



MDR/XDR-TB Colour Test for drug susceptibility testing of *Mycobacterium tuberculosis*, Northwest Ethiopia

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ARTICLE INFO

Article history:

Received 2 October 2019

Received in revised form 27 October 2019

Accepted 30 October 2019

Keywords:

MDR/XDR-TB Colour Test
Drug susceptibility testing
Drug-resistant
Thin-layer agar
Tuberculosis

ABSTRACT

Background: Appropriate technology tests are needed for *Mycobacterium tuberculosis* drug-susceptibility testing (DST) in resource-constrained settings. This study was performed to evaluate the MDR/XDR-TB Colour Test (a colour platethin-layer agar test; TB-CX) for *M. tuberculosis* DST by directly testing sputum at University of Gondar Hospital.

Methods: Sputum samples were each divided into two aliquots. One aliquot was mixed with disinfectant and applied directly to the TB-CX quadrant petri-plate containing culture medium with and without isoniazid, rifampicin, or ciprofloxacin. Concurrently, the other aliquot was decontaminated with sodium hydroxide, centrifuged, and cultured on Löwenstein–Jensen medium; the stored *M. tuberculosis* isolates were then sub-cultured in BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 for reference DST. **Results:** The TB-CX test yielded DST results for 94% (123/131) of positive samples. For paired DST results, the median number of days from sputum processing to DST was 12 for TB-CX versus 35 for LJ-MGIT ($p < 0.001$). Compared with LJ-MGIT for isoniazid, rifampicin, and multidrug-resistant tuberculosis, TB-CX had 59%, 96%, and 95% sensitivity; 96%, 94%, and 98% specificity; and 85%, 94%, and 98% agreement, respectively. All ciprofloxacin DST results were susceptible by both methods.

Conclusion: The TB-CX test was simple and rapid for *M. tuberculosis* DST. Discordant DST results may have resulted from sub-optimal storage and different isoniazid concentrations used in TB-CX versus the reference standard test.

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Introduction

The emergency of multidrug-resistant tuberculosis (MDR-TB) and pre-extensively drug-resistant (Pre-XDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), together with TB and HIV co-

infection, necessitates affordable appropriate technology techniques for the rapid detection and drug-susceptibility testing (DST) of *Mycobacterium tuberculosis* (World Health Organization, 2018). This may reduce morbidity, mortality, transmission, and amplification of drug resistance (Raviglione, 2006; Minion et al., 2010; Jeon, 2015). Ethiopia is one of the triple burden countries with frequent TB, MDR-TB, and TB–HIV co-infected patients (World Health Organization, 2018).

The World Health Organization (WHO) has approved the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) to detect rifampicin (RIF) resistance directly from specimens, as well as line-

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probe assays (LPA) such as GenoType MTBDRplus (Hain Lifescience, Nehren, Germany) to improve MDR-TB detection (World Health Organization, 2008; World Health Organization, 2012). In addition, the GenoType MTBDRsl assay (Hain Lifescience) is available for the rapid detection of second-line TB drug resistance, but is not routinely performed for patients in Ethiopia (World Health Organization, 2008; World Health Organization, 2013).

Conventional *M. tuberculosis* DST using solid media such as Löwenstein–Jensen (LJ) medium is slow, whereas liquid medium-based methods such as the Mycobacteria Growth Indicator Tube 960 system (MGIT) (BD Diagnostics, Sparks, MD, USA) can provide first- and second-line DST results approximately 10 days after *M. tuberculosis* is isolated in sufficient concentrations for indirect DST; however, MGIT is prohibitively expensive in resource-constrained settings (Martin et al., 2009).

Thin-layer agar (TLA) has been in use for *M. tuberculosis* colony and micro-colony detection for many years (Welch et al., 1993; Mejia et al., 1999; Satti et al., 2010; Shibabawa et al., 2019). The MDR/XDR-TB Colour Test (TB-CX) is a non-commercial, in-house TLA technique in which sputum mixed with disinfectant without centrifugation or other processing (or sub-cultured *M. tuberculosis* isolates) is inoculated onto selective TLA containing STC redox indicator (2,3-diphenyl-5-(2-thienyl)tetrazolium chloride). This indicator causes a colour change in the medium when bacteria grow, making positive results apparent by examination with the naked eye (Lee et al., 2006; Shibabawa et al., 2019). Four TB-CX petri-plate quadrants are inoculated concurrently to test for TB and also provide concurrent DST (Robledo et al., 2008; Tovar et al., 2010; IFHAD, 2011; Toit et al., 2012; Zhang et al., 2018; Mekonnen et al., 2019; Shibabawa et al., 2019).

