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The Colour Test for drug susceptibility testing of *Mycobacterium tuberculosis* strains

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SUMMARY

SETTING: Tartu, Estonia.

OBJECTIVE: To assess the performance and feasibility of the introduction of the thin-layer agar MDR/XDR-TB Colour Test (Colour Test) as a non-commercial method of drug susceptibility testing (DST).

DESIGN: The Colour Test combines the thin-layer agar technique with a simple colour-coded quadrant format, selective medium to reduce contamination and colorimetric indication of bacterial growth to simplify interpretation. DST patterns for isoniazid (INH), rifampicin (RMP) and ciprofloxacin (CFX) were determined using the Colour Test for 201 archived *Mycobacterium tuberculosis* isolates. Susceptibilities were compared to blinded DST results obtained routinely using the BACTEC™ Mycobacteria Growth Indicator Tube™ (MGIT) 960 to assess performance characteristics.

RESULTS: In all, 98% of the isolates produced interpretable results. The average time to positivity was 13 days, and all results were interpretable. The Colour Test detected drug resistance with 98% sensitivity for INH, RMP and CFX and 99% for multidrug-resistant tuberculosis. Specificities were respectively 100% (95%CI 82–100), 88% (95%CI 69–97) and 91% (95%CI 83–96) and 90% (95%CI 74–98). Agreement between the Colour Test and BACTEC MGIT 960 were respectively 98%, 96%, 94% and 97%.

CONCLUSION: The Colour Test could be an economical, accurate and simple technique for testing tuberculosis strains for drug resistance. As it requires little specialist equipment, it may be particularly useful in resource-constrained settings with growing drug resistance rates.

KEY WORDS: Estonia; fluoroquinolones; multidrug-resistant tuberculosis; thin-layer agar

APPROXIMATELY 0.5 million cases of multidrug-resistant tuberculosis (MDR-TB) are reported each year, and extensively drug-resistant TB (XDR-TB) has been recorded in a substantial number of countries worldwide.[1] Despite a reported small decrease in TB incidence in some countries, the overall proportion of MDR-TB disease is rising, and many of the settings with high rates of MDR-TB have limited resources in terms of detection of TB and drug-resistant disease.

There is a global need for new reliable and affordable methods of the detection of drug resistance, as rapid identification of MDR- and XDR-TB is vital for the prompt initiation of adequate treatment and interruption of further transmission of resistant strains.[2,3] Three main drug susceptibility testing (DST) strategies are currently in routine use: on solid culture, on liquid culture and molecular methods. Traditional solid culture-based DST, which is less costly than liquid-based methods, can take weeks to yield reliable results. The BACTEC™ Mycobacteria Growth Indicator Tube™ 960 (MGIT; BD, Sparks, MD, USA) liquid culture is a rapid and highly sensitive DST method.[4–9] Although the MGIT system is currently considered the gold standard in DST, it nevertheless has the following disadvantages: 1) the requirement for dense liquid cultures for diagnosis; 2) difficulty in obtaining specialist reagents, particularly in smaller low-income countries; and 3) high costs, which prohibit its use in low-income countries. Molecular tests also require expensive equipment,[10–15] and most also require specialised expertise. New methods are therefore needed for low-income countries with a high incidence of TB and a growing problem of drug resistance.

Mycobacterium tuberculosis microcolony detection on thin-layer agar (TLA) has been in use for a long time.[16–18] It has been evaluated on primary specimens against other culture methods such as MGIT and Löwenstein-Jensen (LJ) media, with excellent results.[19–23] TLA is an inexpensive non-commercial technique, requiring only a 37°C incubator and a light microscope to confirm diagnosis. The TLA

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MDR/XDR-TB Colour Test has recently been developed in collaboration with the Foundation of Innovative New Diagnostics for the identification of *M. tuberculosis* complex and detection of resistance to isoniazid (INH), rifampicin (RMP) and ciprofloxacin (CFX).[24] Although the use of CFX is not currently recommended for the treatment of MDR-TB, several studies have shown a high degree of cross-resistance between fluoroquinolones (FQs).[25,26]

