pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* clinical isolates from the southeast region of Brazil

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**Objectives**: To investigate the presence of mutations in the *pncA* gene in 31 pyrazinamide-resistant *Mycobacterium tuberculosis* and 5 susceptible strains. MICs and pyrazinamidase (PZase) activity were also determined.

**Methods**: All 36 *M. tuberculosis* clinical isolates were genotyped by mycobacterial interspersed repetitive units (MIRUs) and most were also typed by spoligotyping. The MIC value necessary to inhibit 99% of the resistant mycobacterial isolates was determined by microplate Alamar Blue assay (MABA) and by Löwenstein–Jensen assay (LJA). The PZase activity was measured by pyrazinamide deamination to pyrazinoic acid and ammonia, and the entire *pncA* sequence including the 410 bp upstream from the start codon was determined by DNA sequencing of purified PCR products.

**Results**: Of the 31 isolates resistant to pyrazinamide, 26 (83.9%) showed at least one mutation in the *pncA* gene or in its putative regulatory region. Among the 22 different mutations detected in the *pncA* gene and in its regulatory region, 9 (40.9%) mutations (consisting of six substitutions, two insertions and one deletion) have not been described in previous studies. Three pyrazinamide-resistant isolates, confirmed by MIC varying from 800 to 1600 mg/L, carried the wild-type *pncA* sequence and retained PZase activity.

**Conclusions**: These results contribute to the knowledge of the molecular mechanism of pyrazinamide resistance in Brazil and also expand the profile of *pncA* mutations worldwide. The MABA was successfully used to determine the MICs of pyrazinamide.

Keywords: Alamar Blue, MIRU, pyrazinamidase

**Introduction**

Pyrazinamide, a nicotinamide analogue, is an essential first-line drug in combination with isoniazid and rifampicin for tuberculosis short-course chemotherapy. Pyrazinamide appears to kill at least 95% of the semi-dormant bacterial population persisting in a low-pH environment since its activity is present only in the acidic environment found in active inflammation.¹² Brazil has the 15th highest number of tuberculosis cases in the world.⁴ In the most recent nationwide investigation, resistance to one or more drugs was reported in 10.6% of cases. The primary drug resistance rate was 8.6%, and the acquired resistance rate was 22.1%.⁴ Owing to the fact that conventional culture-based methods for pyrazinamide susceptibility testing require ≥2 months (from the isolation of mycobacteria to the susceptibility test) and demonstrate inconsistent results among laboratories,⁵ a

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nationwide investigation into pyrazinamide resistance in Brazil is difficult.

Pyrazinamide is a prodrug that must be activated by bacterial pyrazinamidase (PZase) to the active form pyrazinoic acid (POA), which is toxic to Mycobacterium tuberculosis. Although a specific target for POA remains unknown, it has been suggested that POA accumulation results in a pH reduction which leads to a non-specific inhibitory effect on cellular metabolism. Recently, it has been shown that POA can disrupt M. tuberculosis membrane potential, affecting the transport function at acid pH.

*M. tuberculosis* PZase enzyme is encoded by the *pncA* gene. Mutations in the *pncA* gene causing pyrazinamide resistance have been well characterized and are located along the entire *pncA* gene open reading frame as well as in its putative regulatory region. Mutations located in the *pncA* gene may be responsible for different protein conformations, leading to a failure of the key-lock recognition mechanism between the enzyme and its substrate. However, if the mutation occurs in the putative gene regulatory region, the RNA polymerase may not bind the promoter, thus preventing gene transcription. In both cases, the PZase will not be able to activate pyrazinamide.

Mutations associated with pyrazinamide resistance *in vitro* include nucleotide substitution (both transversion and transition), leading to codon changes and subsequent amino acid substitution, and insertions and deletions of one or more bases, causing a frameshift modification in the gene open reading frame. The complete deletion of the *pncA* gene has also been described. The mechanism for such a wide variety of mutations is not yet clear; therefore it is possible that the *pncA* gene is located in a hotspot region for mutation in the mycobacterial genome. Some authors have mentioned the detection of resistant strains that maintain PZase activity, suggesting that other mechanisms may be involved in pyrazinamide resistance.

Conventional pyrazinamide susceptibility testing by agar proportion or by Lowenstein–Jensen proportion methods is labour intensive and they may exhibit high discordance rates among different laboratories. A novel microplate method (the microplate Alamar Blue assay (MABA)) might be a good choice for drug susceptibility analysis in *M. tuberculosis*, given its rapidity, low technology and cost. Other systems, such as the BACTEC 460TB system, BACTEC MGIT 960 Pyrazinamide and VersaTREK (formerly ESP Culture System II), are commercially available, including for pyrazinamide resistance detection, but they are expensive and impractical to use in developing countries where the prevalence of tuberculosis is high.

