Comparative Diagnostic Accuracy of Mean and Lowest Cycle Threshold Levels in Xpert MTB/RIF Assay and Molecular Epidemiology of Missing Probes In Rifampicin Resistant Tubercular Cases From A Tertiary Care Hospital

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ABSTRACT

Background: The comparative diagnostic accuracy of mean and lowest Ct values needs to be evaluated for the assessment of mycobacterial burden in tubercular cases. Mutation in any codon of 81 base-pair core regions prevents the hybridization of one or more of five overlapping Probes A-E in Xpert MTB/RIF assay indicated by “missing probe.” Molecular epidemiology of missing probes may prove useful in tracing the source of infection and selection of a more suitable drug regimen for treatment.

Methods: This study included 65 rifampicin resistant cases and an equal number of rifampicin sensitive cases detected by Xpert MTB/RIF assay. Only samples tested positive for tubercular bacilli were included. The information regarding the tubercular load, Ct values of five probes targeting the rpoB gene, lowest Ct value among the five probes, missing probe in rifampicin resistant cases and time taken for the entire cycle were recorded in each case.

Results: Lowest Ct is a stronger indicator of tubercular load than the mean Ct value. E probe was found to be missing in majority (64.6%) of the cases, followed by A (6.2%), B and D (4.6%), C (1.5%) probes. In 7.6% cases, more than one probe was missing. None of the probe was missing in 10.6% of rifampicin resistant cases.

Conclusions: Lowest Ct value was found to be a better tool than mean Ct value for the determination of mycobacterium burden. Molecular epidemiology of missing probes could be useful in the development of new probes for the detection of rifampicin resistance.

KEYWORDS: CBNAAT, Xpert MTB/RIF, Tuberculosis, Cycle Threshold, Missing Probe, Rifampicin Resistance

INTRODUCTION

Tuberculosis (TB) is the major cause of mortality from a single infectious agent. South-East Asia alone accounted for 44% of the total global load of TB amongst various World health organization (WHO) regions. India ranked first by contributing 27% of global burden\textsuperscript{[1]}. In 2018 India contributed 27% to the total global drug-resistant TB load that was higher in comparison to the 24% contributed in the previous year\textsuperscript{[1, 2]}. As per WHO, despite the intensified efforts, the incident cases reported lag behind the estimated new cases. India alone accounts for 25% of the gap in estimated and reported incident TB cases worldwide. Therefore, it is crucial that India further escalates its capacity for early diagnosis of TB cases\textsuperscript{[1]}. Insufficient rapid detection and subsequent delay in effective management leads to failure in breaking the chain of ongoing transmission of TB in community. It further predisposes for the development of secondary resistance in tubercle bacilli. Current situation warrants not only the rapid detection of TB cases, but also the early and accurate determination of drug resistance in these cases. Conventional diagnostic techniques for the detection of \textit{Mycobacterium tuberculosis} (MTB) are incapable of providing rapid diagnosis, besides being cumbersome and lacking in sensitivity. Xpert MTB/RIF or CBNAAT (cartridge based nucleic acid amplification test) has emerged as an efficient point-of-care test. Xpert MTB/RIF assay can be directly performed on clinical samples.
with minimal hands-on training and as the sample reagent used in this assay is tuberculocidal, it further addresses the major biosafety concerns [4]. In December 2010, WHO endorsed the utilization of Xpert MTB/RIF assay for the rapid detection of TB. Later in 2013, WHO also approved its usage in specific types of extra pulmonary and pediatric TB disease [2].

Xpert MTB/RIF assay has an analytical detection limit of 131 colony forming units/ ml of sputum sample (95% CI 106–176) [5, 6]. As per WHO, the quantitative Xpert cycle threshold (Ct) could be utilized to evaluate the infectious TB cases for contact tracing in settings where smear microscopy is abandoned [4]. The correlation between Xpert Ct values and mycobacterial load is well documented [6, 7]. However, to the best knowledge of the authors, all the previous studies have either used mean Ct values or lowest Ct values to evaluate the association with bacillary load and none has compared these two to determine the more suitable parameter. This study was initiated with the hypothesis that both the measures i.e. mean Ct values and lowest Ct values, are equally strong indicator of tubercular load. Determination of tubercular load may help in assessing the severity & prognosis of disease and in addition can aid in evaluating the risk of TB transmission [8, 9].

