Multi-centre study to establish interpretive criteria for clofazimine drug susceptibility testing

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ABSTRACT

Objective: We undertook a multi-centre study to establish the clofazimine (CFZ) critical concentration (CC) for drug susceptibility testing (DST) of *M. tuberculosis* on the MGIT 960 system using minimum inhibitory concentration (MIC) distribution and genotypic analysis of the *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 was determined (n=70); in Phase II, a tentative CC for CFZ (n=55) was determined; and in Phase III, the proposed CC was validated using DR-TB clinical isolates stratified by *Rv0678* mutation (n=85).

Result and conclusion: The MIC distribution of CFZ for laboratory and clinical pan-susceptible strains ranged between 0.125 to 0.5 µg/ml. The MIC values of DR-TB isolates used for phase II ranged between 0.25 to 1 µg/ml, and a CC of 1 µg/ml was proposed. Validation of CC in phase III showed overlap between the probably-susceptible and PR *Rv0678* mutants at 1 µg/ml. Thus, we recommend a CC of 1 µg/ml and additional testing at 0.5 µg/ml defining an intermediate category. This is the first comprehensive study to establish a CC for routine phenotypic DST of CFZ using the MGIT 960 system to guide therapeutic decisions.

INTRODUCTION

Clofazimine (CFZ) is used as a standard component for the treatment of leprosy [1] and repurposed for the treatment of drug-resistant TB (DR-TB). It is a riminophenazine agent, which was initially developed for the treatment of TB in the 1950s [2-4]. However, due to poor *in-vivo* results of initial studies, CFZ was thought to be ineffective in the treatment of TB [5]. The renewed interest in the use of CFZ resulted from the findings of the Bangladesh study, which demonstrated successful outcome of shortened DR-TB treatment regimens containing CFZ [6]. Subsequent studies have confirmed these results leading to the endorsement of the short CFZ-containing MDR regimen by World health organization (WHO).

Clinical resistance to CFZ is difficult to ascertain as it is administered as part of combination therapy but nonetheless is 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 *M. tuberculosis* isolates and even with this approach,

published literature is very limited. A breakpoint of 1 µg/ml was proposed for detecting resistance to CFZ using the MGIT 960 system in a small local study in the Netherlands including only 26 MDR/XDR-TB isolates [8]. Currently, no critical concentration (CC) for CFZ testing has yet been defined by CLSI or FDA while the WHO has recently determined a CC of 1 µg/ml, based on small studies and unpublished data [9].

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

With increasing use of CFZ in the treatment of patients with MDR/XDR-TB, a reliable drug susceptibility testing (DST) method is needed. In this multi-centre study, we sought to establish the CFZ CC for DST of *M. tuberculosis* on the MGIT 960 system using the WT MIC distributions and evaluation against the presence or absence of an *Rv0678* mutation.

MATERIALS AND METHODS

Study design and setting

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

In phase I, we determined the MIC range and tentative CC for CFZ using laboratory isolates (pan-susceptible H37Rv [ATCC 27294] and *in-vitro*-selected *Rv0678* mutants) as well as pan-susceptible clinical isolates. In phase II, we determined the MIC distribution and proposed the tentative CC using clinically-resistant isolates. In phase III, we validated this proposed CC. Ethics approval was not required for this laboratory-based study, which used anonymised stored clinical isolates.

MIC testing and drug preparation

Clofazimine MIC testing was performed using the MGIT 960 system following the standard protocol for DST of first-line drugs (Becton Dickinson, USA). The CFZ powder was obtained from Sigma Aldrich (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 subsequent 1:10 dilution was prepared from the thawed stock solution. Thereafter, the test concentrations were made by two-fold serial dilutions ranging between 0.06 and 4.0 µg/ml (working solutions). All the dilutions were made in DMSO. Any leftovers of working solutions were discarded. *M. tuberculosis* H37Rv was included for each batch as a control at all sites. The MIC was determined to be the lowest drug concentration that inhibited a strain.