To improve our knowledge of pyrazinamide resistance mechanisms in *M. tuberculosis* strains isolated in Brazil, we screened the *pncA* gene, measured the PZase activity and determined the MICs of pyrazinamide in 36 (5 pyrazinamide-resistant and 31 pyrazinamide-resistant) *M. tuberculosis* clinical isolates from São Paulo and Paraná states, Brazil.

**Materials and methods**

*Mycobacterial isolates, drug susceptibility testing and PZase assay*

The 31 pyrazinamide-resistant *M. tuberculosis* isolates (including 17 multidrug-resistant isolates and 5 resistant to pyrazinamide only) and 5 pyrazinamide-susceptible *M. tuberculosis* isolates examined in this study were obtained from the culture collections of the Instituto Adolfo Lutz of Ribeirão Preto, the Instituto Adolfo Lutz of Sorocaba and the Instituto Clemente Ferreira in São Paulo state, and the Clinical Bacteriology Laboratory, Department of Clinical Analysis, State University of Maringa, Paraná state, Brazil.

Susceptibility to pyrazinamide was determined by the 10% proportion method in Löwenstein–Jensen medium (Difco Laboratories, Detroit, MI, USA; pH 5.2) containing pyrazinamide 100 mg/L, which is the critical concentration. All isolates were maintained by subculture on Löwenstein–Jensen medium at 8°C and in Middlebrook 7H9 (Difco Laboratories) with oleic-acid-albumin–dextrose–catalase (OADC) enrichment (BBL/Becton-Dickinson, Sparks, MD, USA) at –20°C.

All the 36 *M. tuberculosis* isolates were typed by the mycobacterial interspersed repetitive units (MIRUs) method in order to confirm the strains individuality. The PZase activity was assayed qualitatively as described by Wayne with Dubos broth medium (Difco Laboratories) containing pyrazinamide 100 mg/L.

**Determination of pyrazinamide MIC**

The MIC values were determined using solid Löwenstein–Jensen medium and a Middlebrook 7H9 broth MABA. For the Löwenstein–Jensen assay (LJA), *M. tuberculosis* isolates were first cultured in Middlebrook 7H9 supplemented with OADC for 10 days. A 10^-2 culture dilution was prepared in 0.1% Tween 80 (Sigma, St. Louis, MO, USA) and plated in Löwenstein–Jensen medium (pH 5.2) containing different concentrations of pyrazinamide (from 12.5 to 800 mg/L). The strains were also cultured in Löwenstein–Jensen medium without pyrazinamide at pH 5.2 (low-pH growth control) and pH 6.8 (strain growth control). The tubes were incubated for 28 days at 35°C. The MIC was defined as the lowest pyrazinamide concentration that inhibited >99% of the growth when compared with the control without pyrazinamide at pH 5.2. Pyrazinamide 100 mg/L was considered the critical concentration, as approved for drug susceptibility testing in Brazil.

In the MABA, the isolates were cultured in Middlebrook 7H9 supplemented with OADC (pH 6.0) containing pyrazinamide concentrations ranging from 12.5 to 1600 mg/L. The critical concentration was pyrazinamide 100 mg/L, as recommended for the BACTEC method. The MIC was defined as the lowest pyrazinamide concentration that prevented a colour change from blue to pink on visual observation. In both the MABA and LJA methods, the pyrazinamide-resistant *M. tuberculosis* strain H₃₇Rv (ATCC 27294) was used as control.

**Genomic DNA preparation, PCR and DNA sequencing**

Chromosomal DNA was extracted from isolates cultured for 30 days at 35°C in Lowenstein–Jensen medium as described by Gonzales-y-Merchand et al. and modified by Cardoso et al. A 1200 bp segment including the entire *pncA* gene open reading frame as well as its putative regulatory region was amplified by PCR using forward (pncAS, 5’-CCGGGAGCAGCATTACGATACG-3’) and reverse (pncAR, 5’TGGAAAGGCAACCCGAGA-3’) primers (GenBank accession number BX845278). PCR amplifications were carried out in a thermocycler system PTC 200 (MJ Research Inc., Waltham, MA, USA). Reaction mixtures contained 1.0 μL of template DNA, 200 μM deoxynucleotide triphosphates, 100 nM PCR primers, 1 U Taq DNA polymerase (Biotools do Brasil Ltda., Engenho Novo, RJ, Brazil) and 5% (v/v) DMSO in 1x PCR buffer. The reaction mixtures were incubated at 94°C for 5 min, followed by 25 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, followed by a final extension of 5 min at 72°C. The PCR products were purified using Nucleospin gel cleaner (Macherey-Nagel, Düren, Germany).

