Validation of the GenoType® MTBDRplus Ver 2.0 Assay for Detection of Rifampicin and Isoniazid Resistance in Mycobacterium tuberculosis Complex Isolates at UZCHS-CTRC TB Research Laboratory

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Abstract

Background: Multidrug-resistant tuberculosis (MDR-TB) is a public health concern globally. MDR-TB is defined as resistance to rifampicin (RIF) and isoniazid (INH), the two-major anti-TB first-line TB treatment drugs. Rapid identification of MDR-TB can contribute significantly to the control of TB. The GenoType® MTBDRplus Ver 2.0 assay is a molecular assay used to detect genetic mutations that result in RIF and INH resistance. The aim of this study was to validate the performance of the GenoType® MTBDRplus Ver 2.0 assay for the detection of INH and RIF resistance. Methods: Fifty-five stored Mycobacterium tuberculosis isolates were tested using both the mycobacterial growth indicator tube (MGIT), antimicrobial susceptibility testing (AST), and the GenoType® MTBDRplus Ver 2.0 assay. The MGIT AST was done according to the BBL MGIT AST SIRE system with RIF and INH final critical concentrations of 1.0 μg/ml and 0.1 μg/ml, respectively. The GenoType® MTBDRplus assay (Hain Lifescience, Germany) was performed following the manufacturer’s instructions. Results: The GenoType® MTBDRplus Ver 2.0 assay had a sensitivity, specificity, positive predictive value, and negative predictive value of 100% for INH and RIF resistance. The intra-assay precision for the assay was 100%. Conclusion: The GenoType® MTBDRplus Ver 2.0 assay’s sensitivity and specificity show that the assay is highly accurate for the detection of RIF and INH resistance and thus can be used as an alternate platform due to its shorter results turnaround time.

Keywords: Drug resistance tuberculosis, drug susceptibility testing, GenoType® MTBDRplus, Mycobacterium tuberculosis

Introduction

Tuberculosis (TB) is one of the top ten causes of death globally and is now ranked above HIV/AIDS as the leading cause of death from a single infectious agent.[1] The World Health Organization (WHO) has estimated that approximately 10 million people developed TB in 2017 worldwide.[1] Multidrug-resistant tuberculosis (MDR-TB) is fast becoming a major public health problem especially in developing countries and is defined as resistance to rifampicin (RIF) and isoniazid (INH) which are the major first-line anti-TB drugs.[2,3] In 2008, the WHO recommended the use of molecular line probe assays (LPAs) for the rapid detection of MDR-TB.[4]

The gold standard for drug susceptibility testing of TB is phenotypic drug susceptibility testing (DST) using solid or liquid culture media inoculated with the drugs. The use of phenotypic DST takes a considerably long period of time from several weeks for broth-based culture and up to 2 months for solid cultures to obtain the results.[2,5‑7] Mycobacterial growth indicator tube (MGIT) containing Middlebrook 7H9 is a broth-based...
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culture medium, and Löwenstein–Jensen (LJ), Middlebrook 7H10, and 7H11 are solid culture media used for culture and phenotypic DST. The increase in cases of MDR-TB has necessitated the need for a timely identification of these drug-resistant strains to effectively manage the disease and reduce its spread.[8] Faster molecular methods have been developed to detect resistance using genotyping instead of phenotypic. These molecular tests are based on the identification of gene mutations associated with drug resistance, and one such test is the GenoType® MTBDRplus assay.[2,5,9]

At the UZCHS-CTRC TB laboratory, the BD BBL™ MGIT™ AST SIRE system is the gold standard for the detection of susceptibility of Mycobacterium tuberculosis (MTB) complex to streptomycin (STR), INH, RIF, and ethambutol (SIRE). The system is a rapid manual qualitative nonradiometric method which provides the results within the same time frame (3–14 days) as the BACTEC 460TB system but sooner than the solid based proportion method after culture.[10] However, the use of GenoType® MTBDRplus assay can further reduce the turnaround time to 1–2 days after culture. Therefore, the UZCHS-CTRC has introduced the use of GenoType® MTBDRplus Ver 2.0 (Hain Lifescience, Nehren, Germany) assay to improve patient management.

