Evaluation of tuberculosis diagnostic tools, with extending MODS assay use to second line susceptibility testing

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ABSTRACT

Tuberculosis diagnosis and drug susceptibility testing (DST) are considered a priority for prompt initiation of effective therapy, increasing the chance of cure, decreasing the development of resistance, and reducing transmission.

Aim: Our objective was to evaluate currently applied diagnostic tools for tuberculosis including microscopic examination, GeneXpert, culture, and microscopic observation drug susceptibility (MODS) assay, investigating MODS assay usage for second line DST against culture based methods.

Material and Methods: In this study the 120 sputum samples collected from suspected cases were over one year duration from December 2018 to January 2020. The samples were subjected to ZN microscopic examination, GeneXpert, MODS assay, and culture for detection of mycobacteria. Moreover, resistance to 5 drugs: isoniazid, rifampicin, ofloxacin, levofloxacin, and amikacin were tested using MODS against the proportion method.

Results: The sensitivity and specificity of the MODS assay were similar culture method with the advantage of obtaining the results in a median time of 10.7 days. Whereas the specificity of ZN and GeneXpert was high among untreated cases and decreased in subjects with a history of treatment. Monoresistance was the most common form of resistance detected among new cases followed by multidrug resistance, with a categorical agreement between the two methods above 90% for all tested drugs.

Conclusions: MODS assay is an attractive option once standardized for second line susceptibility testing and GeneXpert assay is of high sensitivity for rapid detection of MTB and RIF resistance especially in treatment naive cases.

KEY WORDS

M. tuberculosis – culture – DST – MODS assay – GeneXpert

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INTRODUCTION

The early diagnosis of tuberculosis (TB) and drug susceptibility testing (DST) are currently an essential demand by the World Health Organization's (WHO) End Tuberculosis Strategy to ensure prompt and effective therapy [1]. Currently, the available guidelines for the first and second line DST weather on solid or liquid media are time-consuming taking up to 6 weeks with the possibility of consequent delay in proper treatment initiation, resulting in disease progression and ongoing transmission of resistant strains. To cut down the turnaround time, numerous commercial molecular assays and broth-based systems were developed. Nevertheless, these methods are relatively expensive, need specific infrastructure and training, placing them out of reach of laboratories in most developing countries [2].

Microscopic observation drug susceptibility (MODS) assay was established as a non-commercial test which has been successfully implemented in settings with limited resources for the detection of MDR TB in 2010, with a promising potential for its use in second-line DST [3, 4].

MODS is a phenotypic assay that detects *M. tuberculosis* and drug susceptibility directly from sputum. It entails culturing a decontaminated sample using liquid media to detect Mycobacterium microcolonies growth with an inverted light microscope; direct DST is performed simultaneously with obtainable positive results within 2 weeks [5].

GeneXpert MTB/RIF is one of the numerous molecular methods developed for the rapid detection of *M. tuberculosis* and RIF's resistance by PCR amplification of the 81-bp fragment of the rpoB gene followed by probing the gene for mutations linked with RIF drug resistance rapidly in almost 2 hours [6, 7]. It has been approved by the WHO in 2010 and endorsed for the screening of MDR-TB in high prevalence and in developing countries.

The objective of the current study was to evaluate TB diagnosis and detection of resistant strains by MODS against the Lowenstein Jensen (LJ) culture-based method after screening the samples by ZN and

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GeneXpert, MODS use was extended to the detection of resistance to fluoroquinolones and kanamycin.

MATERIALS AND METHODS

Study Design, Setting and Subjects

This is a cross-sectional study conducted at the TB laboratory in the Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Alexandria, and TB laboratory at El Maamora Chest Hospital, over one year duration from December 2018 to January 2020. The study was carried on 120 patients clinically and radiologically suspected as pulmonary tuberculosis including: new cases, default cases, relapse or treatment failure cases, attending El Maamora Chest Hospital. Ethical approval was given by the Alexandria University Faculty of Medicine Ethics Committee and the Egyptian Ministry of Health Ethics Committee. (NO:8-2018/10).