**Mycobacterial isolates, drug susceptibility testing and PZase assay**

M. tuberculosis was maintained on Middlebrook 7H9 supplemented with OADC (pH 6.0). The control without pyrazinamide at pH 5.2 served as control. The MIC was defined as the lowest pyrazinamide concentration that inhibited >99% of the growth when compared with the control without pyrazinamide at pH 5.2. Pyrazinamide 100 mg/L was considered the critical concentration, as approved for drug susceptibility testing in Brazil.
1 min at 64°C and 1 min at 72°C, completed by an additional 10 min at 72°C. The pncA PCR products were run on Tris-borate-EDTA-0.8% agarose gel and purified using a Concert Rapid Gel Extraction kit (Gibco BRL, Los Angeles, CA, USA) according to the manufacturer’s instructions. The purified PCR products were sequenced in an ABI Prism 3100 DNA analyzer (Applied Biosystems Inc., Foster City, CA, USA). Sequencing reactions were carried out with the Big Dye Terminator Cycle Sequencing DNA sequencing kit (Applied Biosystems Inc.) using the primers pncAS and pncAR and a new set of internal primers: pncA1 (5'-GCCAACAGTTCATCCCCGTT-3'), pncA3 (5'-CTTCGAGGCGCTGTACGCT-3'), pncA3R (5'-CATCACGTCGTTGCGAATCCAA-3') and pncA4R (5'-CCCGAAGTCTTAGGGAGGCGCT-3') (Figure 1) (GenBank accession number BX842578). The sequence data were analysed using ABI Prism DNA Sequencing Analysis Software version 3.6 NT (PE Applied Biosystems Inc.) and the results were compared with the pncA published sequence (GenBank accession no. U59967).

Results and Discussion

All the 31 resistant and 5 susceptible M. tuberculosis isolates examined were typed by MIRU, and 25 of these isolates (including resistant and susceptible ones) were also typed by spoligotyping. Among the 25 strains typed by spoligotyping, 10 different patterns could be detected. Six of the spoligotype patterns occurred only once, whereas four patterns occurred in clusters ranging from two to nine isolates. The clustered isolates could be separated in some cases by the difference in MIRU profile and in other cases by the presence/absence or the type of pncA mutation. Two isolates presented the same MIRU profile but different spoligotyping patterns; one of these was pyrazinamide susceptible and the other was pyrazinamide resistant with an A3P pncA mutation. Six isolates presented the same spoligotyping pattern and MIRU profile but five different pncA mutations. Only two of these isolates had the same genotype according to both methods and the same mutation (314insC). All the 11 isolates that were typed only by the MIRU genotyping assay presented different profiles (Table 1).

For all the isolates, whether susceptible or resistant to pyrazinamide, the MIC value was determined by the MABA and LJA methods. Despite the fact that we analysed a small number of isolates with different pH media (5.2 for LJA and 6.0 for MABA), it was possible to verify that the isolates exhibited similar MIC values when tested by MABA and LJA. Only one exception occurred, with an isolate that presented an MIC of >1600 mg/L for MABA and 400 mg/L for LJA. It is possible that this isolate was more sensitive to low pH than the others and had a lower growth rate in the LJA medium (Table 1). There were no strains that presented an MIC value compatible with susceptibility for one method and with resistance for the other, and no false-positive or false-negative results were observed. In our study the MABA showed many advantages over the LJA because it is simple and fast (7 days for MABA and 28 days for LJA after the mycobacteria isolation) and the mycobacterial growth can be quantified by fluorometry, spectrophotometry or visual inspection.31 In order to confirm these interesting results, further studies with a larger number of M. tuberculosis isolates should be carried out.

In this study we have not observed the relationship between pyrazinamide MIC values and mutation frequency or position. However, it was observed that every isolate that presented a pncA alteration lacked PZase activity (Table 1). This suggests that the enzymatic activity is very sensitive to sequence alterations in any protein region.

