# GenoType MTBDR*plus*Verson-2 for rapid detection of Rifampin and Isoniazid resistant *Mycobacterium Tuberculosis*

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## ABSTRACT

GenoType MTBDR*plus* is a molecular assay for the detection of *Mycobacterium tuberculosis* complex (MTBC) and drug resistance. This study aimed to determine diagnostic performance of the MTBDR*plus* version-2 in routine laboratory condition. In this cross-sectional study, sputum samples of 242 clinically suspected cases of pulmonary tuberculosis were tested by smear microscopy,MTBDR*plus* and culture(proportional drug susceptibility testing). The sensitivity and specificity of Geno Type MTBDR*plus* for MTBC detection was found to be 94.5% and 95.5% respectively. Likewise, sensitivity of it's for rifampin (RMP) and isoniazid (INH) resistant detection was 94.6% and 80.4% respectively. And, specificity was 98.5% and 98.5% respectively. Mutations in the rpoB gene were identified in 53 of 60 RMP resistant samples with predominant (60.37%) mutation in S531L. Similarly, mutation in katG encoding region and inhA promoter region were identified in 45 of 61 INH resistant samples with predominant (82.22%) mutation in katG encoding region. MTBDR*plus* version-2 is a rapid and highly sensitive test for the detection of *M. tuberculosis* strains and provides additional information on RMP and INH resistance status, which can easily be included in routine laboratory work flow.

**Keywords:** *M. tuberculosis*,MTBDR*plus* version-2, katG, inhA

## INTRODUCTION

Tuberculosis (TB) is an important public health problem. The effective control of TB is based on prompt diagnosis followed by the rapid implementation of adequate anti-tubercular therapy.[1]Multidrug-resistant (MDR) *M. tuberculosis* strains (resistant at least to rifampin (RMP) and isoniazid (INH)) have emerged worldwide and seriously challenged TB control and prevention programs.[2] Diagnoses on the basis of clinical findings and sputum smear microscopy is not sufficient to cover all the infected cases because of their lower sensitivity and specificity. Hence, it ultimately demands highly sensitive and specific laboratory diagnostic tools. Culture, though sensitive and specific, take tiring turnover time for proper outcome. So, the overall scenario warrants rapid detection of MDR-TB which ensures treatment with appropriate drugs, thereby reducing morbidity, mortality, economic costs, and further transmission of infection.[3]

Line probe assays for fast and reliable detection of *M. Tuberculosis* complex (MTBC) and resistance to RMP and INH have recently been endorsed by the WHO.[4] The commonly used MTBDR*plus* version 1 (HainLifescience, Nehren, Germany), which detects RMP and INH resistance only from smear-positive and cultivated speciemens, limited in respect to smear negative.[5,6] To overcome this limitation, the MTBDR*plus* (version 2) test (HainLifescience, Nehren, Germany) was further improved to detect mycobacteria and their resistance status against RMP and INH in smear-positive, smear-negative, and culture-positive specimens as well. As there are hardly published data of the ability of this assay in Nepal, we aim at determining the ability of commercially available MTBDR*plus*(v2.0) for direct detection of MTBC and mutations conferring resistance to RMP and INH directly.

**MATERIALS AND METHODS:** This cross sectional study was conducted between March to August 2013 at German-Nepal tuberculosis project (GENETUP), Kathmandu, Nepal. Patients (participants) suspected of having pulmonary TB based on clinical signs/ symptoms, chest X-ray findings and individuals on anti-TB treatment were included and asked for one spontaneously expectorated sputum sample. Laboratory tests (Auramine stained smear, culture, GenoType MTBDR*plus*)were performed at National Reference Laboratory German Nepal tuberculosis Project, Kalimati, Kathmandu, Nepal using WHO guidelines.[4] All sputum samples were decontaminated using N-acetyl –L- cysteine- NaOH within 24hours of receipt in the laboratory. After centrifugation, the pellet was suspended in 1.5ml of phosphate buffer. An auramine stained smear of the decontaminated

sediment was examined by fluorescence microscopy and graded according to WHO criteria.[4] An aliquot of the decontaminated specimen was cultured on Lowenstein-Jensen solid medium. Identification of *M. tuberculosis* was made by growth rate, colony morphology and P- nitrobezoic acid (PNB) susceptibility. Testing for susceptibility to INH and RMP was performed by proportional drug susceptibility testing using critical concentrations of 0.2 µl/ml for INH and 40 µl/ml for RMP.

