Comparison of three commercial molecular assays for the detection of Rifampicin and Isoniazid resistance among *Mycobacterium tuberculosis* isolates in a high HIV prevalence setting

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Abstract

In a head to head comparison of the MTBDR*plus* Version 2.0 (Hain Lifescience), Xpert® MTB/RIF (Cepheid) and the Anyplex™ MTB/NTM (Seegene) assays we demonstrate equal sensitivity (59/61; 96.7%) and specificity (53/54; 98.1%) for detecting rifampicin resistance with further analysis of discordances. The Xpert does not detect Isoniazid resistance while Anyplex showed high false positivity.

Text

There are a limited number of commercial molecular assays available for the rapid detection of drug resistant tuberculosis, and currently only two are WHO endorsed for this purpose: the GenoType MTBDR*plus* Version 1.0 (Hain Lifescience GmbH, Germany) and the Xpert® MTB/RIF (Cepheid, USA) (1-3). The MTBDR*plus* has been further optimised and this new version (2.0) can now be performed on both smear positive and smear negative clinical specimens as well as cultured isolates according to the manufacturer (4,5).

The Anyplex™ MTB/NTM (Seegene, Korea) assay has not undergone WHO review for use in detecting drug resistant TB but is widely used. It is a multiplex real-time PCR assay capable of distinguishing between *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria (NTM) while allowing for the amplification of drug resistance related gene (*rpoB*, *katG* and *inhA*) sequences simultaneously (6,7).

In this study we evaluate the performance characteristics of these three molecular assays for the detection of drug resistant tuberculosis by performing a head to head comparison of these technologies.

This is a retrospective laboratory based evaluation study. Cultured isolates were collected from the TB laboratory, Diagnostic Division of the Department of Medical Microbiology, Tshwane Academic Division, National Health Laboratory Services (NHLS). The MTBDR*plus* version 1.0 result, performed as part of routine laboratory testing formed the basis of stratification into 50 MDR, 30 fully susceptible, 20 mono-resistant (10 rifampicin and 10 isoniazid resistant) and 20 isolates with undefined mutations. The 20 isolates with undefined mutations, having a wild type missing with no corresponding mutation band on MTBDR*plus* version 1.0 were further characterized by means of Sanger sequencing.

These isolates were collected consecutively to make up a total of 120 which was calculated to provide the required sample size for the comparative evaluation. Four isolates from this sample subset were excluded as they were duplicates and another had poor banding repeatedly on the MTBDR*plus* version 2.0. A total of 115/120 (96%) cultured isolates were analysable across all three systems.

The MTBDR*plus* Version 2.0, Xpert® MTB/RIF (GXP) (G3 cartridge) and the Anyplex™ MTB/NTM (Anyplex) were performed according to manufacturer's instructions. For the GXP 1 ml of cultured isolate was used and sample reagent buffer added in a 2:1 ratio.

Extracted DNA templates of discordant and isolates with undefined mutations based on Version 1 were amplified utilising the primers as previously described for *rpoB, katG* and *inhA* (8). Cycle sequencing utilising the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI prism, USA) was performed according to manufacturer's instructions. The sequence of the PCR amplified DNA was then determined utilising the ABI 3500 XL (Applied Biosystems, USA) genetic analyser. Single nucleotide polymorphisms and indels were detected using the CLC Genomics Workbench (Qiagen, Germany). All variants were evaluated using the Dream DB database and high confidence mutations regarded as resistant (9). For the comparison of the three molecular assays we used a molecular gold standard of the original MTBDR*plus* version 1.0 mutation result as well as sequencing results as mentioned before with the sequencing result regarded as final.

Of the 115 isolates evaluated 61 were rifampicin resistant and 59 were isoniazid resistant based on the molecular gold standard. All three the assays had exactly the same sensitivity (59/61; 96.7%) and specificity (53/54; 98.1%) for the detection of rifampicin resistance. However the discordances were not the same and are shown in Table 1.

Two cases of rifampicin resistance were not detected by both the MTBDR*plus* Version 2.0 and the GXP, both of which had L533P mutations in the rifampicin resistance determining region upon sequencing. When repeated using the most recent release of the Version 2.0 assay as well as the GXP(G4 cartridge) these mutations were detected by the MTBDR*plus* Version 2.0 in codon 530-533 of the *rpoB* region (missing wild type 8 band with no corresponding mutation band) and by the the GXP(G4 cartridge – probe E not binding). This mutation has been associated with discordant genotypic and phenotypic susceptibility results, notably when utilising the widely employed broth-based phenotypic susceptibility testing methods, hence the L533P mutation was thought to be a silent mutation, with no impact on

Table 1: Resolution of discordant rifampicin results by sequencing of *rpoB* region

