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pncA Mutations in Pyrazinamide-Resistant *Mycobacterium tuberculosis* Isolates in Portugal

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The nucleotide sequences of the *pncA* genes within 55 multidrug-resistant pyrazinamide-resistant *Mycobacterium tuberculosis* clinical isolates were determined. Fifty-three out of the 55 isolates were pyrazinamidase (PZase) negative. Four strains contained a wild-type *pncA* gene, and PZase activity was undetectable in two of these strains. Seven of the 18 identified *pncA* mutations found have not been described in previous studies.

Portugal remains the country with the highest rate of notified cases of *Mycobacterium tuberculosis* in the European Union. In 2002, the Portuguese Health Authorities reported a tuberculosis (TB) incidence of 39.5 cases per 100,000 people nationwide and that 2.3% of primary TB cases involved multidrug resistance (MDR-TB cases) (Programa Nacional de Controlo da Tuberculose, Ponto da situação epidemiológica e de desempenho em 2002, available at the Direcção Geral de Saúde website [http://www.dgsaude.pt]). According to the National Laboratory Surveillance System for Resistance TB, 4,170 isolates from notified cases were tested for drug susceptibility between 2000 and 2001. Of these isolates, only 56.5% were tested for pyrazinamide (PZA) resistance (3). One hundred sixty-two isolates (6.9%) were resistant to PZA, including 1.3% that were monoresistant.

Recently, the *M. tuberculosis* pyrazinamidase (PZase) gene (*pncA*) was identified (14). Mutations in the *pncA* gene are considered the major mechanism of PZA resistance in *M. tuberculosis* (14), but resistant strains containing the wild-type gene have been described, suggesting additional resistance mechanisms besides a lack of PZase activity (16).

In an attempt to define the molecular basis of PZA resistance and to expand the profile of *pncA* mutations worldwide, we determined the nucleotide sequences of the *pncA* genes of 55 clinical isolates of *M. tuberculosis* initially found to be resistant to PZA and compared the PZase activities of these strains. These strains, isolated in the years 2000 and 2001, were collected in several hospital units in the Lisbon, Portugal, area. Fifty-five MDR-TB isolates and eight susceptible *M. tuberculosis* isolates were tested for susceptibility to PZA by the BACTEC MGIT 960 method with BACTEC MGIT 960 PZA test medium at a reduced pH of 5.9 and a 100-μg/ml concentration of PZA (Becton Dickinson) according to the manufacturer's manual. Almost all (53 of 55) PZA-resistant isolates were also PZase negative, and there was production of PZase in all PZA-susceptible isolates.

The 55 isolates had previously been subjected to IS*6110* restriction fragment length polymorphism (RFLP-IS*6110*) analysis with restriction enzyme PvuII and a 245-bp IS*6110* probe according to the international standard recommendations (17) and analyzed with Bionumerics software (Applied Maths, Inc., Kortrijk, Belgium).

Qualitative PZase activity analysis was performed with Dubos broth agar containing 100 μg of PZA/ml and 2 mg of sodium pyruvate/ml as described by Wayne (18).

Mutations in the sequence of the *pncA* gene were identified by comparison with the wild-type *M. tuberculosis* H37Rv *pncA* gene sequence (14) by using PCR sequencing. The entire *pncA* open reading frame, as well as 124 bp of the upstream sequence and 59 bp of the downstream sequence, was amplified by PCR by using the primers and conditions described by Morlock et al. (11). A 744-bp PCR product was generated with primers *pncA*-11 and *pncA*-8. Amplifications were carried out with a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer Corp., Foster City, Calif.). The PCR-purified PCR product was subjected to a sequencing reaction by using a BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase. The *pncA* amplicons were sequenced by using four internal primers according to Morlock et al. (11): *pncA*-10 and *pncA*-2R to sequence nucleotide residues 20 to 406 of the 744-bp amplicon, and *pncA*-6 and *pncA*-9 to sequence residues 318 to 720. Sequences were analyzed with BioEdit software (version 5.0.9.1; T. A. Hall Software). Every time a strain had no mutation in the *pncA* gene or in the upstream and downstream sequences, the sequence analysis was repeated.

