Original Article

Rapid Detection of *M. tuberculosis* and Its Resistance to Rifampicin and Isoniazid with the *mfloDx*™ MDR-TB test

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Abstract

Background: Rapid detection of tuberculosis (TB) and its resistance are essential for the prompt initiation of correct drug therapy and for stopping the spread of drug-resistant TB. There is an urgent need for increased use of rapid diagnostic tests to control the threat of increased TB and multidrug-resistant TB (MDR-TB). **Methods:** EMPE Diagnostics has developed a multiplex molecular diagnostic platform called *mfloDx*™ by combining nucleotide-specific padlock probe-dependent rolling circle amplification with sensitive lateral flow biosensors, providing visual signals, similar to a COVID-19 test. The first test kit of this platform, *mfloDx*™ MDR-TB can identify *Mycobacterium tuberculosis* (MTB) complex and its clinically significant mutations in the *rpoB* and *katG* genes and in the *inhA* promotor contributing resistance to rifampicin (RIF) and isoniazid (INH), causing MDR-TB. **Results:** We have evaluated the performance of the *mfloDx*™ MDR-TB test on 210 sputum samples (110 from suspected TB cases and 100 from TB-negative controls) received from a tertiary care center in India. The clinical sensitivity for detecting MTB compared to acid-fast microscopy and mycobacteria growth indicator tube (MGIT) cultures was 86.4% and 84.9%, respectively. All the 100 control samples were negative indicating excellent specificity. In smear-positive sputum samples, the *mfloDx*™ MDR-TB test showed a sensitivity of 92.5% and 86.4% against MGIT culture and Xpert MTB/RIF, respectively. The clinical sensitivity for the detection of RIF and INH resistance in comparison with MGIT drug susceptibility testing was 100% and 84.6%, respectively, while the clinical specificity was 100%. **Conclusion:** From the above evaluation, we find *mfloDx*™ MDR-TB to be a rapid and efficient test to detect TB and its multidrug resistance in 3 h at a low cost making it suitable for resource-limited laboratories.

Keywords: Lateral flow, multidrug-resistant-tuberculosis, *mfloDx*, *Mycobacterium tuberculosis*, padlock probe, rolling circle amplification

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Introduction

Tuberculosis (TB) is a communicable disease and one of the leading causes of death worldwide, with an estimated total of 10.6 million people developed active disease and 1.3 million people died in 2022.[1] Most TB cases in 2021 were in the World Health Organization (WHO) regions of Southeast Asia (45%), Africa (23%), and the Western Pacific (18%), with smaller shares in the Eastern Mediterranean (8.1%), the Americas (2.9%) and Europe (2.2%). Although TB is curable, improper or delayed detection of it and its drug resistance lead to the development of further antibiotic resistance, which remains a challenge for effective treatment. Prompt detection of resistance strains often referred to as drug-resistant TB (DR-TB) is crucial, since they worsen the clinical conditions of the patient, continue to spread in the community, and become a socioeconomic challenge. According to Silva *et al*.,[2] a staggering number of 23.8 million TB deaths and a loss of $13.1 trillion can be expected because of the COVID-19 pandemic.

Conventional microbiological techniques use sputum smear microscopy to detect acid-fast bacilli (AFB) in clinical specimens followed by culture and drug susceptibility testing (DST) through culturing the bacteria in the presence of TB drugs, in strict biosafety laboratories-3 (BSL-3) for up to 8–12 weeks.[3,4] The

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rapid molecular diagnostic tests for the identification of TB and drug resistance have consequently an implementation priority[5] in clinical diagnostics because they can provide results in around 2 h. The commercial nucleic acid amplification tests (NAATs) such as Xpert *Mycobacterium tuberculosis* (MTB)/rifampicin (RIF) assay (Cepheid, USA),[6-10] GenoType MTBDRplus line probe assay (LPA; Hain Lifescience, Germany/Bruker, USA),[11-13] and TrueNAT MTB/MTBplus/MTB-RIF (Molbio Diagnostics, India)[14-17] have been studied widely and recommended as molecular WHO-recommended rapid diagnostic tests for TB diagnosis. The WHO endorsed Xpert and TrueNAT tests for the detection of TB and RIF resistance and LPA for testing additional isoniazid (INH) resistance.[18-21] Xpert MTB/RIF assay simultaneously detects both MTB complex and RIF resistance within two hours[7] while LPA detects MTB, resistance to RIF and resistance to INH in 48 h.[13] TrueNAT MTB/MTB plus detects MTB in 35 min followed by reflex testing for resistance to RIF which takes an additional 60 min, resulting in a total runtime of ˜2 h to detect MTB and RIF resistance.

