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Validation of the FluoroType® MTBDR assay using respiratory and lymph node samples

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ABSTRACT

Background: Tuberculosis (TB), especially drug-resistant TB, is a global public health problem. This study aimed to validate a new molecular diagnostic test, the FluoroType® MTBDR.

Method: Samples underwent routine diagnostic procedures (fluorescence microscopy, culture, species differentiation and phenotypic drug susceptibility testing). Left over samples stored at −20° underwent DNA extraction using the Fluorolyse® kit, followed by FluoroType® MTBDR and Genotype MTBDR*plus* testing.

Results: A total of 350 respiratory and 59 lymph node samples were included in the study; 71 respiratory and 16 lymph node samples were culture positive for *M. tuberculosis* complex (MTBC). The sensitivity of the FluoroType® MTBDR to detect MTBC DNA was 91.4% (95%CI 82.3–96.8%), 68.4% (95%CI 43.4–87.4%) and 62.5%, (95%CI 35.4–84.8%) for respiratory, smear negative respiratory and lymph node samples respectively. The correlating sensitivities of the GenoType MTBDR*plus* were 85.9% (95%CI 75.6–93.0%), 52.6% (95%CI 28.9–75.6%) and 56.3% (29.9–80.2). Sensitivity of the FluoroType® MTBDR to detect RMP and INH resistance for respiratory samples was 96.5% (95%CI 82.2–99.9) and 70% (95%CI 45.7–88.1), respectively. The GenoType MTBDR*plus* revealed sensitivities of 97.1% (95% 85.1–99.9) 70.6% (95%CI 52.5–84.9) for detection of RMP and INH resistance. Indeterminate results were 13/64 (20.3%), 23/64 (35.9%) and 16/64 (25.0%) for *rpoB*, *katG* and *inhA* using the FluoroType® MTBDR.

Conclusion: The FluoroType® MTBDR has a high sensitivity to detect MTBC DNA. However, the high proportion of indeterminate results across all three genes needs to be addressed.

1. Introduction

Global incidence of tuberculosis (TB) is slowly declining. However, multidrug-resistant (MDR)-TB caused by *Mycobacterium tuberculosis* complex (MTBC) resistant to at least isoniazid (INH) and rifampicin (RMP), the backbone drugs in the first-line TB regimen, challenges TB-control [1,2]. Recently extensively drug-resistant (XDR)-TB, defined as MDR-TB with additional resistance to a fluoroquinolone and one of the second line injectables has emerged and so far been reported from 77 countries [2,3]. Treatment of MDR-TB requires the lengthy use of less effective and more toxic second-line drugs [4–7]. The most recent global data show only 50% treatment success and 190,000 death among patients with M/XDR-TB [2].

Rapid and accurate drug susceptibility testing (DST) is important to ensure swift initiation of effective therapy and prevent further transmission. Mycobacterial culture followed by conventional phenotypic DST (pDST) using liquid or solid culture takes several weeks. Molecular methods for the detection of MTBC and mutations associated with resistance to antituberculous drugs have the potential to reduce diagnostic delays from weeks to several hours [8].

In 2010 the WHO recommended the use of molecular tests for diagnosing TB for the first time. The Xpert® MTB/RIF assay was recommended "as the initial diagnostic test in individuals suspected of MDR-TB and/or HIV-associated TB" [9,10]. Since then a policy update recommends the use of Xpert® MTB/RIF for diagnosis of smear negative disease [10]. The test is highly specific (99%) and sensitive (89%), requires minimal hands-on time and provides a result for MTBC detection and RMP-resistance within 2 h [11].

Alternative molecular methods aimed at detecting resistance are line-probe assays (LPA), Sanger targeted sequencing or next generation

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sequencing (NGS) [12–14]. In comparison to the Xpert® MTB/RIF assay the most commonly used LPAs GenoType® MTBDR*plus* and –*sl* require more hands-on time and sensitivity (85%) is generally lower [15]. However, the LPAs detect the most frequent mutations associated with resistance to RMP as well as INH, ethambutol, fluoroquinolones and injectable agents [12,15]. While the Xpert MTB/RIF and LPAs can be applied to primary samples, Sanger sequencing and NGS currently require a MTBC culture isolate.

The FluoroType® MTBDR is a new assay detecting the presence of MTBC DNA and mutations conferring INH- and RMP-resistance. Combining a Linear-After-The-Exponential (LATE)–PCR and lights-on/lights-off probe detection technology, the system identifies mutations in regions of the genes *rpoB*, *katG* and *inhA* that are associated with RMP and INH-resistance [16,17]. Currently 54 mutations can be identified specifically by automatic analysis of melting curves. Compared to the LPAs the FluoroType® MTBDR hands-on time is decreased, results are available more rapidly, risk of DNA contamination is reduced and results are interpreted automatically [18,19].

We recently compared pDST, GenoType® MTBDR*plus* (Hain Lifescience, Nehren, Germany) and FluoroType® MTBDR (Hain Lifescience, Nehren, Germany) using cultures positive for MTBC. The specificity of the FluoroType® MTBDR for detection of INH and RMP resistance was 100% (95%CI 96.0–100%) while the sensitivity was 91.7% for INH and 98.9% for RMP [19]. In this study we aimed to determine the sensitivity and specificity of the FluoroType® MTBDR for MTBC detection using respiratory and lymph node samples.

