Original Article

Rapid Detection of *In Vitro* Antituberculous Drug Resistance Among Smear-Positive Respiratory Samples Using Microcolony Detection-Based Direct Drug Susceptibility Testing Method

Irim Iftikhar, Seema Irfan, Joveria Farooqi, Zahida Azizullah, Rumina Hasan

Department of Pathology and Laboratory Medicine, Aga Khan University Hospital, Karachi, Pakistan

Abstract

Background: With the rise in multidrug-resistant tuberculosis, there is a search for newer techniques that will rapidly detect drug-resistant *Mycobacterium tuberculosis*. Although molecular techniques can detect resistance, culture is still considered gold standard, especially in resource-limited settings where quick, cheap, and easy techniques are needed. The aim of the study was to evaluate microcolony method thin layer agar (TLA) for quick detection of resistance against the first- and second-line antituberculous drugs in clinical isolates. This was a cross-sectional study performed at Aga Khan University Hospital. **Material and Methods:** A total of 87 Z-N stain smear-positive pulmonary samples were received and indirect drug susceptibility test (ID-DST) was performed using Lowenstein–Jensen and mycobacteria growth indicator tube. Direct DST was performed using TLA on 7H10 agar. TLA was observed twice weekly under microscope for 4 weeks. Sensitivity, specificity, and accuracy were calculated for TLA using indirect susceptibility method as the gold standard. Level of agreement was calculated using Kappa score. **Results:** TLA showed sensitivity of 89% and 95.2% for isoniazid and rifampicin, while for ethionamide, ofloxacin, and injectable aminoglycosides, it was 96.6%, 92.1%, and 100%, respectively. Specificity for the first-line drugs was >95% while second-line drugs ranged from 70% to 100%. Mean time to positivity was 10.2 days by TLA as compared to 43.1 days by ID-DST. **Conclusions:** TLA is a quick and reliable method in identifying resistance, especially in resource-limited settings. However, additional liquid culture can be set up as backup, especially in patients on therapy to avoid false negative results.

Keywords: First and second antituberculous agents, microcolony method, multidrug-resistant tuberculosis

Introduction

Key factors for the management of tuberculosis (TB) involve early diagnosis and treatment. In the era of rapid diagnosis, culture-based methods still remain the gold standard for detection and susceptibility testing.[1] Routine indirect drug susceptibility test (ID-DST) testing takes weeks to report.[2] However, direct DST (DDST) methods performed directly on specimen, such as thin layer agar (TLA), can reduce the detection time. Robledo *et al*. evaluated TLA for detection of multidrug-resistant (MDR)-TB and found 100% sensitivity and specificity.[3] The World Health Organization (WHO) suggests that TLA shows promising results for rapid DST but does not endorse it due to lack of data regarding generalizability and detection of antimicrobial resistance other than isoniazid (INH) and rifampicin (RIF), especially in patients under treatment.[4] In the present study, we aim to evaluate the performance of TLA in smear-positive pulmonary cases as a DDST method to detect resistance amongst first-line and second-line antituberculous drugs.

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Material and Methods

Patient recruitment and sample collection

This study was conducted from January 2014 to December 2014 at Aga Khan University Hospital (AKUH), Clinical Laboratories. Inclusion criteria consisted of patients older than 13 years and Ziehl–Neelsen (ZN) smear-positive pulmonary samples for acid-fast bacilli (AFB). A total of 87

Address for correspondence: Dr. Seema Irfan, Department of Pathology and Laboratory Medicine, Aga Khan University Hospital, Supariwala Building, 1st Floor, Karachi, Pakistan.
E-mail: seema.irfan@aku.edu

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respiratory samples were received from 77 patients. Thirty-two patients (32/77) were registered with the (Directly Observed Treatment Short Course) program at the Ojha Institute of Chest Diseases and were suspected MDR-TB cases, either proven by GeneXpert MTB/RIF® (Cepheid) or through failure to respond to the first-line therapy. Respiratory samples fulfilling the smear positivity criteria were submitted for culture and sensitivity in the AKUH, Clinical Laboratory. The research officer responsible for performing and reading TLA-DDST was initially trained and was blinded from IDDST results. Ethical approval was taken from Ethical Review Committee, AKU, and Institutional Review Board, Dow University of Health Sciences.