Isolate	Hain V2	Seegene	GXP	Sequencing
40	S	R	S	Poor sequencing
41[a]	R	S	R	Insertion TTC 432-433
92[b]	R	S	S	Wild type
99[c]	R	S	R	SNP 1862 c>t His>Tyr 445his526tyr
87[d]	S	R	S	SNP 1184 t>c leu>pro 452leu533pro
117[e]	S	R	S	SNP 1184 t>c leu>pro 452leu533pro

a: Isolate 41: MTBDR*plus* V 2.0 mutation in codon 510-519(*rpoB*WT 3 missing), no mutation band, GXP probe B not binding

b: Isolate 92: MTBDR*plus* V 2.0 S531L mutation, no wild type missing, possibly mixed infection

c: Isolate 99: MTBDR*plus* V 2.0 mutation in codon 526-529(*rpoB*WT 7 missing) & H526Y mutation, GXP probe D not binding

d: Isolate 87: Repeat on MTBDR*plus* V2.0 (later version) = mutation in codon 530-533 (*rpoB*WT 8 missing); repeat on GXP G4 cartridge probe E not binding

e: Isolate 117: Repeat on MTBDR*plus* V2.0 (later version) = mutation in codon 530-533(*rpoB*WT 8 missing) repeat on GXP G4 cartridge probe E not binding

the efficacy of rifampicin (10) and has also been shown to be missed by the G3 cartridge (11,12). Upon retrospective review of our laboratory records these two isolates tested rifampicin susceptible utilising the MGIT 960 system. However, recent data has

demonstrated this L533P mutation to be associated with low-level resistance which is clinically significant and retesting using the latest versions of the assays has addressed this short coming (13-15).

One isolate was detected as falsely rifampicin resistant by the MTBDR*plus* Version 2.0 assay against the molecular gold standard displaying a S531L mutation and wild type banding pattern at codon 530-533 suggesting a mixed population. This isolate was recorded as rifampicin resistant according to MGIT 960 phenotypic drug susceptibility testing performed as part of the routine laboratory testing further supporting the findings on the line probe assay. However, no mutations were detected utilising Sanger sequencing. Interestingly the GXP showed binding of all probes, but this may be a masking effect of a wild type strain in a mixed population which is a limitation of the assay design (16). Both the line probe assay and phenotypic susceptibility testing on the other hand are known to be able to detect mixed populations (17) and thus the specificity of the MTBDR*plus* V 2.0 may be falsely low in our study.

The Anyplex assay detected one case of false rifampicin resistance and two cases of false rifampicin susceptibility. Of the two false susceptible cases one had a H526Y mutation and the other an insertion TTC at codon 514 on sequencing. The insertion TTC at codon 514 of the *rpoB* gene has been described in various studies (18-22) though at low frequency; 2.5% of rifampicin resistant isolates in one study and 4% in another (18,23). This mutation has been associated with varying levels of rifampicin resistance with corresponding rifampicin minimum inhibitory concentrations (MIC) of 16 to >256 µg/ml (20,22). The second mutation that was not detected by the Anyplex assay was a H526Y mutation. This is a commonly occurring mutation associated with drug resistance and thus has important treatment implications if not detected (24). The case of false rifampicin resistance could not be resolved

by means of Sanger sequencing as this isolate displayed poor sequencing despite repeat attempts, however both the MTBDR*plus* V 2.0 and the GXP remained wild type upon repeat testing.

No comparison for the detection of isoniazid resistance was possible for the GXP assay as this does not form part of the test. The MTBDR*plus* Version 2.0 and the Anyplex both had sensitivities of 100% for the detection of isoniazid resistance mutations at codon 315 for the *katG* gene and *inhA* promoter region. The Anyplex assay had poor specificity of 82.4% and falsely reported 10 isolates that were defined as wild type as having mutations present in the *inhA* and/or *katG* region. All 10 isoniazid discordant isolates were wild type on sequencing and also had all wild type bands present on MTBDR*plus* V 2.0.

The three molecular assays under evaluation in this study performed well for the detection of rifampicin resistance. The MTBDR*plus* V 2.0 and GXP (G3 cartridge) had good performances and the later versions of both addressed the shortcomings of the earlier versions. The MTBDR*plus* Version 2.0 was superior to the Anyplex overall for the diagnosis of MDR TB. The main drawback of the GXP assay is the lack of isoniazid susceptibility testing.

Strengths of this study are the large numbers of resistant isolates evaluated as well a direct comparison between all three systems. Limitations of this study are the restrictive genotypic standard which is expected to miss 10-15% of Isoniazid resistance (25), lack of sequencing and phenotypic drug susceptibility data for all isolates as well as assays not being performed on direct samples. The Anyplex assay has the advantage of detecting NTMs but this was not assessed. However in the South African context this is of lesser concern, but may be of importance in specialised populations.

Molecular assays can detect non-viable mycobacteria which can lead to problems in interpretation by clinicians however they do offer faster clinically actionable results. The three assays showed overall good performance for the detection of drug resistant TB with each having its own strengths and weaknesses.

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