Are WHO Approved Nucleic Acid Amplification Tests Causing Large-scale “False Identification” of Rifampicin-resistant Tuberculosis?: Programmatic Experience from South India

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Abstract

Introduction: The nucleic acid amplification tests (NAATs): Line probe assay and GeneXpert (Xpert) have evolved as the primary tool for identification of rifampicin (RIF)-resistant (RR) tuberculosis (TB) worldwide, primarily because of the ease and speed. We rechecked RR isolates identified by NAATs from presumptive RR TB cases belonging to South India by the Revised National TB Control Program, India using multiple RIF concentrations on Bactec MGIT system and compared the mutation patterns with the resistance levels. Methodology: Standard protocol for Bactec MGIT system as given by the manufacturer modified for the multiple RIF concentrations was used. All the retests were done in a certified BSL3 laboratory. Results: We found that there is a mismatch of up to 20% (RIF breakpoint 0.5 mg/L); the NAATs probably overidentifying RR TB. Half of the cases with mismatch showed a sub-breakpoint rise in resistance level (0.125 mg/L to 0.5 mg/L RIF). Discussion and Conclusion: The probable reasons for the mismatch are “sub-breakpoint low-level resistance mutants,” hetero-resistant bacterial populations, and other inherent test limitations along with the low RR TB prevalence in South India (<5%) among “presumptive multidrug-resistants.” This could be due to the incomplete selection pressure by an inadequate RIF exposure caused by various factors including a low-RIF dosage being used widely and poor Directly observed treatment. To prevent the false diagnosis of RR TB in a massive scale when using NAATs, we may need to enforce a carefully targeted testing approach and a phenotypic susceptibility testing with multiple RIF concentrations for confirmatory purposes.

Keywords: GeneXpert, Line probe assay, MGIT 960, positive predictive value, rpoB Resistance Determining Region

INTRODUCTION

Nucleic acid amplification tests (NAATs) for simultaneous identification of Mycobacterium tuberculosis (TB) complex and rifampicin resistance (RR) directly from samples are fast becoming the backbone of TB control programs worldwide. These tests are fast, safe, convenient, and accurate but expensive.[1]

Revised National Tuberculosis Control Program (RNTCP), India accepted NAATs; line probe assay (LPA) – Genotype MTBDR plus and GeneXpert (Xpert) MTB/RIF, a few years back as the preferred tests for identification of multidrug-resistant/RIF resistant (MDR/RR) TB from probable MDRTB cases. As of 2015, around fifty LPA laboratories, 26 Liquid culture (MGIT) laboratories, and 119 Xpert sites were established.[2] The Government of India is in the process of installing more Xpert machines all over the country.

Detection of RR by these genotypic tests is based on identifying mutations in Resistance Determining Region (RDR) in mycobacterial rpoB gene, (an 81 bp core region, 507–533 codons) where 96% of the mutations associated with RR is located.[3-7] LPA divides this region into eight overlapping segments/wild
type sequences (WT1 – codons 505–509, WT2 – codons 510–513, WT2/3 – codons 510–517, WT3/4 – codons 513–519, WT4/5 – codons 516–522, WT5/6 – codons 518–525, WT7 – codons 526–529, and WT8 – codons 530–533) and identifies mutations by detecting insignificant/absent amplification of these normal wild-type sequences utilizing the conventional amplification and reverse hybridization with specific probes. In addition, LPA includes provision for identification of four numbers of the well-characterized RR-associated mutated sequences (Mut1 – D516V, Mut2A – H526Y, Mut2B – H526D, and Mut3 – S531L) in the same areas, which is particularly useful, when encountered with hetero-resistant (of mutated and wild) bacterial populations. As per the LPA (Version 2) package insert, mutations in WT8 and WT3 segments without the presence of Mut 3 and Mut 1, respectively, are not resistant unless confirmed by a phenotypic test.