The Colour Test in its current version tests susceptibility to CFX as a proxy for the identification of FQ resistance. The test is based on the TLA method, with the addition of an oxidation-reduction indicator: 2, 3 diphenyl-5-(2-thienyl) tetrazolium chloride (STC),[27,28] and colour-coded quadrants to simplify the reading of the DST results. The addition of STC to the media results in the growth of red TB colonies and thus makes them visible to the naked eye at early stages of growth. This makes daily checking of the plates faster, as there is no need to check every plate under the microscope. A microscope is still needed, however, to confirm a positive diagnosis of *M. tuberculosis* based on colony morphology. The Colour Test was extensively tested for the rapid diagnosis of TB directly from sputum samples.[29] However, no data are available on its performance on cultures, although this might be necessary should the test be applied at a reference level using isolates grown from different types of specimens.

The present study evaluates the performance characteristics and feasibility of the Colour Test in the identification of TB from cultures, concurrently with the detection of MDR-TB strains and resistance to CFX, against a gold standard of conventional phenotypic DST using MGIT.

MATERIALS AND METHODS

Study material

Routine strains archived during the years 2004–2010 were selected sequentially from the most recently archived isolates to older ones at the National TB Reference Laboratory, Tartu University Hospital, Tartu, Estonia. All of the isolates included had been previously identified as *M. tuberculosis* by AccuProbe (GenProbe Inc, San Diego, CA, USA) or GenoType® MTBC (Hain Lifescience, Nehren, Germany), and had RMP and INH DST results available that had been obtained using MGIT. All MDR-TB strains were tested using MGIT for susceptibility to second-line drugs. Twenty of the selected non-MDR-TB strains did not have second-line DST results.

Of the 201 isolates used in the study, 30 were non-MDR-TB and 171 were MDR-TB strains: 82 of the MDR-TB isolates were also resistant to ofloxacin (OFX), and 40 of these were XDR-TB strains, i.e., further resistant to at least one of the injectable drugs (amikacin, kanamycin or capreomycin).

Staff participating in the study were blinded to the original phenotypical DST results. Blinding was performed by an independent staff member not involved in the testing.

Introduction of the test

Before the start of the study, the Colour Test method was standardised using 10 strains from routine laboratory work with known first- and second-line DST results. The best fitting dilutions of the inoculum were determined using several dilutions with sterile water of McFarland 1 standard: 1/10, 1/100, 1/1000 and 1/10 000. It was found that McFarland 1 standard dilutions at 1/10, and sometimes 1/100, had the optimal colony count (50–500 colonies in the control quadrant); consequently, both these dilutions were used in this study to plate the 201 isolates on Colour Test plates.

A new investigator from the field site (National TB Reference Laboratory, Estonia) underwent a 3-day training course in the Colour Test technique at the UK Health Protection Agency National Mycobacterium Reference Laboratory prior to the study. Post-training photos of plates were taken and sent electronically to London and Peru, and the plates were re-read to guide and monitor the success of initial training and to monitor and correct errors.

Panel preparation

Strains were subcultured on LJ media using 250 μl of thawed isolate. At the same time, the initial cryovial was checked for contamination by plating on blood agar and incubating at 37°C for 48 h. The presence of acid-fast bacilli on positive LJ slopes was confirmed by Ziehl-Neelsen staining.[30]

Drug susceptibility testing

First- and second-line DST was performed using the automated liquid MGIT system according to the manufacturer's guidelines and World Health Organization recommended drug concentrations: 0.1 μg/ml for INH, 1.0 μg/ml for RMP[31] and 2.0 μg/ml for OFX.[32] DST was performed at the National TB Reference Laboratory, Tartu University Hospital.

Colour Test method

Middlebrook 7H11 agar was used to prepare the Colour Test plates. The medium was supplemented with oleic acid albumin dextrose complex (OADC) supplement (10%), Mycobacteria Selectatab (Kirchner, Mast Laboratories Ltd, Merseyside, UK) and 50 μg/ml of STC (TCI Europe, Zwijndrecht, Belgium). Quadrant petri dishes were prepared with approximately 4 ml of agar per quadrant, one with 0.2 μl/ml INH and green food colouring, one with 1 μl/ml RMP and yellow food colouring, and one with 2 μl/ml CFX and blue food colouring. Food colouring was supplied by Dr Oetker (Leeds, UK). All food colouring was filter-sterilised using a 0.22 μm filter (Millipore, Billerica, MA, USA). The remaining quadrant contained no drug

concentration and acted as the control for growth detection. The plates were screened for contamination every time the medium was prepared. Internal quality control was performed with a susceptible (H37Ra) and a resistant strain (laboratory origin).

