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The value of microscopic-observation drug susceptibility assay in the diagnosis of tuberculosis and detection of multidrug resistance

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Inexpensive, rapid, and reliable tests for detecting the presence and drug susceptibility of *Mycobacterium tuberculosis* complex (MTBC) are urgently needed to control the transmission of tuberculosis. In this study, we aimed to assess the accuracy and speed of the microscopic-observation drug susceptibility (MODS) assay in the identification of MTBC and detection of multidrug resistance. Sputum samples from patients suspected to have tuberculosis were simultaneously tested with MODS and conventional culture [Löwenstein-Jensen (LJ) culture, BACTEC MGIT™ 960 (MGIT) system], and drug susceptibility testing (MGIT system) methods. A total of 331 sputum samples were analyzed. Sensitivity and specificity of MODS assay for detection of MTBC strains were 96% and 98.8%, respectively. MODS assay detected multidrug resistant MTBC isolates with 92.3% sensitivity and 96.6% specificity. Median time to culture positivity was similar for MGIT (8 days) and MODS culture (8 days), but was significantly longer with LJ culture (20 days) ($p < 0.0001$ for both comparisons). Median time to availability of the susceptibility results was significantly ($p < 0.0001$) shorter with MODS assay (8 days) than MGIT system (20 days). In conclusion, MODS is an inexpensive and rapid test with good performance characteristics for direct diagnosis of tuberculosis and detection of multidrug resistance.

Key words: *Mycobacterium tuberculosis*; multidrug resistant tuberculosis; isoniazid; rifampicin.

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The global burden of tuberculosis (TB) remains high with 10.4 million TB cases estimated to have occurred in 2015, and approximately 480 000 people developed multidrug resistant TB (MDR-TB) globally. Although TB is slowly declining each year, death toll from the disease is still unacceptably high with an estimated 1.4 million people died from the disease in 2015 (1). To end TB worldwide, World Health Organization (WHO) set global TB targets in 1990 in the context of millennium development goals with 2015 the deadline. The target of stopping and reversing TB incidence was achieved in each of the six WHO regions; however, the targets of reducing the prevalence and mortality by half were not met in three (African, Eastern Mediterranean, and European) and two (African and European) of the regions, respectively (2). Turkey is a middle-income country in the European region; however, with the emergence of the Syrian civil war, Turkey has become a major destination and transit country for immigrants and currently harboring 2.7 million Syrian refugees. According to the latest information published by International Organization for Migration, nearly 3.5 million refugees and migrants reside in Turkey (3, 4). Thus, immediate actions need to be taken to prevent the transmission of TB by implementing improved diagnostic tools and effective treatment strategies.

Commercial liquid culture systems and molecular line probe assays have been endorsed by WHO. However, these assays are expensive and require sophisticated laboratory infrastructure. Therefore, their use has been restricted in many resource-limited settings where the need is greatest (5). Several non-commercial culture and drug susceptibility testing (DST) methods have been developed aimed to

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be used in resource-limited settings. One of the most advanced methods is MODS assay, which is a manual liquid culture technique based on microscopic detection of mycobacterial growth in drug-free and drug-containing media.(5). Incorporation of drugs permits rapid and direct DST concurrently with the detection of mycobacterial growth (6).

Smear microscopy continues to be the most common method used in TB diagnosis worldwide (1). According to the recent national TB report, in more than half (≈59%) of the TB laboratories in Turkey, the only microbiological test performed was smear microscopy and the capacity for DST was available in only 20% (7). In this study, we investigated the accuracy and speed of the MODS assay in the diagnosis of TB and MDR-TB through detection of MTBC and determination of its susceptibility to isoniazid (INH) and rifampicin (RIF) in sputum samples obtained from TB suspected patients from Turkey.

MATERIALS AND METHODS

This study was conducted in İstanbul, Turkey, from April 2014 through December 2014. Sputum samples from TB suspected patients were collected in İstanbul University, İstanbul Faculty of Medicine, Department of Medical Microbiology and Yedikule Pulmonary Diseases and Pulmonary Surgery Training and Research Hospital. Sputum samples were transported to the mycobacteriology laboratory at İstanbul University, İstanbul Faculty of Medicine, Department of Medical Microbiology where microbiological assays were performed.

The results of the MODS assay and reference tests were read and interpreted by different investigators, with the readers being unaware of the results of the other test.

