

Thin-layer agar for detection of resistance to rifampicin, ofloxacin and kanamycin in *Mycobacterium tuberculosis* isolates

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SUMMARY

BACKGROUND: In low-income countries there is a great need for economical methods for testing the susceptibility of *Mycobacterium tuberculosis* to antibiotics.

OBJECTIVE: To evaluate the thin-layer agar (TLA) for rapid detection of resistance to rifampicin (RMP), ofloxacin (OFX) and kanamycin (KM) in *M. tuberculosis* clinical isolates and to determine the sensitivity, specificity and time to positivity compared to the gold standard method.

METHODS: One hundred and forty-seven clinical isolates of *M. tuberculosis* were studied. For the TLA method, a quadrant Petri plate containing 7H11 agar with RMP, OFX and KM was used. Results were compared to the Bactec MGIT960 for RMP and the proportion method for OFX and KM.

RESULTS: The sensitivity and specificity for RMP and OFX were 100% and for KM they were 100% and 98.7%, respectively. The use of a TLA quadrant plate enables the rapid detection of resistance to the three anti-tuberculosis drugs RMP, OFX and KM in a median of 10 days.

CONCLUSION: TLA was an accurate method for the detection of resistance in the three drugs studied. This faster method is simple to perform, providing an alternative method when more sophisticated techniques are not available in low-resource settings.

KEY WORDS: tuberculosis; drug resistance; thin layer agar

THE EMERGENCE of multidrug-resistant tuberculosis (MDR-TB, defined as resistance to both isoniazid [INH] and rifampicin [RMP]) and, more recently, extensively drug-resistant tuberculosis (XDR-TB, defined as MDR-TB with additional resistance to any fluoroquinolone [FQ] and to at least one of three injectable second-line anti-tuberculosis drugs used in treatment: capreomycin [CPM], kanamycin [KM] or amikacin [AMK]), together with TB and human immunodeficiency virus (HIV) co-infection, have stressed the need for improved technologies for rapid detection of drug resistance in TB.¹ Culture and drug susceptibility testing (DST) of *Mycobacterium tuberculosis* take several weeks due to the slow growth of the bacteria. Early detection of drug resistance could optimise treatment, improve the outcome for patients with drug-resistant TB and prevent the transmission of MDR-TB.

Treatment of MDR-TB is complicated, as it requires the use of second-line drugs that are less effective and more toxic, demanding longer treatment periods.

This situation poses a serious problem for low-income countries, especially in regions with a high prevalence of HIV infection. To achieve the ambitious new goals to treat MDR-TB recommended by the World Health Organization (WHO), laboratories must develop the capacity to perform DST of first- and second-line drugs to detect MDR- and XDR-TB using rapid methods.² Fully automated commercial systems such as the Bactec Mycobacteria Growth Indicator Tube 960 (MGIT960) have proved their reliability in the rapid detection of resistance to first- and second-line drugs, with results available within on average 10 days; however, they require heavy and costly equipment that is either not universally available or is not suitable for poor countries. Several new methods have been developed to reduce the diagnostic time, such as the microscopic observation drug susceptibility assay (MODS), the nitrate reductase assay (NRA) or the colorimetric redox-indicator assay.^{3–6} One of the new low-cost methods for the diagnosis of TB is thin-layer agar (TLA), which is able to detect growth within 10 days

while also allowing the initial identification of *M. tuberculosis* based on its characteristic cording morphology when observed microscopically.^{7,8} TLA is performed on solid media, and it does not require sophisticated equipment.

Resistance to RMP can be considered a surrogate marker for MDR-TB, while KM and ofloxacin (OFX) are key second-line drugs for the treatment of TB in MDR-TB cases.¹ The aim of the present study was to evaluate TLA for rapid detection of resistance to RMP, OFX and KM in *M. tuberculosis* and to determine the sensitivity, specificity and the time to detection of TLA compared to the gold standard method.