Strain inocula for plating were prepared in a 50 ml centrifuge tube containing two drops of sterile distilled water and approximately 10 2-mm glass beads. Multiple *M. tuberculosis* colonies grown on LJ medium were added to the water. The tube was vortexed for 30 s and allowed to stand for 20 min. The inoculum was diluted and plated on Colour Test plates using two drops per quadrant. The plates were sealed with parafilm (American National Can Company, Norwalk, CT, USA) and placed in a sealed zip-lock plastic bag for safety reasons. Plates were incubated at 37°C in room air.

The plates were read visually every other day until at least 50 colonies appeared in the drug-free control quadrant. Growth was detected as red colonies due to a redox reaction with STC. The presence of *M. tuberculosis* was confirmed using a conventional light microscope (magnification 50×). A strain was classed as resistant to a drug when >1% of colonies appeared in a drug quadrant compared to the control quadrant. Colour Test plates were also re-read 21 days after inoculation.

Statistical power and sampling

The number of selected isolates with different DST patterns was based on power calculations that assumed comparable sensitivity and specificity for standard phenotypic testing for all tested drugs based on the published data of DST testing on TLA plates directly with specimens, i.e., not less than 85% for both sensitivity and specificity.

Statistical analysis was performed using Microsoft Excel (Microsoft, Redwoods, WA, USA) and STATA (StataCorp LP, College Station, TX, USA). The main outcome measures were the performance characteristics of the Colour Test assay: total agreement between experimental and the reference tests, sensitivity, specificity, likelihood ratios for a negative and a positive result (LR− and LR+) and the cost of a Colour Test plate in Estonia.

Ethics statement

As no patient information was used in this study, ethics approval was not sought.

RESULTS

The retail cost for materials for one Colour Test plate using local suppliers for all the reagents was 2.4€ (US$3.1). The resistant and susceptible control strains always yielded correct results, and none of the Colour Test plates were contaminated. Standardisation of the Colour Test method was required to exclude unnecessary dilutions that did not yield enough colonies in the control quadrant or were too dense. No uncertain results in interpreting DST from the Colour Test plates were therefore obtained during this study.

Of the 201 selected isolates tested on the Colour Test plates, 98% gave interpretable results. Four strains showed insufficient growth (<50 colonies in the control quadrant) or no growth on the Colour Test plates despite several attempts. The results from 197 strains were thus used for analysis. To evaluate CFX susceptibility, strains with available MGIT DST results for second-line drugs were used (n = 177). The mean time to detection was 13 days (range 6–21), and all results were interpretable.

The Table demonstrates test performance parameters. The total agreement for individual drugs between

Table Performance characteristics of the thin-layer agar MDR/XDR-TB Colour Test (n = 197 strains)

	MGIT susceptible	DST resistant	Sensitivity % (95%CI)	Specificity % (95%CI)	Total agreement % (95%CI)	LR+ (95%CI)	LR− (95%CI)
INH Colour Test							
Susceptible	19	3	98 (95–100)	100 (82–100)	98 (96–100)	0	0.02 (0.01–0.05)
Resistant	0	175					
RMP Colour Test							
Susceptible	22	4	98 (94–99)	88 (69–97)	96 (93–99)	8.14 (2.81–23.5)	0.03 (0.01–0.07)
Resistant	3	168					
CFX Colour Test							
Susceptible	89	2	98 (91–100)	91 (83–96)	94 (89–97)	10.6 (5.69–19.8)	0.03 (0.01–0.11)
Resistant	9	77					
MDR-TB Colour Test							
Susceptible	27	2	99 (96–100)	90 (74–98)	97 (94–99)	9.88 (3.38–28.9)	0.01 (0–0.05)
Resistant	3	165					
MDR-TB+CFX− Colour Test							
Susceptible	89	2	97 (91–100)	90 (82–95)	93 (88–96)	9.65 (5.35–17.4)	0.03 (0–0.11)
Resistant	10	76					

