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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 multilocus 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

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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* promoter region mutated strains had the −15 C-T mutation. For the *ahpC-oxyR* intergenic region, the most common mutation was 46 G-A (4 of 6 strains). Four strains had mutations in *katG* in addition to *ahpc-oxyR* mutations and 1 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