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Short Communication

Mycobacterial interspersed repetitive unit typing and mutational profile for multidrug-resistant and extensively drug-resistant tuberculosis surveillance in Portugal: a 3-year period overview

Carla Silva[a], João Perdigão[a], Luísa Jordão[b], Isabel Portugal[a,*]

[a] Centro de Patogénese Molecular/URIA, Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
[b] Departamento de Doenças Infecciosas, Instituto Nacional de Saúde Dr Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisbon, Portugal

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ABSTRACT

Multidrug tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) cases constitute a serious health problem in Portugal, of which the majority of isolates belong to the Lisboa family and the Q1 cluster, highly related to the Lisboa family. Here we sought to investigate the molecular basis of resistant TB as well as to determine the prevalence of specific drug resistance mutations and their association with MDR-TB and/or XDR-TB. In total, 74 *Mycobacterium tuberculosis* clinical isolates collected in Lisbon Health Region were genotyped by 24-loci mycobacterial interspersed repetitive units–variable number of tandem repeats (MIRU-VNTR), and the mutational profile associated with first- and second-line drug resistance was studied. Seven new mutations were found, whilst the remaining 28 mutations had been previously associated with drug resistance. None of the mutations was specifically associated with MDR-TB. The mutational patterns observed among isolates belonging to Lisboa3 and Q1 clusters were also observed in isolates with unique MIRU-VNTR patterns but closely related to these strains. Such data suggest that the genotyping technique employed discriminates isolates with the same mutational profile. To establish the most adequate genotyping technique, the discriminatory power of three different MIRU-VNTR sets was analysed. The 15-loci MIRU-VNTR set showed adequate discriminatory power, comparable with the 24-loci set, allowing clustering of 60% and 86% of the MDR-TB and XDR-TB isolates, respectively, the majority of which belonged to the Lisboa3 and Q1 clusters. From an epidemiological standpoint, this study suggests combined mutational and genotyping analysis as a valuable tool for drug resistance surveillance.

1. Introduction

Tuberculosis (TB) remains one of the major causes of morbidity and mortality worldwide. Despite the existence of an efficient anti-TB treatment, the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) has become a great threat to TB control. In 2012, Portugal reported a TB incidence rate of 21.6 cases per 100 000 inhabitants, the highest incidence rate among Western European countries [1]. Portugal also recorded a high number of MDR-TB and XDR-TB cases, particularly in Lisbon city, which compromises the efforts to manage TB control in the country [2].

Most MDR-TB strains circulating in the Lisbon area belong to a particular family of genetically related strains, the Lisboa family, and also to a genetically close cluster, known as Q1 cluster, both detected in a MDR-TB outbreak in the 1990s [3]. Lisboa family and Q1 cluster strains have had a high correlation with multidrug resistance ever since and their evolution to XDR-TB has also been reported [2].

Isoniazid, rifampicin, ethambutol and pyrazinamide constitute the first-line drugs for TB treatment. Multiple drug treatment allows effective bacillary elimination, although the development of MDR-TB, i.e. resistant to at least isoniazid and rifampicin, made necessary the introduction of second-line drugs in TB therapy. Second-line TB treatment includes fluoroquinolones and injectable drugs such as kanamycin, amikacin and capreomycin. The emergence of XDR-TB strains, i.e. MDR-TB with additional resistance to fluoroquinolones and at least one second-line injectable drug (amikacin, kanamycin and capreomycin), turns TB in to a virtually

* Corresponding author. Tel.: +351 21 794 6439; fax: +351 21 793 4212.
E-mail address: isabel.portugal@ff.ul.pt (I. Portugal).

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incurable infection and hampers approaches to manage TB infection.

Multiple factors are involved in the emergence of drug resistance in *Mycobacterium tuberculosis*. Selection of MDR strains occurs mostly when inappropriate treatment is given or due to reduced patient compliance, along with the singular length of chemotherapy required for TB treatment. Such factors, together with mycobacterial features, e.g. the complex cell envelope structure and efflux mechanisms, contribute to a higher acquisition of drug resistance [4–6]. Given the clonal nature of the *M. tuberculosis* genome and the fact that drug resistance in TB results from key chromosomal mutations [5], knowledge of the mutations associated with resistance to the drugs available for TB treatment is crucial not only for the implementation of correct therapeutic schemes but also for investigating mycobacterial resistance mechanisms, which may allow the development of new drugs.

The main goal of this study was to determine the mutations associated with drug resistance in isolates circulating in Lisbon Health Region in Portugal. Since the majority of MDR-TB and XDR-TB belong to specific groups and families, we also aimed to determine whether a given mutation could be associated with MDR-TB and XDR-TB. Therefore, a group of 74 *M. tuberculosis* clinical isolates collected in Lisbon Health Region were genotyped and the mutational profile for first- and second-line drugs for resistant isolates was studied.

2. Materials and methods

2.1. Bacterial growth and drug susceptibility testing

A total of 74 *M. tuberculosis* clinical isolates (33 MDR-TB) referenced by the Portuguese Epidemiological Surveillance System (VigLab-Tuberculose), collected in Lisbon Health Region between 2007 and 2009, were analysed. *Mycobacterium tuberculosis* samples were cultured in Lowenstein–Jensen slants (BD Diagnostic Systems, Sparks, MD) and were incubated at 37 °C for 2–3 weeks until colonies were visible. Isolates were tested for first-line drug susceptibility by the BACTEC 960™ MGIT™ or BACTEC 460™ (BD Diagnostic Systems) method, using critical concentrations according to the manufacturer's instructions. MDR-TB isolates were also tested for second-line drug susceptibility using a radiometric BACTEC 460™ system to amikacin (1 mg/L), capreomycin (2.5 mg/L), ethionamide (5 mg/L), ofloxacin (2 mg/L), kanamycin (30 mg/L), para-aminosalicylic acid (1 mg/L) and cycloserine (40 mg/L) (all from Sigma–Aldrich, St Louis, MO).

