Sample 2	Sample 3
Site 1	0.5	0.25	0.25
Site 2	0.5	0.5	0.5
Site 3	0.25	0.5	0.5
Site 4	0.125	0.125	0.25

Sequencing of Rv0678

Whole-genome sequencing or targeted sequencing (*Rv0678*) of phase III isolates was performed using Illumina platforms (MiSeq or Next500; San Diego, CA, USA) at sites 1–3. Resequencing analyses of *Rv0678* were performed for variant calling using CLC Genomics Workbench v 10 (Qiagen, Venlo, The Netherlands) against the H37Rv Sanger reference genome (Genebank NC000962.3). Variants were called if they were present at a minimum frequency of 30% of the sequence reads at that position.

At site 4, *Rv0678* and part of the intergenic region between mmpS5 and *Rv0678* were amplified and sequenced using the same primers. To analyse the resultant sequences, the *Rv0678* sequence from *M. tuberculosis* H37Rv was taken as the reference (http://tuberculist.epfl.ch).

RESULTS

Phase I

The MIC distribution of CFZ for the H37Rv strain at the four sites ranged between 0.125 μg/ml and 0.5 μg/ml, with a modal MIC of 0.5 μg/ml. The variations between triplicate testing for H37Rv per site differed by a maximum of one dilution, indicating excellent reproducibility (Table 1).

Due to technical problems with the diluent used for drug preparation at site 2, data on pan-susceptible clinical isolates were excluded from the analysis. Among the 27 pan-susceptible clinical isolates from the three sites, one isolate yielded invalid results after repeat testing. MIC results were available for 26 isolates. The MIC values ranged from 0.25 μg/ml to 0.5 μg/ml, with the exception of one isolate, which had an MIC of 1 μg/ml (Figure 1).

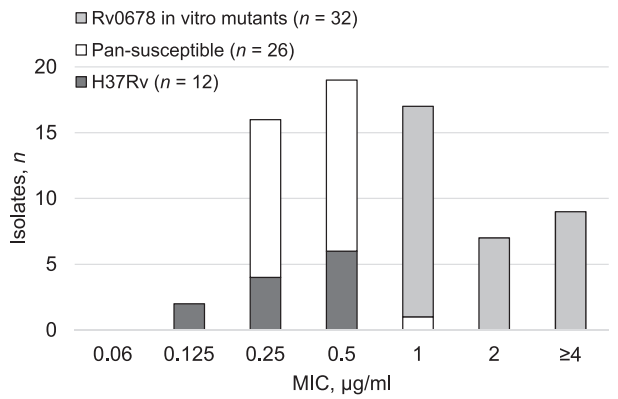

Figure 1 Clofazimine MIC distribution for H37Rv, pan-susceptible and in vitro-selected *Rv0678* mutant isolates (in μg/ml). MIC = minimum inhibitory concentration.

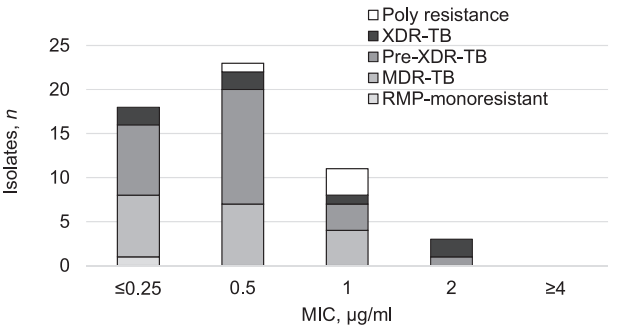

Figure 2 Clofazimine MIC distribution for MDR/XDR-TB isolates in study phase II ($n = 55$). MIC = minimum inhibitory concentration; XDR-TB = extensively drug-resistant tuberculosis; MDR-TB = multidrug-resistant TB; RMP = rifampicin.

The distribution of MICs of CFZ for in vitro-selected *Rv0678* mutants ranged between 1 μg/ml and 4 μg/ml. A tentative CC of 0.5 μg/ml was determined based on inhibition of >95% of the pan-susceptible isolates; the MIC of the in vitro-selected *Rv0678* mutants was consistently above this concentration upon visual inspection of the plot.