Microbiological Analysis of Study Samples

Sputum samples obtained from each patient were routinely analyzed by the Zeihl-Neelsen (ZN) smear microscopy, then processed by the N-acetyl-l-cysteine decontamination method [8]. Followed by culturing on LJ media [9] and performance of MODS assay for detection of MTB growth and Drug Susceptibility Testing (DST)[10]. Furthermore, sputum specimen was analysed also by GeneXpert MTB/RIF for detection of *M. tuberculosis* and genes of rifampin resistance [11].

Mycobacterium Tuberculosis Culture

Decontaminated samples were centrifuged (at 3000× g for 15 min) and resuspended in 2 ml of Middlebrook 7H9 plus "OADC" (oleic acid, albumin, dextrose, and catalase) and antimicrobial supplement PANTA (polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, and Azlocillin) (BD, Sparks, MD, USA). The mixture turbidity was adjusted to McFarland standard (number 1) and was used to inoculate LJ and perform direct MODS assay. The isolates from LJ culture were then used for DST by the 1% proportion method as the gold standard.

Drug susceptibility testing (DST)

1% proportion Susceptibility Method

The isolated *M. tuberculosis* strains and *M. tuberculosis* H37Rv (ATCC 27294) were subjected to drug susceptibility testing (DST) against isoniazid (INH), rifampicin (RIF), ofloxacin (OFX), levofloxacin (LEV), and kanamycin (KAN) using the standard 1% proportion method [12]. The drug concentrations used are the critical concentration recommended by WHO for DST using L.J; 0.2 mg/L for INH, 40 mg/L for RIF, 4 mg/L for OFX, 2 mg/L for LEV, 30 mg/L for KAN [13].

Microscopic observation drug susceptibility (MODS)

The sediment of the NALC processed sputum specimens were used for *M. tuberculosis* growth detection and DST by MODS, against rifampicin (1 µg/ml), isoniazid (0.4 µg/ml) according to published standard operating procedures for the 1st line drugs [14], with minor modifications to include 2nd line drugs: ofloxacin (2 µg/ml), levofloxacin (1 µg/ml) and kanamycin (5 µg/ml) in accordance with the recommendation of Trollip AP et al. [4] and WHO critical concentration for 2nd line drugs for liquid medium [13].

Briefly, each sputum sample was processed in six wells containing Middlebrook 7H9 broth in a sterile 24- well tissue culture plate. The first two wells in each column were drug free (control), in the other four wells, either rifampicin, isoniazid, ofloxacin, levofloxacin or kanamycin were added at a critical concentration of 1 µg/ml, 0.4 µg/ml, 2 µg/ml, 1 µg/ml and 5 µg/ml respectively. Plates were enclosed in plastic bags then incubated at 37 °C to be examined under an inverted microscope for mycobacterial growth by X40 magnification from day 5 of incubation with onward daily reading till 15th day of incubation, then reading was repeated on day18 and day 21. Each plate contained a negative control and positive control (*M. tuberculosis* H37Rv (ATCC 27294) reference strain). In the occurrence of rapid overgrowth or clouding indicating bacterial or fungal contamination; the stored original sample was retrieved for decontamination and cultured. A strain was accepted as susceptible to a drug if the drug containing well showed no growth while the control drug free well showed a minimum of two or more microcolonies (≥ 2 cfu) as cord-like structures. On the other hand, a strain was considered resistant to a drug if cord-like structures were detected in both the control wells and drug-containing wells [14, 15].

Sputum specimens were examined by **GeneXpert-MTB/RIF** (GX assay), present in TB laboratory in El Maamora Chest Hospital, to detect mycobacterium tuberculosis and resistance to rifampicin directly from sputum samples. The GX assay was applied similar to manufacturer instructions [40]. Amplification and quantification of the DNA by real time PCR specified whether MTBC was detected or not, also detection of rpoB mutations using molecular beacons was carried out to report MTB RIF resistance as detected, not detected, or indeterminate [16].

Statistical analysis

Chi-square and Fisher exact tests were used to compare the frequency and percentage among groups. The level of significance was defined as $p < 0.05$. The obtained data were analysed for statistical significance using SPSS version 25. Accuracy measures (sensitivity, specificity, positive and negative predictive values) of the evaluated tests for *M. tuberculosis* detection were determined using L.J culture and 1% proportion method as gold standards for the reference diagnosis.