Over 50 mutations, either high or low level, have been documented in rifampicin resistance determining region. Mutations at specific codons 511 (probe A), 516 & 518 (probe B), 522 (probe C) and 533 (probe E) are associated with low level rifampicin resistance whereas mutations at codons 513 (Probe B), codon 526 (probe D) and 531 (probe E) impart high level rifampicin resistance [10, 11]. This further helps us in inferring that Probes B and E detect both low and high level rifampicin resistance. Probe A and Probe C detect low level resistance whereas Probe D identifies high level rifampicin resistance [12]. The knowledge about the missing probe in any TB case is useful in identification of mutation in specific codon that may further facilitate the selection of a more suitable drug regimen for treatment [12]. Furthermore, the study of recent trends in mutations may aid in development of newer and more effective probes for the detection of rifampicin resistance in TB cases. Therefore, in this study we have assessed the diagnostic accuracy of Xpert MTB/RIF mean vs lowest cycle threshold levels. The molecular epidemiology of missing probes in rifampicin resistant tubercular cases has also been evaluated.

MATERIALS AND METHODS:

This cross-sectional study was carried over duration of one year in the Microbiology Department and DOTS center of a tertiary care hospital of Delhi. Only the samples tested positive for tubercular bacilli by Xpert MTB/RIF assay were included in the present study. Study included 65 rifampicin resistant cases and an equal number of rifampicin sensitive cases detected by Xpert MTB/RIF assay. Systematic random sampling was performed. Every third rifampicin resistant and every sixth rifampicin sensitive case detected by Xpert MTB/RIF assay was enrolled in the study till equal number of rifampicin resistant and rifampicin sensitive cases was achieved within the study duration due to logistic constraints. In accordance with RNTCP guidelines, samples from the suspected cases of TB were tested by Xpert MTB/RIF assay (Cepheid Inc., Sunnyvale, California) following the manufacturer’s instructions [13, 14]. The Xpert MTB/RIF assay is based on the principle of heminested real-time PCR and is capable of simultaneously detecting the presence of MTB-complex and rifampicin resistance within a short duration of 2hrs after loading the sample. Xpert MTB/RIF is an automated test in which sample processing, PCR amplification and detection are all integrated into a single self-enclosed test unit known as the Xpert MTB/RIF cartridge. The primers here amplify a portion of rpoB gene carrying the 81 base pair core region linked with rifampicin resistance in Mycobacterium tuberculosis. Five overlapping molecular beacons (Probes A-E), jointly probe the entire 81-bp core region [15, 16]. Each molecular beacon has a distinct fluorophore. The hybridization and emission of positive signal byno less than two out of five probes detects the presence of MTB complex. Mutation in any codon of 81 base-pair core region prevents the hybridization of one or more probes in Xpert MTB/RIF assay as indicated by “missing probe”. The presence of one or more “missing probes” or the existence of a difference greater than 3.5 amongst first and the last Ct value detects the presence of rifampicin resistance [17]. The quantitative assessment of mycobacterial load was performed by measuring the cycle threshold (Ct) values of five probes targeting the rpoB gene [18]. Ct value was defined as the number of PCR cycles needed for each individual probe to be considered positive and is inversely proportional to the mycobacterial load present in a given clinical sample [19].

Analysis: Information regarding the tubercular load, Ct values of five probes targeting the rpoB gene, lowest Ct value among the five probes, missing probe in rifampicin resistant cases and time taken for the entire cycle were recorded in each case. The mean Ct value of five probes (A, B, C, D and E) was also determined in every case. Data generated during study was entered in excel sheet and analyzed. Statistical analysis was performed using linear regression to calculate the coefficient of determination (R2) to establish the correlation between two variables. Data points of mean and lowest Ct values were plotted to find outside the range points across various categories of tubercular load to determine the success rate of either of the method.

RESULTS

On the basis of number of data points lying outside the ranges across various categories of tubercular load, it was established that lowest Ct is a stronger indicator of tubercular load than the mean Ct value Tables 1, 2, 3 and 4 and Figures 1 and 2)
Table 1: Testing suitability of mean Ct value as an indicator for different categories of tubercular load

<table>
<thead>
<tr>
<th>Category</th>
<th>Very Low</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of tests</td>
<td>32</td>
<td>37</td>
<td>28</td>
<td>33</td>
</tr>
<tr>
<td>Tests outside the range</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Tests inside the range</td>
<td>32</td>
<td>33</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Success rate</td>
<td>100%</td>
<td>89%</td>
<td>89%</td>
<td>70%</td>
</tr>
</tbody>
</table>

Table 2: Testing suitability of lowest Ct value as an indicator for different categories of tubercular load