The capacity and requirements for instrumentation and skilled personnel of currently available NAATs severely limit the prompt detection of multidrug-resistant TB (MDR-TB) in resource-limited settings. Therefore, a proficient method that can address the molecular detection challenges of MDR-TB is required to provide easily interpreted first-hand information about TB and MDR-TB. The *mfloDx*™ diagnostic platform[22] developed by EMPE Diagnostics AB, Sweden is based on two well-established technologies: padlock probe (PLP)-dependent rolling circle amplification (RCA), an isothermal nucleic acid amplification method,[23-25] and sensitive lateral flow nucleic acid biosensor chemistry (signal development readout). *mfloDx*™ MDR-TB (EMPE Diagnostics AB, Stockholm, Sweden and EMPE Diagnostics Private Limited, Hyderabad, India) is a molecular test developed to detect the presence of MTB (by capturing the conserved internal transcribed spacer genomic deoxyribonucleic acid [DNA] region) and clinically significant hotspot mutations in *rpoB, katG*, and *inhA* coding for resistance to major first-line antibiotics RIF and INH. The *mfloDx*™ MDR-TB test can be used directly on sputum samples and the technique is easy to perform and has a turnaround time of 3 h. The results are read using the lateral flow cassettes which consist of wild type (WT) and mutant type (MUT) windows.

The present study aimed to evaluate the *mfloDx*™ MDR-TB test for rapid and simultaneous detection of MTB, RIF, and INH resistance from direct sputum samples in comparison with mycobacteria growth indicator tube (MGIT) culture as a gold standard, MGIT DST for RIF, INH resistance detection and Xpert MTB/RIF as a comparator molecular test for the detection of MTB and RIF resistance.

Methods

The study was conducted at the Department of Microbiology, Christian Medical College (CMC), Vellore, India. The study was approved by the Institutional Ethics Committee (IRB No. 12191 [DIAGNO] dated August 28, 2019) at CMC, Vellore. An informed consent was obtained from the study participants.

Inclusion criteria

Patients with a clinical or bacteriological diagnosis of pulmonary TB, aged >18 years, willing to provide informed consent, and willing to provide sputum were included in the study.

Exclusion criteria

Patients on anti-TB treatment for 14 or more consecutive days and not willing to provide informed consent were excluded from the study.

Sputum collection

Two hundred and ten consecutive sputum samples were included in the study. All participants enrolled in the study provided spontaneously expectorated sputum samples. However, a minimum of 1 ml of sputum sample was collected to obtain reliable results.

Fluorescence microscopy

Fluorescence staining was performed on all the sputum specimens to look for the presence of AFB and reported according to the WHO guidelines for interpreting smear microscopy.[26]

Mycobacteria growth indicator tube culture and mycobacteria growth indicator tube drug susceptibility testing

After processing for smear microscopy, 1 mL of the sputum samples were decontaminated by the standard N-acetyl L-Cystine (NALC)-sodium hydroxide (NaOH) method.[26] The decontaminated pellet was divided into two parts: the first part was used as inoculum for MGIT culture. All MGIT culture-positive specimens were tested with the MGIT TBc ID kit[26] to confirm the identification of MTB and proceeded with MGIT DST[26] for RIF and INH.

Xpert *Mycobacterium tuberculosis*/rifampicin

Gene Xpert MTB/RIF test was performed as per the manufacturer's instruction for all the sputum samples.

mfloDx™ multidrug-resistant-tuberculosis

The second part of the decontaminated sample was resuspended well in 200 µL of sample preparation buffer and heat lysed at 95°C for 20 min. Since the *mfloDx*™ MDR-TB test does not require DNA extraction/purification, 5 µL of lysed supernatant was used to detect MTB and its wild-type as well as, resistance mutations in *rpoB*, *katG* and *inhA*, coding for RIF and INH, respectively [Table 1].

