Pyrosequencing for rapid detection of tuberculosis resistance to Rifampicin and Isoniazid in Syrian and Lebanese clinical isolates

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ABSTRACT

Background: Rapid and accurate techniques are always welcomed for the detection of resistant strains of Mycobacterium tuberculosis MTB.

Objectives: The objective of this study is to evaluate the pyrosequencing technology for the detection of MTB resistance to Rifampicin (RIF) and Isoniazid (INH) in Syrian and Lebanese clinical strains; 66 strains resistant to INH, among them 56 resistant also to RIF, were tested.

Methods: Four pyrosequencing assays were optimized and applied to the following loci: rpoB, rpoB RIF resistance-determining region, katG, the promoter regions of inhA and ahpC-oxyR intergenic region.

Results: The prevalence of mutations on codon 315 of the katG gene, inhA and ahpC-oxyR were 42.4%, 21.2% and 9.0%, respectively, which make an overall sensitivity of 72.6% for INH resistance. All RIF-resistant strains contained at least one non-synonymous codon change in the sequenced rpoB region (507–533) relative to the ATCC reference strain. The RIF drug resistance region (RRDR) sequencing identified 96 modified codons representing 34 different mutations.

Conclusions: The high sensitivity and the short turnaround time combined with multiplex sequencing of several isolates in parallel make pyrosequencing an attractive method for drug resistance screening for MTB.

Introduction

Currently, tuberculosis (TB) in Lebanon is highly affected by the Syrian crisis. Before the beginning of this crisis in 2011, the estimated prevalence of TB in Lebanon was 19 per 100,000 population. The Lebanese National Tuberculosis Programme (NTP) indicates that currently over half of the people referred to the NTP for investigations and treatment are non-Lebanese nationals [1]. As of August 2013, according to the NTP, 100 Syrian refugees have been diagnosed with TB in Lebanon, including 3 cases of multidrug-resistant TB (MDR-TB). Furthermore, during the first half of 2014, 61 cases...
of all TB forms were identified among Syrian nationals, representing 17% of all TB cases notified [1]. The NTP in Lebanon follows DOTS (Directly Observed Treatment Strategy), which has a high treatment success rate among Lebanese nationals. However, the treatment success rate is below that desired among the non-Lebanese patients due to their departure during the treatment [1]. MDR-TB, which is defined as being resistant to at least INH or RIF – the two most powerful first-line anti-TB treatment drugs – is emerging [2]. Weakened or disrupted services for the Syrian refugees can complicate diagnosis and treatment, potentially leading to an increase in MDR-TB. INH-resistant strains most commonly carry mutations on the katG gene, on the promoter region of the inhA gene and on the ahpC-oxyR intergenic region [3]. Mutations in several other genes may also lead to INH resistance, but are less common [4]. RIF-resistant strains serve as a surrogate marker for MDR-TB detection [5]. Resistance in RIF has been attributed to mutations within an 81-bp RRDR of the rpoB gene, corresponding to codons 509–533 [5]. Mutations outside of RRDR have also been reported, with a frequency of <2% [6]. Culture-based drug susceptibility testing (DST) may take 4 weeks from specimen collection to results. At present, pyrosequencing had become a novel approach used for the detection of TB infection and resistance. The objective of this study is to evaluate the pyrosequencing technology for the detection of MTB resistance to RIF and INH in the Syrian and Lebanese clinical strains.

**Materials and methods**

**Bacterial strains**

66 strains of MTB resistant to INH, among them 56 resistant also to RIF, were tested. These clinical isolates were provided by the Medical Biotechnology Section of the National Commission for Biotechnology in Syria and the health and environment microbiology laboratory at Azm Center for Research in Biotechnology at the Lebanese University between July 2003 and October 2005 [7,8]. The drug resistance pattern of the Syrian samples was previously established according to the recommendations of the National Committee for Clinical Laboratory Standards [8]. Antibiotic susceptibility testing of the Lebanese strains was performed using a BBL™ MGIT™ (Mycobacteria Growth Indicator Tube) AST system (Becton Dickinson, Franklin Lakes, NJ), based on comparing the growth of the MTB strains in a drug-containing tube with that of a drug-free tube [7]. All isolates were stored at 80°C. The reference strain H37Rv (ATCC 25177) was used as a control for the wild-type sequence.