The TB-CX colour test has been shown to determine resistance to isoniazid (INH), rifampicin (RIF), and ciprofloxacin (CFX), and the presence of MDR-TB, with excellent sensitivity, specificity, and agreement with the MGIT reference standard method (Toit et al., 2012). The present study was conducted to evaluate the performance of the TB-CX test when used to directly test patient sputum, against the reference standard, MGIT phenotypic DST results. The TB-CX test provides susceptibility results for CFX as a proxy for the detection of fluoroquinolones (FQs) resistance, due to a high degree of cross-resistance between the FQs, although CFX is not currently recommended for the treatment of MDR-TB patients (Devasia et al., 2009; Von Groll et al., 2009).

There is a need to describe the comparative merits of these tests to inform their use in high-burden TB settings with limited

resources. The purpose of the present study was to assess the yield, speed, and accuracy of performing DST for INH, RIF, and CFX using the TB-CX test directly on sputum samples, compared with the reference standard indirect DST using MGIT.

Methods

Study design and period

A comparative cross-sectional study was conducted between March 2016 and August 2017 at the University of Gondar Hospital, Ethiopia. A total of 200 study participants who were ≥ 15 years of age and who had bacteriologically confirmed or clinically diagnosed pulmonary TB were enrolled. The study enrolment method and the TB-CX and LJ results have been described previously (Shibabawa et al., 2019).

Specimen processing

One sputum sample was collected from each patient and divided into two aliquots. One aliquot was mixed with disinfectant and applied directly to the TB-CX plate, as described previously (Shibabawa et al., 2019). Concurrently, the other aliquot was decontaminated with sodium hydroxide, centrifuged, and cultured on LJ medium; *M. tuberculosis* colonies were then sub-cultured for MGIT DST.

TB-CX DST

The TB-CX test was prepared as described previously (Toit et al., 2012). The first quadrant is the control and contains no drugs (i.e., to detect any *M. tuberculosis*, whether drug-resistant or susceptible). The second quadrant contains green colouring with 0.2 $\mu\text{g}/\text{ml}$ of INH, the third quadrant contains yellow colouring with 1 $\mu\text{g}/\text{ml}$ of RIF, and the fourth quadrant contains blue colouring with 2 $\mu\text{g}/\text{ml}$ of CFX. Two drops of sputum–disinfectant mixture (Toit et al., 2012) were added to each quadrant using a disposable plastic pipette; one drop to each quadrant before adding the second drop to each quadrant to improve mixing and balance the inoculum (Figure 1).

After inoculation, the TB-CX test was permanently double-sealed with Parafilm and then placed in a Ziploc bag and incubated at 37 °C. Plates were read three times per week for 6 weeks until >10 colonies had appeared in the drug-free control quadrant. *M.*