MGIT = Mycobacteria Growth Indicator Tube; DST = drug susceptibility testing; CI = confidence interval; LR+ = likelihood ratio for a positive result (indicates how much more likely a strain with resistance to a given drug will have a positive [i.e., resistant] result on the Colour Test plate than a strain susceptible to that given drug); LR− = likelihood ratio for a negative result (indicates how much more likely a strain susceptible to a given drug has a negative [i.e., susceptible] result on the Colour Test plate, compared to a strain that is resistant to that given drug); INH = isoniazid; Colour Test = thin-layer agar MDR/XDR-TB Colour Test); RMP = rifampicin; CFX = ciprofloxacin; MDR-TB = multidrug-resistant tuberculosis; MDR+CFX− = multidrug- and ciprofloxacin-resistant.

the MGIT and the Colour Test varied between 96% (95% confidence interval [CI] 93–99) and 98% (95%CI 96–100) for RMP and INH, respectively, and 94% (95%CI 89–97) for CFX. Overall agreement for detection of MDR-TB was 97% (95%CI 94–99), while it was lower (93%, 95%CI 88–96) for the detection of resistance to all three drugs simultaneously. Test sensitivity was 97% (95%CI 91–100) for detecting resistance to all three drugs at the same time; 98% for detecting resistance to RMP (95%CI 94–99), INH (95%CI 95–100) or CFX (95%CI 91–100); and 99% (95%CI 96–100) for detecting MDR-TB. Specificities varied between 88% (95%CI 69–97) for RMP and 100% (95%CI 82–100) for INH. Specificity for FQs was 91% (95%CI 83–96). The Figure shows the Colour Test colony counts for each of the drugs, demonstrating that there were few borderline results.

Colour Test re-readings on day 21 had no significant effect on INH or RMP results; however, late growth in the CFX quadrant caused a significant decrease in DST agreement with MGIT ($P = 0.03$, data not shown).

