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Evaluation of FluoroType MTB for direct detection of *Mycobacterium tuberculosis* complex and GenoType MTBDRplus for determining rifampicin and isoniazid resistance

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ABSTRACT

In recent years, several molecular methods have been introduced for diagnosis of *Mycobacterium tuberculosis* complex (MTBC), and detecting the drug resistance in clinical specimens. The FluoroType MTB (FT MTB) assay uses real-time polymerase chain reaction (PCR) to detect MTBC in clinical specimens. GenoType MTBDRplus is a line probe assay which detects MTBC, as well as rifampicin, and isoniazid resistance. In this study, the diagnostic performances of FT MTB and GenoType MTBDRplus were evaluated. In total, 247 specimens (124 respiratory, 123 non-respiratory) were analyzed comparing mycobacterial growth methods and FT MTB. GenoType MTBDRplus was used for the specimens positive for MTBC. In all, 23 (9.3%) of 247 specimens were positive for both the culture and FT MTB assay; therefore, the GenoType MTBDRplus assay was performed on 23 clinical specimens. The results were concordant with the drug susceptibility test results. The FT MTB assay provided quick and reliable direct detection of MTBC from the clinical specimens with high sensitivity (95.8%) and specificity (100%). Although the performance of GenoType MTBDRplus was problematic in clinical specimens with mycobacterial levels below the detection limits of the assay, it was a reliable test for cultivated specimens.

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Introduction

Globally, tuberculosis (TB) is still a major health problem. In 2016, 1.3 million people died due to TB infections, and 10.4 million people were estimated to have fallen ill with TB. Moreover, 10% of the individuals newly diagnosed with TB in 2016 were HIV-positive. To fight this burden, detection and treatment gaps need to be addressed, funding gaps should be closed and new tools must be developed [1].

Drug-resistant TB continues to threaten the global control programme, and remains a major public health issue in many countries. In 2016, approximately 490,000 cases of multidrug-resistant TB (MDR-TB) were estimated to have occurred. Worldwide, 4.1% of the newly diagnosed TB cases and 19% of the previously treated cases have MDR-TB. An estimated 240,000 people died of MDR-TB in 2016 and an estimated 6.2% of those people with MDR-TB had extensively drug-resistant TB (XDR-TB) [1].

MDR-TB is defined as resistance to both isoniazid (INH) and rifampicin (RIF), while XDR-TB is defined as MDR-TB with additional resistance to any fluoroquinolone and to at least one of three injectable drugs:

capreomycin, kanamycin or amikacin. In the case of RIF resistance, approximately 95% of the strains have a mutation within the 81 bp region of the *rpoB* gene. INH resistance is more complex and caused by mutations in one or more genes, such as the genes encoding catalase-peroxidase (*katG*) and the enoyl-acyl-carrier protein reductase enzyme (*inhA*) [2,3].

Bacteriological culture still the reference standard for detecting TB, but the results can take weeks to obtain. This testing requires a laboratory with specialized equipment, highly trained staff and an efficient transport system to ensure the viability of the specimens. The phenotypic drug susceptibility test (DST) on cultured specimens is the conventional method for detecting the resistance to first and second-line TB drugs. Today faster commercial liquid culture systems are available for these tests [3].

In recent years several molecular methods have been introduced for the diagnosis of *Mycobacterium tuberculosis* (MTB) and the rapid detection of the drug resistance. Among these are the nucleic acid amplification tests, including the Amplified *M. tuberculosis* Direct Test (Gen-

Probe Inc. San Diego, CA, USA), Probetec (BD, Sparks, MD, USA) and the LAMP assay (Eiken Chemical Co. Ltd., Tokyo, Japan). There are also line probe assays such as the GenoType MTBDRplus (Hain Lifescience GmbH, Nehren, Germany), INNO LIPA Rif.TB assay (Innogenetics, Ghent, Belgium) and Hyplex TBC Test (BAG Healthcare, Lich, Germany). The real-time polymerase chain reaction (PCR) systems include COBAS TaqMan MTB (Roche, Basel, Switzerland), Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) and FluoroType MTB (FT MTB) (Hain Lifescience GmbH, Nehren, Germany) [4,5]. With these assays, it is possible to detect mycobacteria or predict drug resistance within one working day. Despite the advantages of these molecular tests, conventional microscopy and culture remain necessary for monitoring the patient's response to treatment [1].

The FT MTB is a semi-automated assay, using real-time PCR to detect *M. tuberculosis* complex (MTBC) from respiratory and non-respiratory clinical specimens based on HyBeacon fluorescence technology. HyBeacon probes are single-stranded oligonucleotides labeled with fluorophores, and the hybridisation of these probes with the amplified DNA causes an increase in fluorescence. Further discrimination of the target sequences is performed with a melting curve analysis. This assay is performed using a Fluorocycler (Hain Lifescience GmbH, Nehren, Germany) [4,6].