Xpert utilizes a cartridge-based nucleic acid real-time amplification technology which checks for mutations in five overlapping segments across the same 81 bp RDR named A, B, C, D, and E. An absent amplification of any segment or a late amplification signified by an increase in the number of cycles >4, compared to other segments (being a real-time PCR) points to the presence of a mutation. The performance of Xpert (and probably LPA) is reported to be inferior to traditional phenotypic susceptibility tests in heterogeneous bacterial populations as they need higher mutant proportions depending on the mutation site and type, for identification. In addition, there has not been any mentioning of any exceptions among mutations as in LPA.

We tried to assess the “positive predictive value” [PPV] of LPA and Xpert in real life settings of RNTCP in South India by rechecking stored isolates from NAATs identified RR cases. We used multiple RIF concentrations using MGIT 960 for rechecking, assuming it as the reference standard.

**Methodology**

As part of the study, two separate sets of isolates from samples were selected.

- Samples received at our laboratory for second-line drug susceptibility testing (DST) from those patients identified as RR by Xpert (Cepheid, Sunnyvale, CA, USA) done at Tamil Nadu state, from July 2014 to June 2015
- Samples identified as RR by LPA (Hain Lifesciences GmbH, Nehren, Germany) done at our laboratory (samples from Kerala state and Tamil Nadu) from January to June 2015.

The stored isolates from the above two groups which were susceptible to RIF (MGIT 1 mg/L) were retested with multiple RIF concentrations (1.0, 0.5, 0.25, and 0.125 mg/L) for detecting resistance following standard MGIT 960 (Becton Dickinson, Maryland, USA) procedure as per the package insert, modified for the multiple concentrations. The “patterns” of mutations were identified from the NAATs test details and patient details including treatment were recovered from the culture requests.

**Results**

Of the total 139 culture positives of 163 RR samples identified by Xpert, 137 were recovered successfully from stored isolates. Similarly, 190 of 191 stored isolates were successfully recovered of the total 219 RR samples identified by LPA.

To accommodate the chance of error in the process, we took resistance to RIF at and between 0.5 and 1 mg/L as “borderline resistant” and included it as resistant for all comparisons along with the standard breakpoint of 1 mg/L.[10]

MGIT found 29 isolates (21%) as RIF susceptible of the 137 Xpert RR isolates. In comparison, 33 isolates were MGIT RIF susceptible (17%) out of 190 isolates with mutated rpoB RDR identified by LPA. However, after removing the “WT8 mutations other than Mut 3 type” from the list as advised by the LPA package insert, the susceptible figures for LPA group were corrected to 14 of 159 isolates (9%). We also got one isolate with WT3 mutation which was subsequently proven resistant with a standard breakpoint of 1 mg/L. A good number of isolates were susceptible to RIF even at 0.125 mg/L; 18 out of 137 (13.1%) from the Xpert group and 10 out of 190 (5.3%) from the LPA group [Figure 1].

LPA strips were available (for rechecking) for all the 190 isolates selected. Copies of the automated Xpert results with probe details were available only for 75 of the 137 isolates. The rest were provided as a report from the testing laboratory without the details. The isolates with the probe details were considered for further comparison [Tables 1 and 2].

Some difference in pattern proportions was expected between the 8 probed LPA (WT1 to WT8) and the 5 probed Xpert (A to E) because of the results extracted from two different sets of samples and different overlaps between probes. Maximum number of mutations among LPA group were at WT8 (129/190; 87 WT8 absent-Mut3+, 31 WT8 absent, and 11 Mut3) followed by WT7 (34/190; 21 WT7 absent Mut2+, 11 WT7 absent,
This matched well with the Xpert group having a majority of mutations at probe site E (45/75) and probe site D (15/75), which corresponds, somewhat to WT8 and WT7 of LPA. The similar proportion of mutations in the groups matched with most of other published studies from diverse geographies and probably is a universal trend among mycobacterial populations. [3-6,11] Mutations at WT1 were absent in our data, while WT2 (5/190 and extra 4 numbers in combination with WT3) in LPA and Site A (9/75) in Xpert, reported significant numbers of mutation.

Isolated mutant bands present in spite of all the wild-type bands present among LPA group, due to hetero-resistant populations were not rare - 14 (7.4%). Eleven of that belonged to Mut3, two to Mut2, and one to Mut1.