The *mfloDx*™ MDR-TB test requires only standard laboratory equipment and infrastructure such as a thermal cycler, heat block, small tabletop centrifuge, and a vortex mixer, to perform the test. The test consists of 6 steps after DNA extraction, namely preamplification, PLP capturing the specific targets, purification of ligated circles using magnetic beads, RCA

of the circles, restriction and digestion of the amplified single-stranded concatemers and finally, development of visual signals on the lateral flow cassettes. This test kit provides confirmatory results in a qualitative "YES" or "NO" format, within 3 h by producing red color lines [Figure 1].

As shown in Figure 1 above, the window on the left-hand side of the cassette (marked as "WT") indicates MTB identity and the detection of the WT allele of the resistance-detecting codons. The right-hand side window of the cassette (marked as MUT) indicates the detection of mutations in the respective genes and the hybridization control which is an indicator of the functionality of the visualization solution. The internal control band visible at the bottom of both the cassette windows is the indicator for the successful completion of all the steps of the *mfloDx*™ MDR-TB test.

The clinical sensitivity and clinical specificity for the detection of MTB, RIF, and INH resistance of the *mfloDx*™ MDR-TB test was compared with the conventional gold standard culture test (MGIT DST) and to a WHO-recommended molecular test (Xpert MTB/RIF).

Results

Two hundred and ten sputum samples were analyzed with three different tests: namely MGIT culture, Xpert MTB/RIF, and *mfloDx*™ MDR-TB according to standard laboratory practice and manufacturer's instructions. As shown in Table 2, the smear positivity rate in this study was 28.1% (59/210), while the MGIT culture positivity was found to be 35.2% (74/210). The molecular test, Xpert MTB/RIF showed positivity in 52.4% for MTB detection (110/210) while *mfloDx*™ MDR-TB test showed positivity in 34.3% (72/210) of sputum samples.

Among the 59 smear-positive sputum samples, 51 were MTB positive and 8 were negative by *mfloDx*™ MDR-TB test. Out of the 151 smear-negative samples, 21 were positive and 130 were negative by the *mfloDx*™ MDR-TB test. The clinical sensitivity and specificity of the *mfloDx*™ MDR-TB test against smear were 86.4% and 86.1%, respectively [Table 3].

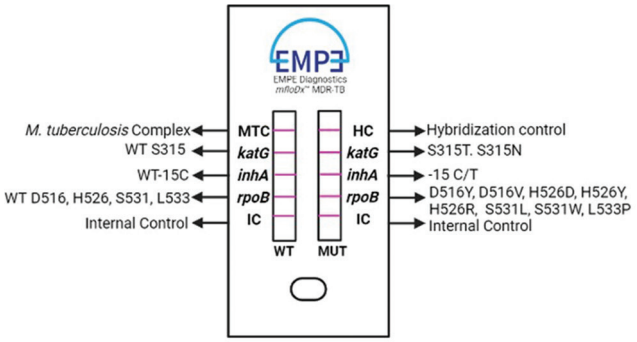

Figure 1: Representative illusion of the *mfloDx*™ MDR-TB lateral flow cassette. WT indicates *Mycobacterium tuberculosis* complex identity and the detection of the wild-type allele of the resistance-coding gene. Mutant type (MUT) indicates detection of mutations in the respective genes and the hybridization control. IC indicatesthe detection of internal control in both wild type and MUT. MTC: *Mycobacterium tuberculosis* complex, WT: Wild type, HC: Hybridization control, IC: Internal control, MUT: Mutant type, *M. tuberculosis*: *Mycobacterium tuberculosis*

Detection of *Mycobacterium tuberculosis*

Among the 73 MGIT culture MTB-positive samples, 62 were MTB positive and 11 were MTB negative by the *mfloDx*™ MDR-TB test. Out of the 137 MGIT culture-negative samples, 10 were MTB positive and 127 were MTB negative by the *mfloDx*™ MDR-TB test. Thus, the clinical sensitivity and specificity of the *mfloDx*™ MDR-TB test against MGIT culture was found to be 84.9% and 92.7%, respectively.