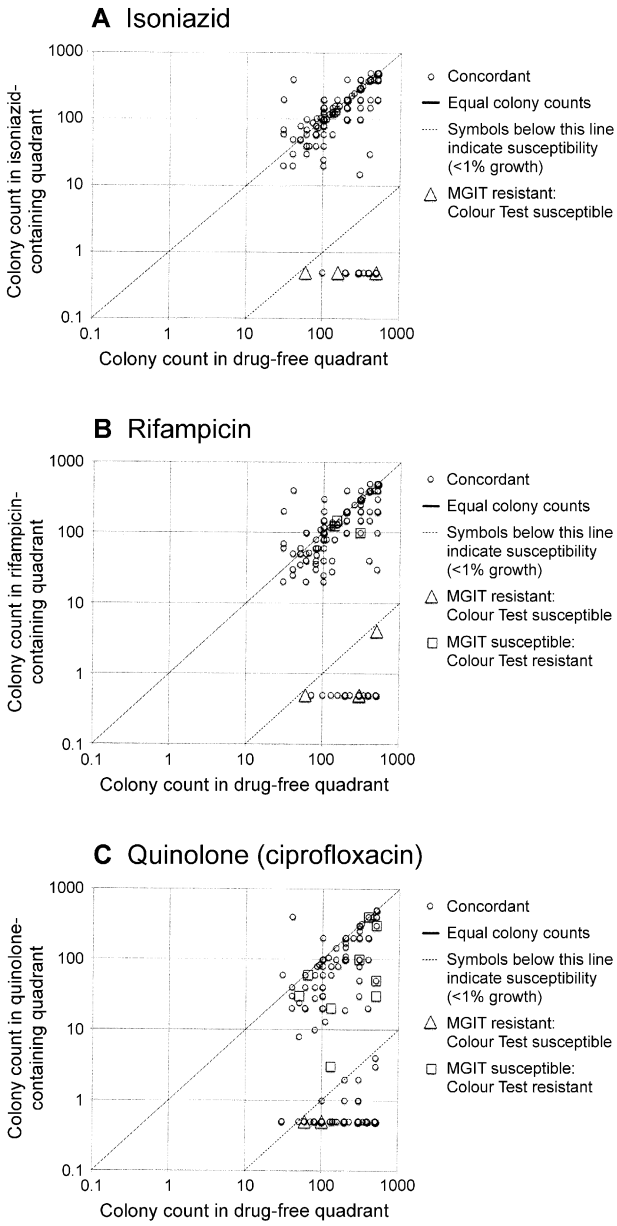

Figure Colour Test colony count results. The graphs show the number of *M. tuberculosis* colonies visible to the naked eye on the day that each test became culture-positive. Drug resistance results that were concordant and discordant with the MGIT test are indicated by different symbols (see legend). Strains were considered susceptible to a drug if it prevented more than 99% of tuberculosis growth (dotted line). MGIT = Mycobacteria Growth Indicator Tube.

DISCUSSION

This is the only study so far to pragmatically evaluate the performance of the Colour Test for the detection of resistance to INH, RMP and CFX on clinical isolates as an alternative to commercial testing methods. This inexpensive, non-commercial test allows for the diagnosis of MDR-TB; moreover, it can serve as an exclusion test for XDR-TB by analysing resistance to CFX.

Our study demonstrated good performance of the Colour Test. The test proved inexpensive; however, appropriately designed cost-effectiveness studies are needed to assess this formally. The cost of retail materials needed to produce one Colour Test plate was 2.4€ when all reagents were purchased in Estonia; this is similar to estimates published in 2009 by Martin et al. for TLA.[20] Although, the Colour Test DST performance time was somewhat slower than the MGIT DST at 13 days, it was significantly faster than other solid-media DST.[6]

In a routine diagnostic laboratory, it was feasible to introduce the Colour Test method within a relatively short period of time. The addition of Selectatab to the Colour Test media did not inhibit the growth of TB, and none of the plates were contaminated throughout the study. The addition of STC to the media made the red colonies visible to the naked eye in early stages of growth, and the addition of food colouring to the detection quadrants facilitated rapid preparation and easier reading of the plates. The prior standardisation of the dilutions used allowed for easy interpretation of the DST results.

The high total agreement between MGIT DST and excellent LR− and LR+ values suggests that this technique can serve as an accurate and promising test. The FQ DST agreement between the Colour Test and MGIT was highest when the Colour Test was read on the day that TB growth was first detected; interpretation should therefore not be delayed after this time. The test shows excellent sensitivity for all the drugs tested, and the specificity was excellent for INH, while the specificity for RMP and CFX was lower than expected. The MGIT DST results were obtained prior to cryopreservation, whereas the Colour Test results were obtained after cryopreservation and subculture of the TB strain. This methodological issue may

have contributed to the discrepancies observed, and future research should ideally compare different tests performed concurrently on the same passage of a strain. The lower specificity for RMP and a wide CI might result from a very low proportion of susceptible strains in the panel (only 25/197 strains were RMP-susceptible).

One of the study limitations was the indirect comparison of the test performance in detecting sensitivity to FQs. Possible absence of complete cross-resistance between CFX and OFX might have resulted in the lower specificity for this group of drugs. However, evidence of cross-resistance has been reported elsewhere;[25,26] the results presented are therefore somewhat controversial. Due to technical and financial constraints, no sequencing of the tested isolates was conducted. Further modifications to the Colour Test methodology permitting further testing for OFX or moxifloxacin, as performed by Martin et al. in 2009, are needed.[33]

Overall, the results of this study demonstrate similar good performance characteristics when used indirectly on isolates compared to previous studies with its progenitor, the TLA test, on direct specimens.[22,23] The advantages of the indirect method are possible application using cultures grown from all types of pulmonary and extra-pulmonary biological specimens, and as a replacement or back-up for commercial methods. Another advantage of this method is that it is relatively safe in environments where the relative risk of infection with *M. tuberculosis* cultures is high.[34] For the Colour Test technique, isolates are plated in a biological safety cabinet, and the plate is sealed and bagged securely; consequently, if the plate is dropped, no culture can escape.

This study suggests that, with appropriate training and careful attention to methodology, the Colour Test is a reliable technique. It is relatively rapid, with high accuracy for MDR-TB detection, and is suitable for low-income countries as a rapid and more economical alternative to liquid culture systems. Direct testing on different types of primary specimens could be of even higher diagnostic value to MDR-TB suspects. The Colour Test should therefore be further evaluated in high-resistance settings using direct specimens, as this could further illustrate the advantages of this safe and economical test.