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Taking the proportion method as the reference procedure Categorical agreement for RIF, INH, OFX, LEV, and KAN testing by MODS were calculated as percentage of strains yielding the same result category (sensitive/resistant) when compared to the standard procedure a minimum of 90% score is required to denote agreement. Errors detected were categorized further as either major error (ME) when there is a false resistant result and is calculated as (major errors/Total susceptible strains x100) or very major error (VME) when there is a false sensitive result and is calculated as (very major error/Total resistant strains x100) [17].

RESULTS

Out of the 120 patients clinically and radiologically suspected of pulmonary tuberculosis, 47 (39.2%) were confirmed by LJ culture to have pulmonary TB. Among which 34 new cases were further categorized as 31(25.8%) newly diagnosed and 3 (2.5%) new cases on 1st line treatment with delayed smear conversion, 13 previous treatment cases were further categorized as; 7 (5.8%) defaulters, 4 (3.3%) relapse and 2 (1.7%) treatment failure cases.

ZN smear examination displayed positive results for AFB in 45/120 (37.5%) samples while LJ culture revealed positive results in 47/120 (39.2%) samples, as for MODS assay 51/120 (42.5%) samples were positive for *M. tuberculosis* and finally, 72/120 (60%) were positive by GeneXpert.

Table 1 shows the results of evaluation of direct ZN smear, MODS assay, and GeneXpert for detection of *M. tuberculosis* in sputum specimens against LJ culture as a gold standard. Figure 1 shows the relation between positive results among the 4 used methods.

Regarding types of patients, out of 72 specimens that were positive by GeneXpert, 52 specimens were also positive by combining culture results for MODS and/or LJ culture. These 52 specimens were obtained from 35 (67.3%) new cases, 4 (7.7%) new cases on 1st line treatment with delayed smear conversion, 7 (13.5%) default cases, 4 (7.7%) relapse cases, 2 (3.8%) treatment failure cases. While out of 20 specimens that were positive by GeneXpert but culture negative, 2(10%) were new cases, 11(55%) were new cases on 1st line treatment with delayed smear conversion, 4(20%) defaults cases, and 3(15%) relapse cases. The effect of therapy on culture results among GeneXpert positive sputum specimens showed a statistically significant difference (*MCP = 0.00)*.

To remove the effect of therapy on ZN and GeneXpert specificity result against LJ culture, we attempted to recalculate its specificity in the 82 new cases without treatment exposure, from which 31 cases were LJ positive, GeneXpert specificity increased to 88.24% and ZN specificity increased to 100%.

Out of the 47 isolated strains tested for susceptibility to INH, RIF, OFX, KAN, and LEV, 17/47 strains showed resistance; Monosresistance was detected in 12/17 (70.6%) strains: one strain was resistant to INH isolated from a new case, 7 strains were resistant to OFX isolated from 5 new cases, 1 default case and 1 relapse, the remaining 4 strains showed monoresistance to KAN and were all isolated from new cases. Combined resistance to OFX and LEV was detected in only one (5.9%) strain isolated from a new case on 1st line treatment with delayed smear conversion, while multi drug resistance was detected in 4 (23.5%) strains; 3 were INH, RIF, OFX resistant isolated from 2 new cases and one relapse, while one strain was INH, RIF, KAN resistant isolated from a new case, 42 strains were found sensitive to INH and RIF. Concordance between proportion method and MODS for detection of MDR strains was 100%.