MATERIALS AND METHODS

Strains

One hundred and forty-seven clinical isolates of *M. tuberculosis*, obtained from the collection at the Institute of Tropical Medicine in Antwerp, Belgium, were studied: 122 were MDR and 25 were drug-susceptible. The reference strain *M. tuberculosis* H37Rv (American Type Culture Collection number 27294), was used as the reference susceptible control. All isolates were freshly subcultured on Löwenstein-Jensen (LJ) medium before being tested by the different methods.

Antimicrobial agents

All drugs were in chemically pure powder. RMP (Sigma-Aldrich NV/SA, Bornem, Belgium) was prepared at a concentration of 10 mg/ml in methanol, filter sterilised and frozen at -20°C until use. Ofloxacin was obtained from Sigma-Aldrich (St Louis, MO, USA), and kanamycin monosulfate from ICN Biomedicals Inc. (Aurora, OH, USA). KM was dissolved in sterile distilled water, and OFX was dissolved in 0.1 N NaOH; both drugs were filter sterilised. Stock solutions were made at 1 mg/ml and stored at -20°C for no more than 6 months.

Drug susceptibility testing

DST for RMP was performed in MGIT960 according to the manufacturer's instructions (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). The proportion method (PM) was performed on 7H11 agar for OFX and KM according to the standard procedure with the following recommended critical concentrations: OFX 2 $\mu\text{g}/\text{ml}$, and KM 6 $\mu\text{g}/\text{ml}$ (National Committee for Clinical and Laboratory Standards).⁹ Results were read 21 days after incubation at 37°C in 5% CO_2 atmosphere.

TLA

A quadrant Petri plate was used and prepared with 5 ml of 7H11 agar per compartment. One of the compartments served as the growth control (GC), another with 1 $\mu\text{g}/\text{ml}$ RMP, the third compartment with 2.0 $\mu\text{g}/\text{ml}$ OFX and the last one with 6 $\mu\text{g}/\text{ml}$ KM.

We first standardised the TLA using different concentrations of the inoculum. To avoid false susceptible results, we chose a higher concentration of the mycobacterial suspension as compared to the standard method (dilution 1:100). The inoculum was prepared from fresh LJ medium resuspended in sterile distilled water, adjusted to a McFarland turbidity No. 1, and diluted 1:50. Ten μl of the inoculum was inoculated on each compartment. Plates were sealed with parafilm and incubated at 37°C in a 5% CO_2 incubator. Plates were read twice weekly for 21 days using a conventional microscope (objective 10 \times). Once the GC compartment was positive, resistance was defined as any growth appearing in the compartments with drugs as compared to the GC compartment. A susceptible strain was defined as no growth appearing in the compartments with drugs compared to the GC. Growth detection results in TLA were read blind to the results in MGIT960 and the PM.

RESULTS

In a first step, the TLA method was standardised with a panel of eight strains with a known resistance profile. As standardisation of the inoculum is important, the actual inoculum size was determined using different concentrations: McFarland 1 diluted in 1/100, 1/50 and 1/10. Different volumes of the inoculum—100, 50, 25 and 10 μl —were inoculated in the central area of the TLA plate containing the Middlebrook 7H11 agar and the corresponding drugs. Full concordance of results was observed using an inoculum corresponding to a McFarland 1 diluted 1/50 using 10 μl for inoculation.

All 147 clinical isolates of *M. tuberculosis* were tested for susceptibility to RMP, OFX and KM. With TLA, results were available on an average of 10 days of incubation compared to 3 weeks for OFX and KM and 8 days for MGIT960. The Table shows the DST results obtained by the gold standard method and TLA. Among the 147 isolates, 122 were resistant to

Table Results of the 147 *M. tuberculosis* isolates tested by the gold standard method and the TLA plate for RMP, OFX and KM

		Gold standard		Sensitivity %	Specificity %	Accuracy %
		S	R			
RMP	S	25	0	100	100	100
	R	0	122			
OFX	S	95	0	100	100	100
	R	0	39			
KM	S	77	0	100	98.7	99.3
	R	1	67			

TLA = thin-layer agar; RMP = rifampicin; OFX = ofloxacin; KM = kanamycin; S = susceptible; R = resistant.