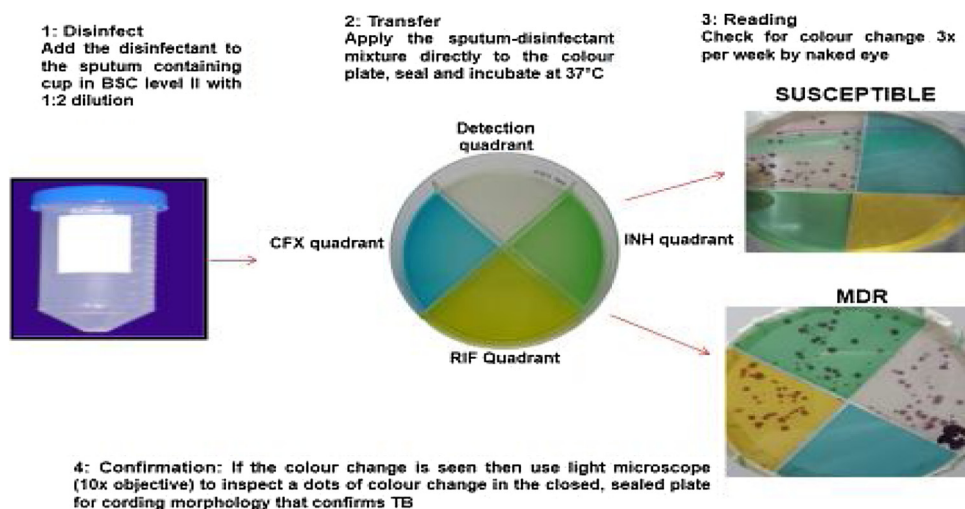


Figure 1. MDR/XDR-TB Colour Test sputum processing and detection, and final confirmation of the dots with cording formation characteristics of *Mycobacterium tuberculosis* complex.

tuberculosis growth was detected when red colonies were seen with the naked eye due to the redox reaction with STC. Cording colony morphology was then confirmed using a conventional light microscope (10 × objective) without opening the TB-CX plate or the bag containing it.

Once the control quadrant was positive, resistance was defined as any growth appearing concurrently in the quadrants with drugs as compared to the no drug control quadrant. A susceptible strain was defined as >10 colonies first growing on the no-drug control quadrant without any concurrent growth in the drug-containing quadrants. The TB-CX protocol recommends that DST is immediately interpreted and no subsequent assessments are made once growth of 11 or more colonies is observed in the no-drug control quadrant (IFHAD, 2011). However, for exploratory purposes, we additionally re-inspected each TB-CX plate until 6 weeks of incubation had been completed.

MGIT DST

MGIT SIRE kits were used following the manufacturer's instructions, with critical concentrations of 0.1 µg/ml, 1 µg/ml, and 2 µg/ml for INH, RIF, and CFX, respectively (Mycobacteriology Laboratory Manual, 2014). CFX was obtained from Sigma-Aldrich, dissolved in sterile distilled water, and filter sterilized through a 0.22 µm membrane. All stock solutions were stored at –20 °C for 6 months.

Stored *M. tuberculosis* complex (MTBC) colonies were sub-cultured and incubated in the MGIT instrument at 37 °C until the instrument indicated a tube was positive. The tube was then removed and checked for pure MTBC growth by Ziehl–Neelsen positivity, blood agar plate negativity, and MPT64 antigen (Capilia TB) test positivity. If the sample was positive for MTBC, then DST was performed using one 7-ml MGIT tube as a control and another tube for each of INH, RIF, and CFX. When 1% or more of the test population grew in the presence of the critical concentration of the drug, an isolate was defined as resistant (Mycobacteriology Laboratory Manual, 2014).

HIV testing

HIV counselling and testing were provided for all participants following the algorithm (KHB/STAT-PAK/Unigold) recommended by the Federal Ministry of Health of Ethiopia.

Quality control

For each TB-CX batch, quality control was performed using susceptible, MDR, and CFX-resistant *M. tuberculosis* strains at The Ohio State University, USA. As the TB-CX plates were then shipped at ambient temperature, upon arrival in Ethiopia, quality control was repeated for each batch using susceptible *M. tuberculosis* H₃₇R_v on all quadrants. Two batches of TB-CX plates that passed quality control in Ohio but then failed quality control after shipping to Ethiopia were discarded. All TB-CX were read blind to the results of the reference standard method. MGIT DST quality control used drug-susceptible H₃₇R_v and INH, RIF, and/or CFX known drug-resistant *M. tuberculosis* strains.

Statistical analysis

Data were entered into EpiData v. 3.1 (EpiData Association, Odense, Denmark) and analysed using IBM SPSS Statistics version 20 (IBM Corp., Armonk, NY, USA). The time for TB-CX DST was recorded from sputum processing until the growth of 11 or more colonies was observed in the drug-free control quadrant, allowing DST interpretation according to the TB-CX protocol (IFHAD, 2011).