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

Each site tested the pan-susceptible H37Rv in triplicate using their own stock. 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 was tested at 0.06 to 1 µg/ml and 0.25 to 4 µg/ml, respectively. The MIC distribution for H37Rv, pan-susceptible clinical and *in-vitro*-selected *Rv0678* mutant strains were plotted. The tentative CC was determined as the concentration that inhibited 95% of the susceptible isolates. A plot of this tentative CC of CFZ was visually inspected in comparison to the *in-vitro*-selected *Rv0678* mutants as an additional confirmation.

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

The MIC distribution of 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-TB (XDR-TB) drugs. Five concentrations from 0.25 to 4.0 µg/ml were tested.

Phase III: Validation of the Critical Concentrations

Each site independently tested local MDR/XDR isolates not included in the previous phases. In addition, all isolates underwent sequencing to detect mutations in the *Rv0678* gene. Isolates with WT *Rv0678* were classified as probably-susceptible (PS) while isolates with with resistance-associated *Rv0678* mutations were considered probably-resistant (PR). Isolates were

classified as PS if mutation was found that has not been previously described, and the MICs were in the WT range while PR if the MICs were in the non-WT range. If, however, they were singleton mutants they were classified as PR.

Test 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 that inhibited 95% of the PS isolates. The number and proportion of isolates classified as susceptible using the final CC was evaluated against the PR isolates harbouring *Rv0678* mutation. If the MICs of more than 20% of the PR *Rv0678* mutants overlapped the CC, an intermediate (I) category would be proposed to minimise errors in reporting.

Sequencing of Rv0678 gene

Whole Genome Sequencing or targeted sequencing (*Rv0678*) of phase III isolates was performed using Illumina platforms (MiSeq or Next500) for sites 1-3. Resequencing analysis of the *Rv0678* gene was performed for variant calling using CLC genomics workbench v. 10 (Qiagen, 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.

For Site 4, the *Rv0678* and part of the intergenic region between mmpS5 and *Rv0678* was amplified and sequenced using the same primers. For analysis of the sequences, *Rv0678* sequence from *M. tuberculosis* H37Rv was taken as a reference (http://tuberculist.epfl.ch).

RESULTS

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

Table 1: MIC of Clofazimine in MGIT960 for H37Rv

Laboratory	H37RV		
	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

Due to technical problems with the diluent used for the drug preparation at site 2, data for the 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 to 0.5 µg/ml, with the exception of one isolate having an MIC of 1 µg/ml (Fig 1).

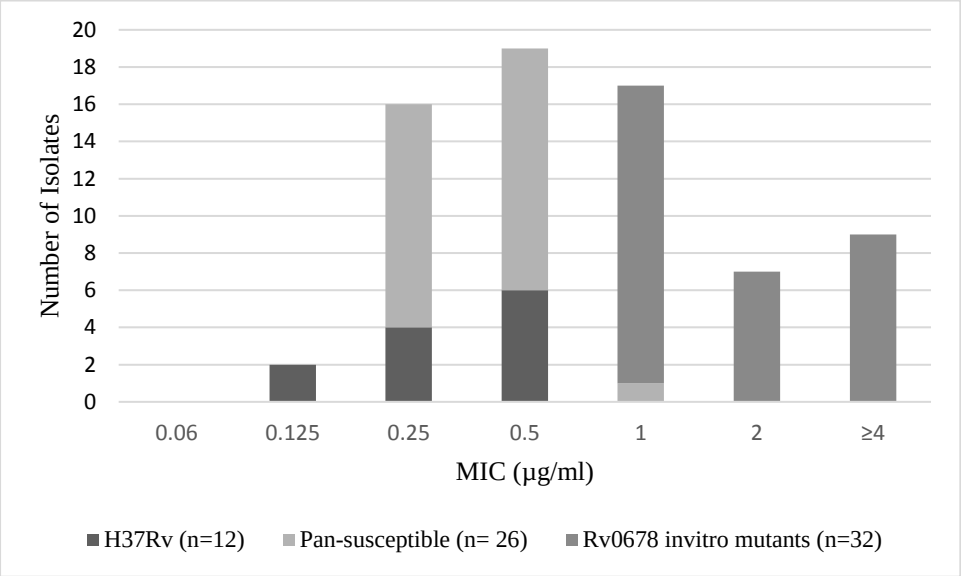

Figure 1: CFZ MIC distribution for H37Rv, pan-susceptible and in vitro-selected Rv0678 mutant isolates (µg/ml)