GenoType® MTBDRplus assay (Hain Lifescience, Nehren, Germany) is a qualitative deoxyribonucleic acid (DNA) strip test developed for the identification of MTB complex and its resistance to RIF and/or INH. The assay uses polymerase chain reaction (PCR) and hybridization to detect wild type genes as well as rpoB genes associated with RIF resistance and inhA which confer INH resistance. Almost all RIF resistant strains harbor mutations in the rpoB genes which encode the β subunit of the RNA polymerase. Mutations in the katG which encodes catalase peroxidase and inhA which encodes the NADH enoyl ACP reductase are related to the high-level and low-level INH resistance, respectively.[2,9]

GenoType® MTBDRplus Ver 2.0 assay was released to replace the version 1.0 platform which had performance problems.[11] In other settings, the GenoType® MTBDRplus assay was evaluated for its performance on smear-positive, smear-negative clinical specimens and cultivated samples with other methods such as BACTEC 460TB, MGIT 960 systems, and the 1% proportion method on LJ medium.[2-5,10,12-14] This study validated the performance of GenoType® MTBDRplus Ver 2.0 assay against the BD BBL™ MGIT™ AST for the detection of RIF and INH resistance on stored MTB complex isolates.

**Methods**

**Mycobacterium tuberculosis isolates**

Fifty-five MTB complex strain isolates were obtained from the National Microbiology Reference Laboratory (NMRL), a referral laboratory in Harare, Zimbabwe, for the northern region TB control program serving an estimated 7 million population. These isolates were subcultured onto LJ culture media and incubated at 37°C. The cultures took between 7 and 14 days to obtain positive results. After growth, the isolates were tested for RIF and INH resistance using the BD BBL™ MGIT™ AST SIRE system and GenoType® MTBDRplus Ver 2.0 assay.

**Conventional drug-susceptibility testing using BD BBL™ MGIT™ AST SIRE system**

After the cultures became positive, they were tested for DST according to the BD BBL™ MGIT™ AST SIRE system within 7 days. For each sample, 4 ml of Middlebrook 7H9 broth was put in a sterile universal bottle with 8–10 glass beads. A 10 μl sterile inoculating loop was used to scrape colonies from the LJ medium. The colonies were suspended in the broth to a turbidity of 1.0 McFarland standard. The suspension was vortexed for 2–3 min to break the large colonies. The suspension was then allowed to sit for 20 min to let the sediment settle at the bottom of the tube. The supernatant was carefully transferred to another sterile universal bottle and allowed to sit for another 20 min. The supernatant was then transferred to a third sterile universal bottle and adjusted to 0.5 McFarland. The adjustment was done by comparing the turbidity of the suspension with the turbidity of the McFarland turbidity standard made by combining 1% sulfuric acid (H₂SO₄) and 1% anhydrous barium chloride (BaCl₂) in the following volumes: for 1.0 McFarland (9.95 ml H₂SO₄ and 0.05 ml BaCl₂) and for 0.5 McFarland (9.9 ml H₂SO₄ and 0.1 ml BaCl₂). For susceptibility testing, 1 ml of the 0.5 McFarland-adjusted suspension was diluted in 4 ml of sterile saline to make 1:5 dilution.[11,15]

The BD BBL™ MGIT™ AST SIRE system is based on growth of the MTB strain in a drug-containing tube compared to a drug-free tube. For the purposes of this study, only RIF and INH were used for the DST. A set of three tubes was labeled, one as growth control (GC), second as RIF, and a third as INH. The drugs are available in a lyophilized state in the following approximate formula: RIF 200 μg and INH 20 μg. The lyophilized drugs were reconstituted with 4 ml of sterile distilled water to make the drug concentration of 50 μg/ml and 5 μg/ml, respectively. In each of the three tubes, 0.5 ml of BBL MGIT OADC enrichment was added. OADC enrichment contains oleic acid, albumin, dextrose, and catalase which are essential for the rapid growth of mycobacteria. No drug was inoculated in the GC tube, and by using sterile pipette tips, 0.1 ml of the drug-suspension was aseptically inoculated in the respective tubes and their final critical concentration in the MGIT tubes were 1.0 μg/ml for RIF and 0.1 μg/ml for INH; 0.5 ml of the 1:5 organism suspension was inoculated into each of the three tubes. The tubes were incubated at 37°C and read daily from the 3rd day after inoculation. MTB complex strain ATCC 27294 known to be susceptible to both RIF and INH was used as a positive control.[11,15]

