Pyrosequencing: a rapid and effective sequencing method to diagnose drug-resistant tuberculosis

Aishwarya Govindaswamy, 1,* Dhananjayan Sakthi, 2 Rekha Pai, 2 Lakshmanan Jeyaseelan 3 and Joy Sarojini Michael 1

Abstract

Purpose. This study was undertaken to evaluate the efficiency of the pyrosequencing (PSQ) assay for the rapid detection of resistance to rifampicin (RIF), fluoroquinolones (FQs) and second-line injectables (SLIs) such as capreomycin (CAP) and kanamycin (KAN) in Mycobacterium tuberculosis (Mtb) clinical isolates.

Methodology. Pyrosequencing is a simple and accurate short read DNA sequencing method for genome analysis. DNA extraction from Mtb clinical isolates was performed using Tris-HCl buffer and chloroform. The rpoB (RIF), gyrA (FQs) and rrs (aminoglycosides) genes were amplified, followed by sequencing using the PyroMark Q24 ID system. The PSQ results were compared with the results from the conventional drug susceptibility testing performed in the laboratory.

Results. The sensitivity of the PSQ assay for the detection of resistance to RIF, FQ, CAP and KAN was 100 %, 100 %, 40 % and 50 %, respectively. The specificity of the PSQ assay was 100 %.

Conclusion. The PSQ assay is a rapid and effective method for detecting drug resistance mutations from Mtb clinical isolates in a short period of time.

INTRODUCTION

Drug-resistant tuberculosis (TB) is a major global public health issue and causes millions of deaths each year worldwide [1]. The emergence of drug-resistant strains has reduced the viable treatment options and threatens to make TB a highly lethal disease. In the World Health Organization (WHO) Annual Global TB Report 2016 it was estimated that there were 10.4 million TB cases. Drug resistance surveillance data shows that 3.9 % of new and 21 % of previously treated TB cases were estimated to have involved rifampicin (RIF)- or multidrug-resistant tuberculosis (RR-TB, MDR-TB). MDR-TB accounts for 3.3 % of new TB cases. About 9.5 % of MDR-TB cases show additional drug resistance, leading to extensively drug-resistant TB (XDR-TB) [2, 3].

In India, where drug-resistant TB is on the rise, there is an urgent requirement for a rapid, low-cost and robust test for the detection of drug resistance that should be at the very least, complementary to existing standards of diagnosis [4]. Bacteriological methods are highly sensitive and specific and are considered to be the gold standard for drug susceptibility testing (DST). However, these methods are labour-intensive and time-consuming for the detection of resistance to most of the anti-TB drugs [5]. The advent of nucleic acid amplification tests (NAATs) has revolutionized the diagnosis of drug-resistant tuberculosis, leading to more rapid diagnosis and prompt patient management. Cartridge-based nucleic acid tests (CBNAAT), such as the Xpert MTB Rif assay, can detect both Mycobacterium tuberculosis (Mtb) and rifampicin resistance in 2 hours and can be performed directly on clinical specimens. The other WHO-approved molecular test is the line probe assay (Hain’s MTBDRplus and MTBDRsl), which detects mutations causing resistance to isoniazid, rifampicin, fluoroquinolones (FQs) and second-line injectable (SLI) agents [6]. The drawback of CBNAAT and the line probe assay is that they have fixed targets for detection and cannot detect all mutations in that region or mutations outside the target region. Sequencing methods can overcome these limitations and extend rapid testing to all the anti-TB drugs and can be applied in a clinical setting [7, 8].
This study aims to evaluate the pyrosequencing (PSQ) assay for the rapid molecular detection of drug-resistant tuberculosis. *Mtb* isolates from laboratory-confirmed cases of MDR-TB and XDR-TB were studied. The results from the PSQ assay were correlated with those from conventional gold standard drug susceptibility testing.

**METHODS**

**Clinical isolates**

This was a prospective study that was performed over a period of 22 months using *Mtb* isolates obtained from patients with TB. Approval for the study was obtained from the Institutional Review Board, Christian Medical College, Vellore (IRB Min. no. 9131, dated 12th November 2014). The study isolates comprised MDR-TB/XDR-TB and susceptible *Mtb* strains. The clinical samples from patients with suspected TB were received for processing in the microbiology laboratory as part of routine diagnostics for mycobacteria culture and drug susceptibility testing. Consecutive *Mtb* isolates that were both resistant and sensitive in the first- and second-line DST results were selected and evaluated by PSQ assay. Mutations were studied by PSQ on 57 *Mtb* isolates for RIF resistance, 50 for FQ resistance and 51 for SLI drug resistance. Xpert MTB/RIF assay results were available for 56 out of 57 isolates on those that were tested for RIF resistance.

**Drug susceptibility testing method**

The drug susceptibility testing was performed using the 1% agar proportion method based on standard laboratory protocols [9]

**DNA extraction**

Genomic DNA was extracted using the method described by [Jureen et al.](#). The extracted DNA was quantified by measuring the optical density at 260 nm using the nanodrop method. The extracted DNA of the isolates was diluted to around 20–40 ng µl⁻¹ and stored at −20°C for further molecular work-up.