### GenoType MTBDRplus Test:

The Geno Type MTBDR*plus* line probe assay was carried out according to the manufacturer's instructions. Briefly, 500 µl of decontaminated sample material was transferred into a 1.5 ml screw capped tube and centrifuged for 15 min at 10,000 *g*. The pellet was resuspended in 100 µl of an alkalinelysis buffer and incubated for 5 min at 95°C in a water bath. Subsequently, 100 µl of neutralization buffer was added to lysate, vortexed, and centrifuged for 5 min. Volume of 5µl supernatant was directly used for polymerase chain reaction (PCR).The PCR mixture was prepared by mixing 10 µl of amplification mix A [AM-A; 10X buffer, nucleotides, and DNA polymerase] and 35 µl of amplification mix B [AM-B; MgCl2, the biotinylated primers, and dye]. Then 5 µl of the purified DNA was added. PCR amplification was done using the PCR program recommended by the manufacturer's instructions: i) 15 min incubation at 95°C, where the activation of the polymerase takes place; ii) 20 cycles of 30 s at 95°C and 2 min at 65°C; and iii) 30 cycles of 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C. The final cycle consisted of 8 min at 70°C. Amplified DNA hybridization and detection were performed with a Twin Cubator (Hain, Lifescience, Nehren, Germany). The hybridization procedure included the following steps: chemical denaturation of the amplification products at room temperature for 5 min, hybridization of single stranded biotin-labeled amplicons to membrane-bound probes at 45ºC for 30 min, stringent washes, an addition of streptavidin- alkaline phosphatase (AP) conjugate at room temperature for 30 min and AP staining reaction to detect colorimetric bands.

**Interpretation:**The developed strips were pasted in the designated field by aligning the bands CC (Conjugate control) and AC (Amplification control) with the respective lines on the sheet, provided with kit. The resistance was determined and noted down in the respective column. Each strip has a total of 27 reactions zones: six controls (conguate, amplification, *M. tuberculosis* complex, ropB, katG and inhA), eight rpoBwild-type: (WT) and four mutant (MUT) probes, one katGWT and two MUT probes, and others two inhA wild-type and four mutant probes. Results were interpreted according to the manufacturer's instructions.

**Data analysis:**The performances of the Geno Type MTBDR*plus* test were compared to that of a conventional DST for detection of RMP and INH resistance. The sensitivity, specificity, and accuracy of the assays were calculated. Data was analyzed using SPSS 16.0. Comparisons were performed using *Z* test to compare two proportion taking 95% CI. Statistical significance was defined as a p value of 0.05.

**Ethical consideration:** The study was approved by the Research and Ethical Sub Committee of Nepal Medical College and Teaching Hospital. Patients were informed regarding the purpose of the study and possible uses of their samples in research purpose. The participants have provided an informed consent as well.

## RESULTS

Out of a total of 242 sputum samples evaluated, result displayed that128(52.90%) of the samples were culture positive, 110 (45.45%) were culture negative and 4 (1.65%) cultures were contaminated and hence excluded from the study. Out of 238 samples, 121 (50.84%) were confirmed TB positive and 105 (44.12%) negative by both culture and MTBDR*plus* tests. Of the culture positive specimens, 7 (2.94%) were MTBDR*plus* negative. From among the ones that were MTBDR*plus* positive, 5 (2.10%) were negative by culture. No significant difference (p=0.521) was found on comparing the two results(table 1). Similarly, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of MTBDR*plus* assay were found to be 94.5%, 95.5%, 96.0%, and 93.8% respectively.