The sensitivity of MODS assay for INH, RIF and LEV testing was 100% when compared to proportion

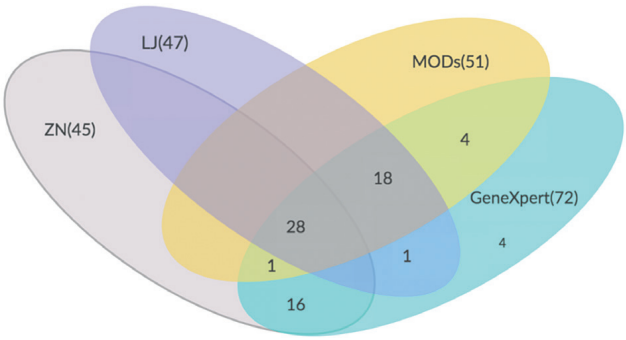

Figure 1. Venn diagrams for the number of positive samples by different tests (ZN, LJ, MODS, GeneXpert)
Twenty eight samples were positive by all tests, 18 were positive by LJ, MODS and GeneXpert, 1 sample was positive by ZN, MODS &, GeneXpert, 16 were positive by ZN & GeneXpert, 4 samples were positive by MODS & GeneXpert, 1 was positive by LJ & GeneXpert and finally 4 were positive only by GeneXpert.

Table 1. Evaluation of direct ZN smear, MODS assay, and GeneXpert for diagnosis of tuberculosis in 120 suspected cases

	Sensitivity [%]	Specificity [%]	Overall accuracy [%]	PPV [%]	NPV [%]
Direct ZN smear	59.56	76.71	70	62.22	74.67
MODS	97.87	93.15	95	90.19	98.55
GeneXpert	100	66.67	79.17	65.28	100

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method where (5, 4, 1) isolates found resistant by proportion were also resistant by MODS. As for the specificity, only RIF showed 100% specificity, unlike INH and LEV both had one extra isolate resistant only by MODS yielding a specificity of 97.56% and 97.78% with a major error of 2.4% and 2.2%.

Regarding ofloxacin results, out of the 11 isolates resistant by the proportion method, 9 were resistant by MODS and 2 were sensitive yielding a sensitivity of 81.82% with a very major error of 18.18%, the specificity was 97.14% as one isolate was resistant only by MODS. Finally for kanamycin out of 4 isolates resistant by proportion method 3 only were resistant by MODS with a very major error of 25% while one isolate was resistant by MODS only yielding a specificity of 97.62%. The Categorical agreement was found acceptable between the two methods for each drug tested as shown in table 2.

The time consumed to reach a positive result by the two culture methods is shown in Figure 2. Results obtained by MODS required a minimum of 7 days and up to 21 days, with a mean time of 10.69 ± 1.892 days, which was much shorter compared to LJ that ranged from 21–43 days with mean average of 33.04 ± 4.369 days. The time difference between both methods was found to be statistically significant (p = 0.00). This was the time needed for detection of MTB and DST simultaneously by MODS while for LJ it was the time needed for isolation of MTB only. Further DST by proportion methods needed extra time ranging from 21–32 days with an average of 28 days.

The liquid media was more prone to contamination as the present study showed a contamination rate of 6.67% with MODS assay compared to 2.5% with LJ medium.

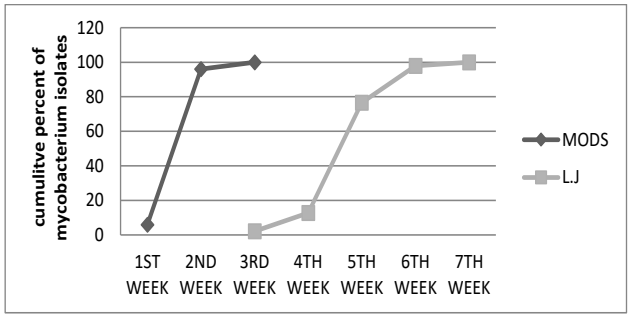

Figure 2. Cumulative percent of the time to culture positivity for MODS assay and LJ culture

As for detection of rifampicin resistance using GeneXpert; out of 72/120 sputum specimens positive for MTB, 65/72(90.2%) specimens showed no RIF resistance while 3/72(4.2%) samples had indeterminate RIF susceptibility and 4/72 (5.6%) were found resistant to RIF. These 4 samples grew isolates that were also found to be RIF resistant by proportion method and by MODS assay giving a 100%, sensitivity, specificity, PPV, NPV, and accuracy of GX MTB/Rif for detection of RIF resistance.