**Gene amplification**

The insertion sequence 'IS6110' was used as the molecular target for the identification of the *Mtb* complex. The molecular targets used for the determination of drug resistance were: RIF, the rifampicin resistance-determining region (RRDR) of *rpoB*; FQs, the quinolone resistance-determining region (QRDR) of *gyrA*; SLI drugs [capreomycin (CAP) and kanamycin (KAN)], the *rrs* and *eis* promoter regions. The primers and cycling conditions used were those described by [Lin et al.](#) [11]. Amplification was carried out in a Veriti Thermal Cycler (Applied Biosystems, CA, USA).

The PCR products were electrophoresed on a 2% agarose gel containing 0.5 µg ml⁻¹ freshly prepared ethidium bromide. The gel was visualized by ultraviolet radiation using Quantity One (version 4.6.2) software in the gel documentation system (Bio-Rad, Hercules, CA, USA). Isolate amplicons showing discrete bands at 110 bp for IS6110, 182 bp for the *rpoB* gene, 225 bp for the *gyrA* gene, 180 bp for the *rrs* gene and 86 bp for the *eis* promoter gene were taken up for PSQ assay.

**Pyrosequencing analysis**

Four PSQ assays were optimized for the following loci: *rpoB*, *gyrA*, *rrs* and the *eis* promoter region. The preparation of the template and sequencing was performed with a PyroMark Q24 ID System (Qiagen). The sequencing primers and the nucleotide dispensation order used were as described by [Lin et al.](#). The sequences obtained from each sequencing primer were gathered and compared in BioEdit version 7.2.5 with the H37RA wild-type sequence (NCBI reference sequence: NC_009525.1) as the standard reference sequence. The presence of mutation in the amino acid sequence of a strain was considered to indicate resistance.

**RESULTS**

**Rifampicin resistance**

Among the 57 *Mtb* isolates on which PSQ was performed, the assay detected mutations in 40 *Mtb* isolates that were also phenotypically resistant to RIF. Among these there were six unique mutations and combinations of mutations across the RRDR region in the RIF-resistant isolates. The most predominant mutation observed in the *rpoB* gene was the ‘TCG531TTG’ mutation, which was detected in 33 (82%) of the 40 RIF-resistant isolates.

In addition, PSQ picked up mutations in three isolates that were phenotypically sensitive. The mutations observed in these isolates were at codon 511 (Leu511Pro). The performance of the PSQ assay for the detection of RIF resistance in comparison to phenotypic DST is summarized in Table 1.

**PSQ in comparison with the Xpert MTB/RIF assay**

Xpert MTB/RIF assay results were available for 56 *Mtb* isolates. The agreement between the Xpert MTB/RIF assay and the PSQ assay was found to be 98.2% (kappa=0.953, *P* value <0.001). One isolate was determined to be susceptible by the Xpert MTB/RIF assay and resistant by the PSQ. This isolate had a mutation in codon 531 (TCG531TTG), but it was considered to be resistant by the conventional 1% proportion method of DST testing.

**Fluoroquinolone resistance**

FQ DST results were available for 50 *Mtb* isolates. PSQ detected mutations in 28 *Mtb* isolates that were also phenotypically resistant to FQ. PSQ detected a total of six unique mutations across the *gyrA* gene (QRDR). Twenty resistant isolates showed mutations in the 94th codon of *gyrA*: 15 GAC→GCC, 2 GAC→GCG, 1 GAC→TAC, 1 GAC→CAC and 1 GAC→AAC. Eight isolates showed a 90 GCG→GTG mutation. The performance of the PSQ assay for the detection of FQ resistance in comparison to phenotypic DST is summarized in Table 2.
Second line injectable drug resistance

For the SLI drugs, DST results were available for 51 Mtb isolates, among which 15 were phenotypically resistant to CAP, 14 were phenotypically resistant to KAN and 10 were resistant to both of the SLIs (CAP and KAN). Among the 10 Mtb isolates, 5 had a 1401 A–G mutation and 1 had a 1402 C–T mutation in the \( rrs \) gene region as evaluated by PSQ. The remaining five Mtb isolates did not show any mutation in the \( rrs \) gene segments evaluated, suggesting that mutations might exist in other sites of the \( rrs \) gene. Thus the \( eis \) promoter gene region was also evaluated by the PSQ assay. The PSQ detected mutation in the \( eis \) promoter region of one of the isolates, C-12T, which was susceptible to CAP and resistant to KAN.

The performance of the PSQ assay for the detection of SLI resistance in comparison to phenotypic DST is summarized in Table 3.

The sensitivity and specificity of the various PSQ subassays are listed in Table 4.