Of the 127 smear positive samples, 110 (86.61%) were positive by both culture and MTBDR*plus* tests and 10 (7.87%) were negative by the two tests. While 4 (3.15%) of the ones that were positive by culture showed negative with MTBDR*plus* and 3 (2.36%) culture negative were positive with MTBDR*plus*. On comparing MTBDR*plus* with culture result from smear positive sample, no significant difference (p=0.4367) was found (Table 1). Similarly, sensitivity, specificity, PPV and NPVof MTBDR*plus* assay for smear positive samples were found to be 96.5%, 76.9%, 97.3%, and 71.4% respectively.

Out of 111 smear negative samples processed; 11 (9.90%) samples were reported positive by both culture and MTBDR*plus*, and 95 (85.60%) were proven negative by both. It was seen that 3 (2.70%) culture positive were MTBDR*plus* negative and 2 (1.80%) culture negative were MTBDR*plus* positive. No significant difference (P=0.524) was seen on comparing these two tests. Sensitivity, specificity, PPV and NPV of MTBDR*plus* assay for smear negative samples were found to be 78.6%, 97.9%, 84.6%, and 96.9% respectively (Table 1).

**Table 1:** Comparision of MTBDR*plus* assay with culture result for the detection of MTBC

| Type of Sputum Specimen | MTBDR*plus* | Culture | | P value |
|---|---|---|---|---|
| | | Positive | Negative | |
| Both smear positive and smear negative | MTBC positive | 121 | 5 | 0.5206 |
| | MTBC negative | 7 | 105 | |
| Smear Positive | MTBC positive | 110 | 3 | 0.4367 |
| | MTBC negative | 4 | 10 | |
| Smear negative | MTBC positive | 11 | 2 | 0.5241 |
| | MTBC negative | 3 | 95 | |

Note: MTBC- *Mycobacterium tuberculosis* complex

**Detection of rifampin and isoniazid resistance:** Table 2 shows the detection of drug resistance by Geno Type MTBDR*plus* and conventional DST method. Out of 121 samples analyzed for RMP resistance, 53 (43.80%) were resistant and 64 (52.90%) were sensitive to it as detected by both of tests. All 3 (2.47%), which were detected as resistant to RMP by conventional DST were identified as sensitive by Geno Type MTBDR*plus* assay. Only 1 (0.83%) specimen that was reported as sensitive to RMP by DST was identified as resistant by MTBDR*plus* assay. Sensitivity, specificity, PPV, NPV and accuracy of the MTBDR*plus* assay for the detection of RMP resistance was 94.6%, 98.5%, 98.1%, 95.5% and 96.69% respectively. There was no significant difference (p=0.687) between these test assays.

Out of 121 samples analyzed for INH, 45 (37.19%) were resistant and 64 (52.90%) were sensitive to it by both tests. All 11 (9.09%), which were resistant to INH by conventional DST were identified as being sensitive by GenoType MTBDR*plus* assay. And 1 (0.83%), which was identified as sensitive by conventional DST was identified as being resistant by MTBDR*plus.* Similarly sensitivity, specificity, PPV, NPV and accuracy of the MTBDR*plus*assay for the detection of Isoniazid resistance were 80.4%, 98.5%, 97.8%, 85.3% and 90% respectively. There was no significant difference (P=0.138) between these two assays.