2.2. Mycobacterial interspersed repetitive units–variable number of tandem repeats (MIRU-VNTR) genotyping

The 74 *M. tuberculosis* clinical isolates as well as the reference strains *Mycobacterium bovis* BCG and *M. tuberculosis* H37Rv were genotyped by a 24-loci MIRU-VNTR technique as previously described by Supply et al. [7]. Based on the MIRU-VNTR profile of each isolate, a dendrogram was constructed with the MIRU-VNTR*plus* web application (http://www.miru-vntrplus.org) using Dsw measure of genetic distance, and clustering was performed by the unweighted pair-group method with arithmetic average (UPGMA). A cluster group was defined when 100% similarity of the MIRU-VNTR pattern is observed.

2.3. PCR amplification and sequencing

Mycobacterium tuberculosis isolates resistant to at least one drug ($n = 52$) were screened for mutations in genes associated with resistance to first-line drugs: *katG* and *mabA–inhA* regulatory region for isoniazid; the rifampicin resistance-determining region of the *rpoB* gene for rifampicin; *rrs* and *rpsL* genes for streptomycin; the *embB* gene for ethambutol; and finally, the *pncA* gene for pyrazinamide.

Twenty-four MDR-TB isolates with second-line drug susceptibility test (DST) data available were screened for mutations in genes associated with resistance to second-line drugs: the quinolone resistance-determining region of the *gyrA* gene was analysed for fluoroquinolones (ofloxacin); and *tlyA*, *rrs* and *eis* genes were analysed for injectable second-line drugs (capreomycin, amikacin and kanamycin).

Genomic regions were amplified by PCR, purified and sequenced as previously described [2,8]. All sequences were further analysed with CLC Sequence Viewer 6.0 (CLC Bio, Aarhus C, Denmark) and BioEdit Sequence Alignment Editor v.7.2.3 (Ibis Biosciences, Carlsbad, CA) using *M. tuberculosis* H37Rv as the reference strain.

3. Results and discussion

3.1. MIRU-VNTR genotyping

Seventy-four clinical isolates collected in Lisbon Health Region as well as the reference strains *M. bovis* BCG and *M. tuberculosis* H37Rv were genotyped by 24-loci MIRU-VNTR (Fig. 1). Among the isolates studied, 20 isolates were distributed between four MIRU-VNTR clusters and the remaining isolates presented distinct MIRU-VNTR patterns. The major clusters were verified as belonging to clusters Lisboa3 and Q1, which were previously characterised by Portugal et al. [3]. These two groups shared >85% MIRU-VNTR similarity between them and together comprised 48.5% of the MDR-TB (16/33) and 71.4% of the XDR-TB (10/14) isolates.

3.2. Mutation analysis in genes associated with resistance to first- and second-line drugs

From the 74 clinical isolates, 22 (29.7%) were fully susceptible, with the remaining 52 isolates showing resistance to at least one first-line drug. Globally, twelve different resistance patterns were observed, five of which were MDR-TB, including a total of 33 MDR-TB isolates. Among the latter, 24 isolates were resistant to all first-line drugs. The hotspot regions associated with drug resistance to first-line drugs were screened and the mutations found are presented in Table 1. The most prevalent mutations found among these isolates were: C-15T (24/40; 60.0%) in the *mabA–inhA* regulatory region of *inhA* and S315T (5/40; 12.5%) in the *katG* gene for isoniazid resistance; S531L (27/34; 79.4%) in the *rpoB* gene for rifampicin resistance; K43R (21/41; 51.2%) in the *rpsL* gene for streptomycin resistance; M306V (13/26; 50.0%) in the *embB* gene for ethambutol resistance; and L120P (6/31; 19.4%) and V125G (6/31; 19.4%) in the *pncA* gene for resistance to pyrazinamide. Moreover, seven new mutations were found: S460N and 1420delT in *katG*; P224L in *rrs*; and 390InsA, H71Q, 412del3bp and 438del13bp in *pncA* [9].

Of the 33 MDR-TB analysed isolates, only 24 had second-line DST information. Among the resistant isolates ($n = 20$), 10 distinct resistance profiles were observed, from which 8 fit the XDR-TB definition. The mutational profile for genes associated with resistance to second-line drugs were analysed in the 15 MDR-TB isolates resistant to either a second-line injectable drug and/or fluoroquinolone plus an MDR-TB isolate genetically close to the Q1 cluster, which is resistant to all first-line drugs. The most prevalent mutations observed were: S91P (8/16; 50.0%), followed by D94G (5/16; 31.3%) and D94A (3/16; 18.8%) in the *gyrA* gene for resistance to fluoroquinolones; Ins755GT (7/9; 77.8%) in the *tlyA* gene for resistance to capreomycin; A1401G (3/3; 100%) in the *rrs* gene for combined resistance to amikacin, kanamycin and capreomycin; and G-10A (10/10; 100%) in the *eis* gene for resistance to kanamycin. Noteworthy, the mutations D94A, A1401G and G-10A were observed