The time for MGIT DST was recorded from sputum processing until sufficient LJ growth, plus sub-culturing time of LJ colonies in MGIT tubes, plus the time taken for MGIT DST. The Wilcoxon rank test for paired samples was used to compare the time to resistance detection for TB-CX versus LJ-MGIT, excluding TB-CX with uninterpretable DST results. Key proportions were reported with their 95% confidence interval (95% CI) and a *p*-value of <0.05 was considered statistically significant. Sensitivities, specificities, and predictive values (negative, NPV; positive, PPV) were calculated for the detection of drug resistance by TB-CX versus MGIT using MedCalc version 11.6.1 (MedCalc Software, Maria-kerke, Belgium).

Results

Clinical characteristics

Two hundred participants were enrolled. Their median age was 30 years and 61% were 15–34 years old. Most participants were male (63%), urban dwellers (57%), and literate (57%). Of the 200 participants, 26% had a previous history of anti-TB treatment, 13% were HIV-positive, 4% had a history of contact with a RIF mono-resistant/MDR-TB patient, 14% had a family history of TB, and 58.5% were Ziehl–Neelsen smear-negative (Shibabawa et al., 2019).

DST yield

Of the 200 patients enrolled, 66% (*n* = 131) had a positive TB-CX test and 68% had a positive LJ culture. One specimen with interpretable TB-CX DST had contaminated LJ culture and so was excluded from the analysis. Insufficient growth in the drug-free quadrant (<11 colonies) for interpretable DST occurred in 6% (95% CI 2–10%; 8/131) of positive TB-CX, which were excluded from the analysis. All LJ-positive samples had interpretable MGIT DST. Overall, 65% (*n* = 130) had positive culture by both TB-CX and LJ culture (Shibabawa et al., 2019). This study compared DST results by TB-CX versus LJ-MGIT for the 94% (122/130) with paired results for both tests.

DST speed

The time from sputum processing to TB-CX DST was a median 12 days (interquartile range 9–16 days, range 6–26 days), which was significantly faster than LJ-MGIT DST at a median 35 days (interquartile range 29–39 days, range 22–72 days) (*p* < 0.0001) (Figure 2).

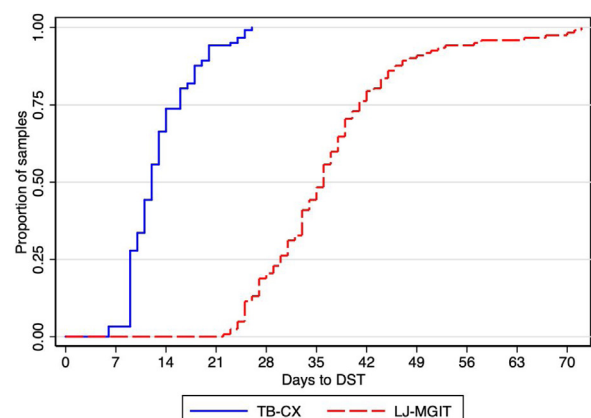


Figure 2. Comparison of the turnaround time in days to DST between the TB-CX test and LJ-MGIT.

DST accuracy

By MGIT, the prevalence of INH resistance was 30% (37/122) and RIF resistance was 19% (23/122). [Figure 3](#) shows the colonies observed in the TB-CX test control and drug-containing quadrants.

The TB-CX sensitivity was 59%, 96%, and 95% for detecting resistance to INH, RIF, and MDR-TB, and specificity was 96%, 94%, and 98%, respectively. All isolates were susceptible to CFX with both methods. The agreement between the TB-CX and MGIT was 85%, 94%, 100%, and 98% for INH, RIF, CFX, and MDR-TB, respectively ($p < 0.001$) ([Table 1](#)). No TB-CX had contamination preventing interpretation, although 1.5% (3/200) of culture-negative TB-CX were partially contaminated.

TB-CX plate re-reading at ≥ 1 week after completing the per-protocol interpretations for susceptible isolates revealed later growth in 15 quadrants for INH, 14 for RIF, and none for CFX up to 6 weeks.

Discussion

Appropriate technology diagnostic methods are needed for low-income countries with a high incidence of TB and drug resistance. The present study evaluated the performance of the MDR/XDR-TB colour test for direct detection from sputum specimens of resistance to INH, RIF, and CFX, as an alternative method in a resource-limited country, Ethiopia.