Among the LPA and Xpert groups, mutations at WT2 and Site A reported the highest “error percentages” compared to MGIT; 80% (4/5 cases) and 78% (7/9 cases), respectively, were RIF susceptible by MGIT.

WT3 in LPA, site of a known silent mutation [12,13] had only one case reported in our study, which turned out to be resistant by MGIT. However, mutations in both WT3 and WT4 accounted for 8 cases, 5 out of that (62.5%) were RIF susceptible.

LPA WT8 and Xpert probe E from the two groups had most numbers of “errors” (21; 16% and 11; 24%, respectively) as these were the most common mutations encountered. Errors in other sites were numerically insignificant in this study similar to other studies about such “disputed” mutations in rpoB RDR.[6,14-16]

Xpert identified only nine mutations among the 75 by a significant difference in a number of cycles (≥4 cycles) criteria. Out of that 6 (66.7%) gave RR levels of ≥0.5 mg/L.

Based on our LPA data, an absent wild-type band along with the presence of the corresponding mutant band was always associated with RR ≥1 mg/L (112/112), while a mutant band present without a corresponding absent WT has a lower possibility of RR (11/14). WT7 without Mut2 gave a better association with resistance (9/11 above RIF 0.5 mg/L) compared to absent WT8 without Mut3 (12/31 above RIF 0.5 mg/L).

A detailed comparison of RR and susceptible isolates (by MGIT) based on the available data on duration of exposure to treatment [Table 3] did not produce any clear results as majority of the samples received (275/318, 86%) were claimed to be from patients, already well exposed to TB drugs (New cases under first-line treatment for >4 months and retreatment cases). However, MGIT INH resistance (0.1 mg/L) among the RR (by MGIT) group was much higher at 90% (234/252) compared to 74% (40/54) among RIF sensitive group, which

| Table 1: Different mutation sites identified by line probe assay and the corresponding rifampicin resistance levels |
|---------------------------------------------------------------|
| Mutation site | Total isolates | 1 mg/L | 0.5 mg/L | 0.25 mg/L | 0.125 mg/L | <0.125 mg/L |
|----------------|----------------|--------|----------|-----------|-----------|-----------|
| WT8 - Mut3 +   | 87             | 87     | -        | -         | -         | -         |
| WT7 - Mut2 +   | 21             | 21     | -        | -         | -         | -         |
| WT3,4 - Mut1 + | 4              | 4      | -        | -         | -         | -         |
| WT2,3 -        | 3              | 3      | -        | -         | -         | -         |
| WT4/5,6 -      | 5              | 4      | 1        | -         | -         | -         |
| WT7 -          | 11             | 6      | 3        | 1         | 1         | -         |
| Mut1/2/3 +     | 14             | 11     | -        | -         | -         | 3         |
| WT3 -          | 1              | 1      | -        | -         | -         | -         |
| WT1 -          | -              | -      | -        | -         | -         | -         |
| WT3,4 -        | 8              | 2      | 1        | 4         | 1         | -         |
| WT2 -          | 5              | -      | 1        | -         | 1         | 3         |
| WT8 -          | 31             | 10     | 2        | 12        | 3         | 4         |
| Total          | 190 (100)      | 149 (79) | 8 (4)  | 17 (9)    | 6 (3)     | 10 (5)    |
| Total (corrected): All except WT8 - | 159 (100) | 139 (87) | 6 (4)  | 5 (3)     | 3 (2)     | 6 (4)     |

