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DIAGNOSTICS

Resistance to pyrazinamide in Russian *Mycobacterium tuberculosis* isolates: *pncA* sequencing versus Bactec MGIT 960

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SUMMARY

Resistance to pyrazinamide (PZA) may impact clinical outcome of anti-tuberculosis chemotherapy. PZA susceptibility testing using MGIT 960 is not reliable and little information is available on the prevalence of PZA resistance in Russia. A collection of 64 clinical isolates of *Mycobacterium tuberculosis*, including 35 multidrug resistant and extensively drug-resistant (MDR/XDR), was analyzed for PZA resistance using MGIT 960, Wayne test, and sequencing of PZA resistance genes *pncA*, *rpsA* and *panD*. In addition, we analyzed 519 MDR-TB strains for susceptibility to PZA by MGIT 960. Sequencing of *pncA* revealed 17 of 25 (68%) MDR strains and all 10 XDR strains harboring *pncA* mutations. A correlation of $\varphi = 0.81$ between MGIT 960 and *pncA* sequencing was observed. Mutations in *rpsA* and *panD* not associated with PZA resistance as defined by MGIT 960 were identified. We found 1 PZA-resistant strain without mutations in known PZA resistance genes. Almost 73% of MDR-TB strains isolated in Moscow, Russia, were PZA-resistant by MGIT 960 testing of 519 MDR-TB clinical isolates. Further studies are needed to determine the role of *rpsA* and *panD* mutations in possible low-level PZA resistance and to identify the molecular basis of new PZA resistance in the isolate without known PZA resistance mutations.

1. Introduction

Tuberculosis (TB) is a major global health problem with an estimated 9.0 million new TB cases and 1.5 million TB deaths in 2013 [1]. Pyrazinamide (PZA) is a key first-line drug in TB chemotherapy, which when introduced shortened modern anti-TB therapy to 6 months due to its sterilizing ability [2]. PZA is recommended for treatment of both drug-susceptible and drug-resistant TB cases [3].

PZA is a unique drug, lacking activity against replicating *Mycobacterium tuberculosis*, but killing only non-growing persister bacilli [4]. PZA is a prodrug, which needs to be activated by *M. tuberculosis* enzyme pyrazinamidase (PZase) into its active form – pyrazinoic acid (POA) [5]. Mutations in gene *pncA* encoding PZase are the major mechanism of PZA resistance in *M. tuberculosis* [5–7]. One of the probable mechanisms of action of PZA, is the disruption of membrane potential by the protonated POA, leading to the inhibition of major cell processes [4]. Recently, S1 ribosomal protein (RpsA) was identified as a molecular target for POA [8]. Mutations in *rpsA* gene were found in some PZA-resistant *M. tuberculosis* isolates lacking mutations in *pncA* [8,9]. RpsA is a key protein of trans-translation, a mechanism for saving stalled ribosomes or damaged mRNA, essential for dormant and persister cells [10,11]. Another gene involved in PZA resistance is *panD*, encoding an aspartate decarboxylase involved in pantothenate and coenzyme A biosynthesis [12].

The phenotypic testing for PZA susceptibility can show both false-positive and false-negative results [13]. Up to 40% of false-positive resistance can be obtained on Bactec MGIT 960 [14].

Abbreviations: H, isoniazid; R, rifampicin; E, ethambutol; PZA, Z, pyrazinamide; Eto, ethionamide; Am, amikacin; Cm, capreomycin; Lfx, levofloxacin; PZase, pyrazinamidase; POA, pyrazinoic acid.

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Simons et al. validated a method of using both Bactec MGIT 960 and *pncA* sequencing for determining PZA resistance [15]. Also *rpsA* sequencing should be applied for more confident results [8,9].

The PZA resistance may highly impact the clinical outcome in case of MDR-TB treatment [16]. Russia has a significant MDR-TB problem [17]: an estimated of 19% of new-TB cases and 49% of previously treated TB are MDR in Russia [1]. Although PZA resistance in MDR-TB (ZR-MDR-TB) in some parts of the world has been found to vary between 10 and 85% [16], and even some data was reported from different regions of Russia [18,19], no such information is available for MDR-TB strains in Moscow.

The aim of this study was to address resistance to pyrazinamide in *M. tuberculosis* isolates from Russia by different techniques (phenotypic DST using Bactec MGIT 960, the Wayne PZase test and *pncA*, *rpsA* and *panD* sequencing), and analyze the prevalence of ZR-MDR-TB in Moscow, Russia.

2. Materials and methods

2.1. Setting of the study

M. tuberculosis cultures were obtained from the Central TB Research Institute (CTRI), Moscow Russia, where the DST, the Wayne PZase test and the DNA isolation were conducted. *pncA*, *rpsA* and *panD* amplification and preparation for sequencing were done at the Vavilov Institute of General Genetics, Moscow, Russia. An approval by the Institute Ethical Committee of CTRI was obtained for this study on July 15, 2013 (receipt #2013-1).