Reading was done by placing the tube into a BD BACTEC MicroMGIT Fluorescence Reader starting with the GC tube. On
day 3 of incubation, the GC tube was placed in the reader, and a result that showed any value between 1 and 13 (black in color) was considered negative and if a value was between 14 and 20 (red in color), the result was positive. The drug-containing tubes were only read from the day the GC tube became positive and for up to two additional days. The drug-containing tube that showed negative results within the 3 days of reading was interpreted as sensitive and a tube that showed positive results at any one of the 3 days of reading was interpreted as resistant.

GenoType® MTBDRplus Ver 2.0 assay

The GenoType MTBDRplus Ver 2.0 assay was performed according to the manufacturer’s instructions. The assay is based on a strip technology, and three steps are involved in the whole procedure namely: DNA extraction from the MTB complex strain isolates, multiplex PCR with biotinylated primers, and reverse hybridization. For each of the three genes: RpoB for RIF resistance, inhA for low-level INH resistance, and katG for high-level INH resistance, the assay tests for the presence of wild-type (WT) and mutant (MUT) probes. Each strip has 27 reaction zones including the conjugate control (CC), amplification control (AC), MTB complex (TUB), WT, and MUT zones.

DNA was extracted using the GenoLyse® kit which comprises of Lysis buffer (A-LYS) and neutralization buffer (A-NB). For each isolate, a loopful of colonies was taken from the LJ culture and suspended in 100 μl of A-LYS in a sterile 2 ml cryotube and vortexed. The suspension was then incubated at 95°C for 5 min. After 5 min of incubation, 100 μl of A-NB was added to the mixture of A-LYS and isolate and vortexed for 5 s. The cryotubes were then centrifuged for 5 min at a speed of 25380 g. After centrifugation, the supernatant which is the DNA solution was transferred into another sterile 2 ml cryotube, stored at −20°C until ready for PCR.

All the reagents needed for amplification are included in the Amplification Mix-A (AM-A) and Amplification Mix-B (AM-B). For each sample, 10 μl of AM-A which contains 10 × buffer, nucleotides, and DNA polymerase was mixed in a PCR tube with 35 μl of AM-B containing Magnesium Chloride (MgCl₂), biotinylated primers, and dye. 5 μl of the DNA solution was added to the master mix to give a total volume of 50 μl. For positive control, 5 μl of MTB strain ATCC 27294 was added to the mixture, and for negative control, 5 μl of sterile distilled water was used. Since the sample was from cultivated isolates, the “MDR CUL” amplification protocol was used to amplify the PCR mixture using GTQ Cycler-96. The PCRs consisted of 1 cycle of 15 min of denaturing at 95°C, followed by 10 cycles of 30 s at 95°C and 2 min at 65°C, followed by 20 more cycles of 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C, with a final extension of 1 cycle at 70°C for 8 min.