2. Methods

Decontaminated respiratory samples and lymph nodes stored at −20° for a maximum of 3 months were included in the study if the volume was at least 500 µl. The study was conducted between August and December 2017 and aimed to include a total of 350 respiratory samples. To ensure a sufficient number of MTBC culture positive respiratory samples, all respiratory samples revealing growth of MTBC were included. Culture negative MTBC respiratory samples were chosen at random from samples submitted during the study. All lymph node samples with sufficient volume submitted from August to December 2017 were included in the study. Most of the lymph nodes samples were tissue samples, as left-over volumes from aspirates were insufficient. Samples sent to the National Mycobacterial Reference Laboratory (NRL) in Borstel, Germany for fluorescence microscopy and mycobacterial culture as part of the routine diagnostic service were included. At the time of the study the majority of samples submitted to the NRL were from Germany. In addition samples from patients diagnosed with RMP resistance by Xpert MTB/RIF in Sierra Leone were also processed at the NRL. Samples from German patients were only tested using the Xpert MTB/RIF if requested by the clinician. Only one sample was included per patient. Respiratory samples were decontaminated using a CE marked NALC-NaOH decontamination kit (MycoDDRe, IMMY, Norman, OK, USA) and re-suspended in 2 ml phosphate buffer. Lymph node specimens were minced and suspended in phosphate buffer. Mycobacterial cultures included two solid cultures (Loewenstein-Jensen, Stonebrink) and a liquid culture (Mycobacterial growth indicator tubes (MGIT), Becton Dickison, Sparks, Md., USA) incubated at 37° for 8 and 6 weeks respectively for all samples. In addition for lymph node samples cultures on 7H10 and MGIT were incubated for 8 and 6 weeks at 30°.

pDST, GenoType MTBDR*plus* version 2 and FluoroType® MTBDR were performed as by the manufacturers instructions and previously described [19]. The MGIT 960-Isoniazid-Rifampicin-Ethambutol (IRE) kit (Becton Dickison) was used for pDST according to the manufacturer's instructions. The critical concentrations of Rifampicin and Isoniazid were 1 µg/ml and 0.1 µg/ml, respectively [20]. DNA was extracted using the Fluorolyse® kit (Hain Lifescience, Nehren, Germany); the same DNA extract was used to perform both molecular tests. Smear, mycobacterial culture and pDST results were extracted from the laboratory information system.

The FluoroType software automatically analyses melting curves and computes results as “no MTB complex DNA detected”, “MTB complex DNA detected”, “not interpretable” in case of unspecific peaks, or “invalid” in case of failure of positive/negative or amplification controls. For MTBC DNA positive samples, the FluoroType software provides information on wildtype, specified and unspecified *rpoB, katG* and *inhA* mutations. If neither the wildtype nor mutations are unambiguously identified the FluoroType software provides an “indeterminate” result.

Interpretation of the GenoType MTBDR*plus* results was performed according to the manufacturer's instructions. The scientist performing the molecular tests was blinded with regards to the phenotypic results.

For discordant resistance results either between the two molecular methods or the molecular and phenotypic method sequencing in the key regions of *rpoB, katG, inhA* and *aphC12* was performed using cultured MTBC isolate. If the Genotype MTBDR*plus* showed a missing wildtype band, but not mutation band Sanger sequencing in the respective key region was performed to determine the specific mutation. Sanger sequencing was performed using an ABI 3130xl genetic analyzer (Applied Biosystems) and an ABI BigDye Terminator cycle sequencing kit (version 3.1) according to the manufacturer's instructions.

Sensitivity and specificity for MTBC detection together with corresponding 95% confidence intervals of the FluoroType® MTBDR and the GenoType MTBDR*plus* were calculated separately for respiratory and lymph node samples using mycobacterial culture as the reference standard. Sensitivity was also calculated stratified by smear status. To determine the accuracy of the FluoroType® MTBDR assay and the GenoType MTBDR*plus*, results were compared with pDST results. Sensitivity and specificity were calculated for RMP and INH separately. Sensitivity was defined as the proportion of isolates correctly determined as resistant by the molecular test compared to the pDST results. Specificity was defined as the proportion of isolates correctly determined as susceptible by the FluoroType® MTBDR compared to the pDST results. Concordance between FluoroType® MTBDR and the Genotype MTBDR*plus* results was calculated using Kappa statistics. The proportion of indeterminate results for each of the three gens (*rpoB, katG, inhA*) was calculated for the FluoroType® MTBDR and the GenoType MTBDR*plus*. All statistical analysis were performed using Stata 14.0.

Information was extracted from the laboratory information system anonymously. The ethics committee of the University of Luebeck approved the study. Individual consent was not sought as no additional patient information was obtained.

3. Results

3.1. Respiratory samples

A total of 350 samples were included; among those 71 and 279 tested culture positive and negative for MTBC. Among the 71 MTBC culture positive samples 19 were smear negative and 52 positive (3 + n = 20, 2 + n = 16, 1 + n = 7, scanty n = 9). The majority of the MTBC positive cultures (n = 65) were identified as *M. tuberculosis*, 6 as *M. africanum*. The pDST pattern of the culture isolates was as follows: 28 were INH and RMP susceptible, 5 were INH mono-resistant, 2 RMP mono-resistant, 35 were MDRs and one was a mixture of an MDR and fully susceptible isolate.

The FluoroType® MTBDR revealed invalid results for 8 samples: one of them turned out to be MTBC culture positive and 7 were culture negative.

The sensitivity and specificity of the FluoroType® MTBDR to detect MTBC DNA was 91.4% (64/70, 95%CI 82.3–96.8%) and 99.6% (271/272, 95%CI 98.0–100%) (Table 1). Sensitivity for smear negative samples was 68.4% (13/19, 95%CI 43.4–87.4%). For the GenoType MTBDR*plus* sensitivity and specificity to detect MTBC DNA was 85.9%