This study was reviewed and approved by İstanbul University İstanbul Faculty of Medicine Ethics Committee.

Laboratory methods

Detection of MTBC

Sputum samples were decontaminated and digested by sodium hydroxide-N-acetylcysteine method. The concentrated sediment of the sample was used for microscopic examination of Ehrlich-Ziehl-Neelsen (EZN) stained sputum smears (8). The resuspended sediment was divided into two aliquots: one was used for reference culture methods [LJ culture using LJ slants (BD, Sparks, MD, USA) and automated liquid culture using BACTEC MGIT 960 system (BD)] according to published guidelines (8) and manufacturer's instructions, and the other was used in MODS assay.

EZN staining was used to confirm the growth of mycobacteria in all positive MGIT tubes and LJ slants. After validation of mycobacterial growth with EZN staining, MPT64 antigen detection using BD MGIT TBc Identification Test (TBc ID) (BD, Sparks, MD, USA) was performed. GenoType Mycobacterium CM/AS kit (Hain Lifesciences, Nehren) was applied when TBc ID test was negative.

The MODS assay was performed in accordance with the published protocol (9). For each patient sample, four wells of the 24-well tissue culture plate were used: two drug-free wells, one well containing INH at 0.4 μg/mL, and another well containing RIF at 1 μg/mL. A negative control column was included in every plate. Plates were examined daily from days 5 to 14, on alternate days from days 15 to 21, under an inverted light microscope at 10× objective for the presence of characteristic tangled growth (Fig. 1) of MTBC in the drug-free wells. If there was no evidence of growth, with characteristic morphology, by day 21, the culture was considered negative. Fungal or bacterial contamination was recognized by rapid overgrowth or clouding; if detected, the stored portion of the original sample was decontaminated and cultured.

Drug susceptibility testing

Indirect DST was performed on all MTBC strains isolated with standard culture, using BACTEC MGIT 960 system (Becton Dickinson, Sparks, MD, USA). The DST protocol, as provided by the manufacturer, was strictly adhered to and an automated result was provided by the MGIT

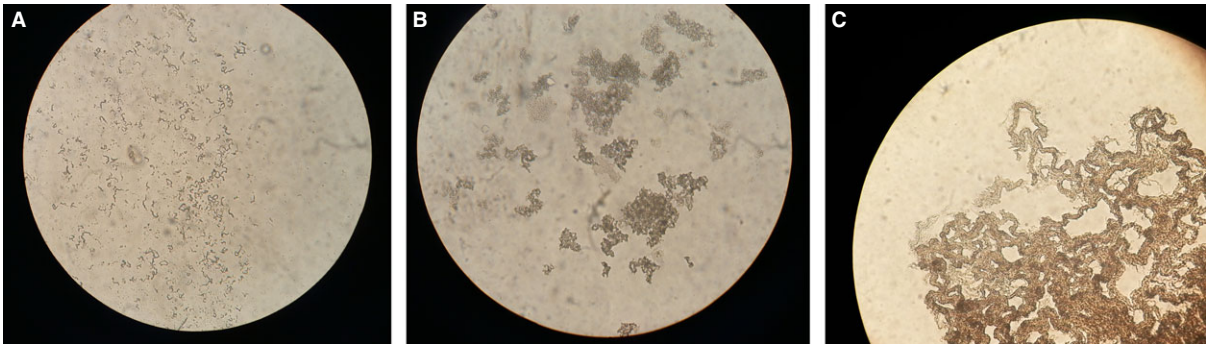

Fig. 1. Characteristic serpentine structure of MTBC in MODS culture plate as seen under an inverted light microscope (10 × objective) after 7 days (A), 14 days (B), and 21 days (C) of incubation. MTBC, *Mycobacterium tuberculosis* complex; MODS, microscopic-observation drug susceptibility.

machine. The final critical concentration of INH was 0.1 μg/mL and of RIF was 1 μg/mL.

Direct DST was performed with MODS assay for INH and RIF. Growth in drug-free control wells, but not in drug-containing wells, indicated a susceptible strain, and growth in drug-free and a drug-containing well indicated resistance to that drug. If mycobacterial growth was detected in both INH and RIF containing wells, the strain was identified as MDR. Drug-sensitive (*M. tuberculosis* ATCC H37Rv), INH resistant (*M. tuberculosis* ATCC 35822), and RIF resistant (*M. tuberculosis* ATCC 35838) control strains were inoculated in a separate plate on each processing day. If the results for the control strains were not as expected, the results for all samples plated at the same time were considered invalid and the stored portions of the original samples were re-processed using freshly prepared antibiotic solutions.