The time for detection of resistance to INH, RIF, and CFX by TB-CX was faster compared to LJ-MGIT DST (median 12 vs. 35 days), avoiding the pre-isolation culture step before performing MGIT DST. MGIT DST was performed using stored *M. tuberculosis* colonies that were sub-cultured using MGIT tubes, which might have delayed the time to MGIT DST. The results of the present study are comparable to those of other studies that have reported a median time for TB-CX detection of drug resistance of 10 days and 11 days ([Robledo et al., 2008](#); [Martin et al., 2009](#)) and high accuracy of MDR-TB detection.

The addition of STC to the medium made the red colonies visible to the naked eye during the early stages of growth and made the interpretation of the TB-CX test DST easy. No contamination on the drug-containing quadrants was noted for the 122 *M. tuberculosis* isolates throughout the study. However, 1.5% (3/200) of samples were contaminated from TB-CX test-negative drug-containing quadrants.

The TB-CX test showed excellent sensitivity for RIF and MDR-TB detection, while the sensitivity for INH resistance detection was lower than expected. The specificity was excellent for all drugs and MDR-TB detection, as in studies conducted in different areas ([Robledo et al., 2008](#); [Martin et al., 2009](#); [Toit et al., 2012](#); [Ardizzoni et al., 2015](#)). The sensitivity of the TB-CX test for INH (59%) was comparable to that reported in one study by Hussain et al. (71%) ([Hussain et al., 2012](#)), but lower as compared to other reports ([Robledo et al., 2008](#); [Martin et al., 2009](#); [Toit et al., 2012](#);