DISCUSSION

Phenotypic methods for diagnosis of tuberculosis and susceptibility testing still take the upper hand as molecular methods are not available for all agents nor are they used on a wide scale in the developing world to which the high burden countries belong, besides the fact that molecular tests may not detect all types of resistance or new mutation [18].

Table 2. Evaluation of MODS assay against proportion method for DST

MODS Assay	Proportion method		Categorical agreement	ME	VME
	Resistant	Sensitive			
INH **Resistant**	5	1	97.9%	1	–
Sensitive	0	40			
RIF **Resistant**	4	0	100%	–	–
Sensitive	0	42			
OFX **Resistant**	9	1	93.6%	1	2
Sensitive	2	34			
LEV **Resistant**	1	1	97.8%	1	–
Sensitive	0	44			
KAN **Resistant**	3	1	95.6%	1	1
Sensitive	1	41			

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In the current study ZN smear and GeneXpert showed low specificity of 76% and 66.67% compared to previous work [19, 20] as 17/45 (37.8%) smear positive samples were LJ culture negative, all of which were positive by GeneXpert and 1 case was also positive by MODS assay. Ideally, the proportion of smear positive culture negative specimens should be less. However, the TB lab of El-Maamora Chest Hospital is considered as a referral lab for tuberculous cases from the whole of Alexandria and nearby governorates, which are often referred from peripheral units after starting anti-tuberculosis therapy. All ZN positive culture-negative specimens, GeneXpert positive seen in this study belonged to cases already exposed to anti-TB drugs, (17 isolates were detected from 2 default cases, 3 relapse cases, and 12 new cases on 1st line anti TB drugs with delayed smear conversion) this exposure to treatment renders the bacilli dead or damaged in the tissue so they can still be detected by ZN stain or GeneXpert molecular test, but cannot grow on culture [21, 22]. The low specificity of GeneXpert agreed well with Meawed T. E. et al. [19, 23] reporting 75% for detection of MTB in sputum samples from retreatment patients in Egypt. Moreover, Theron G. et al [24] concluded that patients with previous tuberculosis are at a higher risk of false positive GeneXpert results especially when with chest radiology discordant with active infection. We attempted to recalculate the specificity of ZN and GeneXpert from new cases only and it increased to 100% and 88% respectively, endorsing the effect of treatment on these two methods. The only advantage of Gene Xpert MTB/RIF assay in retreated TB cases even if bacilli are damaged depends on its ability to detect rifampicin related mutations and resistance.

Regarding the low sensitivity of ZN sputum smear in our study, this is a well-known drawback of ZN since a count of 10^4/ml AFB is needed to yield a positive smear result [25]. Yet it still and will remain an essential tool due to its low cost, short turnaround time and high specificity, especially among new cases.

MODS sensitivity, specificity, PPV, and NPV reported in the current work for detection of *M. tuberculosis* were in agreement with other studies worldwide [26–28]. In our study, there were 5 positive cases by MODS assay which were negative by LJ culture. Among these 5; one case was smear-positive and GeneXpert positive, and 4 others were smear negative but were positive by GeneXpert, suggesting a false-negative LJ culture in these 5 cases. Moore D. A. et al. [10] have also reported higher sensitivity of MODS, this is explained by the greater sensitivity of liquid over solid media culture for TB detection, because of the constituents of Middlebrook $7H_9$ making it more enriched plus the added OADC supplement. Cross-contamination from another positive specimen or control strain during the time of inoculation is unlikely as they were all positive by GeneXpert.

Another great advantage for MODS is that the time to culture positivity was significantly shorter (10.7 days) in comparison to LJ culture (33 days). Moore D. A. et al. [10] have also reported a faster growth rate of 7 days for MODS than that of the MGIT liquid culture and LJ culture. In a study from India, the turnaround time of culture positivity by MODS was 10.3 days similar to our result (10.69) and the contamination rate was 7% similar to that reported in our study (6.67%) [29]. Moreover, MODS assay revealed DST results on the same day of MTB detection while LJ culture needed more time (average 28 days) after isolation of *M. tuberculosis* for applying the proportion method to obtain DST results.