**Table 2:**Comparision of Geno Type MTBDR*plus* assay with conventional DST for rifampin and isoniazid resistance.

| Genotype MTBDR*plus* | Conventional DST | | P vaue |
|---|---|---|---|
| | Resistant | Sensitive | |
| $RMP^r$ | 53 | 1 | 0.6871 |
| $RMP^s$ | 3 | 64 | |
| $INH^r$ | 45 | 1 | 0.1381 |
| $INH^s$ | 11 | 64 | |

Note: $RMP^r$–rifampin resistance, $RMP^s$ – rifampin sensitive, $INH^r$– Isoniazid resistance, $INH^s$ – Isoniazid sensitive

**Mutation on rpoB gene associated with Rifampicin resistance:** Mutation in the rpoB genes were identified in 53 of 60 RMP resistant samples. Out of 60, four samples did not give MTBC and three gave wild type pattern on MTBDR*plus* assay. Of the 53, nine different hybridization patterns with nine specific mutations were observed on 81 bp hot spot region of rpoB gene with 32(60.37%) samples showing the specific nucleotide exchange TCG to TTG, resulting in the amino acid exchange of serine to leucine. Only 1 (1.88%) sample had a mutation located in the region 526, resulting in the aminoacid exchange of histidine to tyrosine. Similarly, 1 (1.88%) sample had a mutation located in the region 516, resulting in the aminoacid exchange of aspartic acid to valine. 19 (35.84%) samples had mutation of unknown type but within the codons 505-533 (Table 3).

**Table 3:** Pattern of mutation on rpoB gene

| rpoB gene pattern | Amino acid change on rpoB gene | RMP result | No (%) isolates |
|---|---|---|---|
| ΔWT1 & ΔWT2 | Unknown | $RMP^r$ | 1 (1.88%) |
| ΔWT2 & ΔWT3 | Unknown | $RMP^r$ | 1 (1.88%) |
| ΔWT3 | Unknown | $RMP^r$ | 2 (3.77%) |
| ΔWT3 & ΔWT4 | Unknown | $RMP^r$ | 1 (1.88%) |
| ΔWT7 | Unknown | $RMP^r$ | 2 (3.77%) |
| ΔWT8 | Unknown | $RMP^r$ | 12(22.64%) |
| ΔWT3 & ΔWT4, MUT1 | D516V | $RMP^r$ | 1 (1.88%) |
| ΔWT7, MUT2A | H526Y | $RMP^r$ | 1 (1.88%) |
| ΔWT8, MUT3 | S531L | $RMP^r$ | 32 (60.37%) |
| Total | | | 53 (100%) |

Note: ΔWT- missing of wild type, MUT- mutation; $RMP^r$- rifampin resistance, D- aspartic acid, V- valine, H- histidine, Y- tyrosine, S- serine, L- leucine

**Mutation on katG encoding region and inhA promoter region associated with Isoniazid resistance:** Mutation on katG encoding region and inhA promoter

region were identified in 45 of 61 INH resistant samples. Out of 61,5 did not give MTBC and 11 gave wild type pattern on MTBDR*plus* assay. Genotype MTBDR*plus* reported 82.22% (37/45) of specimens exhibiting mutations in katG alone, 8.89% (4/45) showing mutation in inhA alone and 8.89% (4/45) displaying mutation in both katG and inhA. The mutation patterns identified as conferring INH resistance included katGmut1, katGmut2 and inhA mut1. None of the inhA mut2, inhA mut3A and inhA mut3B were identified in this study (Table 4).

**Table 4:** Pattern of amino acid change and mutation on katG encoding region and inhA promoter region associated with Isoniazid resistance.

| INH resistance gene pattern | Amino acid changed | No (%) of isolates |
|---|---|---|
| ΔWT of katG gene, MUT2 | S315T2 | 1 (2.22%) |
| ΔWT of katG gene, MUT1 | S315T1 | 36 (80%) |
| ΔWT1 of inhA gene, MUT1 | C15T | 4(8.89%) |
| ΔWT of katG gene, MUT1 and ΔWT1 of inhA gene, MUT1 | S315T1, C15T | 4 (8.89%) |
| Total | | 45(100%) |

Note: ΔWT- missing of wild type, MUT- mutation, S-serine, T-threonine, C-cysteine

## DISCUSSION

The first version of MTBDR*plus* was validated for smear-positive pulmonary samples, and it could be proven that both the detection of MTBC and of RMP and INH resistance was high (sensitivity of 98.1% for the detection of RMP resistance and 84.3% for the detection of INH resistance).[6] However, the usefulness of smear-negative samples with this test was limited. The new MTBDR*plus*(v2.0) test supposed to have higher analytical sensitivity, and could be used for both smear positive and negative specimens. The current study compares the utility of this test in an endemic setting of Nepal.