The *mfloDx*™ MDR-TB test showed 72 positives and 38 negatives among the 110 xpert MTB/RIF positive samples. All negative GeneXpert samples were negative also by *mfloDx*™ MDR-TB test. As can be seen in Table 3, the clinical sensitivity and specificity of *mfloDx*™ MDR-TB test showed 65.5% and 100%, respectively, against the Xpert MTB/RIF.

Detection of resistance

MGIT DST

Among the 73 culture-positive TB samples, 58 samples were tested for RIF and INH. Out of these 58, only 7 were found to be resistant for both RIF and INH (MDR-TB), while 1 sample was found to be RIF monoresistant and 6 were INH monoresistant. The remaining 44 were sensitive to both RIF and INH.

Table 1: Mutations detected by *mfloDx*™ multidrug-resistant tuberculosis test

Gene	Codon	Mutation	Aminoacid change
rpoB	516	G/T	D516Y
	516	A/T	D516V
	526	C/G	H526D
	526	C/T	H526Y
	526	A/G	H526R
	531	C/T	S531L
	531	C/G	S531W
	533	T/C	L533P
katG	315	G/C	S315T
	315	G/A	S315N
inhA	−15	C>T	-

Table 2: Distribution of results for 210 sputum samples

Test	Overall samples (*n*=210)	
	Positive, *n* (%)	Negative, *n* (%)
Smear	59 (28.1)	151 (72)
MGIT culture	74 (35.2)	136 (64.7)
Xpert MTB/RIF	110 (52.4)	100 (47.6)
mfloDx™ MDR-TB test	72 (34.3)	138 (65.7)

TB: Tuberculosis, MTB/RIF: *Mycobacterium* TB complex/resistance to rifampicin, MDR-TB: Multidrug-resistant TB, MGIT: Mycobacteria growth indicator tube

Table 3: Comparison of the *mfloDx*™ multidrug-resistant tuberculosis test against smear, mycobacteria growth indicator tube, and Xpert *Mycobacterium tuberculosis* complex/resistance to rifampicin

Smear versus *mfloDx*™ MDR-TB			MGIT versus *mfloDx*™ MDR-TB			Xpert MTB/RIF versus *mfloDx*™ MDR-TB		
***mfloDx*™ MDRTB (*n*=210)**	**Smear**		***mfloDx*™ MDR-TB (*n*=210)**	**MGIT**		***mfloDx*™ MDR-TB (*n*=210)**	**Xpert MTB/RIF**	
	Positive	**Negative**		**Positive**	**Negative**		**Positive**	**Negative**
Positive	51	21	Positive	62	10	Positive	72	0
Negative	8	130	Negative	11	127	Negative	38	100
Sensitivity (%)	86.4		Sensitivity (%)	84.9		Sensitivity (%)	65.5	
Specificity (%)	86.1		Specificity (%)	92.7		Specificity (%)	100.0	

TB: Tuberculosis, MTB/RIF: *Mycobacterium* TB complex/rifampicin, MDR-TB: Multidrug-resistant TB, MGIT: Mycobacteria growth indicator tube

Xpert MDTB/RIF

Among the 110 MTB-positive samples, 97 were RIF susceptible and 13 were RIF resistant.

mfloDx™ MDR-TB

Out of the 72 TB-positive samples, 7 were found to be resistant to RIF and INH, indicating that they were MDR-TB. While 1 was monoresistant to RIF, 5 were monoresistant to INH, and 56 were susceptible to both RIF and INH. The remaining 3 samples were indeterminate for RIF (did not show any band in WT or MUT), of which 2 were additionally indeterminate for INH. These samples are under further investigation by sequencing.