### DISCUSSION

Pyrosequencing is a rapid, robust and high-throughput real-time sequencing method that is easy to perform. In this method, multiple \( Mtb \) isolates can be sequenced simultaneously in a 24-well plate. The assay is capable of simultaneously detecting \( Mtb \) and the most common mutations conferring phenotypic resistance to RIF, FQs and SLI agents. It can also be used to detect novel mutations within the targeted gene regions, while it can differentiate between silent and missense mutations that are not associated with resistance. The accuracy of PSQ is comparable to that of other sequencing methods, such as Sanger sequencing, and the sequence information provided by the PSQ assay enables users to know the minimum inhibitory concentrations (MICs) of the drugs with respect to each mutation, thus guiding treatment decisions [12, 13].

For the detection of RIF resistance, the sensitivity and specificity of the PSQ assay were 100 % (91.19 %–100 %) and 100 % (69.15 %–100 %), respectively, which was in concordance with previously published studies [11, 14, 15]. The sensitivity and specificity of the PSQ assay in comparison with the Xpert MTB/RIF assay were 100 % and 93.33%, respectively. PSQ detected mutation at codon 531 (TCG531TTG) in one of the isolates, which was determined to be susceptible by the Xpert MTB/RIF assay. However, it was found to be resistant by the gold standard conventional DST testing. Studies have shown that the Xpert assay failed to detect RIF resistance *in vitro* when <90 % of the organisms in the sample were RIF-resistant [16]. The PSQ assay detected mutation in all of the RIF-resistant isolates (\( n = 40 \)) included in the study. Notably, in our study the majority of \( Mtb \) clinical isolates (82 %) had mutations in codon 531, which is similar to the findings of previously published reports [10, 17, 18]. Additionally, PSQ also detected mutation (CTG511CCG) in three isolates that were determined to be susceptible. This mutation has already been documented as one of the disputed mutations conferring ‘low-level’ RIF resistance [19]. Since these mutations are already known to have inconsistent RIF-DST results, they were excluded for the specificity analysis in our study [19, 20].
Among the 50 *Mtb* isolates evaluated by PSQ, 28 had mutations in the gyrA region. Two studies have shown that 60% to 90% of *Mtb* clinical isolates with FQ resistance have mutations in the QDRDR of gyrA, commonly in codons 88, 90, 91 and 94 [21, 22], which is similar to the results in our study, where roughly 55% of the mutations occurred in the 94th codon region.

With regard to the SLIs, our study showed 100% specificity for the detection of CAP and KAN resistance. However, the sensitivity was low for the detection of CAP resistance (40%). For KAN, there was an increase in the sensitivity to 50% due to the addition of the eis promoter gene in the PSQ assay. Other studies have also shown similar increases in sensitivity with the inclusion of the eis promoter gene in the PSQ assay [23, 24]. The lower sensitivity rates of PSQ for the detection of SLIs (CAP and KAN) reflect our incomplete understanding of the resistance mechanisms associated with the SLIs investigated in the study. Among the 51 *Mtb* isolates evaluated by PSQ assay, the most common mutation observed in our study was 1401 A–G mutation (72%), followed by 1402 C–T mutation (14%), which is similar to the results of other studies [11, 14].

The limitations of the PSQ assay, which restrict its use for routine diagnostics, include its high cost and the technical expertise required to perform it. To conclude, the PSQ assay is a rapid and effective method for the identification of the drug resistance of *Mycobacterium tuberculosis* to RIF and FQs, but further studies need to be performed to look for mutations in other SNPs that are associated with drug resistance to SLI agents. Larger studies need to be performed using direct clinical specimens, which could be valuable in a clinical setting for appropriate patient management.

Acknowledgement
The authors gratefully acknowledge the Institutional Review Board of the Christian Medical College, Vellore (IRB Min. no. 9131, dated 12 November 2014).

Conflicts of interest
The authors declare that there are no conflicts of interest.

References
11. Lin SY, Rodwell TC, Victor TC, Rider EC, Pham L et al. Pyrosequencing for rapid detection of extensively drug-resistant

Table 3. Mutations in the rrs and eis promoter regions and their association with phenotypic DST result

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mutation</th>
<th>No. of isolates</th>
<th>DST Results (CAP)</th>
<th>DST Results (KAN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td>rrs</td>
<td>A1401G</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C1402T</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>eis</td>
<td>C12T</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4. Sensitivity and specificity of the PSQ assay in comparison to the gold standard phenotypic DST

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIF</td>
<td>100 % (87 %–100 %)</td>
<td>100 % (73 %–100 %)</td>
<td>100 %</td>
<td>1</td>
</tr>
<tr>
<td>FQ</td>
<td>100 % (82.8 %–100 %)</td>
<td>100 % (78.9 %–100 %)</td>
<td>100 %</td>
<td>1</td>
</tr>
<tr>
<td>SLI (CAP)</td>
<td>40 % (16.3 %–67.7 %)</td>
<td>100 % (85.8 %–100 %)</td>
<td>82.35 %</td>
<td>0.48</td>
</tr>
<tr>
<td>SLI (KAN)</td>
<td>50 % (23.04 %–76.96 %)</td>
<td>100 % (90.51 %–100 %)</td>
<td>86.27 %</td>
<td>0.59</td>
</tr>
</tbody>
</table>


Five reasons to publish your next article with a Microbiology Society journal
1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.