As in other studies (2, 6, 9–11, 14–16), the frequency of *pncA* mutations in the Portuguese PZA-resistant isolates analyzed was very high, 94%. The results obtained are presented in Table 1. Seven of the 18 identified *pncA* mutations have not been described in previous studies (2, 5, 7, 8, 10, 11, 14–16). There was a thymine-to-cytosine point mutation at position 359, resulting in proline instead of leucine, in 26% of the isolates, making this the most common type of *pncA* mutation in this study. These 13 isolates represented at least two different clones defined by RFLP-IS*6110* analysis (Fig. 1), indicating that they are actually different strains which happened to acquire the same type of mutation. Since *pncA* mutations occur randomly along the whole gene, apparently the same mutations of the *pncA* gene would rarely be present in unrelated isolates. Indeed, the two RFLP patterns belong to cluster A

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TABLE 1. *pncA* nucleotide and amino acid changes in PZA-resistant *M. tuberculosis* clinical isolates from Portugal

Mutation site	Nucleotide change	Amino acid change[a]	No. of isolates
−11	A→G	Mutation in promoter	2
2	T2C	Met1→Thr*	1
137	C137T	Ala46→Val	1
152	A152C	His51→Pro	1
193	A insertion	Frameshift	1
212	A212G	His71→Arg	3
250	C insertion	Frameshift*	4
286	A286G	Lys96→Glu	3
290	T insertion	Frameshift*	1
359	T359C	Leu120→Pro*	13
374	T374G	Val125→Gly*	8
391	GG insertion	Frameshift	1
395	G395A	Gly132→Asp	1
406	G406C	Asp136→His	1
421	C421T	Gln141Stop	1
439	CG insertion	Frameshift*	3
476	T476C	Leu159→Pro*	5
511	G511A	Ala171→Thr	1

[a] An asterisk indicates a new mutation not reported in previous studies (2, 5, 7, 8, 10, 11, 14–16).

(13) now known as cluster Lisboa, a cluster of highly related strains that are responsible for the majority of MDR-TB in Portugal. The fact that this most common mutation has not been described in other studies is in agreement with the observation that cluster Lisboa is almost limited to Portugal, also being found in some neighboring countries (RFLP-IS*6110* patterns of these strains were compared with those in the international MDR-TB database established at the Rijksintituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands).

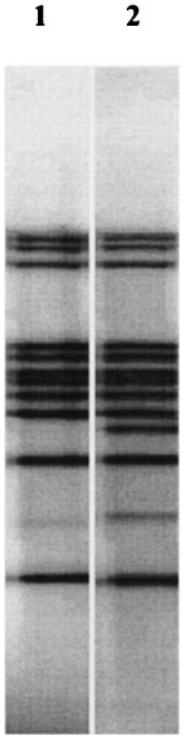

FIG. 1. RFLP-IS*6110* patterns of strains from cluster Lisboa. Lane 1, Lisboa3; lane 2, Lisboa2.

Four strains had no mutation in the *pncA* gene, even though they were resistant to PZA. Further sequence analysis of the *pncA* upstream region, which contains the putative *pncA* promoter, also failed to reveal any mutations for these strains. Two of these strains were positive for PZase activity, considered a rare event because we have found only one report of a case of a PZA-resistant strain without a *pncA* mutation (2). This finding indicates a possible alternative mechanism of PZA resistance that does not affect PZase activity or expression but is not very important in PZA resistance. These two strains present similar RFLP patterns and belong to a two-strain cluster. For the other two strains without *pncA* mutations, PZase activity was undetectable. We retested these four strains without *pncA* mutations for PZA susceptibility by using the BACTEC MGIT 960 PZA with 100-, 300-, and 900-μg/ml concentrations of PZA. PZase activity was also retested. The two PZase-positive strains had relatively low PZA MICs (100 to 300 μg/ml). One of the initial resistant strains confirmed to be PZase negative by the assay was found to be resistant to a 100-μg/ml concentration of PZA but susceptible when 300 μg/ml was used. This result may be an indication that a markedly low MIC (100 μg/ml) should be used for determining that these strains are truly phenotypically resistant. These findings support some previous reports (2, 4) that suggested the use of 300 instead of 100 μg of PZA per ml in the single-concentration qualitative test. It is also possible that this strain remained negative for PZase activity due to the poor sensitivity of the method used, as the relationship between PZA susceptibility and positive PZase activity is well established (19). The other PZase-negative strain had an MIC of over 900 μg/ml. PZA-resistant strains that were PZase negative were also found by others (1, 2, 8, 11, 12). Cheng et al. (2) suggested that PZA resistance is due to a *pncA*-regulatory gene and that mutation of this gene can affect the expression of *pncA*. Nevertheless, it can be noted that there was a strong correlation between loss of PZase activity and PZA resistance, making the determination of PZase activity an indirect measure of PZA susceptibility.

In this study, we have shown that most of the PZA-resistant *M. tuberculosis* strains analyzed, isolated in Portugal, carry mutations in the *pncA* gene, despite the fact that the number of strains in which no mutations were found still makes phenotypic tests necessary for the detection of PZA resistance. Although the high diversity of *pncA* mutations already described could be useful as a marker in tracing the outbreak or transmission of PZA-resistant *M. tuberculosis* isolates, one should be cautious in interpreting *pncA* mutation results for epidemiologic purposes.

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