<table>
<thead>
<tr>
<th>Load</th>
<th>Very Low</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of tests</td>
<td>32</td>
<td>37</td>
<td>28</td>
<td>33</td>
</tr>
<tr>
<td>Tests outside the range</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Tests inside the range</td>
<td>0</td>
<td>36</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>Success rate</td>
<td>100%</td>
<td>97%</td>
<td>96%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 3: Correlation of mean / Lowest Ct with time taken for RR and RS profiles (n=130)

<table>
<thead>
<tr>
<th>Case</th>
<th>RR</th>
<th>RS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean CT</td>
<td>Lowest CT</td>
<td>Mean CT</td>
</tr>
<tr>
<td>RR</td>
<td>0.25%</td>
<td>0.14%</td>
</tr>
</tbody>
</table>

Table 4: Distribution of probes missing among the various tubercular load categories in rifampicin resistant cases (n=65)

<table>
<thead>
<tr>
<th>Missing Probe</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
<th>Very Low</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>9</td>
<td>13</td>
<td>14</td>
<td>6</td>
<td>42</td>
</tr>
<tr>
<td>AB</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AE</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BE</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DE</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

Many studies have used mean Ct values to compare the diagnostic utility of GeneXpert Ct values with smear microscopy and culture as a measure of tubercular load \[4, 20\]. However, Fradejas I et al. have utilized lowest Ct values for the prediction of smear status \[18\]. Despite our best efforts we could not find the studies comparing the mean and lowest Ct values to determine the more suitable parameter for the assessment of mycobacterial burden in TB disease. In the present study, lowest Ct was found to be
In the present study, the most frequent (64.6%) probe responsible for rifampicin resistance was Probe E. Our findings are in concordance with other studies that have also reported Probe E being the commonest mutation accountable for rifampicin resistance. Probe E determines the mutation in rpoB gene wherein lysine substitutes for serine (TCG to TTG). In our study, second most common rpoB mutation was located in the region of Probe A (6.2%) followed by Probe D and Probe B regions (4.6% each). However, in a study by Kanade S et al, Probe D was the second most common (4.3%) mutation followed by Probe B (3.79%) and Probe A (2.91%). The overall frequency of mutation detected by absence of Probe B in our study was comparable to this study. Kaur R et al in their study reported mutation at Probe B (20.8%) being second most frequent, followed by Probe D (13.8%) and Probe A (7.7%). Mutation at Probe C was responsible for rifampicin resistance in fewer cases. Similar observation was also documented by other studies. These lower mutation rates at Probe C genetic region are probably due to its lower susceptibility to mutation or being confronted to lesser selection pressure. In present study, more than one probe was missing in 7.6% of rifampicin resistant cases. The previous studies have reported a lower frequency for the same. Kanade S et al. have reported more than one missing probe in 2.04% cases. Ullah A et al. have reported combination of probes missing in 1.2% cases.

This increase in frequency of more than one probe missing in recent times probably points towards the adaptability of Mycobacterium tuberculosis to enhanced drug presence. In our study, most common combination of missing probes was AB (3.1%) followed by other combinations of AE, BE and DE at equal frequencies of 1.5% each. However, Kanade S et al. have reported DE (1.16%) being the most frequent combination followed by AB (0.73%) and Reddy R et al. have documented AD & AE (1.2% each) as the commonest combinations followed by BD (0.6%). In addition, two studies have also observed a combination of ADE, triple probes missing. Although, in our study we did not observe missing triple probes in any combination. All the five probes were present in 10.6% of rifampicin resistant cases wherein the CT interval was above 3.5 cycles. Another study has reported presence of all probes in 3.2% of rifampicin resistant cases. The understanding about the variation in trend of mutations can help in evolution of newer probes for detecting rifampicin resistance in TB cases.

CONCLUSION

Xpert MTB/RIF assay is crucial for the achievement of global targets concerning early detection and efficient management of TB cases. The simultaneous detection of mycobacteria and rifampicin resistance in a short duration of approximately 2 hours enables the early recognition of multidrug resistant TB. Ct value detected by this assay can be useful for detecting the tubercular load. Lowest Ct value was found to be a better tool than mean Ct value for the determination of mycobacterial burden. Commonest rpoB mutation was located in the region of Probe E. Molecular epidemiology of missing probes may prove useful in tracing the source of transmission in these drug resistant cases and can aid in development of new probes for the detection of rifampicin resistance. Further, studies involving the larger number study subjects and different geographical areas are needed to substantiate the findings of this study.

REFERENCES


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