**Polymerase chain reaction**

The primers used to amplify and sequence the katG gene, the promoter region of the inhA gene and the ahpC-oxyR intergenic region were synthesized according to Zhao et al. [9], and those for RRDR were synthesized according to Bravo et al. [10] by ThermoScientific, USA (Table 1). Amplification of the target regions was performed in a thermal cycler (MyCycler C1000; Bio-Rad). The efficacy of the amplification was determined by gel electrophoresis. The polymerase chain reaction (PCR) products obtained from this step were used for pyrosequencing.

**Pyrosequencing**

Sample preparation for pyrosequencing was performed according to the manufacturer’s instructions. Single-stranded DNA amplicons were prepared semi-automatically using a Vacuum Prep Tool and Vacuum Prep Worktable (Biotage, Uppsala, Sweden). A 20 µl aliquot of biotinylated PCR product was immobilized onto 4 µl of streptavidin-coated Sepharose® High Performance Beads (Amersham Biosciences, Piscataway, NJ, USA) with 26 µl of binding buffer, pH 7.6 (10 mM Tris–HCl, 2 M NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.1% Tween 20) and incubated at 65°C under agitation at 1400 rpm for at least 5 min. Double-stranded DNA immobilized on Sepharose beads was washed with 70% ethanol and denatured with 0.2 M NaOH. Unbound single-stranded DNA was washed with 0.1 M Wash buffer (0.1 M Tris–HCl [pH 7.6]). The beads carrying single-stranded DNA amplicons were suspended in 38.4 µl of annealing buffer, pH 7.6 (20 mM Tris-acetate, 5 mM Mg-acetate) containing 200 nmol of sequencing primers (Table 1). The single-stranded DNA was annealed to the sequencing primer at 80°C for 2 min followed by incubation for 2 min at room temperature. The single-stranded PCR products were sequenced using the PyroMark™ Q96 ID System (Biotage). To identify point mutations, sequences from clinical isolates were compared with that of wild-type MTB ATCC 25177. An internal control was also used to validate the results.

**Ethics approval**

The research was approved by the responsible institutional ethics committee of the Lebanese University.

**Results**

For the INH resistance, the results (Table 2) showed that among the 66 isolates, 28 (42.4%), 14 (21.2%), and 6 (9%) had mutations on codon 315 of the katG gene, on the promoter region of the inhA gene and on the ahpC-oxyR intergenic region, respectively. The overall sensitivity for INH resistance detection by pyrosequencing is 72.6%. The most common katG mutation was Ser315Thr (92.8%). All inhA mutations were silent mutations, and one strain had both katG and inhA mutations.

Of the 56 RIF-resistant clinical isolates analyzed, all strains contained at least 1 non-synonymous codon change in the sequenced rpoB region (507–533) relative to the ATCC reference strain (Table 3). The pyrograms indicated the presence of 97 modified codons representing 34 different codon changes (Table 3). One codon change was a consequence of a single base pair deletion. Five codon changes resulted in silent mutations through nucleotide substitutions, and the
rest resulted in missense mutations. All silent mutations were accompanied by non-silent mutations. Codon changes occurred primarily at codons 531 (37/97: 38%), 533 (28/97: 29%) and 526 (9/97: 9%). The mutation S531 L (TGG/TTG) was by far the most frequent (35 patients: 35/56: 63%). Fig. 1 shows a representative DNA sequence analysis of 81-bp RRDR core region of rpoB gene of RIF-resistant isolates of MTB.

<p>| Table 1 – Primer sequences for PCR and pyrosequencing. |</p>
<table>
<thead>
<tr>
<th>Genes</th>
<th>F (5’-3’)</th>
<th>R-biotinylated (5’-3’)</th>
<th>Sequencing primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG</td>
<td>TCGCGATGAGCGT-ACAGC</td>
<td>CCGCACGGAGGTC-AGTGC</td>
<td>CATACGACCTCGATGC</td>
</tr>
<tr>
<td>inhA</td>
<td>TTACGCTCGAGGAA-TACCG</td>
<td>CTGAACCGGATACGAA-TGGG</td>
<td>GCCGGG</td>
</tr>
<tr>
<td>ahpC-oxyR</td>
<td>CGGACTCTCGAACC-ACTG</td>
<td>CCTCATCATAAGG-GACAATG</td>
<td>CATTTGTTCGACAT</td>
</tr>
<tr>
<td>rpoB RDR</td>
<td>GGAGCGGAGCAC-ACCAGG</td>
<td>AGGCGAGTACGAC-CTGATTT</td>
<td>1-CCAGAAACGAGCT</td>
</tr>
</tbody>
</table>