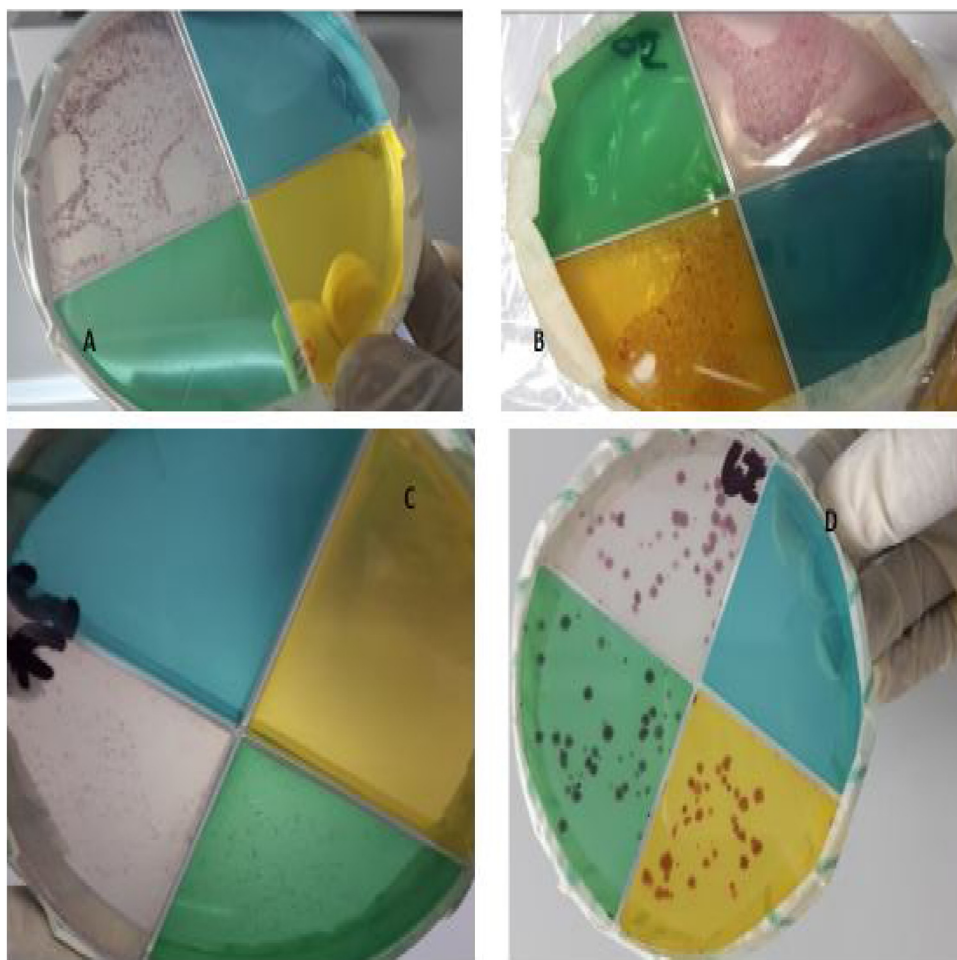


Figure 3. Growth was observed as red dots, indicating colonies in the growth control and/or drug-containing quadrants. (A) Growth only in the control quadrant (white/clear), indicating susceptibility to all drugs. (B) Growth in the control quadrant and RIF quadrant (yellow), indicating resistance to RIF and susceptibility to INH and CFX. (C) Growth in the control quadrant and INH quadrant (green), indicating resistance to INH and susceptibility to RIF and CFX. (D) Growth in the control quadrant and in the RIF and INH quadrants, indicating MDR-TB (resistant to both RIF and INH) with susceptibility to CFX (blue quadrant).

Table 1Performance characteristics of the MDR/XDR-TB Colour Test ($n = 122$ isolates).

TB-CX test	MGIT DST		Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Total agreement % (95% CI)*
	S	R					
INH							
S	82	15	59 (42–75)	96 (90–99)	88 (68–98)	85 (76–91)	85 (76–91)*
R	3	22					
RIF							
S	93	1	96 (78–100)	94 (87–98)	79 (59–92)	99 (94–100)	94 (87–98)*
R	6	22					
CFX							
S	122	0	–	100 (100)	0	100 (100)	100 (100)*
R	0	0					
MDR-TB							
S	98	1	95 (77–100)	98 (93–100)	91 (72–99)	99 (94–100)	98 (93–100)*
R	2	21					

S, Susceptible; R, Resistant; MGIT, Mycobacteria Growth Indicator Tube; DST, Drug susceptibility testing; RIF, Rifampicin; INH, Isoniazid; CFX, Ciprofloxacin; MDR-TB, Multi-drug resistant tuberculosis; CI, Confidence interval; NPV, Negative predictive value; PPV, Positive predictive value.

* Statistically significant agreement exists between the two tests; $p < 0.001$.

Mekonnen et al., 2019). This could be explained by the fact that the present study involved the direct inoculation of sputum, which may have reduced the sensitivity of the TB-CX test compared to indirect inoculation with isolates. Deviations from the recommended TB-CX protocol, such as shipping the TB-CX at ambient temperature and using the TB-CX for up to 4 months after preparation instead of the recommended 66 days (IFHAD, 2011), may have contributed to the suboptimal sensitivity. However, discordant DST results were probably principally explained by our use of different INH concentrations in TB-CX versus the reference standard test. Unlike RIF, which usually clearly indicates dichotomous resistance or susceptibility, INH resistance is a continuum with frequent intermediate results, so our use of different INH concentrations in the TB-CX test and MGIT test would be expected to cause some discrepant results. In an experiment that will be reported separately comparing TB-CX results with a reference standard assay that determines minimum inhibitory concentration to INH, we found that TB-CX sensitivity for detecting INH resistance was 93% when compared with the same INH concentration in the reference standard. However, when compared with the next dilution of INH in the reference standard, the sensitivity of the TB-CX to detect INH resistance fell to 70%. Thus, in the present research, the use of different INH concentrations in the TB-CX versus the MGIT reference standard may fully explain the low INH sensitivity that was observed. This finding suggests that the concentration of INH used in the TB-CX should be reconsidered (IFHAD, 2011).

Except for low INH DST sensitivity, the agreement between the TB-CX test and MGIT DST was excellent for the individual drugs and MDR-TB detection, which is comparable to the results of other studies (Martin et al., 2009; Toit et al., 2012; Ardizzone et al., 2015). This method might be a priority for TB prevention and care by providing earlier results directly from specimens, when compared to conventional methods, and reducing the use of empirical treatment resulting from the delay of conventional methods for TB DST. Moreover, it could be of added value in TB patient follow-up and monitoring of treatment in smear-positive cases and provide information on culture conversion in smear-negative cases.