Definitions and outcome measures

A MODS culture with corded growth was considered to be false positive if the companion MGIT/LJ culture grew a *Mycobacterium,* which was identified as non-tuberculosis mycobacteria (NTM). Moreover, a negative MODS culture was considered to be false negative if MTBC was isolated from the companion MGIT/LJ culture.

A positive reference result was defined as a positive culture on either LJ or MGIT culture. The primary outcome measures were sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), agreement and turnaround time (TAT) of the MODS assay compared with standard reference methods for detection of MTBC, and its susceptibility to INH and RIF. TAT was defined as the time from specimen processing to the time of culture positivity and DST result. TAT for indirect DST included the time to culture positivity of the primary culture.

Statistical analysis

Chi-square and Fisher exact tests were used to compare the frequency and percentage among groups. To examine diagnostic agreement and evaluate the level of concordance among two methods, kappa coefficient was used. However, a *t*-test was used to compare the means of variables of two groups that had a normal distribution. When necessary, nonparametric Mann–Whitney *U* test and Wilcoxon test were used to compare two independent and two dependent groups, respectively.

The level of significance was defined as $p < 0.05$. Statistical analysis was performed with SPSS version 17.5.

RESULTS

Detection of *Mycobacteria*

A total 331 sputum samples were collected from 241 patients suspected to have TB. Of these 331 samples, 74 (22.4%) were smear positive and 257 (77.6%) were smear negative.

Mycobacterium tuberculosis complex was detected in 74 samples (22.4%). Of these, 69 were smear positive and 5 were smear negative. NTM was detected in one smear positive (*M. abscessus*) and 4 smear negative (2 *M. abscessus*, 1 *M. fortuitum*, and 1 unspecified) samples (Fig. 2).

MGIT culture was the most sensitive culture method, detecting MTBC in all 74 samples, and no additional strains could be isolated with other culture methods. MODS culture was the second most sensitive method detecting MTBC in 71 (68 smear positive, 3 smear negative) samples. LJ culture was the least sensitive culture method detecting MTBC in 64 (62 smear positive, 2 smear negative) samples. Overall, detection rates were 22.4% (74/331), 21.5% (71/331), and 19.3% (64/331) for MGIT, MODS, and LJ culture, respectively.

False-positive results were obtained with MODS in three (one smear positive and two smear negative) samples (3/331), due to cord forming NTM (*M. abscessus*). These three strains were resistant to

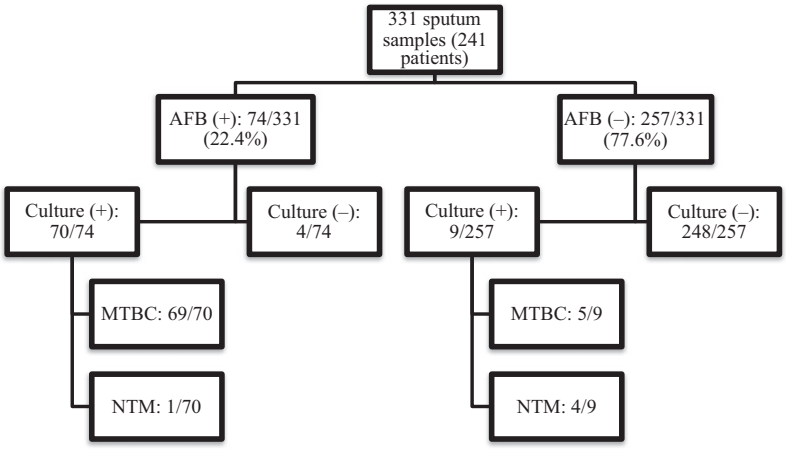

Fig. 2. Smear and culture results for 331 sputum samples obtained from 241 patients suspected to have tuberculosis. AFB, acid-fast bacteria; MTBC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculosis *Mycobacteria*.

both INH and RIF, and therefore misclassified as MDR-TB. Cording acid-fast bacteria (AFB) was also observed in the EZN-stained slides prepared from MGIT cultures of these samples; however, the TBc ID test was negative. MODS failed to detect MTBC in three (one smear positive and two smear negative) samples (3/331; Table 1). Time to culture positivity in MGIT culture of these two smear-negative samples were 21 and 30 days, and also cording AFB was not observed in the EZN-stained slides prepared from MGIT cultures of these samples. Nevertheless, MTBC was detected with MODS in other samples of these patients.