RIF: Rifampicin

| Table 2: Different mutation sites identified by GeneXpert and the corresponding rifampicin resistance levels |
|---------------------------------------------------------------|
| Mutation site | Total isolates | Isolates resistant at RIF 1 mg/L | Isolates resistant at RIF 0.5 mg/L | Isolates resistant at RIF 0.25 mg/L | Isolates resistant at RIF 0.125 mg/L | Isolates resistant at RIF <0.125 mg/L |
|----------------|----------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| A              | 9              | 2                               | -                               | 1                               | 1                               | 5                               |
| B              | 4              | 3                               | 1                               | -                               | -                               | -                               |
| C              | 2              | 2                               | -                               | -                               | -                               | -                               |
| D              | 15             | 11                              | 2                               | 1                               | -                               | 1                               |
| E              | 45             | 33                              | 1                               | 2                               | 1                               | 8                               |
| Total (%)      | 75 (100)       | 51 (68)                         | 4 (5)                           | 4 (5)                           | 2 (3)                           | 14 (19)                         |

RIF: Rifampicin
Table 3: Comparison of mycobacteria growth indicator tube rifampicin resistant and susceptible groups among nucleic acid amplification test rifampicin-resistant isolates based on available treatment history

| GeneXpert/LPA resistant | MGIT susceptible (%) | MGIT resistant (%) |
|-------------------------|----------------------|--------------------|
| Failures                | 22 (37.9)            | 72 (27.7)          |
| Relapses                | 13 (22.4)            | 93 (35.8)          |
| Defaults                | 15 (25.9)            | 60 (23.1)          |
| New cases <4 months    | 8 (13.8)             | 35 (13.5)          |
| Total                   | 58 (100)             | 260 (100)          |
| INH resistance (by MGIT 0.1 mg/L) | 40 of 54 (74), 3 not available | 234 of 252 (90), 8 not available |

MGIT: Mycobacteria growth indicator tube, INH: Isoniazid, LPA: Line probe assay

was statistically significant ($P < 0.0001$, odds ratio = 0.2198 with 95% confidence interval 0.1013–0.4769).

Eight of the samples identified RR by Xpert were received from HIV-positive patients. Four of them were culture positive, and all were RIF susceptible by MGIT. The only HIV positive detected by LPA as RR was from a health worker, which was also found resistant by MGIT.

**Discussion**

The comparison clearly shows that there is a clear mismatch in the detection of RR between the NAATs and MGIT more toward mutations on either ends of rpoB core RDR. The inclusion of the four well-characterized mutant probes and the recommended phenotypic confirmation test for other mutations in WT8 and WT3 is apparently giving the LPA a higher PPV; 91% versus 80% for GeneXpert as per our data. This supposed advantage of LPA against GeneXpert, levels out in situations without a phenotypic DST availability as the mutation in WT8 (without Mut3) is frequent (31/190, 16.3%) and 12 (38.7% or 6.3% of total) out of that were clear RR which would have been missed without the phenotypic DST.

Understandably, a phenotypic test with multiple concentrations is likely to be the preferred test than repeat testing by LPA/ GeneXpert (or any test for identifying mutation) in situations associated with such disputed/silent mutations.

We are not sure of the reasons behind the apparent poor correlation of phenotypic DST even at <0.5 mg/L to treatment status. Over half of RIF susceptible by MGIT 960 were susceptible even to 0.125 mg/L. A part of the above can be attributed to the known tendency of automated liquid culture DST systems such as MGIT to miss slow-growing mutants in hetero-resistant populations.[17] However, perhaps, there are more important underlying reasons for this in our context.

RNTCP offers RR identification to presumed RR TB patients comprising mostly nonresponders/late responders under treatment or retreatment patients. In this study, failures (29.6%), relapses (33.3%), and lost to follow-ups (23.6%) accounted for the vast majority of the 318 samples received with treatment history. The relative abundance of “sub-breakpoint low-level RR mutants” (or presence of hetero-resistant bacterial populations) may be due to the poor selection pressure by an inadequate exposure to RIF. Indeed lost to follow-ups and relapse cases (together >55% in this study) are very heterogeneous broad groups associated with variable drug exposure. Apart from poor DOT, some of the other probable reasons for suboptimal RIF exposure are low-RIF dosing by the program (450 mg of RIF on alternate days for patients up to 60 kg body wt) or by the doctor, poor intestinal absorption of RIF due to ingested food/beverages[18] or chronic inflammatory diseases, [19] other concurrent illness (such as HIV and diabetes mellitus), and alcohol abuse. The lower INH resistance among the phenotypically RIF susceptible isolates compared to the phenotypically RR ones (74%–90%, respectively) in our data which was statistically significant ($P < 0.001$) also points to inadequacies in Anti TB Treatment.