Hybridization was performed using a TwinCubator prewarmed to 45°C. The hybridization buffer (HYB) and stringent wash solution (STR) were prewarmed to 37°C–45°C before use. For each strip, 10 μl of conjugate concentrate was diluted in a sterile tube with 990 μl of conjugate buffer (CON-D). In a separate tube, 10 μl of substrate concentrate (SUB-C) was diluted with 990 μl of substrates buffer (SUB-D) for each strip. In each of the wells of a 12 well tray, 20 μl of the PCR product was mixed with 20 μl of denaturation solution (DEN) and incubated for 5 min at room temperature. After incubation, 1 ml of prewarmed HYB was added to each well and mixed carefully until the solution was homogenous. A prelabeled strip was then placed in each well, completely covered by the solution, and allowed to incubate while on the TwinCubator for 30 min at 45°C. At the end of the 30 min of hybridization, all the HYB solution was aspirated using a sterile pasteur pipette. Then, 1 ml of STR was added to each strip and incubated for 15 min at 45°C on the TwinCubator. The STR was then removed, and 1 ml of Rinse solution (RIN) was added to each strip and incubated for 1 min. The RIN was removed, 1 ml of diluted conjugate was added to each strip and allowed to incubate for 30 min. The solution was removed, and the test strips were rinsed twice by using a RIN for 1 min at each wash, followed by sterile distilled water for 1 min. All solutions were removed between washes. One milliliter of diluted substrate was then added to each strip and incubated for up to 20 min. The diluted substrate was removed, and the reaction was stopped by rinsing twice with sterile distilled water. The test strips were then affixed to the GenoType R MTBDRplus Ver 2.0 assay evaluation sheet provided in the kit, for interpretation of results. To determine the intra-assay precision (parallel testing), 10 isolates and 2 controls were set up in triplicates and tested under identical conditions on three different days.

Interpretation of the assay results was performed according to the package insert. For accurate evaluation, a provided template was used to align the CC, AC, and the TUB with the respective bands on the sheet. The presence of WT bands with the absence of the MUT band was interpreted as sensitive. The absence of a WT band and the presence of a MUT band for a specific gene on the strip were interpreted as resistance. A strain that revealed the presence of reaction to both a MUT band and the corresponding WT band was considered heteroresistant.

Ethical considerations

Approval to conduct this study was granted by the Department of Laboratory Services in the Ministry of Health and Child Care on 21 March 2018. The study specimens were deidentified and individual informed consent was not required in this study.

Results

DST was done on 55 isolates using the GenoType MTBDRplus Ver 2.0 assay and the BD BBL™ MGIT™ AST method. Out of the 55 isolates that were tested, 28 (50.9%) were susceptible to RIF, 3 (5.5%) were RIF mono-resistant strains, and 24 (43.6%) were MDR strains. Of these isolates, 35 (63.6%) were susceptible to INH and 20 (36.4%) were resistant to INH and were all MDR strains [Table 1].

The results of the validation of the performance of the GenoType MTBDRplus Ver 2.0 assay in detecting RIF and INH resistance are summarized in Table 2. The sensitivity,
specificity, positive predictive value, and negative predictive value of GenoType MTBDRplus Ver 2.0 assay were 100% for the detection of both RIF and INH resistance. All parallels showed identical banding patterns and comparable signal strength, representing 100% intra-assay precision.

Mutations associated with rifampicin and isoniazid drug-resistant tuberculosis

We also determined the frequency of mutations associated with RIF and INH drug-resistant TB. Table 3 summarizes the distribution of different banding pattern in drug-resistant isolates.

| Gene | Missing band | Gene region | Mutation present | RIF monoresistance | INH monoresistance | MDR | Total |
|------|--------------|-------------|------------------|--------------------|--------------------|-----|-------|
| rpoB | WT8          | 530-533     | S531L            | 2                  | 21                 | 23  |       |
|      | WT7          | 526-529     | H526D            | 1                  | 1                  | 2   |       |
|      | WT2, WT3     | 510-517     | UKM              | 0                  | 0                  | 1   |       |
|      | Present WT8  |             | S531L*           | 0                  | 0                  | 1   |       |
| Total|              |             |                  | 3                  |                     | 24  | 27    |
| katG | WT           | 315         | S315T1           | 0                  | 18                 | 18  |       |
|      | Present      |             | S315T1*          | 0                  | 1                  | 1   |       |
| Total|              |             |                  | 0                  |                     | 19  | 19    |
| inhA | WT1          | -15/-16     | C15T             | 0                  | 0                  | 0   |       |
|      | WT2          |             |                  | 0                  | 0                  | 0   |       |
| Total|              |             |                  | 0                  |                     | 1   | 1     |