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RÉSUMÉ

CONTEXTE : Tartu, Estonie.
OBJECTIF : Evaluer les performances et la faisabilité de l'introduction d'un test agar en couche mince MDR/XDR-TB Colour Test (Colour Test) comme méthode non commerciale pour tester la sensibilité à l'égard des médicaments (DST).
SCHÉMA : Le Colour Test combine la technique sur agar en couche mince avec un format simple en quadrant avec code des couleurs et milieu sélectif pour réduire la contamination et indication colorimétrique de la croissance bactérienne pour simplifier l'interprétation. Les types de résistance aux médicaments pour l'isoniazide (INH), la rifampicine (RMP) et la ciprofloxacine (CFX) ont été déterminés au moyen du Colour Test sur 201 isolats archivés de *Mycobacterium tuberculosis*. Les sensibilités ont été comparées en aveugle avec des résultats de la DST obtenus en routine au moyen du BACTEC™ Mycobacteria Growth Indicator Tube™ (MGIT) 960 afin d'évaluer les caractéristiques de performance.
RÉSULTATS : Des résultats interprétables ont été obtenus pour 98% des isolats. La durée moyenne avant positivité a été de 13 jours et tous les résultats ont été interprétables. Le Colour Test a interprété la résistance aux médicaments avec une sensibilité de 98% pour l'INH, la RMP et la CFX et de 99% pour la tuberculose multirésistante. Les spécificités ont été respectivement de 100% (IC95% 82–100), de 88% (IC95% 69–97), de 91% (IC95% 83–96) et de 90% (IC95% 74–98). La concordance entre le Colour Test et le BACTEC MGIT 960 a été respectivement de 98%, 96%, 94% et 97%.
CONCLUSION : Le Colour Test pourrait être une technique économique, précise et simple pour tester les souches de bacilles tuberculeux concernant leur résistance aux médicaments. Comme il n'exige que peu d'équipement spécialisé, il peut être particulièrement utile dans des contextes à ressources limitées où les taux de résistance aux médicaments sont croissants.

RESUMEN

MARCO DE REFERENCIA: Tartu, en Estonia.
OBJETIVO: Evaluar el rendimiento diagnóstico y la factibilidad de introducir la MDR/XDR-TB Colour Test (Colour Test) como un método no comercial de estudio de la sensibilidad a los medicamentos (DST).
MÉTODO: La Colour Test asocia la técnica en agar de capa delgada en un formato sencillo de cuadrantes codificados por color, con un medio selectivo que disminuye la contaminación y un indicador colorimétrico de crecimiento bacteriano a fin de simplificar la interpretación. Se determinaron los perfiles de sensibilidad a isoniazida (INH), rifampicina (RMP) y ciprofloxacino (CFX) mediante la Colour Test en 201 aislados de *Mycobacterium tuberculosis*. Se compararon estos resultados de sensibilidad con los resultados anónimos obtenidos por el método corriente BACTEC™ Mycobacteria Growth Indicator Tube (MGIT™) 960, a fin de evaluar la eficacia de la prueba.
RESULTADOS: Se obtuvieron resultados interpretables con el 98% de los aislados. El lapso promedio hasta la obtención de un resultado positivo fue 13 días y todos los resultados se pudieron interpretar. La Colour Test detectó la resistencia con una sensibilidad de 98% para INH, RMP y CFX y de 99% en la TB-MDR. La especificidad fue 100% para INH (IC95% 82 a 100), 88% para RMP (IC95% 69 a 97), 91% para CFX (IC95% 83 a 96) y 90% en la TB-MDR (IC95% 74 a 98). La concordancia entre la Colour Test y el método BACTEC MGIT 960 fue 98%, 96%, 94% y 97%, respectivamente.
CONCLUSIÓN: La Colour Test podría constituir una técnica económica, precisa y sencilla de determinación de la resistencia de las cepas de *M. tuberculosis* a los medicamentos. Dado que no precisa materiales especializados, la prueba podría ser útil en los entornos con restricción de recursos y tasas progresivas de farmacorresistencia.