Laboratory procedure

Standard protocol

All pulmonary samples (sputum, tracheal aspirate, and bronchial lavage) were decontaminated using N-acetyl-L-cysteine-sodium hydroxide with a final concentration of 1.25%, and centrifugation was done for 20 min at 3000 g. The sediment was then resuspended 1–2 ml phosphate buffer saline with final volume of 3 ml. One drop of decontaminated sample was used to prepare smear, and ZN staining was performed to see number of AFB per high power field (×1000). Using standard laboratory protocol, Lowenstein–Jensen (LJ) and mycobacteria growth indicator tube (MGIT) were inoculated. Identification of *Mycobacterium tuberculosis* (MTB) was on the basis of ZN staining colony morphology and inhibition to para-nitrobenzoic acid (PNB). Indirect sensitivity testing was performed on 7H10 agar using final critical concentrations of 0.2 μg/ml INH, RIF 1 μg/ml, ofloxacin (OFX) 2 μg/ml, amikacin (AK) 4 μg/ml, kanamycin (KM) 5 μg/ml, ethionamide (ETH) 5 μg/ml, and capreomycin (CAP) 5 μg/ml in accordance with the WHO recommendations.[1] Resistance was defined as growth on drug-containing quadrants >1% as compared to the number of colonies growing on the drug-free quadrant. Susceptibility was defined as growth on the control quadrant with no growth or <1% on the drug-containing quadrant.

Thin layer agar method

TLA-DDST method is a microcolony direct detection method on solid culture, based on inoculation of specimens after decontamination into drug-free and drug-containing media, followed by microscopic examination of early growth. Based on number of AFB seen on smear, the residual portion of decontaminated sample underwent dilutions in Middlebrook 7H9 broth according to the AFB seen on smear: <25 AFB/field, no dilution; 25–250 AFB/field, 10–1 dilution; and >250 AFB/field, 10–3 dilution.[3] The TLA-DDST used the same principle of agar proportion method with standard critical drug concentration. For preparation of TLA-DDST, four quadrant plates with 7H10 with oleic albumin dextrose catalase (OADC, BD Diagnostics, Sparks, MD, USA) and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BD Diagnostics, Sparks, MD, USA) were used. For each specimen, three TLA plates were used with each quadrant containing 5 ml agar: First plate had one growth control (GC) drug-free quadrant, one with 0.5 mg/ml PNB (Sigma USA) for MTB identification and INH and RIF in the 3rd and 4th quadrant, respectively. Second plate had GC, OFX, ETH, and CAP in separate quadrants. The last plate received the two drugs (GC, AK, and KM) in each of three quadrants while 4th one remained empty. After inoculation, TLA plates were sealed and incubated at 36°C with 5% CO_2 atmosphere and read twice a week with conventional microscope at ×100 magnification for up to 4 weeks. Growth in GC quadrant and inhibition in PNB quadrant along with cord formation was considered positive for MTB. Once GC quadrant was considered positive (≥2 colony forming units); other compartments were evaluated for growth. Resistance was defined as any growth in drug-containing quadrant as compared to GC quadran as shown in Figure 1. MTB was considered to be susceptible if there was growth in GC quadrant but no growth in drug containing quadrant as shown in Figure 2. Scant breakthrough growth in drug-containing wells after prolonged incubation >1 week was not considered indicative of resistance.[5] *M. tuberculosis* H37Rv (ATCC 27294) and a known MDR-TB (laboratory's internal quality control strain), previously confirmed on IDDST, were used as control strain.

Data analysis

The data were entered and coded into Microsoft Excel® 2010 and transferred to StataCorp LP Statistics/Data Analysis StataCorp (Texas, US) for analysis. Sensitivity, specificity, and accuracy were calculated for TLA using indirect susceptibility method as gold standard. Percentages of discrepancies (very major, major) were calculated for TLA as compared to the gold standard method. Errors were categorized as very major if the isolate was resistant according to gold standard method and sensitive when tested by TLA and major if sensitive according to gold standard method and resistant when tested by TLA. Level of agreement was calculated using Kappa score with range from 0 to 1, where 1 was referred perfect agreement between two categories (sensitive and resistant) of TLA and gold standard test. The following standards were used for the strength of agreement for the Kappa coefficient: 0–0.0099 as poor;

