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Genotypic detection of rifampicin-resistant *M. tuberculosis* strains in Syrian and Lebanese patients

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Summary
Setting: The incidence of multi- and extensively drug-resistant TB cases is increasing in many countries. Resistance to rifampicin is widely considered a surrogate marker for multiple drug resistant TB. No efforts have been made to identify and quantify the drug-resistant genotypes in the Syrian and Lebanese communities.
Objective: The genotypic characterization of rpo B mutations in the rifampicin drug-resistance region (RRDR) of resistant *Mycobacterium tuberculosis* isolates in Syrian and Lebanese patients.
Design: The pyrosequencing technique was applied to DNA derived from the *M. tuberculosis* isolates of 56 patients.
Results: RRDR sequencing identified 97 modified codons representing 35 different mutations; 31 (34%) of the 97 modifications were novel and have not been previously reported. The changes were mostly within codons 531 (37/97: 38%), 533 (28/97: 29%) and 526 (9/97: 9%). Additionally, 30 (54%) isolates had multiple codon changes.
Conclusion: This study indicates the importance of the RRDR hotspot region for the detection of rifampicin resistance in MTB clinical isolates from Syrian and Lebanese patients. However, new mutations and mutations in other locations within the RRDR were also observed. The vast majority (95%) of the studied isolates from this pool of patients contained mutations in codons 531 and/or 533.

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Introduction

Approximately 95% of all of tuberculosis cases occur in developing countries, where the disease has typically remained endemic [1]. In recent years, a dramatic increase in the number of cases of drug-resistant infections has occurred. The number of multi- and extensively drug-resistant cases (MDR, XDR) was estimated to be approximately 440,000 in 2008, with 150,000 deaths [2]. MDR TB is thought to emerge in patients either through exogenous infection by resistant strains or through the endogenous emergence of mutations due to suboptimal treatment [3,4]. The treatment of resistant TB is medically difficult, economically expensive and has adverse health effects for patients [5,6]. Despite extensive treatment measures, levels of mortality are still high. However, mortality has decreased significantly [7] in recent years following the introduction of several measures, including the application of molecular diagnostic techniques [8], strain identification [9] and the investigation of transmission [10,11].

The combination of rifampicin and isoniazid is the backbone of first-line and short-course chemotherapy. Rifampicin, a macrocyclic antibiotic, targets mycobacterial DNA-dependent RNA polymerase, a complex oligomer composed of four different subunits (α, β, β' and σ, which are encoded by rpo A, rpo B, rpo C and rpo D, respectively). Rifampicin binds specifically to the rpo B-expressed subunit and suppresses the initiation step of transcription [12]. Resistance to rifampicin results from spontaneous mutations, which occur at a rate of 10^8. These mutations have been widely shown to localize to the rpo B region, primarily in codons 507—533. This 81-bp region is called the RIF resistance-determining region (RRDR). Resistance to rifampicin is largely considered a surrogate marker for MDR TB due to its association with other drug resistance phenotypes [13].

Pyrosequencing technology has recently been used to characterize the genotypes of resistant tuberculosis strains [14—16]. Pyrosequencing chemistry differs in several aspects from standard sequencing. For example, fluorochromes and radioactivity are not used, and no postreaction step is required when using this technique [17]. The technology enables the rapid prediction of mutations and is suitable for the simultaneous screening of short sequences in large numbers of samples. It is therefore a proven, reliable and high-throughput assay for the rapid and specific analysis of rifampicin-resistant *M. tuberculosis* strains [18].

The presence of drug-resistant tuberculosis in Syria and Lebanon is known [19]. However, no efforts have been made to identify and quantify the drug-resistant genotypes in this community. In this study, pyrosequencing was used to fully characterize the RRDR mutations prevalent in *M. tuberculosis* isolates obtained from Syrian and Lebanese patients for the first time.

Materials and methods

Bacterial strains

A total of 56 clinical rifampicin-resistant *Mycobacterium tuberculosis* isolates (resistant) were selected. These clinical isolates were provided by the Medical Biotechnology Section of the National Commission for Biotechnology in Syria and the Azm Center for Research in Biotechnology and Its Applications at Lebanese University. The isolates were derived from 45 Syrian, 7 Lebanese and 4 Iraqi (living in Syria) patient samples collected between July 2003 and October 2005 from all Syrian and Lebanese provinces (muhafazat) [20,21]. The drug resistance pattern of the Syrian samples was previously established according to the recommendations of the National Committee for Clinical Laboratory Standards [21] and that of the Lebanese samples was also previously established [20]. All isolates were stored at −80 °C. The reference strain H37Rv (ATCC 25177) was used as a control for the wild-type sequence. The research was approved by the responsible institutional ethics committee.

DNA extraction

DNA extraction was performed with maximum precautions under a biosafety class two hood according to [20]. The isolates were incubated in a water bath at 80 °C for approximately 30 min to kill the bacteria and then centrifuged for 10 min at 8000 rpm. TE buffer containing 1% Triton X-100, 0.5% Tween 20, 10 mM Tris—HCl pH 8.0 and 1 mM EDTA was added to the pellet. The rest of the procedure was performed according to the instructions provided with the Qiagen DNA Blood Mini Kit (Qiagen, Germany) with one minor modification: the incubation period at 37 °C was 2 h instead of 90 min.

PCR

The primers used to amplify and sequence the rifampicin resistance-determining region (RRDR) were synthesized according to [22] by Thermo