Phase II

The CFZ MIC results for MDR/XDR isolates was available only for sites 1, 2 and 3. Among the 55 clinical isolates tested, 7 were XDR-TB, 25 were pre-XDR-TB (defined as TB with resistance to isoniazid and rifampicin [RMP] and either a fluoroquinolone or a second-line injectable agent, but not both), 18 were MDR-TB, one was RMP-monoresistant and four had poly resistance. The MIC of CFZ for these isolates ranged between 0.25 μg/ml and 1 μg/ml at sites 1 and 3. At site 2, the MIC ranged between 0.25 μg/ml and 2 μg/ml. The MIC distribution and comparison of different CCs for MDR/XDR-TB isolates is shown in respectively Figure 2 and Table 2. Using a tentative CC of 0.5 μg/ml, only 74.5% (41/55) of the isolates were found to be susceptible. However, at a tentative CC of 1 μg/ml, 94.6% (52/55)

Table 2 Comparison of critical concentration cut-off points and resistance categorisation for clofazimine in multidrug-resistant and extensively drug-resistant tuberculosis isolates

		Tentative critical concentration							
		⩽0.25 μg/ml		0.5 μg/ml		1 μg/ml		2 μg/ml	
Site	*n*	S	R	S	R	S	R	S	R
Site 1	19	8	11	14	5	19			
Site 2	16	3	13	8	8	13	3	16	
Site 3	20	7	13	19	1	20			

S = susceptible; R = resistant.

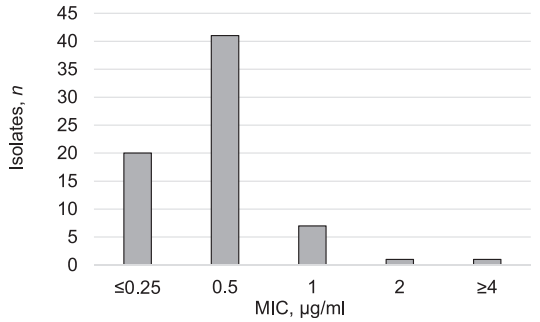

Figure 3 MIC distribution of wild-type clofazimine. MIC = minimum inhibitory

of the isolates were inhibited, with only 5.4% (3/5) of isolates at site 2 showing growth at this concentration.

Phase III

All four sites participated in the validation of the proposed CC in phase II. Of 88 isolates phenotypically tested during this phase, three isolates were excluded due to sequencing failure. Of the remaining isolates, 82.3% (70/85) were PS isolates and 17.6% (15/85) were PR isolates harbouring the *Rv0678* mutation. Of the PS isolates, 87% (61/70) had an MIC of $\leqslant$0.5 μg/ml, while 10% (7/70) had an MIC of 1 μg/ml (Figure 3). The remaining 2.9% (2/70) of the isolates had MICs of >1 μg/ml. Among the PR isolates with *Rv0678* mutations, 53.3% (8/15) had an MIC of >1 μg/ml, while 33.3% (5/15) had an MIC of 1 μg/ml, and 13.3% (2/15) had an MIC of $\leqslant$0.5 μg/ml (Table 3). Three isolates harbouring a V3I mutation all had an MIC of <0.25 μg/ml, and were therefore categorised as wt.

DISCUSSION

Our study was the first comprehensive, multicentre study to establish a CC for CFZ using the MGIT960 system. It provides evidence for the WHO-endorsed CC of 1.0 μg/ml,[9] which was based in part on findings from the present study.

The MIC distribution of CFZ was determined using laboratory isolates and clinical *M. tuberculosis* isolates from geographically diverse populations. A tentative CC of 0.5 μg/ml was proposed for susceptible isolates in phase I. However, the MIC range was increased for the MDR/XDR-TB isolates used (0.25 to 1 μg/ml) in phase II. A CC of 1 μg/ml was therefore proposed.