*Heteroresistance, RIF: Rifampicin, INH: Isoniazid, UKM: Unknown mutation, WT: Wild type, MDR: Multidrug resistance, AST: Antimicrobial susceptibility testing

Discussion

In this study, the performance of the GenoType MTBDRplus Ver 2.0 assay for the detection of RIF- and/or INH-resistant strains of MTB was validated on 55 MTB isolates, which were isolated from pulmonary TB patients and stored at the NMRL. The GenoType MTBDRplus Ver 2.0 assay performed had 100% concordance with standard BD BBL MGIT AST SIRE system and had a reduced turnaround time by at least 3 days compared to the manual MGIT AST.

Most studies determined the sensitivity of the GenoType MTBDRplus assay to be between 95% and 100%. However, one study done in Ethiopia on smear-positive and smear-negative sputum specimens had a sensitivity of 88.2% and 60%, respectively. The sensitivity and specificity of 100% for the detection of RIF resistance reported in this study was also obtained in other studies done elsewhere.

In this study, RIF resistance-specific mutation by rpoB MUT probes was observed in 26 out of the 27 RIF resistant isolates. The high RIF resistance-specific mutation on the rpoB gene is consistent with other studies elsewhere. Of the 26 specific
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CONCLUSION

The results of this study show that the GenoType® MTBDRplus Ver 2.0 assay has same sensitivity and specificity for the detection of RIF and INH resistance and MDR-TB as the standard BD BBL MGIT AST SIRE system. Our data support the view that the GenoType® MTBDRplus Ver 2.0 assay can be considered as an alternative to the conventional phenotypic DST for the detection of RIF, INH, and/or multidrug resistance in MTB isolates, with much-desired turnaround time.

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Nil.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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Figure 1: Example of results showing banding patterns and their evaluation obtained with the GenoType MTBDRplus Ver 2.0 assay. Line 1, positive control (Mycobacterium tuberculosis strain, ATCC 27294); line 2, MDR-TB with high-level INH resistance MTB heteroresistant to both RIF and INH with high-level INH resistance; line 3 MTB susceptible to both RIF and INH; line 4, MDR-TB with high-level INH resistance; line 5, MTB RIF resistant and INH susceptible; lines 6–8, MDR-TB with high-level INH resistance; line 9, MTB susceptible to both RIF and INH; line 10, negative control (sterile distilled water). MDR-TB: Multidrug-resistant tuberculosis, INH: Isoniazid, RIF: Rifampicin, MTB: Mycobacterium tuberculosis

mutations of the rpoB gene, 24 (92.3%) had mutations at codon S531L, and the remaining 2 (7.7%) had mutations at codon H526D. A high rate of S531L mutation was also reported in other studies.[20,22,23] The one isolate missing rpoB WT2/WT3 is an example of a rare silent mutation in codon 514 of the rpoB gene, and this was considered resistant because the phenotypic results were resistant.[9]

In this study, the sensitivity and specificity for the detection of INH resistance were 100%. The higher proportion 92.9% of katG mutation in codon S315T1 is comparable to rates reported in other studies and this accounts for more INH resistance in high TB burden countries and is responsible high-level INH resistance.[19,24-29] The 7.1% of the INH resistance due to inhA mutation is responsible for low-level INH resistance, and the low proportion is reported elsewhere.[5,6,20]

Heteroresistance of MTB is the coexistence of susceptible and resistant organisms to anti-TB drugs in the same patient and is considered as a preliminary stage to full resistance.[30] Therefore, in LPAs such as the GenoType MTBDRplus Ver 2.0 assay, heteroresistance is defined by the presence of all WT bands as well as the presence of one or more of the MUT bands as seen on line 2 in Figure 1. In this study, one (3.7%) showed RIF heteroresistance which is lower than 7.7% found by Singhal et al.[30] However, INH heteroresistance was higher in this study 5% than the 1.9% reported in the Singhal et al.’s study.[28] Heteroresistance was only found in one MDR-TB isolate in contrast to a study done in India that reported high levels of heteroresistance.[27]
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