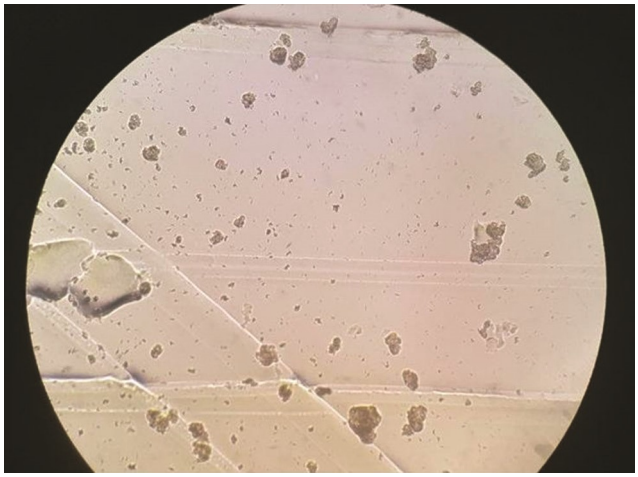

Figure 1: Microcolonies of *Mycobacterium tuberculosis* seen under a microscope (10 × objective) showing characteristic cord formation in positive drug quadrant (resistant) on direct drug susceptibility test.

0.01–0.20 as slight; 0.21–0.40 as fair; 0.41–0.60 as moderate; 0.61–0.80 as substantial; and 0.81–1 as almost perfect.

Results

In this study, 87 samples were obtained from 77 patients; four patients submitted more than one sample. Demographic findings showed a mean age of 36 years with almost equal distribution of male versus females (ratio 1.07). Treatment histories were available for 32/77 patients with 20 on therapy while 12 were either not on any treatment or had received <5 months therapy when they submitted the sample. Mean time to susceptibility reporting of patients on therapy was 11.9 days (95% confidence interval [CI]: 10.2–13.6) by TLA while 43.3 days (95% CI: 40.2–46.4) by indirect method [Table 1].

Out of 87 smear-positive sputum samples, 70 samples grew MTB; the rest of the 17 samples belonged to 11 patients who were all on therapy. For 70 positive samples, susceptibility testing was performed and read by ID-DST using agar proportion method. However, only 64 samples were microscopically readable by direct susceptibility testing using TLA-DDST method. The Kappa score for culture positivity between two tests remained 0.828 (95% CI: 0.61–1.03) which shows very good level of agreement. Rate of contamination for TLA was 3.4% as three samples were contaminated. Out of these, two were completely contaminated due to overgrowth of bacteria and results could not be read. There was one sample in which two quadrants, KM and ETH, got contaminated and these results had to be removed from susceptibility analysis.

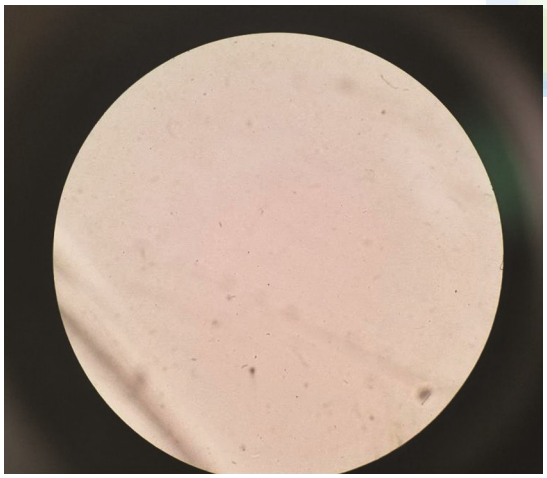

Figure 2: Susceptible drug quadrant showing no growth on direct drug susceptibility test.

Among 64 readable TLA microcolony-based DDST, 45 were identified as MDR-TB cases while 22 were susceptible to first-line TB drugs. Sensitivity and specificity for detection of resistance to INH, RIF, AK, KM, OFX, CAP, and ETH are shown in Table 2. The accuracy for detection of resistance by TLA remained perfect for AK and KM, and above 90% for all other drugs.

The Kappa scores for INH, RIF, OFX, and CAP were >0.8 while >0.6 for ETH, which signifies high level of agreement between the two test methodologies [Table 3]. There was no discrepancy in results of AK and KM; thus, their Kappa scores could not be computed. There were two very major errors observed for RIF and CAP (3.13%), while only one very major error was observed for ETH and OFX (1.58%). Regarding major errors, two were observed in INH and ETH (3.13%), three in OFX (3.17%), and one in RIF (1.5%).

For the 27 samples on which GeneXpert MTB/RIF® (Cepheid) results were available, the agreement between GeneXpert and TLA remained 100% regarding MTB detection, but TLA detection of RIF was 92.59%. There were two samples in which TLA reported false susceptibility to RIF, but GeneXpert as well as IDDST reported resistance.