On comparison with the reference MGIT culture, the MODS assay had a sensitivity and PPV of 96% and a specificity and NPV of 98.8%. The agreement between MGIT and MODS culture was 98.2% (kappa value 0.948). The sensitivity and PPV of MODS assay in smear-positive samples increased to 98.6%. The specificity and NPV of the MODS assay in smear-positive samples could not be assed due to low culture negativity rate (4/74) in reference culture. The specificity and NPV of MODS assay in smear-negative samples increased to 99.2%. The sensitivity and PPV of the MODS assay in smear-negative samples could not be assessed due to low culture positivity rate (5/257) in reference culture (MGIT; Table 2).

Detection of drug resistance

Drug susceptibility testing results were compared in MTBC strains isolated from 71 samples in which MTBC was detected by both MODS and reference culture. When compared with the DST results obtained by MGIT system, MODS detected INH and RIF resistance with 87.1% and 85.7% sensitivity and 100% and 96.5% specificity, respectively. The agreement of MODS with MGIT system was 94.4% (kappa value 0.884) for detection of INH resistance and 94.4% (kappa value 0.822) for detection of RIF resistance. The sensitivity and specificity of MODS in the detection of MDR strains were 92.3% and 96.6%, respectively. Moreover, the agreement of MODS with MGIT system was 95.8% (kappa value 0.863; Tables 3 and 4).

Table 1. Detection of MTBC in sputum samples by MODS culture

	MTBC (+)		MTBC (−)		Total
	AFB (+)	AFB (−)	AFB (+)	AFB (−)	
MODS (+)	68	3	1	2	74
MODS (−)	1	2	3	250	257
Total	69	5	4	252	331

AFB, acid-fast bacteria; MODS, microscopic observation of drug susceptibility; MTBC, *Mycobacterium tuberculosis* complex.

TAT for culture and drug susceptibility testing

The median time to culture positivity was similar for MODS [8 days, interquartile range (IQR) 6–11 days] and MGIT culture (8 days, IQR 6–10.5 days; $p = 0.969$) and significantly ($p < 0.0001$) shorter than LJ culture (20 days, IQR 15–21 days; Fig. 3). The median time from initial sample processing to the results of DST was significantly ($p < 0.0001$) shorter in MODS assay (8 days, IQR 6–11 days) than MGIT system (20 days, IQR 18–23 days; Fig. 4).

DISCUSSION

This study evaluated the performance of MODS assay in the diagnosis of TB and MDR-TB demonstrating that MODS is a reliable method for detecting MTBC and determining its susceptibility to INH and RIF, as well as providing support for expanding its use.

Solid media is the only culture method in many resource-constrained settings. Therefore, in most of the earlier studies, MODS assay was compared with solid culture methods. Similar to these studies (6–14), MODS culture outperformed LJ culture in both detection rates and speed in our study. Only a few studies (11–13, 15) compared MODS with automated MGIT system. Although the sensitivity (range 81–89%) and the specificity (range 92.3–97%) of the assay in these studies were slightly lower than our findings, overall the results were consistent with ours concluding that MODS assay is a reliable method.

Table 2. The performance of MODS assay in detection of MTBC in sputum samples

	MTBC (n/N)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
All samples (n = 331)	71/74	96% (91.5–100)	98.8% (97.5–100)	96% (91.5–100)	98.8% (97.5–100)
Smear positive (n = 74)	68/69	98.6% (95.7–100)	NC	98.6% (95.7–100)	NC
Smear negative (n = 257)	3/5	NC	99.2% (98.1–100)	NC	99.2% (98.1–100)

CI, confidence interval; MTBC, *Mycobacterium tuberculosis* complex; NC, not calculated; n/N, number detected by MODS assay/number detected by reference method; NPV, negative predictive value; PPV, positive predictive value.