2.2. Strain selection

Sixty-four *M. tuberculosis* isolates were selected for this study randomly among those isolated in CTRI in 2013 to form 2 groups based on their drug susceptibility: a larger group of 41 isolates resistant to at least 1 anti-TB drug, and a control group of 23 drug-susceptible isolates. In addition, we analyzed PZA susceptibility among 519 strains with MDR-TB derived from a total of 2257 clinical isolates from CTRI in the period from January 2011 to August 2014 by Bactec MGIT 960 DST (see below).

2.3. Strain storage and culture

M. tuberculosis cultures isolated from patients were stored at −80 °C. The cultures were thawed on ice and subcultured on the automatic growth detection system Bactec Mycobacterial Growth Indicator Tubes (MGIT) 960 (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's manual. All the positive cultures were confirmed to belong to *M. tuberculosis* genus. Ziehl-Neelsen stain was used to detect acid-fast bacilli (AFB). If AFB were detected in a positive MGIT 960, an immune-chromatography express-test “BD MGIT TBc ID” (Becton Dickinson, Franklin Lakes, NJ, USA) was carried out, according to the manufacturer's manual. Samples from Bactec MGIT 960 test-tubes were plated on blood agar and incubated for 24 h at 37 °C. If any growth was detected on blood agar in 24 h, the culture was considered contaminated and eliminated from the study.

2.4. M. tuberculosis DNA isolation

Genomic DNA was isolated from *M. tuberculosis* cultures on a robotized system EVO 150 (Tecan, Männedorf, Switzerland) with “M-Sorb-Tub-Avtomat” kit (Syntol, Moscow, Russia).

2.5. Drug susceptibility tests (DST)

First line drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) susceptibility tests were carried out on the automated system Bactec MGIT 960 according to the manufacturer manual, “SIRE” and “PZA” kits were used. The susceptibility of mycobacteria to second-line drugs (ethionamide, amikacin, capreomycin and levofloxacin) (Sigma, St. Louis, MO, USA) was performed as recommended in Ref. [20].

2.6. PZase activity assay

The PZase activity assay (The Wayne PZase test) [21] was performed with a modification described by Zhang et al. [12], which allows for conducting the test more rapidly in Eppendorf microtubes in liquid medium. *M. tuberculosis* H37Rv was used as positive control and *Mycobacterium bovis* BCG which carries a mutation in *pncA* [5] was used as a negative control.

2.7. pncA, rpsA and panD amplification and sequencing

The gene *pncA* was amplified by PCR and sequenced with primers pncAf (5′-GATCTATCCCGCCGGTTGGGTG-3′, position −137) and pncAr (5′-CCGGTGAACAACCCGACCCAG-3′, position +661). The gene *rpsA* was amplified with primers rpsAf (5′-CCGTCGAGTAGCCTCGTCAGGTA-3′, position −69) and rpsAr (5′-ACGTCGTGGACAGCAACGACTTC-3′, position +1532), two additional primers were synthesized for sequencing – rpsAmf (5′-GGCACCATCCGAAAGGGTGT-3′, position +625) and rpsAmr (5′-GAAACCCGCTCACGGTCCAT-3′, position +818). The gene *panD* was amplified and sequenced with primers panDf (5′-GGCTGCTGGACAACATTGCGATT-3′, position −97) and panDr (5′-GCACGACCTTTGCGTGCTCTTTC-3′, position +504). All primers were designed using Primer-BLAST online-tool [22]. The PCR products were purified with GeneJET PCR Purification kit (Thermo Scientific, Waltham, MA, USA) and Sanger sequenced.

2.8. Statistical analysis

Diagnostic accuracy of the Bactec MGIT 960 was assessed through the binary classification test treating *pncA* sequencing as the gold standard/reference for the assessment. Two main measures of binary classification test – sensitivity and specificity – reflect the ability of the test to properly assign true positive and true negative cases. The classification function is intended to assess the method's ability to properly assign cases to the respective class. Method's sensitivity and specificity constitutes the overall method's accuracy – the share of correctly classified cases and the inverse measure represents the error in prediction of the method.

Complementary test's measures – positive and negative predictive values – serve as the additional measure of diagnostic test performance representing the proportions of correctly classified cases within each class. Evidence shows the relatively low negative predictive value representing relatively high number of false negative cases provided by diagnostic test.

False discovery rate serves as the method for controlling the expected proportion of incorrectly rejected the null hypothesis, i.e. the “false discoveries”. False omission rate, inversely, represents the expected proportion of incorrectly accepted the null hypothesis. The results showed the higher expected frequency to omit the tested case, i.e. to miss hypothesis that should be normally rejected, which may lead to false negative results.

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