Discussion

TB is a worldwide problem. Microbiological identification of TB has a vital role in decreasing the spread of TB in community, and we are in dire need of consistent and quick methods of detection. Multiple rapid molecular diagnostic techniques such as GeneXpert and MTBDR plus have been used for early MDR-TB detection; these molecular techniques have just been approved for detection of RIF and INH resistance while second-line line probe assays have been accepted for quinolones and injectables. However, they do not eliminate the need for culture-based testing, a requirement for assessing microbiological response to therapy. Hence, they can only be used for screening purposes as they do not detect viable organisms. Moreover, newer molecular techniques require specific infrastructure, equipment, skill, and stable source of electricity. ID-DST using culture is still gold standard method in TB diagnostics, with turnaround time of 6 weeks resulting in delay of treatment and increase TB spread in community. Different DDST methods are being established to decrease detection time such as microcolony observation drug

Table 1: Average time for detection by direct susceptibility testing using microcolony thin layer agar method and indirect drug susceptibility testing

Culture yield	TLA days (95% CI)	Indirect DST days (95% CI)	*P*
Mean time to detection of all TLA positive cases	10.2 (9.42-11.11)	43.1 (40.7-45.5)	<0.01
Mean time to detection of MDR-TB cases	10.9 (9.77-12.06)	42.5 (39.4-45.67)	<0.01
Mean time to detection of non-MDR-TB cases	8.9 (7.73-9.97)	43.7 (39.4-48.1)	<0.01
Mean time to detection in patients on therapy	11.9 (10.2-13.6)	43.3 (40.2-46.4)	<0.01

TLA: Thin layer agar, DST: Drug susceptibility testing, CI: Confidence interval, MDR: Multidrug-resistant, TB: Tuberculosis

Table 2: Sensitivity, specificity, and accuracy of thin layer agar for isoniazid, rifampicin, amikacin, kanamycin, ethionamide, capreomycin, and ofloxacin keeping indirect drug susceptibility testing as gold standard

Antimicrobial drugs	TLA	Indirect		Sensitivity (%)	Specificity (%)	Accuracy (%)
		S	R			
INH	S	17	0	89	100	96.87
	R	2	45			
RIF	S	20	2	95.2	95.34	95.31
	R	1	41			
AK*	S	60	0	100	100	100
	R	0	4			
KM*	S	59	0	100	100	100
	R	0	4			
ETH	S	57	1	96.61	75	95.2
	R	2	3			
CAP	S	57	2	100	71.4	96.87
	R	0	5			
OFX	S	35	1	92.1	96.1	93.75
	R	3	25			

*Number of isolates for AK and KM were 63 while they were 64 for rest of the antibiotics. TLA: Thin layer agar, INH: Isoniazid, RIF: Rifampicin, AK: Amikacin, KM: Kanamycin, ETH: Ethionamide, CAP: Capreomycin, OFX: Ofloxacin, S: Susceptible, R: Resistant

Table 3: Categorical agreement between thin layer agar and Indirect susceptibility testing (*P*-value for all Kappa scores was <0.001)

Antimicrobial drugs	Kappa score (95% CI)	Very major error (%)	Major error (%)
INH	0.923 (0.67-1)	0	3.13
RIF	0.895 (0.65-1)	3.13	1.5
OFX	0.872 (0.62-1)	1.5	3.17
CAP	0.816 (0.57-1)	3.13	0
ETH	0.641 (0.39-0.88)	1.58	3.17

Kappa score for AK and KM could not be calculated as there was 100% sensitivity and specifi city. CI: Confidence interval, INH: Isoniazid, RIF: Rifampicin, OFX: Ofloxacin, CAP: Capreomycin, ETH: Ethionamide, AK: Amikacin, KM: Kanamycin

susceptibility (MODS) using 7H9 broth, microcolony detection by TLA, and nitrate reductase method (NRA). Some of these rapid methods such as MODS have been approved as interim solution in resource starved settings.[5] Few studies have been performed to exploit TLA for rapid drug susceptibility reporting. Using TLA-DDST method, mean drug susceptibility turnaround time shortened from 43.1 days by IDDST to 10.2 days. These results are in concordance with different studies, i.e., 7 days reported by Ardizzoni *et al*., 11.2 days by Battaglioli *et al*., and 9.6 days by Alam *et al*.[6-8] Turnaround time for MDR detection also shortened to 10.8 days from 42.7 days by IDDST method.