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

Phase II*:* The MIC results of CFZ for MDR/XDR isolates was only available for site 1, 2 and 3. Among the 55 clinical isolates tested, seven were XDR-TB, 25 were pre-XDR-TB, 18 were MDR-TB, one was rifampicin (RIF) mono-resistant and four had poly-resistance. The MIC of CFZ for these isolates ranged between 0.25 to 1 µg/ml for both site 1 and 3. For site 2, the MIC ranged between 0.25 to 2 µg/ml. The MIC distribution and comparison of different CC for the MDR/XDR isolates is shown in Figure 2 and Table 2, respectively. Using a tentative CC of 0.5 µg/ml, only 74.5% (41/55) of the isolates were found susceptible. However, at tentative CC of 1 µg/ml 94.6% (52/55) of the isolates were inhibited with only 5.4% (3/5) of isolates from site 2 showing growth at this concentration.

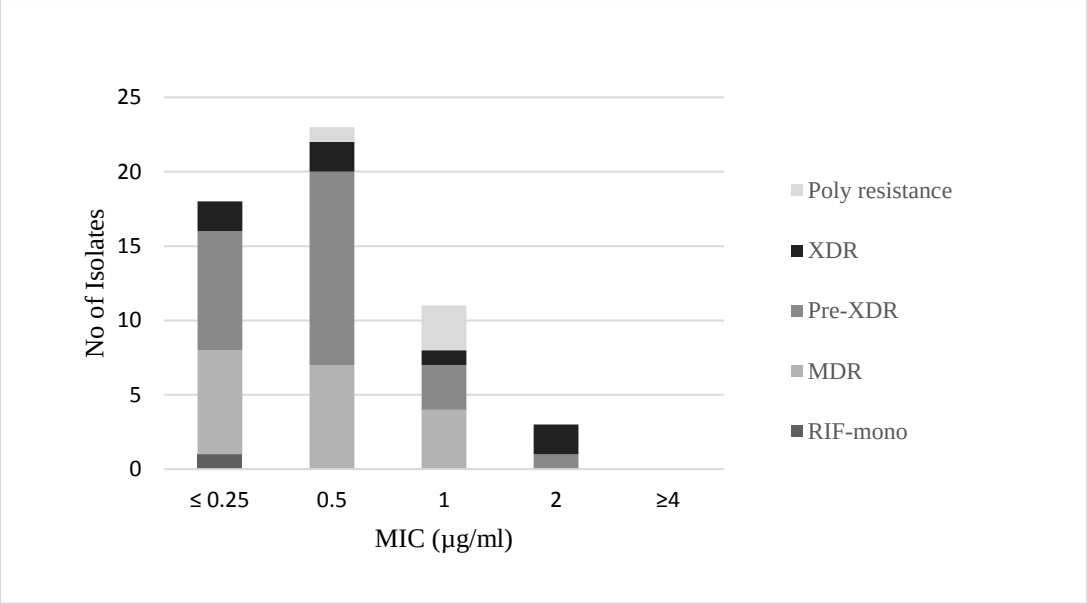

Figure 2: CFZ MIC distribution for MDR/XDR-TB isolates for Phase II (N=55)

Table 2: Comparison of critical concentration cut-offs and resistance categorization for CFZ among MDR/XDR-TB isolates

Sites	N	Tentative critical concentration							
		≤ 0.25		0.5		1		2	
		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