There are concerns about the clinical significance of such “disputed mutations,”[20–23] but there are also reports about getting good results without adverse reactions by increasing the RIF dose; if the serum concentration is low.[21,22,24,25] In this context, we also need to take into account, the renewed interest in increasing the dose of first-line drugs, especially RIF, much higher up to 1200 mg daily which is reported to be very effective at decreasing relapses, increasing cure rates, and shortening the duration of treatment with manageable side effects.[26-31]

The low RR rates among the study participants could also be another contributing factor for the mismatch. Those states in India, which introduced programmatic RR screening and treatment earlier have comparatively lower RR/MDR rates among presumptive RR TB cases, such as Kerala (3.4%), Tamil Nadu (4.3%), Andhra Pradesh (6%), and Gujarat (7.3%).[2] Almost all of these RR/MDR cases were identified by NAATs. The WHO policy update on Xpert MTB-RIF usage 2013[1] cautions the use of Xpert in target populations with low RR prevalence because of poor PPV (<90% PPV among <15% RR prevalence and <70% PPV among <5%). To overcome this, we may need to select target groups with >15% RR or should advise phenotypic confirmation (with multiple concentrations) if the patient is not exposed to RIF “with a reasonable dosage for a reasonable duration (with reasonable certainty) within a reasonable period.”

Of particular concern is the recommendation to use Xpert as the preferred (often exclusive) test to confirm TB among extrapulmonary TB, presumed pediatric TB, and HIV-TB co-infection, where the RR rates could be low depending on the general prevalence of the area (2.2% among new pulmonary TB cases in India).[2] A false resistant result here could bring considerable anxiety to patients, their families, and the clinicians alike, as the phenotypic confirmation takes 6–10 weeks for the result. The situation may get further...
complicated if the culture is negative, as often is the case due to poor bacterial viability, if the TB treatment had already started. Considering the huge numbers of RR TB being identified every year by the NAATs, even a moderately low PPV can lead to a large number of patients being put on probably unnecessary and potentially dangerous, expensive second-line TB drugs. For example, the south Indian states of Kerala, Tamil Nadu, and Andhra Pradesh together identified 3346 RR cases from 67981 MDR suspects in 2014 by NAATs. [2] If the PPV of Xpert in our study of 79% is applicable, 669 patients identified as RR could be phenotypically Rif susceptible (≤0.5 mg/L) and could have a realistic chance of getting cured by “adequate dosages” of first-line drugs itself with superior sterilizing activity.

There were considerable strengths to our study as the tests were carried out in a quality assured and certified laboratory under RNTCP. It was based on real samples identified as RR by Xpert and LPA in a wide geographic area under the program systematically, where no other certified facility was available for phenotypic DST screening.

There were also some weaknesses. The culture and MGIT DST on “GeneXpert identified RR group” were done on a different set of samples received from the patients within 1 month of the diagnosis. Furthermore, DST on solid medium was not attempted.

The study points to the need to have an extensive characterization of mutations and their contribution to Rif resistance among variably exposed bacterial populations.

**Conclusion**

Xpert and LPA have a mismatch of up to 20% with MGIT (RIF 0.5 mg/L); the concerned mutations are more frequently encountered toward both ends of rpoB RDR among the tested samples. The increased frequency of mutations associated with “sub-breakpoint low-level resistance,” hetero-resistant bacterial populations, and inherent limitations of the NAATs and MGIT are probably responsible for the mismatch among our samples. Inadequate drug exposure due to low-RIF dosages, poor DOT, and other issues associated with poor serum drug levels along with the low RR prevalence are the probable reasons. Extensive use of NAATs among such a population without a strictly targeted approach and confirmatory phenotypic tests with multiple Rif concentrations may result in false RR results on a massive scale.

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**Conflicts of interest**

There are no conflicts of interest.

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