Considering DST results, it was observed that monoresistance was more common than combined resistance in new cases (11/17 64%) similar Eufrasio R. et al. [30] whose work showed that monoresistance was the most common form of resistance among new cases, also comparable to Sobhy K. A. et al [31] work in Egypt; with 56.8% monoresistance among new cases. As for MDR 3 cases representing 17.6 % of the 17 resistance cases, and 8.8% out of all 34 new cases in the study, which is higher than the national country average of 1.4% MDR among new cases which may be due to limited study sample compared to national surveillance, however this finding warrants attention [32].

In the current study, MODS had a 100% CA for RIF when compared to the proportion method, the lowest CA of 93% was for OFX. All drugs tested showed a CA of more than 90%. However very major errors were detected for OFX and KAN. Similarly, discrepancies regarding INH, OFX and, KAN resistance were also reported by other studies [19, 26, 27, 33]. Reason for discrepant DST results between MODS and proportion method for tested drugs was unclear, but it may be due to the qualitative nature of the assay, sample processing, or splitting, which can affect the bacillary volume in each inoculum causing discrepancy between MODS assay and proportion method used as the reference.

The highest resistance detected in this study was for OFX, where 11 isolates showed OFX resistance, whether monoresistance or combined, this is in agreement with others who concluded that absence of restrictions on acquiring fluoroquinolones medication and their availability as over the counter medication used to treat various infections as community acquired pneumonia is the main contributor to preexisting ofloxacin resistance especially in new TB cases, this is besides other non-healthcare associated factors that help to add to this resistance including the use of fluoroquinolones in farming [34, 35]. This led to the WHO updated recommendations in 2018, on the use of levofloxacin or moxifloxacin for the treatment of MDR- TB instead of ofloxacin, and drug susceptibility testing of ofloxacin to be eliminated and laboratories

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should changeover to levofloxacin or moxifloxacin testing [13, 36]. This change took place after the initiation of the current study thus we added levofloxacin testing to our work and we are in total agreement with the updated guideline as OFX resistance was high and was not representative of FQ resistance since cross resistance with levofloxacin was only detected in one isolate of the 11 ofloxacin resistant strains when using the above recommended MICs. Suggesting that resistance to ofloxacin does not necessarily exclude susceptibility to levofloxacin or other FQ and should not be a reason for depriving MDR-TB patients of the FQ -including shorter regimen. Javaid A. et al [37] also emphasized that fluoroquinolones should not be used haphazardly for MDR treatment without prior specific testing in areas with indiscriminate accessibility to this group of drugs.

So our results endorse others regarding the fact that the assay can also be used for detection of *M. tuberculosis* resistance to second-line drugs especially if ofloxacin testing is excluded as CA ranged from 100 % to 95.6% for other drugs, with one very major error associated with kanamycin representing 20%, however, this percent is relatively high due to the low number of KAN resistance detected [2, 38].

CONCLUSION

The MODS Assay is a rapid and cost-effective method for detecting *M. tuberculosis* and DST for 1st and 2nd line drugs when compared to culture-based method. With adequate medical technologists' training, the MODS assay can be adopted in developing countries where rapid and inexpensive methods are urgently needed. Since MODS can be implemented directly on samples, this will offer the clinician the value of rapid and simultaneous detection of *M. tuberculosis* and DST results. We also concluded that GeneXpert assay is of high sensitivity for rapid detection of MTB and RIF resistance especially in treatment naive cases, and that extra caution should be taken when interpreting positive results from treatment exposed cases. To sum up combining MODs assay and GeneXpert MTB/RIF assay will definitely improve the detection rate of *M. tuberculosis* and extending MODS' use to second line drugs will help better treatment choices and decisions for MDR-TB cases.

Limitations: The main limitation was lack of growth of a few samples on the reference LJ culture media, which may be due to patients receiving antiTB-drugs, this affected the assessment of other methods in relation to the gold standard used. Raising the point that culture may fail to detect *M. tuberculosis*, makes the presence of a gold standard for diagnosis of tuberculosis difficult. Still, cultures are contemplated to be the best choice. The low proportion of drug-resistant TB detected in this study is considered also a limitation.

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Conflict of interest
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