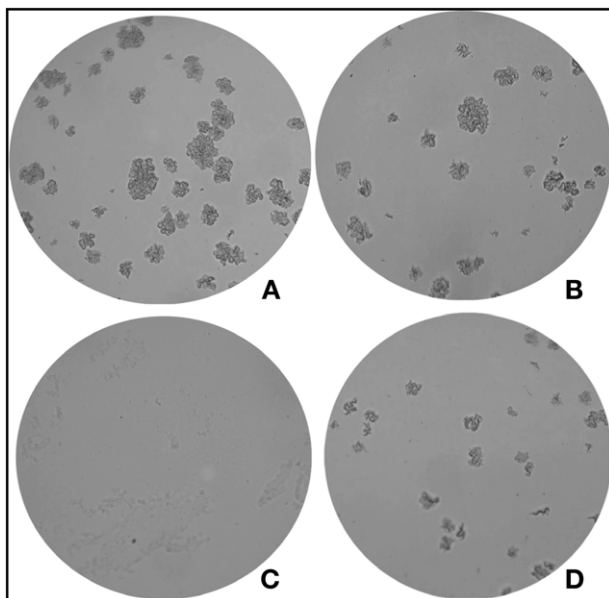


Figure Thin layer agar quadrant plate: **A)** positive growth control; **B)** rifampicin-resistant; **C)** ofloxacin-susceptible; **D)** kanamycin-resistant.

RMP, 39 were resistant to OFX, and 67 were resistant to KM. Full agreement was found between the results obtained for the detection of RMP and OFX resistance. For KM, one discordant result was classified as false resistant by TLA, giving a sensitivity of 100% and a specificity of 98.7%. We repeated the test by both methods and found the same results. We then used the resazurin microtiter assay (REMA) to determine the minimal inhibitory concentration (MIC) of KM for this isolate, according to previously published methodology.¹⁰ The strain had an MIC of 5 µg/ml, which is classified as resistant to KM. The Figure illustrates a strain susceptible to OFX but resistant to RMP and KM.

DISCUSSION

In this study, we have evaluated the TLA on clinical isolates of *M. tuberculosis* for the detection of resistance to RMP, KM and OFX, three key anti-tuberculosis drugs in the treatment of TB and MDR-TB. The development of new methods for TB diagnosis that are able to provide earlier results than the conventional methods is a priority for TB control. With the conventional method performed on LJ or 7H11 medium, results are available only after 1 or 2 months, and thus treatment is usually prescribed empirically. One disadvantage of the commercially available rapid liquid culture techniques, such as the MGIT, is the inability to observe the colony morphology, unlike other solid media. Also, for daily routine use, the high costs could be an obstacle to its widespread implementation. Besides standard laboratory equipment, TLA requires only a standard microscope. Concerns

have been voiced about the use of CO₂, which favours the growth of *M. tuberculosis*. In a study by Schaberg et al., it was found that CO₂ incubation allowed *M. tuberculosis* to be detected only 1 day earlier than when CO₂ was not employed.¹¹ This is an important question that needs to be resolved to avoid the need for expensive CO₂ incubators, and further studies are needed to confirm this observation.

In this study it was important to consider a total inhibition of growth for the definition of susceptibility, to avoid the detection of false-susceptible strains. TLA using a quadrant plate format is an alternative low-cost method that can reduce the time to detection of resistance in *M. tuberculosis* to 10 days compared to several weeks using the conventional proportion method. Microcolonies could be seen under the microscope long before they were observed visually. Although the method is easy to perform, a minimum of training is needed to recognise the growth of *M. tuberculosis* under the microscope and differentiate it from any other bacterial contamination. Characteristic growth cord formation of *M. tuberculosis* was simple to recognise because the study was performed on fresh, pure culture. As regards biosafety, a biological class II cabinet is recommended, as for any culture TB manipulation. TLA can be implemented in an existing TB culture laboratory, and the need for training, evaluation and quality control should be considered. The potential risk of aerosols is reduced in TLA compared to the use of other liquid culture media. Using TLA directly on sputum samples will avoid the pre-isolation culture step that requires at least 2 weeks before performing DST. A recent study evaluated TLA directly on sputum samples for the detection of resistance to RMP and INH.¹² TLA sensitivity and specificity for both drugs was 100%, and time to detection of resistance was 11 days for smear-positives, showing a performance comparable to conventional DST methods. Further operational field studies are needed, especially applying TLA directly on sputum samples, to fully validate its usefulness for the rapid detection of drug resistance in TB.