<p>| Table 2 – Mutations identified within loci associated with resistance to Isoniazid. |
| Codon 315 katG mutations | InhA promoter mutations | ahpC-oxyR intergenic |</p>
<table>
<thead>
<tr>
<th>(Amino acid change); (Nb, %)</th>
<th>(Nb, %)</th>
<th>(Nb, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference strain ATCC 25177</td>
<td>AGC (–)</td>
<td>–</td>
</tr>
<tr>
<td>M. tuberculosis strains (n = 66)</td>
<td>AGC → ACC (Ser → Thr); (26, 39.3%)</td>
<td>–15 C-T; (14, 21.2%)</td>
</tr>
<tr>
<td>AGC → ATC (Ser → Ile); (1, 1.5%)</td>
<td>–</td>
<td>–6 G-A; (1, 1.5%)</td>
</tr>
<tr>
<td>AGC → AAC (Ser → Asn); (1, 1.5%)</td>
<td></td>
<td>–12 G-T; (1, 1.5%)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>42.4%</td>
<td>21.2%</td>
</tr>
<tr>
<td>Total sensitivity</td>
<td>72.6%</td>
<td></td>
</tr>
</tbody>
</table>

<p>| Table 3 – Number and percentage of mutated strains on rpoB region. |</p>
<table>
<thead>
<tr>
<th>Nb of strains (%)</th>
<th>Codon and Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25177</td>
<td>No mutation</td>
</tr>
<tr>
<td>21 (37.5%)</td>
<td>531 TCG → TTG (Ser → Leu)</td>
</tr>
<tr>
<td>5 (8.9%)</td>
<td>531 TCG → TTG (Ser → Leu); 533 CTG → CTG (Leu) → (Arg)</td>
</tr>
<tr>
<td>3 (5.3%)</td>
<td>531 TCG → TTG (Ser → Leu); 533 CTG → CTG (Leu) → (Arg)</td>
</tr>
<tr>
<td>2 (3.5%)</td>
<td>526 CAC → CGC (His) → (Arg); 528 CGC → ACG (Arg) → (Thr); 533 CTG → CCG (Leu) → (Pro)</td>
</tr>
<tr>
<td>2 (3.5%)</td>
<td>526 CAC → GAC (His) → (Asp); 533 CTG → CTG (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>531 TCG → TTG (Ser → Leu); 533 CTG → GGC (Leu) → (Gly)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>526 CAC → CAG (His) → (Gln); 533 CTG → CGG (Leu) → (Pro)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>526 CAC → CGC (His) → (Arg); 529 CGA → CAA (Arg) → (Gln); 533 CTG → CGT (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>531 TCG → ATT (Ser → Ile); 533 CTG → CTG (Leu) → (Pro)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>525 ACC → ATC (Thr → Ile); 531 TCG → TTG (Ser → Leu)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>526 CAC → TGC (His) → (Cys); 533 CTG → CGG (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>G deletion position 15; 533 CTG → CGG (Leu) → (Pro)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>517 CAG → CCA (Gln) → (Pro); 531 TCG → TTG (Ser → Leu); 533 CTG → CCG (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>512 AGC → CTC (Ser → Leu); 513 CAA → CCA (Gln) → (Pro);</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>524 TTG → CTC (Leu) → (Leu); 529 CGA → CCG (Arg) → (Arg); 533 CTG → CTG (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>516 GAC → CTC (Asp) → (Leu); 517 CAG → CAA (Gln) → (Gln); 533 CTG → CGG (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>531 TCG → TTG (Ser → Leu); 533 CTG → GGC (Leu) → (Ala)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>516 GAC → GTC (Asp) → (Val)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>526 CAC → GCA (His) → (Asp); 532 CGG → GCC ( Ala) → (Gly); 533 CTG → CGG (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>511 CTG → CCG (Leu) → (Pro); 533 CTG → CGT (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>517 CAG → AGA (Gln) → (Arg); 531 TCG → TTG (Ser → Leu)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>531 TCG → TTG (Ser → Leu)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>531 TCG → TTG (Ser → Leu); 532 CGG → CGC (Ala) → (Arg); 533 CTG → TGG (Leu) → (Trp)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>513 CAA → CAG (Gln) → (Gln); 533 CTG → CGG (Leu) → (Pro)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>526 CAC → GAC (His) → (Asp)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>531 TCG → TGC (Ser → Cys)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>512 AGC → ACC (Ser → Thr); 531 TCG → TTG (Ser → Leu)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>533 CTG → CGT (Leu) → (Arg)</td>
</tr>
<tr>
<td>1 (1.7%)</td>
<td>512 AGC → TGC (Ser → Ser); 513 CAA → CCA (Gln) → (Pro); 533 CTG → CGG (Leu) → (Pro)</td>
</tr>
</tbody>
</table>
Pyrosequencing performs real-time sequencing of short DNA segments at a speed averaging 1 min per nucleotide. In this study, the pyrosequencing method was evaluated for MTB resistance detection; 66 clinical isolates resistant to INH were subjected to katG, inhA promoter region and ahpC-oxyR intergenic region mutations detection, and 56 strains resistant to RIF were subjected to rpoB region (507-533) mutations detection. This study showed that the pyrosequencing applied to katG, inhA promoter and ahpC-oxyR intergenic region was able to detect a relatively large proportion of INH-resistant strains (72.6%). It is quite possible the presence of other mechanisms of resistance were mediated by the changes in other chromosome loci, e.g., kasA, dfrA and ndh [4]. The use of a single codon 315 katG mutation for INH resistance is not accurate in this country since it will lead to a sensitivity of only 42.4%. The prevalence of mutations in katG varies strongly among different regions of the world. The mutation rate was 98% in Russia [11], 66.7% in Finland [12], 60% in South Africa [13] and China [14], 46% in Switzerland [15] and 37% in Italy [16]. To improve the sensitivity of pyrosequencing for INH detection, 2 loci (inhA promoter and ahpC-oxyR intergenic region) were added. In a study [17] conducted by Huang et al., 18 of 28 MDR-TB isolates possessed ahpC mutations alone or in addition to katG mutations outside of codon 315. Detection of INH resistance from these isolates was missed by the MTBDRplus assay; this demonstrated the significance of adding the ahpC locus for detecting INH resistance. The present study found a rather small increase (9.0%) in sensitivity contributed by ahpC. However, any increase in the detection of INH resistance reduces ineffective INH treatment and prophylaxis and is welcomed by clinicians. A recent study in the United States of America showed a sensitivity of 88.6% by using the 3 loci [18].