De Logu et al. reported the MICs of CFZ to be higher for RMP-resistant/MDR-TB isolates and pyrazinamide resistance than the pan-susceptible H37Rv.[14] This would also be concordant with a study from the Netherlands, which proposed a cut-off point of 1 μg/ml for MDR/XDR-TB using the MGIT960 system.[8]

Subsequent validation of the CC in phase III showed 87% of the isolates with a wt *Rv0678* would be classified as susceptible if a CC of 0.5 μg/ml was used while, at 1 μg/ml, 97% would be susceptible, confirming the proposed CC of 1 μg/ml. However, 33% of the isolates with an *Rv0678* mutation had a MIC of 1 μg/ml, classifying them as susceptible. In addition, 50% of the in vitro-selected *Rv0678* mutants tested in phase I had an MIC of 1 μg/ml. At a CC of 1 μg/ml, the PS and PR *Rv0678* mutants were thus not clearly separated. This problem could be resolved in part by introducing an intermediate category, which may also cover potential low-level resistance even if below the CC. The intermediate category is neither clearly resistant nor susceptible but provides a buffer category. Patients with an intermediate result could therefore be treated, but would need to be monitored, because *Rv0678* is a transcriptional regulator of an efflux pump, and higher MICs and resistance may thus develop upon drug exposure. The clinical relevance of such cases remains to be determined. We therefore propose testing at 0.5 μg/ml and at 1 μg/ml. If *M. tuberculosis* isolates show no growth at 0.5 μg/ml, the isolates are considered susceptible; if the isolates show growth at 0.5 μg/ml and no growth at 1 μg/ml, the isolates are considered intermediate, while growth at 1 μg/ml is considered resistant to CFZ. Despite the intermediate

Table 3 *Rv0678* mutations and corresponding MICs for clofazimine ($n = 15$)

		MIC					
Rv0678 mutation	*n*	$\leqslant$0.25 μg/ml	0.5 μg/ml	1 μg/ml	2 μg/ml	4 μg/ml	>4 μg/ml
Arg132Stp	2						2
Asn4Thr	1			1			
Gly6Trp	1			1			
Leu74Met	1				1		
Gly87Arg	1	1					
Insertion A in codon 92	1				1		
Glu49fs	3			1	1	1	
Ser53Leu	1				1		
Ser2Ile	1					1	
Gly121Arg	2			2			
Glu21Asp	1		1				

MIC = minimum inhibitory concentration.

category, 2/15 (13%) of the PR *Rv0678* mutant isolates with a MIC ⩽0.5 μg/ml would be classified as susceptible. These were, however, singleton mutants, which makes it difficult to interpret their importance. Technical errors cannot be ruled out, as MICs and the sequencing were not repeated in case valid results were obtained. Isolates occurring around the CC should also be further characterised, where available, by assessing a narrower MIC range (e.g., 0.5, 0.75 and 1 μg/ml) and sequencing *Rv0678*.

Variability in *Rv0678* mutations has been observed, and data on their relevance in CFZ resistance are limited. Xu et al. found that all isolates with *Rv0678* mutations ($n = 5$) had an MIC of >1 μg/ml.[15] In our study, most isolates with a *Rv0678* mutation had an MIC of ⩾1 μg/ml. Isolates with Ser53Leu and Ser2Ile mutations had an MIC of respectively 2 μg/ml and 4 μg/ml; these data are consistent with results from a previous study.[16] Two isolates with an Arg132Stp mutation had an MIC of >4 μg/ml, which suggests that the mutation plays a role in resistance. Three isolates had Glu49fs mutations, having MICs of 1, 2 and 4 μg/ml. Two isolates with Gly121Arg mutations had an MIC of 1 μg/ml. An MIC of 1 μg/ml in these cases may thus be related to CFZ resistance. This result has been corroborated by unpublished data (L Rigouts, personal communication) on two in vitro-selected Gly121Arg mutations with MICs of 1 μg/ml and 4 μg/ml. Further worldwide studies with a large number of strains are required to generate more data on the association of specific *Rv0678* and other mutations with MICs and their impact on treatment outcomes.

The present study had several limitations. Replicate testing was not performed for all the isolates used in our study. No information was available on previous CFZ exposure for the clinical isolates included, and the routine isolates in Antwerp were probably not representative of isolates in Belgium, but of isolates from low-income countries as it is a supranational reference laboratory. Furthermore, we did not sequence phase I and II isolates for *Rv0678* or other putative genes.

Despite these limitations, our study had important strengths: the laboratories involved were highly proficient in TB DST. In addition, inclusion of molecular testing for comparisons with phenotypic MICs provided greater understanding of the correlation between the phenotypic and genotypic testing of CFZ.