Our DNA sequencing results support previous findings that most pyrazinamide-resistant M. tuberculosis strains have alterations in pncA16,17,20,23,35,36 and indicate that pncA mutation is the major pyrazinamide resistance mechanism in the Brazilian M. tuberculosis isolates. Among the pyrazinamide-resistant isolates, 26 (83.9%) exhibited 22 different changes in pncA nucleotide sequence (Table 1). The mutations in pncA included seventeen nucleotide substitutions causing amino acid change in 19 isolates, one stop codon in 1 isolate, frameshift mutations caused by two insertions (314insC and 495insC) in 3 isolates and one deletion (315delC) in 1 isolate. The C28T mutation created a stop codon, Q10STOP, resulting in premature protein synthesis termination. Among the 22 different mutations found in the pncA gene and in its putative regulatory region, 9 mutations have not been described in previous studies.12–21,23,35–43 Six of these mutations are single-nucleotide substitutions that occurred in seven isolates; four of these nucleotide substitutions are transversions, and two are transitions. The remaining three mutations are frameshifts; the deletion 135delC was described in one isolate, the insertion 314insC was detected in two isolates, and the insertion 495insC was found in one isolate.

It is generally considered that mutations leading to pyrazinamide resistance are scattered along the pncA gene.5,20 However, some authors have mentioned a certain degree of conservation of pncA mutations at amino acid residues 3–17, 61–76 and 132–142 in the PZase protein.14,18,36 We found that 68.2% of the mutations are located in regions that are different from those reported previously.11,14,36,40 Mutations were observed in the regions between residues 45 and 59 and between residues 96 and 120 in 22.7% of cases each, and 18.2% of the mutations were found between residues 155 and 172. In another pyrazinamide resistance study carried out in Brazil, 61.1% of the nucleotide substitutions occurred in the region between residues 128 and 171,40 but our findings indicate that 45.5% of the mutations occurred in different regions from those mentioned in that study. The fact that these strains were isolated in distinct regions in Brazil may indicate that environmental factors influence the distribution of mutations along the pncA gene. One mutation was located at nucleotide –11 (A>G) in the pncA gene putative regulatory region, and its relation with pyrazinamide resistance has been described previously.15,17,20,23,35,36
**pncA mutations in pyrazinamide-resistant M. tuberculosis**

Table 1. Pyrazinamide MIC, pyrazinamidase activities, genotyping profiles and pncA mutations in 31 pyrazinamide-resistant and 5 pyrazinamide-susceptible *Mycobacterium tuberculosis* isolates and in the H37Rv strain

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>PZA MICs (mg/L)</th>
<th>PZase activity</th>
<th>pncA mutations</th>
<th>Genotyping</th>
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MABA: microplate Alamar Blue assay; PZA: pyrazinamide; PZase: pyrazinamidase; WT: wild type; NP: not performed; NA: not applicable; NG: no growth; (+): positive; (–): negative.

<sup>a</sup>Novel mutations.

<sup>b</sup>Q10<sup>+</sup> means Q10 to STOP point.

One pyrazinamide-resistant isolate failed to show a pncA gene PCR product despite the integrity of the extracted DNA (observed in the agarose gel). However, the PCR for the MIRU genotyping was successful. A mutation in the primer target can be discounted: the pncA gene amplification was tried with another set of primers (pncA 1 mentioned above, and pncA 5R: 5′-TCGCTCACTACA-TCACCGGC-3′) that were more internal than those used before and yet no PCR product was obtained. The amplicon obtained by the pncA gene amplification using the pncA 1 and pncA 5R primers is 751 bp long (Figure 1). The absence of amplified product suggests that the pncA gene may be deleted in this strain.

The mutations –11A>G, G203T, G314A and 314insC were found in two mycobacterial isolates each. The MIRU genotyping analysis demonstrated that the same MIRU type occurred only for the isolates carrying the 314insC mutation. This could illustrate the active transmission of resistant strains since these strains were isolated in close regions of São Paulo state. The remaining isolates that had the same mutations showed different MIRU profiles, indicating that different strains have acquired the same mutations.

Three pyrazinamide-resistant isolates carried the wild-type pncA sequence and retained PZase activity. This finding supports the hypothesis that other mechanisms may be involved in
pyrazinamide resistance—possibly alteration in pyrazinamide uptake, increased POA active efflux or mutations leading to the modification or amplification of an unknown POA target. As expected, the five pyrazinamide-resistant isolates had no mutations in the pncA gene or in its regulatory region and displayed PZase activity (Table 1).

Unfortunately, six M. tuberculosis pyrazinamide-resistant isolates received from the culture collections were non-viable and the MIC and PZase activity could not be determined. However, pncA gene sequencing was carried out for these strains.

The results presented in this study contribute to the knowledge of the molecular mechanisms of pyrazinamide resistance in Brazil as well as to expanding the known profiles of pncA mutations worldwide. The MABA method seems to have great potential and needs further evaluation as a candidate for rapid determination of M. tuberculosis susceptibility to pyrazinamide.

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Transparency declarations

None to declare.

References


Barco et al.