Overall sensitivity, specificity, positive predictive value and negative predictive value of MTBDR*plus* assay were 94.5%, 95.5%, 96.0% and 93.8% respectively in our study. Sensitivity was found to be higher and specificity was slightly lower as compared to Crudu *et al*.[7] This different type of result experience could be because of different modality in specimen choice, as in the study of Crudu *et al*,[7] specimens were mostly smear negative (74%), whereas we included only 48% of smear negative specimens. Although, our sensitivity result was higher to that of Barnard *et al*,[8] but it was not statistically significant difference.

Similarly, sensitivity of this tool was 78.6% in smear negative specimens. This is also comparable, though slightly higher to the sensitivity described by Crudu*et al*[7] and Barnard *et al*.[8] The overall and smear positive specificity of this test in the current study compares less to Crudu *et al*[7] and Barnard *et al*.[8]The reason behind this might be the fact that the study participants were also patients under treatment. Patients under treatment harbor DNA of dead bacteria,that can be detected by PCR while their samples could be microscopic and culture negative at same time.

Results of this study show that MTBDR*plus* assay has good capability for rapid detection of RMP & INH resistant *M. tuberculosis.* Previous studies have demonstrated that the Genotype MTBDR*plus* test (either of any version) had outstanding performance: 91.7% to 100% of sensitivity in detecting RMP resistance, and 34.6% to 94.6% of sensitivity in detecting INH resistance.[8-13]Regarding the detection of RMP and INH resistant *M. tuberculosis*, by MTBDR*plus*(v2.0), sensitivity of this test in our study was comparable to the study of Crudu *et al*[7] for RMP and INH detection. Similarly, result of specificity compares similar in these two studies.

Consistant with previous studiesby Ramaswami *et al*[14] and Ratan*et al*[15] that RMP resistance *M. tuberculosis* isolates worldwide have mutations within the 81 bp core region of the rpoB gene, this study found mutation in this region in 94.64% of RMP resistance isolates. The rpoB codons 531, 526, and 516 are the most frequently mutated codons worldwide, although variations in the relative frequencies of mutations in these codons have been described for *M. tuberculosis* isolates from different geographic locations. The most frequently mutated codon in this study was codon 531 (60.37%), which was similar to those reported in clinical isolates from Nepal[16], India[17], China[18] and other geographical regions.[11,19] Although high frequencies of mutations in codon 516 in clinical isolates have been reported from various parts of Northern India[18], this study found a lower frequency of this mutation (1.88%), which was comparable to that found in a study done in China.[18]

Phenotypically RMP resistance isolates with no rpoB mutations in this study were 5.4%, similar to those reported previously in Nepal.[16,20] Therefore, this finding suggested that majority of RMP resistance isolates in Nepal could be rapidly detected by screening for the most common genetic alterations of the rpoB gene, although the prevalence of isolates lacking mutations also needs to be considered.

Previous studies indicated that INH resistance was mediated by mutations in several genes, most commonly

of inhA.[14,21] Furthermore, this study found that 82.22%, 8.99% and 8.99% of phenotypically INH resistance clinical isolates had point mutations in katG,inhA promoter region, and in both KatG and inhA promoter region respectively, and the frequencies were similar to those reported by other researchers.[5,16,21]Of the isolates 11 (18.03%) INH resistance had no resistance-associated alterations in the two targets analyzed, indicating that resistance in these isolates could be due to mutations present outside of the sequenced area or in other gene (Kas A, ndh or others).[5]

The MTBDR*plus* assay (v2.0) could be a very useful tool for the management of TB because of its use for rapid identification of RMP and INH resistant *M. tuberculosis* in both smear negative and smear positive sputum specimens.

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