There have been a few studies that have evaluated TLA for MTB detection, and in most, the positivity rate of TLA remained higher compared to LJ and MGIT. Robledo *et al*. in 2008 conducted a study on 95 patients and detected MTB in smear-positive cases using TLA (91.3%) and MGIT (96.7%) than with LJ (84.7%).[3] Moreover, a study from Kenya found lower but comparable sensitivity of TLA (74%) as compared to LJ (71%) in smear-negative respiratory samples.[9] Both these studies used clinical specimens and these results are comparable to those that we observed in our study, i.e. 73.5% TLA and 80% IDDST. There were four cases in this study that failed to grow on TLA while we were able to get results on IDDST. Total time for culture positivity and final reporting of DST was very long: mean of 110 days (15.7 weeks). Since treatment history was not available, inhibitory effect of antituberculosis therapy on growth rate cannot be excluded. Thus, TLA-DDST method may be limited by false negative results observed in patients on therapy. There were two specimens in our study that could not be evaluated due to contamination while results were available on IDDST. This shows that optimization of procedure for decontamination of specimens may be necessary, especially if delay is expected and overgrowth of bacteria may have occurred. Moreover, setting liquid culture as backup should be considered as low proportion of viable bacteria in the specimen may require broth medium for better growth enhancement.

Some studies have evaluated first-line TB drugs by this rapid method while there is lack of data for second-line drugs. The sensitivity of detection of resistance against INH and RIF in our study was similar to other studies from Bangladesh, Latin American Countries, and Georgia, which also tested TLA; the rates ranging from 87.5% to 100% for INH and 98.2%–100% for RIF were reported.[3,6,8] These results are comparable with other noncommercial methods: sensitivity of 91%–97% and 98%–99% for INH and RIF by MODS, while 97% for both drugs by NRA.[4,10]

There were two cases in our study that showed susceptibility to RIF by TLA-DDST but were resistant by both IDDST and GeneXpert. This phenomenon can probably be explained by the slow growth of these strains since TLA plates were kept for a short incubation time of 28 days only. There could

also be unreliability in standardizing the inoculum for direct susceptibility testing as we did not know the proportion of live bacteria among the AFB seen on smear.

Sensitivity and specificity for OFX were >90%, while for injectables, AK and KM were 100%. Martin *et al*. worked on MDR-TB isolates using TLA and found 100% sensitivity for OFX and KA but 98.1% specificity of KM. Not much has been published on performance of TLA regarding AK; however, Huang *et al*. using MODS reported sensitivity and specificity for AK >93% and >90% for KM.[11]

In our study, TLA-DDST was 100% sensitive and 71.4% specific for detection of CAP resistance. Moreover, TLA showed 96.61% sensitivity and 75% specificity for ETH resistance. To the best of our knowledge, this is the first study evaluating TLA for CAP and ETH. However, MODS have been evaluated for ETH and CAP and sensitivity and specificity >88.8% which corresponds well with TLA ETH and CAP findings in our study.[11] Although the sensitivity against second-line drugs remains considerably higher, the specificity for ETH and CAP was much lower. This may be due to the smaller sample size, a limitation of this study.

TLA, although a cheap and quick method, has certain drawbacks. It requires microscopic expertise of technical staff in identifying MTB complex from *Mycobacterium* other than TB (nontuberculous mycobacteria). Second, microscopy is a time-consuming procedure as plates have to be observed under microscope for 4 weeks, and it can be difficult in laboratories handling a large volume of culture requests. Finally, MTB strains from patients already on therapy may require longer incubation time up to 12 weeks. In such cases, direct susceptibility testing using TLA may turn out to be false negative due to shorter incubation time (4 weeks), drying of plates incubated for a longer duration, or a very small number of viable bacteria. We recommend that TLA plates of patients with treatment history and smear positivity should be held for longer incubation with liquid culture as back-up.

Small sample size as well as inclusion of only smear-positive specimens was additional limitation; thus, the results will not be applied to smear-negative samples. Moreover, molecular tests on all samples and for detection of resistance against all antibiotics were not available, and so discrepant results could not be resolved except in case of RIF in only a proportion of cases. Further studies are required to establish the generalizability of this test in smear-positive cases and the effect of TLA diagnosis on patient's outcome.

Conclusion

TLA is a quick and reliable test that has an added benefit of providing culture and susceptibility tests at the same time. Moreover, it has significant value in patients requiring monitoring response to therapy as nucleic acid amplification tests have not been approved for this purpose.

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Conflicts of interest

There are no conflicts of interest.

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