The GenoType MTBDRplus version 2 is a line probe assay which detects the MTBC, as well as mutations in the *rpoB* gene for RIF resistance, *katG* gene for high-level INH resistance and *inhA* gene for low-level INH resistance. This test has two steps, PCR amplification and reverse hybridization, and is approved for use in both culture-positive isolates and smear-negative or positive specimens. This assay was endorsed by WHO in 2008 [7].

The first aim of the present study was to evaluate the diagnostic performance of the FT MTB assay for the direct detection of the MTBC in clinical specimens, and compare the results with the reference culture method using Lowenstein-Jensen (L-J) medium and the Bactec MGIT 960 system (BD, Sparks, MD, USA). The second aim was to evaluate the diagnostic accuracy of the GenoType MTBDRplus assay for detecting the RIF and INH resistance of the MTBC in clinical specimens, by comparing the DST results using the Bactec MGIT 960 system.

Materials and methods

Clinical specimens

Specimens from patients with a clinical suspicion of TB or previous TB findings that were sent to the Istanbul Faculty of Medicine, Department of Medical

Microbiology, for routine TB analysis, between June and October of 2012, were included in this study. Ethics committee approval was not required for this research, since the clinical specimens used in this study were obtained as part of the standard patient care, and the data were analyzed anonymously. In total, 247 specimens (124 respiratory: sputum, bronchoalveolar lavage, gastric lavage; 123 non-respiratory: pus, peritoneal fluid, biopsy, urine, ascites, ejaculatory fluid, cerebrospinal fluid) were analyzed.

The specimens were decontaminated using an N-acetyl-L-cysteine-sodium hydroxide solution, neutralized with phosphate buffer and concentrated via centrifugation [8]. The processed specimens were separated into two volumes. The first volume was used for acid-fast bacillus (AFB) microscopy via the Ehrlich-Ziehl-Neelsen (EZN) method, as well as culture in Bactec MGIT 960 liquid medium (BD, Sparks, MD, USA) and L-J solid medium (BD, Sparks, MD, USA). The second volume was used for the FT MTB analysis. The GenoType MTBDRplus assay was performed on those specimens that were found to be positive when using the FT MTB assay. For three of the specimens, the GenoType MTBDRplus assay was performed with DNA extracted from the cultivated strains, because no interpretable results could be obtained with the DNA taken directly from the specimens.

All of the cultures were incubated at 37°C for 6–8 weeks. From the positive Bactec MGIT 960 vials or L-J cultures, EZN stained smears were prepared and an MGIT TBc Identification Test (BD, Sparks, MD, USA) was performed. Drug susceptibility testing was carried out for the MTBC-positive cultures using the MGIT 960 system (BD, Sparks, MD, USA) according to the manufacturer's instructions [9].

DNA preparation from patient specimens

The mycobacterial DNA extraction from the decontaminated patient specimens was performed using a FluoroLyse kit (Hain Lifescience GmbH, Nehren Germany), according to the manufacturer's instructions [10].

DNA preparation from cultivated strains

To prepare the DNA from the cultivated strains, one loopful of cells was suspended in 300 μ L of distilled water, boiled at 95°C for 20 min, sonicated for 15 min and centrifuged for 5 min [11].

FT MTB assay

The FT MTB assay was divided into three steps: DNA extraction, DNA amplification and melting curve analysis.

The last two steps took place in the FluoroCycler instrument (Hain Lifescience GmbH, Nehren, Germany). The PCR mixes were freshly prepared by adding 3 μL of amplification mix A to 7 μL of amplification mix B. Then, 6 μL of the extracted DNA was added. The positive control was included in the kit, and PCR-grade water was used as the negative control. All of the PCR mixes were loaded into the FluoroCycler. The results were provided as 'no MTB complex DNA detected', 'MTB complex DNA detected', 'not interpretable' or 'invalid' [11].

GenoType MTBDRplus assay

The GenoType MTBDRplus procedure was divided into three steps: DNA extraction, multiplex amplification with biotinylated primers and reverse hybridization. The PCR mixes were freshly prepared by combining 10 μL of amplification mix A with 35 μL of amplification mix B. Then, 5 μL of the extracted DNA was added. PCR-grade water was used as the negative control, and the PCR amplification was done using the programme recommended by the manufacturer. After the PCR amplification, the reverse hybridization step was performed using a TwinCubator instrument (Hain Lifescience GmbH, Nehren, Germany). The interpretation of the hybridization results was done according to the manufacturer's instructions [12].