In summary, standardisation of the CFZ DST is important; DMSO was used to avoid solubility issues experienced early on in our study (data not shown). We recommend testing at two concentrations (0.5 μg/ml and 1.0 μg/ml). This approach is different from the WHO recommendation, which proposes a single concentration. Although the criteria for resistance remain the same, our recommendation to include an intermediate category is more conservative and may minimise false-susceptible results. However, given the uncertainty about the correlations between *Rv0678* mutations, phenotypic DST and lack of data correlating *Rv0678* mutations with clinical outcomes, the CC proposed in the present study must be critically re-evaluated in further studies. Furthermore, we recommend that the manufacturer of the MGIT960 system develop ready-to-use kits to perform CFZ testing, as has been done for other drugs. To date, CFZ resistance has not been studied in depth. Our study provides data for routine phenotypic DST for CFZ and information for future research.

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Conflicts of interest: none declared.

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RÉSUMÉ

OBJECTIF : Entreprendre une étude multicentrique pour établir la concentration critique (CC) de la clofazimine (CFZ) pour les tests de pharmacosensibilité (DST) de *Mycobacterium tuberculosis* sur le système MGIT™ 960™ recourant à la distribution de la concentration minimale inhibitrice (MIC) et à l'analyse génotypique des mutations de *Rv0678*.

SCHÉMA : Dans la Phase I de l'étude, la distribution de la MIC des souches de laboratoire (*H37Rv* et mutants sélectionnés in vitro de *Rv0678*) et les isolats cliniques pan susceptibles a été déterminée ($n = 70$); dans la Phase II, une CC tentative pour la CFZ ($n = 55$) a été déterminée; et dans la Phase III, la CC proposée a été validée en utilisant les isolats cliniques de tuberculose résistante (TB-DR) stratifiés par mutation *Rv0678* ($n = 85$).

RÉSULTATS ET CONCLUSION : La distribution des MIC de CFZ pour les souches de laboratoire et les souches cliniques pan susceptibles est allée de 0,125 à 0,5 µg/ml. Les valeurs de MIC des isolats de TB-DR utilisés pour la Phase II allaient de 0,25 à 1 µg/ml et une CC de 1 µg/ml a été proposée. La validation de la CC dans la Phase III a mis en évidence un chevauchement entre les mutants probablement sensibles et probablement résistants de *Rv0678* à 1 µg/ml. Nous recommandons donc une CC de 1 µg/ml et un test supplémentaire à 0,5 µg/ml définissant une catégorie intermédiaire. Ceci est la première étude complète visant à établir une CC pour le DST phénotypique de routine de la CFZ en utilisant le système MGIT960 afin de guider les décisions thérapeutiques.

RESUMEN

OBJETIVO: Emprender un estudio multicéntrico con el objeto de definir la concentración umbral de clofazimina (CFZ) en las pruebas de sensibilidad de *Mycobacterium tuberculosis* en el sistema MGIT™ 960™, mediante la distribución de la concentración inhibidora mínima (MIC) y el análisis genotípico de las mutaciones del gen *Rv0678*.

MÉTODOS: En la Fase I del estudio se determinó la distribución de la MIC de las cepas de laboratorio (*H37Rv* y mutantes *Rv0678* seleccionados in vitro) y en aislados clínicos pansensibles ($n = 70$); en la Fase II, se definió una concentración umbral experimental para la CFZ ($n = 55$); y en la Fase III del estudio se validó la concentración de CFZ propuesta con los aislados clínicos resistentes estratificados (TB-DR) según la mutación en *Rv0678* ($n = 85$).

RESULTADOS Y CONCLUSIÓN: La MIC de CFZ con las cepas de laboratorio y los aislados clínicos pansensibles osciló entre 0,125 µg/ml y 0,5 µg/ml. Los valores de la MIC de los aislados TB-DR utilizados en la Fase II oscilaron entre 0,25 µg/ml y 1 µg/ml y se propuso una concentración umbral de 1 µg/ml. Al validar la concentración umbral durante la Fase III del estudio, se observó una superposición de los mutantes de *Rv0678* probablemente sensibles y los resistentes con la concentración de 1 µg/ml. Por esta razón, se recomienda utilizar una concentración umbral de 1 µg/ml y realizar pruebas complementarias con 0,5 µg/ml que definan una categoría intermedia. Este es el primer estudio exhaustivo encaminado a determinar la concentración umbral en las pruebas fenotípicas corrientes de sensibilidad a CFZ en el sistema MGIT960, con el propósito de orientar las decisiones terapéuticas.