Phase III*:* all four sites participated in the validation of the CC proposed in phase II. Of 88 isolates phenotypically tested during this phase, three isolates were excluded due to sequencing failure. From the remaining, 82.3% (70/85) were PS isolates and 17.6% (15/85) were PR isolates harbouring *Rv0678* mutation. Among the PS isolates, 87% (61/70) had an MIC ≤0.5 µg/ml while 10% (7/70) had MIC of 1 µg/ml (Figure 3). The remaining 2.9% (2/70) isolates had MICs >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 MIC ≤0.5 µg/ml (Table 3). Three isolates harboring a V3I mutation all had an MIC < 0.25 µg/ml and hence were categorized as WT.

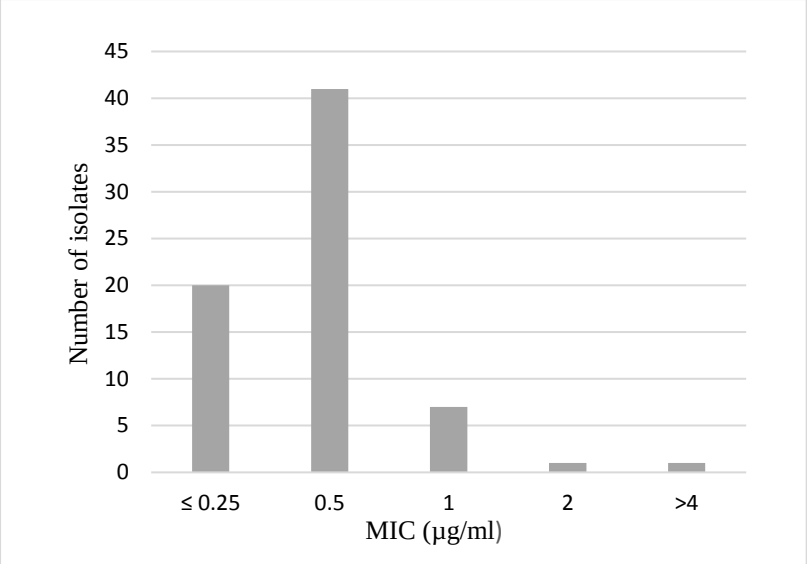

Figure 3: CFZ WT MIC distribution

Table 3: The *Rv0678* mutations and corresponding MICs for CFZ (N=15)

***Rv0678* mutation**	**N**	**MIC**					
		≤ 0.25	**0.5**	**1**	**2**	**4**	**>4**
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				

DISCUSSION

Our study is the first comprehensive, multicentre study to establish a CC for CFZ using the MGIT 960 system, and provides evidence for the WHO endorsed CC of 1.0 µg/ml [9], which was in part based on findings from this 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 (0.25 to 1 µg/ml) used for phase II. Therefore, a CC of 1 µg/ml was

proposed. Similarly, De Logu *et al*. [14] reported CFZ MICs to be higher for isolates with RIF-R/MDR and PZA resistance compared to the pan-susceptible H37Rv. This would also be concordant with the study from the Netherlands, which proposed a breakpoint of 1 µg/ml for MDR/XDR TB using the MGIT 960 method [8].

Subsequent validation of CC in phase III showed 87% isolates with a WT *Rv0678* would be classified as susceptible if a CC of 0.5 µg/ml is used, while, at 1 µg/ml, 97% would be susceptible, confirming the proposed CC of 1 µg/ml. However, 33% of the isolates with *Rv0678* mutation had an 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. Hence, at CC of 1 µg/ml, the PS and PR *Rv0678* mutants are not clearly separated. This problem could be resolved in part by introducing an I category and may also cover potential low-level resistance even if below the CC. Note that the I is neither clearly resistant nor susceptible but provides buffer category. Thus patients with an I result could be treated but need to be monitored as the *Rv0678* is a transcriptional regulator of an efflux pump and thus on drug exposure, higher MICs and resistance may develop. The clinical relevance of such cases remains to be determined. We, thus propose testing at 0.5 µg/ml and 1 µg/ml. If *M. tuberculosis* isolates show no growth at 0.5 µg/ml, the isolate is considered susceptible; if the isolates shows growth at 0.5 µg/ml and no growth at 1 µg/ml, the isolate is considered I, while growth at 1 µg/ml is considered resistant to CFZ. Despite the I category (2/15, 13%) of the PR *Rv0678* mutant isolates with MIC≤0.5 µg/ml would be classified as susceptible. These were however singleton mutants making it difficult to interpret their significance. Technical errors cannot be ruled out since 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 the *Rv0678* gene.