Each strip of the GenoType MTBDRplus assay had 27 reaction bands, including six controls [conjugate, amplification, MTBC (TUB), *rpoB*, *katG*, *inhA* controls], eight *rpoB* wild-type (WT1-WT8) and four mutant probes (*rpoB* MUT D516V, *rpoB* MUT H526Y, *rpoB* MUT H526D, *rpoB* MUT S531L), one *katG* wild-type and two mutant probes (*katG* MUT S315T1, *katG* MUT S315T2), and two *inhA* wild-type and four mutant probes (*inhA* MUT1 C15T, *inhA* MUT2 A16G, *inhA* MUT3A T8C, *inhA* MUT3B T8A). A valid result was indicated by the presence of five control bands on each strip. A missing band in any of the wild-type probes or the presence of a band in any of the mutation probes suggested resistant isolates [12].

Results and discussion

TB is a major global health problem, and millions of people become infected each year. With the new developments in TB diagnostics over the last few years, the use of rapid molecular tests to diagnose TB and drug-resistant TB has been increasing [5]. FT MTB, which is a semi-automated assay using real-time PCR to detect the MTBC in respiratory and non-respiratory clinical specimens, is one of these tests [6]. In addition, GenoType MTBDRplus is a line probe assay

which detects MTBC, as well as mutations in the *rpoB* gene for RIF resistance, and the *katG* and *inhA* genes for INH resistance [13].

With the FT MTB assay, Hofmann-Thiel and Hoffmann [6] analyzed 1,039 respiratory specimens; and the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were found to be 88.1%, 98.9%, 83.8% and 99.2%, respectively. Eigner et al. [4] examined 661 pulmonary specimens and reported an analytical sensitivity of 95.1%, and specificity of 96.4%. Obasanya et al. [14] analyzed 296 respiratory samples and found sensitivity and specificity of 88.6% and 59.7%, respectively. Moure et al. [15] evaluated this test for the detection of MTB complex strains in 17 paraffin-embedded biopsies and reported a sensitivity of 60%, and a specificity of 71.4%. In the present study, out of 124 respiratory specimens, 15 (12.1%) were positive for both the culture and the FT MTB assay. Eleven of the gastric lavage specimens within this group were AFB negative. The FT MTB assay failed to detect the MTBC in one gastric lavage specimen that had a positive culture, but was AFB negative. In the respiratory specimens, the sensitivity, specificity, PPV and NPV were determined to be 94%, 100%, 100% and 99%, respectively (Table 1).

In the present study, 123 non-respiratory specimens were analyzed and eight (6.5%) of them were positive for both the culture and FT MTB assay. One urine specimen from this group was AFB negative. In the non-respiratory specimens, the sensitivity, specificity, PPV and NPV were all 100% (Table 2). With regard to all 247 specimens examined in this study, 23 (9.3%) had both a positive culture and FT MTB assay, while 12 (4.8%) of these specimens were AFB negative (11 gastric lavage, one urine). The overall sensitivity, specificity, PPV and NPV for this assay were 95.8%, 100%, 100% and 99.5%, respectively.

When considering only smear-positive specimens, Hofmann-Thiel and Hoffmann [6] reported the sensitivity, specificity, PPV and NPV to be 100%, while Eigner et al. [4] found the sensitivity of the test to be 100%. In addition, Obasanya et al. [14] reported the sensitivity and the specificity to be 100% and 35.3%, respectively, for the smear positive specimens. In the present study,

Table 1. Comparison of the FT MTB assay with culture results in respiratory specimens.

<i>n</i> = 124	By Bactec MGIT 960	
	Culture positive	Culture negative
Positive with FT MTB	15 (12.1%)	0
Negative with FT MTB	1 (0.8%)	108 (87.1%)
Sensitivity = 94%		PPV = 100%
Specificity = 100%		NPV = 99%

Comparison of the FT MTB assay with culture results in non-respiratory specimens.

<i>n</i> = 123	By Bactec MGIT 960	
	Culture positive	Culture negative
Positive with FT MTB	8 (6.5%)	0
Negative with FT MTB	0	115 (93.5%)
Sensitivity = 100%		PPV = 100%
Specificity = 100%		NPV = 100%

the sensitivity, specificity, PPV and NPV were 100% for both the respiratory and non-respiratory smear-positive specimens. However, there were only four smear-positive respiratory specimens and seven smear-positive non-respiratory specimens. Therefore, more specimens should be evaluated to obtain more reliable data.