Table 3. The value of MODS assay in detection of INH and RIF resistance and MDR strains

	INH	RIF	MDR
Sensitivity (95% CI)	87.1% (75.3–98.90)	85.7% (67.4–100)	92.3% (77.8–100)
Specificity (95% CI)	100% (100–100)	96.5% (91.7–100)	96.6% (91.9–100)
PPV (95% CI)	100% (100–100)	85.7% (67.4–100)	85.7% (67.4–100)
NPV (95% CI)	90.9% (82.4–99.4)	96.5% (91.7–100)	98.3% (94.8–100)
Agreement (95% CI)	94.4% (89–99.7)	94.4% (89–99.7)	95.8% (91.1–100)

CI, confidence interval; INH, isoniazid; RIF, rifampicin; MDR, multidrug resistance; MTBC, *Mycobacterium tuberculosis* complex; NPV, negative predictive value; PPV, positive predictive value.

Table 4. Comparison of susceptibility results obtained with MGIT system and MODS assay

	MGIT-R	MGIT-S	Total
INH			
MODS – R	27	0	27
MODS – S	4	40	44
Total	31	40	71
RIF			
MODS – R	12	2	14
MODS – S	2	55	57
Total	14	57	71
	MGIT-P	MGIT-N	Total
MDR			
MODS – P	12	2	14
MODS – N	1	56	57
Total	13	58	71

N, negative; P, positive; R, resistant; S, sensitive.

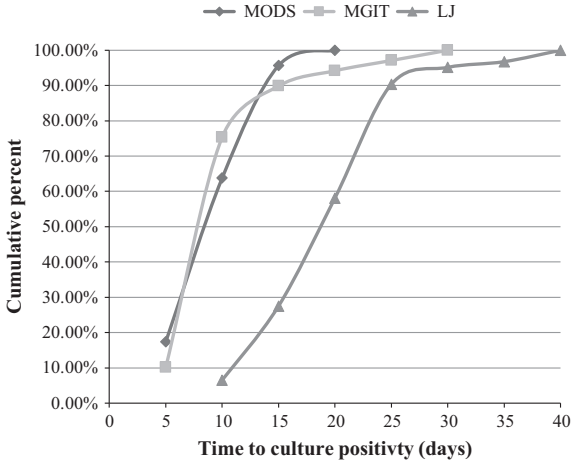

Fig. 3. Time to MTBC growth for MODS and two reference standard culture methods. Median turnaround time for *Mycobacterial* culture was similar for MODS (8 days) and MGIT culture (8 days), but was significantly longer for LJ culture (20 days; $p < 0.0001$ for both comparisons). LJ, Löwenstein-Jensen; MTBC, *Mycobacterium tuberculosis* complex; MGIT, BACTEC MGIT (mycobacteria growth indicator tube) 960 system; MODS, microscopic-observation drug susceptibility.

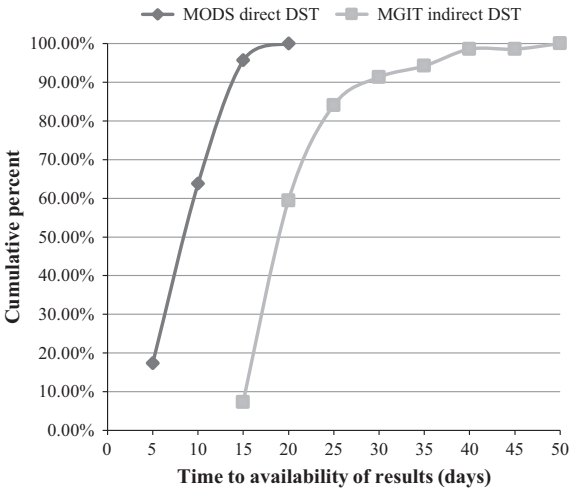

Fig. 4. Time to availability of DST results for MODS assay compared with reference standard MGIT system. Median time to availability of the susceptibility results was significantly ($p < 0.0001$) shorter with MODS assay (8 days) than MGIT system (20 days). DST, drug susceptibility test; MGIT, BACTEC MGIT (*Mycobacteria* growth indicator tube) 960 system; MODS, microscopic-observation drug susceptibility assay.