Variability in *Rv0678* mutations have been observed and there is limited data on their relevance for CFZ resistance. A study by Xu and colleagues [15] found all isolates with *Rv0678* mutation (n=5) having an MIC >1 µg/ml. In our study, the majority of isolates with *Rv0678* mutation had an MIC ≥1 µg/ml. Isolates with Ser53Leu and Ser2Ile mutations had MIC of 2 µg/ml and 4 µg/ml, respectively and is consistent with a previous study [16]. Two isolates with Arg132Stp mutation had MIC >4 µg/ml, suggesting its role in resistance. Three isolates had Glu49fs mutation, having MICs of 1, 2 and 4 µg/ml. Two isolates with Gly121Arg mutations had an MIC of 1 µg/ml. Thus, MIC of 1 µg/ml in these cases may be related to CFZ resistance. This

is corroborated by unpublished data (personal communication Leen Rigouts) of two *in-vitro*-selected Gly121Arg mutations having an MIC of 1 and 4 µg/ml. Further studies with large number of strains collected worldwide are required, to generate more data on association of specific *Rv0678* and other mutations with MICs and their impact on treatment outcomes.

The study has a number of limitations. Replicate testing was not done for all the isolates used in the study. No information was available on previous CFZ exposure for the clinical isolates included and the routine isolates in Antwerp were probably not reflective of Belgium isolates, but more representative of low-income country isolates. Also we did not sequence phase I and II isolates for *Rv0678*, nor did we sequence other putative genes. Despite these limitations, the study has important strengths with laboratories involved highly proficient in TB DST. In addition, the inclusion of molecular testing in comparison with the phenotypic MICs provides greater understanding of correlation between phenotypic and genotypic testing of CFZ.

In summary, standardization of the CFZ DST is important and DMSO should be used a solvent to avoid solubility issues experienced early on in this study (data not shown). We propose to test at two concentrations (0.5 µg/ml and 1.0 µg/ml). This approach is different from WHO recommendation that has proposed a single concentration. Although the criteria for resistance remains the same, our recommendation to include an intermediate category is more conservative and may minimise false susceptible results. However, the proposed CC in this study need to be critically re-evaluated with further studies, given the uncertainty about the correlation between *Rv0678* mutations, phenotypic DST, and lack of data correlating *Rv0678* mutations to clinical outcomes. Furthermore, we recommend that the manufacturer of the MGIT 960 system develops ready-to-use kits to perform CFZ testing as is done for other drugs. To date CFZ resistance has been poorly studied. Our study provides data for routine phenotypic DST for CFZ and information for future research.

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AUTHOR'S CONTRIBUTION

Planned and designed the study: Salman Siddiqi, Nazir A Ismail, Sabine Rüsch-Gerdes, Camilla Rodrigues, Leen Rigouts, and Halima M Said. Performed laboratory work, analysis and interpretation of the data: Halima M Said, Shaheed V Omar, Kanchan Ajbani, Nesline Sukhadiad, Thomas A Kohl, and Maren Diels. Contributed samples/data: Nazir A Ismail, Katarina Kranzer, Sabine Rüsch-Gerdes, Camilla Rodrigues, Leen Rigouts, and Stefan Niemann. Wrote first draft of manuscript: Nazir A Ismail and Halima M Said. All authors provided intellectual input, reviewed and approved the final manuscript.

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