RIF is a powerful drug used for treating TB. The accuracy of molecular testing for RIF resistance is critical, since it may influence early clinical decisions. The frequencies of mutations detected (100%) for rpoB in RIF-resistant isolates by the pyrosequencing assay are consistent with other reports in the literature and demonstrates the importance of the RRDR hotspot region in the resistance of clinical TB isolates. Several studies have indicated that this region is responsible for 90–95% of RIF-resistant cases [19]. Notably, the vast majority of patients (95%) had mutations in codons 531 and/or 533. This could greatly reduce the expense and complexity of future early detection efforts in the local patient pool. The present study also emphasizes the frequency of codon changes at position 533. In clear contrast to previous reports [20,21], the majority of isolates in this study exhibited more than 1 codon change (2–5). Many codon changes involved more than one base pair change. A significant portion appeared to involve a two-base pair inversion, while others were likely to involve multiple base pair substitutions through point mutations. The high GC/AT ratio may contribute mechanistically to the mutability of this hot spot region.

The main advantage of this assay is its rapidity compared with that of conventional testing. This characteristic, coupled with the excellent specificity for detection of resistance to the two most important anti-tuberculosis medications in the majority of cases, makes this assay potentially useful by allowing appropriate treatment several days or weeks sooner than would be possible with conventional testing. Pyrosequencing, however, still cannot supplant conventional phenotypic methods of resistance testing. Where there are multiple genetic bases for resistance or where all the mechanisms of resistance have not been identified, genotypic methods have limitations. For instance, this assay looks for resistance only in the katG, inhA and ahpC-oxyR for INH resistance. For this drug, there remain other genetic mechanisms for resistance outside the three tested loci. Where the genetic
basis for resistance has been identified, it should be possible to
develop genotypic assays to detect resistance. However,
practical considerations limit the numbers of assays that
can be run for each sample, and costs and complexities of
testing additional sites have to be weighed against the incre-
mental benefits of such testing in clinical practice.

Conclusion

Pyrosequencing is a rapid and accurate method for detecting
MTB resistance to Rif and INH. The accuracy of sequencing by
pyrosequencing is comparable to that of Sanger sequencing,
and the availability of sequence information enables users
to study the association of drug minimum inhibitory concen-
trations (MICs) with each mutation. Once sufficient data asso-
ciating specific mutations with MIC distributions have been
accumulated, pyrosequencing may become a useful tool for
predicting the level of drug resistance. Such information
may help guide treatment decisions before the availability of
phenotypic DST results. Based on the outcome of this
study, a larger field trial of this assay is under way in Lebanon.

Conflict of interest

The authors have no competing interests to declare.

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