Eigner et al. [4] reported that 13 of their specimens with cultural growth of nontuberculous mycobacteria (NTM) (one *M. avium*, two *M. goodnae* and 10 *M. intracellulare*) were negative when using the FT MTB assay, so the test showed no cross-reactivity. However, this was one of the limitations of the present study. None of the specimens showed cultural growth of NTM; therefore, we could not evaluate the cross-reactivity of this test. Another limitation of the present study was the small sample size, especially for the non-respiratory specimens, and more studies with a higher number of specimens are needed.

For the GenoType MTBDRplus assay, previous studies have reported the sensitivity, specificity, PPV and NPV values in detecting INH resistance as ranging between 60%–100%, 95.1%–100%, 97.4%–100% and 90.7%–100%, respectively. In addition, this assay's sensitivity, specificity, PPV and NPV values in detecting RIF resistance are in the range of 82.3%–100%, 73.2%–100%, 81%–100% and 98.9%–100%, respectively [16–25]. When the results were evaluated, this assay seemed to be more sensitive for detecting RIF resistance than INH resistance. This is probably because the mutation in the *rpoB* gene related to RIF resistance occurs in a well-defined region, while 10%–25% of the INH resistant strains are thought to have mutations outside the *katG* and *inhA* loci. Efflux systems may also play a role in INH resistance [22]. Javed et al. [26] evaluated the reliability of the GenoType MTBDRplus assay among 100 MDR MTB strains in Pakistan. It was reported that the assay could not detect 30 (30%) isolates resistant to INH and 23 (23%) isolates resistant to RIF. The authors concluded that the resistance in these strains was probably caused by mutations in genes other than those covered by the test, so in high TB incidence countries such as Pakistan, these molecular tests should still be a complement rather than a replacement to conventional DST.

Barnard et al. [17] reported that three out of 51 MTB strains (5.9%) were not detected with the GenoType MTBDRplus assay. In addition, Yadav et al. [25] showed that this assay correctly identified MTB in 242 of 251 (96.4%) culture-positive specimens. Felkel et al. [18] reported four false positive results and a false negative one when comparing them with cultures of patients without a history of MTBC treatment. Ferreira Junior et al. [27] reported that the GenoType MTBDRplus identified two out of 40 (5%) INH resistant and three out of 40 (7.5%) RIF resistant strains as sensitive. In the study reported by Gu et al. [20], with this assay, one out of six (16.6%) RIF resistant strains and one out of seven (14.2%) INH resistant strains were falsely detected as sensitive. Ferro et al. [19] reported seven false negative results among 132 INH resistant strains (5.3%), and five false negative results among 124 RIF resistant strains (4.0%) when using this assay. In the present study, the GenoType MTBDRplus assay was performed on the 23 clinical specimens which were positive in the FT MTB assay. An interpretable result could not be obtained for three of the specimens (intestinal biopsy, urine and gastric lavage), but 20 of them were found to be susceptible to RIF and INH. In the three specimens, the result was only available when the test was performed using the cultivated specimens. An antituberculous drug susceptibility test was performed on 23 of the strains using the Bactec MGIT 960 method, and all of the strains were found to be susceptible to RIF and INH (Table 3). The GenoType MTBDRplus assay provided test results concordant with the DST in 20 specimens for which interpretable results were obtained. However, this small sample size for the GenoType MTBDRplus assay was one of the limitations of this research. For more definitive data, more specimens should be evaluated.

In one study by Cavusoglu et al. [28], interpretable results were not obtained for 10 of their 90 clinical specimens (11.1%). Similarly, in the present study, for three of the 23 specimens (13.1%) (intestinal biopsy, urine and gastric lavage) an interpretable result could not be obtained using the GenoType MTBDRplus assay. For these specimens, the result was only available when the

Table 3. Comparison of the GenoType MTBDRplus assay with the results from the Bactec MGIT 960 drug susceptibility test.

<i>n</i> = 23	By Bactec MGIT 960	
	Resistant to RIF	Susceptible to RIF
Resistant to RIF by GenoType MTBDRplus	0	0
Susceptible to RIF by GenoType MTBDRplus	0	20 (86.9%)
Invalid	0	3 (13.1%)

test was performed with cultivated specimens. This may be because the clinical specimens contained a number of mycobacteria under the detection limit of the test (160 bacteria/mL).


Conclusions

The FT MTB assay provided quick and reliable direct detection of MTBC from clinical specimens, with high sensitivity (95.8%) and specificity (100%). The performance of GenoType MTBDRplus was determined to be problematic, in some instances, with regard to the mycobacterial count in the clinical specimens, but it was found to be a reliable test for the cultivated specimens.

Disclosure statement

The authors declare that they have no competing interests.

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