Drug-resistant TB is a major obstacle in global TB control, and therefore, rapid and accurate tests are needed to identify these cases. MODS can provide susceptibility results simultaneously with culture results. The performance of MODS assay in detecting INH and RIF resistance was investigated in various studies and two meta-analysis (6, 11, 12, 16–21), and MODS assay was found to be an accurate method for detecting drug resistance. According to the pooled estimates of the meta-analysis (16, 19), sensitivity and specificity of the assay ranged between 96–98% and 96–99.4%, respectively, for detecting RIF resistance and ranged between 90–97.7% and 95.8–98.6%, respectively, for detecting INH resistance (16, 19). Most of the earlier studies used proportion method as the reference DST (16, 19). However, only a few studies used automated MGIT system as the reference method for DST, and the sensitivity and specificity of MODS in these studies ranged between 75–93.8% and 92.9–97.4% for detecting INH and 93.6–100% and 96.9–100% for detecting RIF resistance, respectively (17, 20, 21). In our study, the sensitivity of MODS for detection of INH and RIF resistance was slightly lower compared

with most of these earlier studies; however, the specificity was similar. Reasons for the relatively lower sensitivity are unclear, but it may be due to the qualitative nature of the assay, sample storage, processing, or splitting, which can affect the bacillary volume in each inoculum causing a discrepancy between MODS assay and indirect DST used as the reference. Furthermore, the critical concentration used, type of the samples (some studies only included smear-positive samples), and the rate of resistant strains may influence the performance of the test method.

In the studies that used automated MGIT system as reference, MODS assay detected MDR-TB with high sensitivity (range 85.7–95.3%) and the specificity (range 97.5–100%) (17, 20, 21), which was consistent with our results. Moreover, the TAT of MODS culture was similar with MGIT culture and shorter than LJ culture as expected. Apart from one study (21) in which MGIT culture was found to be significantly faster than MODS culture (median TAT 6 days vs 12 days), MODS culture yielded results (median TAT range 6–12 days) at least as fast as reference liquid culture methods (median TAT range 6–16 days) and significantly faster than solid culture (median TAT range 21–30 days) (6, 12, 13, 22).

The major advantage of MODS assay compared with conventional indirect DST is its speed. In studies where primary isolation time was taken into account, the median time to availability of the susceptibility results for conventional indirect tests ranged between 22 and 71 days, whereas for MODS, it ranged between 7 and 9 days (6, 11–13, 20). Only a few studies (17, 20) compared TAT of MODS and MGIT for DST, and only one study (20) included the primary isolation time. In this study (20), the median TAT for primary MGIT culture was found to be 10 days; however, another median 19 days was needed to obtain the DST results with MGIT system. Whereas with MODS assay, DST results were available in a significantly shorter duration (median 8 days).

One limitation of our study was that the culture positivity rate among smear-negative samples was low. Therefore, the sensitivity of MODS assay could not be calculated in smear-negative samples.

An important shortcoming of the MODS assay is its relatively poor ability to discriminate MTBC from cord forming NTM, which was the case in three samples in our study. However, this problem can be addressed with revising the MODS assay to include a microtitter well-containing p-nitrobenzoic (PNB) acid, which specifically inhibits the growth of MTBC (5). In all the three samples, which were falsely identified as MTBC with MODS, the strains were resistant to both INH and RIF, therefore misclassified as MDR-TB. Misclassifying a NTM strain as MDR-TB has serious consequences. Thus, if MODS assay is to be used as the only microbiological diagnostic test for detection of TB, revisions should be made in order to increase the specificity of the test as mentioned above.

WHO published a policy statement regarding the use of non-commercial culture and DST methods including MODS assay and recommended its use under strict laboratory protocols only in reference or national laboratories (5). However, these methods were developed for settings with limited resources where liquid commercial systems and rapid genotypic tests are not available. Thus, restricting the use of rapid non-commercial assays like MODS will not address the problem, and it is important to establish the accuracy of these tests when used by non-expert groups. Therefore, we conducted a study evaluating the diagnostic accuracy of MODS assay in Turkey, where rapid, easy, and cheap methods are urgently needed and the performance of the test was not previously assessed. We compared the results of MODS with both solid (LJ) culture and commercial automated liquid culture and DST (BACTEC MGIT 960 system). In conclusion, we demonstrated that MODS is a rapid, and highly specific and sensitive test for detecting MTBC, and its resistance to INH and RIF. However, due to the risk of misidentifying cord forming NTM as MTBC, we strongly encourage adding a PNB containing well to increase the specificity of the test.

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