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R É S U M É

CONTEXTE : Dans les pays à faibles revenus, des méthodes économiques de détermination de la sensibilité de *Mycobacterium tuberculosis* aux antibiotiques sont nécessaires.

OBJECTIF : Évaluer la méthode thin layer agar (culture sur agar en couche mince ; TLA) pour la détection rapide de la résistance à la rifampicine (RMP), à l'ofloxacine (OFX) et à la kanamycine (KM) dans des isolats cliniques de *M. tuberculosis* et de déterminer, par rapport à la méthode de référence, la sensibilité, la spécificité et la durée avant positivité de la méthode TLA.

MÉTHODES : On a étudié 147 isolats cliniques de *M. tuberculosis*. Pour la méthode TLA, on a utilisé une boîte de Pétri divisée en quatre quartiers préparée avec de l'agar 7H11 contenant les médicaments correspondants RMP, OFX et KM. Les résultats ont été comparés à la méthode

gold standard MGIT960 pour la RMP et à la méthode des proportions sur agar 7H11 pour OFX et KM.

RÉSULTATS : La sensibilité et la spécificité pour RMP et OFX ont été de 100% et pour KM, la sensibilité a été de 100% et la spécificité de 98,7%. L'utilisation de la plaque TLA divisée en quatre quartiers permet la détection rapide de la résistance de *M. tuberculosis* à l'égard des trois médicaments antituberculeux RMP, OFX et KM après une durée médiane de 10 jours.

CONCLUSION : La TLA s'est avéré une méthode précise pour la détection de la résistance aux trois médicaments étudiés. Cette méthode plus rapide, d'emploi aisé, fournit une méthode alternative lorsque des techniques plus sophistiquées ne sont pas disponibles dans des contextes à faibles revenus.

R E S U M E N

MARCO DE REFERENCIA : En países de bajos ingresos existe una necesidad de métodos económicos de evaluación de la sensibilidad de *Mycobacterium tuberculosis* a los medicamentos.

OBJETIVO : Valorar el método de agar en capa delgada (TLA) en la detección rápida de la resistencia de *M. tuberculosis* a rifampicina (RMP), ofloxacino (OFX) y kanamicina (KM) a partir de aislados clínicos, y evaluar la sensibilidad y la especificidad de esta técnica y el lapso necesario hasta obtener un resultado positivo, en comparación con el método de referencia.

MÉTODOS : Se estudiaron 147 aislados clínicos de *M. tuberculosis*. El TLA se realizó en una caja de Petri dividida en cuadrantes preparada con agar 7H11 que contenía el antibiótico correspondiente : RMP, OFX o KM.

Los resultados se compararon con el método de referencia, que para la RMP es el sistema MGIT960 y para el OFX y la KM es el método proporcional en agar 7H11.

RESULTADOS : El método TLA ofreció una sensibilidad y una especificidad para la resistencia a RMP y OFX de 100% y para KM, la sensibilidad fue 100% y la especificidad 98,7%. La utilización de una caja de Petri dividida en cuadrantes favoreció la detección rápida de la resistencia de *M. tuberculosis* a los tres medicamentos antituberculosos con una mediana de 10 días.

CONCLUSIÓN : El TLA fue una técnica precisa en la detección de resistencia a RMP, OFX y KM. Este sistema más rápido es de realización sencilla y representa una opción cuando no se cuenta con métodos sofisticados en los entornos con escasos recursos.
