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Original article

Detection of multidrug-resistant *Mycobacterium tuberculosis* strains isolated in Brazil using a multimarker genetic assay for *katG* and *rpoB* genes

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ABSTRACT

Multidrug-resistant tuberculosis (MDRTB) is a serious world health problem that limits public actions to control tuberculosis, because the most used anti-tuberculosis first-line drugs fail to stop mycobacterium spread. Consequently, a quick detection through molecular diagnosis is essential to reduce morbidity and medical costs. Despite the availability of several molecular-based commercial-kits to diagnose multidrug-resistant tuberculosis, their diagnostic value might diverge worldwide since *Mycobacterium tuberculosis* genetic variability differs according to geographic location.

Here, we studied the predictive value of four common mycobacterial mutations in strains isolated from endemic areas of Brazil. Mutations were found at the frequency of 41.9% for *katG*, 25.6% for *inhA*, and 69.8% for *rpoB* genes in multidrug-resistant strains. Multimarker analysis revealed that combination of only two mutations ("*katG*/S315T + *rpoB*/S531L") was a better surrogate of multidrug-resistant tuberculosis than single-marker analysis (86% sensitivity vs. 62.8%). Prediction of multidrug-resistant tuberculosis was not improved by adding a third or fourth mutation in the model. Therefore, rather than using diagnostic kits detecting several mutations, we propose a simple dual-marker panel to detect multidrug-resistant tuberculosis, with 86% sensitivity and 100% specificity. In conclusion, this approach (previous genetic study + analysis of only prevalent markers) would considerably decrease the processing costs while retaining diagnostic accuracy.

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Introduction

Tuberculosis (TB) remains an important public health concern as about one third of the world population is asymptomatically infected with latent *Mycobacterium tuberculosis*.[1] Multiple health campaigns are being addressed to diminish the incidence of the disease, but TB elimination remains a challenge, due to the emergence of clinical forms of multidrug-resistant strains of *M. tuberculosis*,[1] that are resistant to at least isoniazid (INH) and rifampicin (RIF), the first-line antitubercular (anti-TB) drugs. Beyond increasing TB incidence, mortality, and the rate of initial treatment failure, multidrug-resistant tuberculosis (MDRTB) also increase the cost of TB treatment.[1] For example, while a 6-month regimen of INH + RIF (plus pyrazinamide and streptomycin or ethambutol in the first two months) will cure most cases of non-resistant TB, MDRTB-patients will need at least 12–24 months of therapy with more toxic second-line drugs.[1,2] In addition, MDRTB accounts for up to 84% of the "retreatment" cases of TB.[1] With this perspective, early diagnosis appears to be the best strategy to control MDRTB.[1]

Conventional culture-based drug-susceptibility testing (DST) for TB can take as much as 90 days to complete, due to the slow growth of *M. tuberculosis*.[3] Since diagnostic delay is an arduous obstacle to effective MDRTB care, development and implementation of molecular approaches for rapid detection of MDRTB are needed for attenuating the MDRTB burden. Towards that end, many studies have investigated gene mutations that confer drug resistance in *M. tuberculosis*.[4–6] As a result, many commercial tests have been developed to detect MDRTB and, consequently, the WHO recommends to all governments the gradual implementation of evidence based commercial tests using molecular techniques for diagnosis and control of MDRTB.[1]

Overall, these tests aim to detect mutations in the mycobacterial genes involved in drug metabolism, which may confer phenotypic changes associated with antibiotic resistance. In this way, INH resistance is frequently associated with mutations in the catalase-peroxidase enzyme (encoded by the *katG* gene and involved in metabolic activation of the drug),[5] and/or in the enoyl-ACP reductase gene promoter (*inhA*), required for mycobacterial cell wall biosynthesis.[7] Likewise, resistance to RIF is strongly related to mutations in a region of 81 bp in the *rpoB* gene (encoding the beta subunit of the RNA polymerase, the drug target),[3,8] resulting in decreased affinity of RIF for the active centre of the enzyme.[9] Mutations in several other genes may also lead to INH or RIF resistance but are less frequent.[10–12] Thus, common gene markers for MDRTB are S315T in *katG*, −15C/T (CGT-CAT) in the operator region of *inhA*, and H526D and S531L in *rpoB*.[4,5]

Diagnostic parameters, such as sensitivity and specificity, of commercially available diagnostic tests are poorly understood in a worldwide context.[1] This is of interest since there is evidence that geographic location influences on the strain-to-strain *M. tuberculosis* genetic variability.[13–15] In this context, current gold-standard molecular biomarkers to diagnose MDRTB might not be found at reasonable frequencies to guide anti-TB drug choice in neglected regions such as Brazil. Moreover, it is even possible that non-trivial mutations could turn out to be novel biomarkers for MDRTB diagnosis in such regions. In this regard, we genotyped four mycobacterial SNPs (single nucleotide polymorphisms) in isolates from TB patients of an endemic region of Brazil to investigate their predictive value to diagnose MDRTB, and using these new reference standards as gold-standard. Our aim was to test and validate a rapid and cost-effective real-time PCR assay for detecting RIF and INH resistance.

Methods

Isolation, identification and drug sensitivity tests of M. tuberculosis

M. tuberculosis isolates were considered as MDRTB when resistant to at least INH and RIF (worldwide used as first-line antitubercular drugs), and as sensitive when susceptible to all anti-tubercular drugs. Therefore, mono-resistant strains for INH or RIF, as well as other poly-drug-resistance not including both INH and RIF were not considered for further experiments in our study. These isolates were randomly selected and collected from a repository located at the Central Public Health Laboratory "Prof. Gonçalo Muniz" (LACEN-BA), state of Bahia, Brazil. All isolates from the collection come from sputum of local patients which completed a demographic and clinical questionnaire after signing an informed consent form. All procedures were approved by the Human Ethical Committee of the Universidade Estadual de Santa Cruz (UESC; Ilhéus, Brazil), under protocol number 098/07.

The selected samples were cultivated on Löwenstein-Jensen agar (LJ). Biochemical tests, including nitrate reduction, niacin test and 68 °C catalase inhibition were conducted in order to classify these strains according to the Manual of Tuberculosis Bacteriology.[16] Drug sensitivity tests to INH, RIF, ethambutol (EMB), pyrazinamide (PZA), and streptomycin (SM) were performed using the Canetti (1969) multiple proportional dilution method.[17] The standard strains *M. tuberculosis* H37Rv and 636 were used as sensitive and MDRTB controls, respectively, as recommended by the Brazilian Ministry of Health.[16] The minimum inhibitory concentrations (MIC), defined as the lowest drug concentration showing complete inhibition of bacterial growth, were as follow: INH $\leq$ 0.2 μg/mL, RIF $\leq$ 1 μg/mL, EMB $\leq$ 5 μg/mL, SM $\leq$ 2 μg/mL, and PZA $\leq$ 25 μg/mL. The criterion for drug resistance was growth of $\geq$1% of the bacterial population on media containing the critical concentration of each drug.

Genotyping assay and amplification

Mycobacterial genomic DNA was extracted from the cultured strains according to Van Soolingen et al.[18] The quantity and purity was determined measuring spectrophotometric signals at 260 nm and 280 nm.

Assays were performed, in accordance to Espasa et al.,[19] in a spectrofluorometric thermal cycler (ABI Prism 7500, Applied Biosystems®, Carlsbad, CA, USA), using the primers and probes summarized in Table 1. The *Mycobacterium tuberculosis* Complex-Specific Insertion Sequence IS6110 was also amplified as internal control. Five amplification reactions were

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