Detection of heteroresistant *Mycobacterium tuberculosis* by pyrosequencing

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**ABSTRACT**

The ability of pyrosequencing to detect a resistant minority population of a heteroresistant *Mycobacterium tuberculosis* strain was investigated by performing a titration study. A mutant signal was noted only at 35 to 50% mutant DNA target, which is significantly higher than phenotypic drug susceptibility test methods.
The increasing prevalence of drug-resistant strains of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), demands new measures to expand TB infection control. Rapid and accurate diagnosis of the pathogen, and its drug susceptibility pattern, is essential for timely initiation of effective therapy, and ultimately, control of the disease (1). Various molecular-based methods to detect drug-resistant *M. tuberculosis* have been developed in order to circumvent lengthy phenotypic drug susceptibility testing (DST) (2-6).

DNA sequencing is a robust method to analyze sequence variants and is often considered the gold standard for detection of resistance-associated mutations in *M. tuberculosis* (7). A limitation with sequencing methods, which may not be evident, is the difference in ability to detect a minority population of a heteroresistant strain compared to phenotypic DST methods. A heteroresistant strain refers to a mix of both susceptible and resistant organisms in a bacterial population (8). This can be attained during suboptimal drug treatment (acquired resistance) or due to mixed infection of strains with different susceptibility, e. g. superinfection with a resistant strain in a patient with drug-susceptible TB (9). Chakravorty and colleagues reported that an *rpoB* Sanger sequencing assay required at least 50% mutant DNA target to be detected (10). Phenotypic DST methods commonly define a bacterial population as resistant if 1% or more of the organisms are resistant (11). Different sensitivities for phenotypic and genotypic methods may have implications for the interpretation of the results. Discordant results may not always be due to mutations being present in an unknown or not investigated region of the genome, but may in fact be present in the targeted region. However, due to a low fraction of bacteria harboring a mutation, it may not be able to detect the genotype despite having a resistant phenotype.

The ability of pyrosequencing to detect a minority population of a heteroresistant *M. tuberculosis* strain has not been reported. Therefore, the aim of the study...
was to investigate the sensitivity of an \textit{rpoB} pyrosequencing assay to detect heteroresistant \textit{M. tuberculosis} by performing a titration study of a wild type and a mutant DNA sample.

DNA was isolated as previously described (12). Wild type DNA was extracted from the reference strain \textit{M. tuberculosis} H37Rv ATCC 25618, which was cultured on drug-free Löwenstein-Jensen (LJ) medium prior to DNA extraction. Mutant DNA was extracted from a phenotypically (LJ proportion method (13)) rifampin (RIF) resistant \textit{M. tuberculosis} clinical isolate harboring an \textit{rpoB} S531L substitution (TCG/TTG) after culturing on LJ-medium containing 40 mg/L RIF to eliminate any wild type organisms. In the titration study, the wild type and mutant DNA were mixed in 5 and 10\% increments, ranging from 0 – 100\% of respective target. PCR was performed for each target mix and all PCR reactions were subsequently subjected to an \textit{rpoB} pyrosequencing assay as previously described (5). Pyrograms and sequences were obtained from PyroMark Q96 ID Software 2.5 (Qiagen, Germantown, MD, USA). The software automatically converted pyrogram peaks to a nucleotide sequence.

Figure 1 shows sections, surrounding the S531L mutation, of the pyrosequencing pyrograms for different target mixes. Full pyrograms for all target mixes are available in Figure S1 in the supplementary material. When the pyrograms were investigated by the naked eye, a mutant signal could be noted at presence of 35\% mutant DNA target and became more apparent at 40\% mutant DNA (Figure 1). Automatic interpretation by the pyrosequencing software required 70\% of mutant DNA for detection (Table 1). Presence of 50-65\% mutant DNA yielded an inadequate peak level at the mutant nucleotide position leading to a missing nucleotide at the mutated position. In summary, the assay required at least 50\% of mutant DNA for automatized detection.

Molecular-based methods offer a great chance to improve detection of drug-resistant \textit{M. tuberculosis}; however, their results should be interpreted in the light of their
performance characteristics demonstrated by the laboratory. The ability of different methods to detect a resistant (mutant) minority population in a heteroresistant sample varies, especially between phenotypic and molecular-based methods. The pyrosequencing assay investigated in this study required between 35 and 50% of mutant DNA for detection. A previous study reported a detection limit of 15% for pyrosequencing and 30% for Sanger sequencing for detection of Lamivudine-resistant hepatitis B virus (14). Low sensitivity does not only encompass sequencing but also other molecular-based methods, involving both the GenoType MTBDRplus and GeneXpert MTB/RIF tests (15, 16). This exemplifies the notion that sensitivities varies between different assays and methods. The sensitivity assumingly depends on a number of factors, including the specific methodology itself, the extent of assay optimization, and the presence of different resistance related mutations (15). It is likely that most molecular methods have a limit of detection (LOD) for a minority population that is greater and not equal to the defined LOD of phenotypic methods. Next-generation sequencing (NGS) is currently still quite expensive but could possibly offer a better sensitivity compared to other molecular-based methods due to greater target coverage (17). Thus, NGS could potentially offer a better understanding of the population structure in clinical samples. Digital PCR may be another alternative to detect heteroresistance more precisely (18).

Despite technical limitations, molecular methods designed to detect drug-resistant *M. tuberculosis* offer a much more rapid detection of drug-resistant TB cases than phenotypic DST, and will most likely play an increasing role in TB control programs. The need of molecular diagnostic methods for rapid identification of drug resistance in *M. tuberculosis* is obvious in the light of emerging severely drug-resistant strains. A thorough understanding of the several methodological parameters, including procedural limitations, is critical for proper interpretation of results. Any molecular-based method should be fully validated in terms of both its analytical and its clinical sensitivity and specificity prior to
implementation. Technical limitations however should not lead to cessation of engagement, but should rather be regarded as an encouragement to continue to develop and optimize improved and more sensitive molecular methods.

REFERENCES


FIGURE LEGEND

Figure 1. Sections of pyrograms from the titration study of wild type and mutant Mycobacterium tuberculosis DNA. The box indicates the first and second nucleotide positions of rpoB codon 531. The sequence variants of this codon were TCG (serine) if wild type and TTG (leucine) if mutated. The wild type vs. mutant DNA target ratios were (A) 100/0, (B) 65/35, (C) 60/40, and (D) 0/100.
### Table 1. Pyrogram to sequence conversion by PyroMark Q96

<table>
<thead>
<tr>
<th>Wild type / mutant ratio</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>GGGTTGACCC ACAAAGCGCCG ACTGTCGGGC</td>
</tr>
<tr>
<td>55/45</td>
<td>GGGTTGACCC ACAAAGCGCCG ACTTTCGGGC</td>
</tr>
<tr>
<td>50/50</td>
<td>GGGTTGACCC ACAAAGCGCCG ACTGTT-GGCG</td>
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<tr>
<td>45/55</td>
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<tr>
<td>40/60</td>
<td>GGGTTGACCC ACAAAGCGCCG ACTGTT-GGCG</td>
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<tr>
<td>35/65</td>
<td>GGGTTGACCC ACAAAGCGCCG ACTTGTGGGC</td>
</tr>
<tr>
<td>30/70</td>
<td>GGGTTGACCC ACAAAGCGCCG ACTGTTGCG</td>
</tr>
<tr>
<td>0/100</td>
<td>GGGTTGACCC ACAAAGCGCCG ACTGTTGCG</td>
</tr>
</tbody>
</table>

*Pyrograms presented in Supplementary Figure S1.*