In addition to good specificity and a short time to resistance detection, the TB-CX test has other merits. It does not require additional sophisticated laboratory equipment, colonies are easy to visualize with the naked eye due to the indicator in the medium, and it avoids biosafety risks. A disadvantage of the TB-CX test is that it is laborious, requiring time for repeated observation of *M. tuberculosis* growth in order to report the accurate DST pattern

based on the protocol, which may limit its use to laboratories that process moderate to low numbers of specimens for DST.

Uninterpretable TB-CX DST results due to low bacillary load seen in smear-negative specimens or after an extended sample storage time before specimen processing may cause scanty culture growth, delaying or preventing DST interpretation. This is supported by Banda et al., who stated that mycobacterial viability declined with prolonged storage (Banda et al., 2000).

The TB-CX protocol recommends that after the growth of 11 or more colonies is observed in the drug-free quadrant, then DST should immediately be interpreted and no subsequent assessments made (IFHAD, 2011). We found that re-reading of the susceptible TB-CX after ≥ 1 week sometimes demonstrated delayed growth in drug-containing quadrants that did not indicate drug resistance, demonstrating the importance of following the protocol.

In conclusion, the findings of this study showed that the MDR/XDR-TB Colour Test was simple and rapid for the simultaneous detection of *M. tuberculosis* and INH, RIF, and CFX DST. This method might be a priority for TB prevention and care by providing earlier results directly from specimens when compared to conventional methods and by reducing prolonged empirical treatment, facilitating the early appropriate treatment of patients with MDR-TB in resource-constrained settings. Moreover, it could be of value in following up patients with TB and monitoring treatment, potentially providing information on culture conversion. The TB-CX test format favours its implementation in laboratories without additional mycobacterial diagnostic equipment. Further studies are necessary to assess INH DST reliability either using the same concentration of INH in the TB-CX versus the reference standard test, or a composite reference standard, or ideally using a reference standard test that determines the minimum inhibitory concentration for INH.

Author contributions

AS: Conceived and designed the study, participated in sample collection, performed laboratory experiments, analysed and interpreted the data, wrote the first draft and final write up of the manuscript. BG: Designed the study, analysed and interpreted the data, edited and approved the final manuscript, agreed with the manuscript results and conclusions. HK: Made the colour plates, edited and approved the final manuscript. ET: Edited and approved the final manuscript. JMB: Critically reviewed, edited and approved the final manuscript. CE: Led the team that

developed TB-CX, wrote and shared the TB-CX protocol, critically reviewed, edited and approved the final manuscript. JT: Made the colour plates, analysed and interpreted the data, edited and approved the final manuscript. SW: Designed the study, analysed and interpreted the data, edited and approved the final manuscript, agreed with the manuscript results and conclusions. BT: Designed the study, analysed and interpreted the data, edited and approved the final manuscript, agreed with manuscript results and conclusions.

Ethical approval

Ethical approval was obtained from the University of Gondar Ethics Review Board (Ref. No. O/V/P/RCS/05/19/2016) and a letter of permission was obtained from the hospital director. All participants were volunteers, and sputum samples were collected after obtaining written informed consent from adult participants and also from parents or guardians of those who were <18 years old. The TB-CX results were unvalidated so did not influence the treatment, which followed national protocols. Confidentiality was maintained for all data collected.

Conflict of interest

We declare that we have no competing interests.

Acknowledgements

We thank the University of Gondar for financial support and The Ohio State University for internal funds to Drs Torrelles and Wang. CAE acknowledges funding from the Wellcome Trust; the Joint Global Health Trials consortium award MR/K007467/1, DFID; FIND; the Bill and Melinda Gates Foundation; and the Charity IFHAD: Innovation For Health And Development. We also extend our gratitude to the University of Gondar Hospital TB Culture Laboratory, Amhara Public Health Institute, and Ethiopian Public Health Institute for providing MGIT tubes and MGIT SIRE kits to perform the reference standard method. The funders had no role in the design or analysis of the study.

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