Clinical sensitivity *mfloDx*™ MDR-TB, for the detection of RIF (100%) and INH (84.6%) resistance, in comparison with MGIT test, while the clinical specificity was 100% for both RIF and INH. In comparison with Xpert MTB/RIF, the *mfloDx*™ MDR-TB revealed 89% sensitivity and 100% specificity to RIF resistance. The indeterminate rate for RIF and INH resistance on *mfloDx*™ MDR-TB was 4.2% and 2.8%, respectively.

Discussion

Molecular tests were initially developed for the diagnosis of pulmonary TB and RIF resistance in adults and children. The massive gap in TB detection made worse during the pandemic, has already cost lives, worsened transmission, and derailed years of progress in TB control.[27] A few new diagnostic technologies have been endorsed by WHO during the past 10 years.[18] The amplification and detection of MTB DNA have proven to be highly sensitive and specific. Some amplification technologies have the great advantage of also being able to detect resistance to additional anti-TB drugs.[27] However, each of these tests has its limitations in terms of infrastructure, targets included in the assay, ease of performance, and interpretation of the results.

The *mfloDx*™ MDR-TB test is a unique and rapid near point-of-care test based on the PLP and RCA coupled with lateral flow biosensor for the detection of MTB as well as resistance to RIF and INH. This test provides information about the most common clinically significant mutations causing resistance to RIF and INH, in addition to the detection of TB. Consequently, providing a valuable preview of the drug susceptibility pattern at the early stages of diagnosis could certainly help clinicians start treatment with appropriate antibiotics.[25]

The *mfloDx*™ MDR-TB test showed a sensitivity of 86.4%, 84.9%, 65.5% and specificity of 86.1%, 92.7%, 100% in comparison with smear microscopy, MGIT culture and Xpert MTB/RIF, respectively from both smear-positive and smear-negative samples. In smear-positive sputum samples, the *mfloDx*™ MDR-TB test showed a sensitivity of 92.5% and 86.4% against MGIT culture and Xpert MTB/RIF, respectively. In addition, *mfloDx*™ MDR-TB showed a 100% sensitivity and specificity for RIF susceptibility testing results when compared to MGIT DST. In comparison with Xpert MTB/RIF, *mfloDx*™ MDR-TB showed a sensitivity of 89% for RIF susceptibility testing. Only one sample showed discordant results for RIF resistance in comparison with Xpert MTB/RIF. This sample was susceptible to RIF by both MGIT-DST and *mfloDx*™ MDR-TB but resistant by Xpert MTB/RIF. As for INH susceptibility results, two samples that were resistant with MGIT DST were found to be susceptible to *mfloDx*™ MDR-TB and thereby showed 84.6% sensitivity for INH susceptibility testing. It is well known[28] that in addition to *katG* and *inhA,* there are several other genes such as *oxyR-ahpC, kasA* which code for INH resistance that are not detected by *mfloDx*™ MDR-TB test or other molecular rapid tests. There were 3 RIF indeterminate samples, of which 2 were indeterminate to INH also. All three samples were smear-negative (probably below the detection limit).

Conclusion

To conclude, the *mfloDx*™ MDR-TB test is an open platform test and can be performed in any basic molecular biology laboratory with facilities to do a basic polymerase chain reaction and has a turnaround time of 3 h, which is much shorter than the routine MGIT culture and DST.

Outcome of the study

Despite Xpert MTB/RIF test providing results in a shorter time frame, the *mfloDx*™ MDR-TB has the significant advantage of detecting INH resistance at the same time. In a clinical setting,

this test can be applied to smear-positive samples with excellent sensitivity and specificity.

Rationale of the study

Overall, *mfloDx*™ MDR-TB test addresses the pressing need for improved diagnostic tools that are sensitive, rapid, accessible, and cost-effective, ultimately contributing to more effective TB control and elimination efforts on a global scale.

Limitations of the study

The *mfloDx*™ MDR-TB test relies on DNA amplification. Considering the multitude of MTB genetic variants, it is possible that for some strains the selected mutations will not be detected. This qualitative test can detect DNA from both viable and nonviable bacteria. The test cannot be used to quantitatively monitor the progression of TB or be correlated to the original number of bacteria in the sample.

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Conflicts of interest

There are no conflicts of interest.

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