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Short communication

Peruvian and globally reported amino acid substitutions on the *Mycobacterium tuberculosis* pyrazinamidase suggest a conserved pattern of mutations associated to pyrazinamide resistance

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ABSTRACT

Resistance to pyrazinamide in *Mycobacterium tuberculosis* is usually associated with a reduction of pyrazinamidase activity caused by mutations in *pncA*, the pyrazinamidase coding gene. Pyrazinamidase is a hydrolase that converts pyrazinamide, the antituberculous drug against the latent stage, to the active compound, pyrazinoic acid. To better understand the relationship between *pncA* mutations and pyrazinamide resistance, it is necessary to analyze the distribution of *pncA* mutations from pyrazinamide resistant strains.

We determined the distribution of Peruvian and globally reported *pncA* missense mutations from *M. tuberculosis* clinical isolates resistant to pyrazinamide. The distributions of the single amino acid substitutions were compared at the secondary structure domains level. The distribution of the Peruvian mutations followed a similar pattern as the mutations reported globally. A consensus clustering of mutations was observed in hot-spot regions located in the metal coordination site and to a lesser extent in the active site of the enzyme.

The data was not able to reject the null hypothesis that both distributions are similar, suggesting that *pncA* mutations associated to pyrazinamide resistance in *M. tuberculosis*, follow a conserved pattern responsible to impair the pyrazinamidase activity.

1. Introduction

Tuberculosis (TB) is the major cause of deaths due to a single infectious disease in the world causing 1.6 million deaths annually (Corbett et al., 2003). Pyrazinamide (PZA) is an important first-line drug for TB treatment and appears to be the most potent for killing *Mycobacterium tuberculosis* (MTB) in its latent stage (Girling, 1984; Mitchison, 1985; Heifets and Lindholm-Levy, 1992).

Although PZA is important in TB treatment, mechanisms of resistance are not completely understood (Zhang and Mitchison, 2003). In consensus it is accepted that PZA has to be converted to pyrazinoic acid (POA) by bacterial pyrazinamidase (PZAse), to perform its bactericidal activity against MTB. POA is pumped out of the mycobacterium, and in the presence of an acidic external pH it is protonated. The protonated POA re-enters the mycobacterium and releases the proton, acidifying the mycobacterial cytoplasm. The cytoplasm acidification together with the accumulation of POA lethally disrupts the mycobacterial membrane permeability and transport (Zhang et al., 1999, 2003). The major mechanism of PZA-resistance is considered to be the loss of PZAse activity that is linked to mutations in *pncA*, the PZAse coding gene. The correlation between the presence of *pncA* mutations and PZA-resistant phenotype has been reported between 75% and 97% (Hirano et al., 1997; Scorpio et al., 1997; Sreevatsan et al., 1997; Mestdagh et al., 1999; Cheng et al., 2000; Hou et al., 2000; Park et al., 2001), despite that the PZAse function determined from recombinant enzymes, explained 27.3% of the variability of PZA-resistance level determined by the percentage of growing in BACTEC (Sheen et al., 2009a). This association together with the fact that silent *pncA* mutations are rare is the basis for predicting PZA-resistance based on the identification of *pncA* mutations (Scorpio et al., 1997; Sheen et al., 2009b).

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No crystal structure of MTB PZAse is available, but Du et al. (2001) developed an homology theoretical structure model based on the nicotinamidase/PZAse of *Pyrococcus horikoshii* as a template. In addition, we developed a refined theoretical model available in the Protein Data Bank (PDB ID: 1X8A). According to both models, the secondary structure of the PZAse includes 4 alpha helices, 6 beta strands, and 10 loops. The catalytic center includes an active site (AS) comprised of the residues D8, A134, and C138, and a metal coordination site (MCS) comprised of D49, H51 and H71. The *P. horikoshii* nicotinamidase/PZAse is 37% similar to MTB PZAse with 180 amino acids length; therefore, according to bioinformatics principles these structures should be highly similar, although some significant structural variations may exist in this model compared to the yet undetermined structure of MTB.

The aim of this work was to determine and compare the distribution, at the secondary structure level, of amino acid substitutions of *M. tuberculosis* PZAse from PZA-resistant strains found in Peru with those reported globally.

2. Methods

2.1. pncA mutations

In previous studies we analyzed 108 *M. tuberculosis* clinical strains PZA-resistant confirmed by BACTEC and the PZA susceptible reference strain H37Rv (Sheen et al., 2009a,b). We found that 74 strains (69%) had a single amino acid substitution in the PZAse, comprising 23 mutations: L4S, G24D, D12A, D12G, C14G, Q10R, Y34D, K48T, D49N, G78C, P54L, T76P, P62L, H51R, L85P, H71Y, F94L, L116P, G105D, W119L, D136G, H137P, T135P. Among these, 7 were mutations not previously reported elsewhere: C14G, G24D, K48T, D49N, F94L, L116P, W119L. Six more PZAse substitutions associated to PZA-resistant strains confirmed by BACTEC were included in the Peruvian sample: V7I, V7F, V139A, A134V, T142P, V155A (Escalante et al., 1998). The localization of the mutations within the secondary structure and the compromise of the AS or the MCS were determined.

Globally reported *pncA* mutations ($n = 210$) were obtained by compilation of the missense mutations associated with PZA-resistance, reported in the literature until December 2008 (Hirano et al., 1997; Scorpio et al., 1997; Sreevatsan et al., 1997; Lemaitre et al., 1999; Marttila et al., 1999; Mestdagh et al., 1999; Brown et al., 2000; Cheng et al., 2000; Hou et al., 2000; Bishop et al., 2001; Lemaitre et al., 2001; Park et al., 2001; Endoh et al., 2002; Lee et al., 2002; Suzuki et al., 2002; Huang et al., 2003; Miyagi et al., 2004; Portugal et al., 2004; Post et al., 2004; Tracevska et al., 2004; Denkin et al., 2005; McCammon et al., 2005; Rodrigues Vde et al., 2005; Barco et al., 2006; Martin et al., 2006; Aragon et al., 2007; Sekiguchi et al., 2007; Jureen et al., 2008; Mphahlele et al., 2008).

2.2. Distribution of PZAse amino acid substitutions and analysis

The Peruvian and global amino acid substitutions associated to PZA-resistance were mapped into the PZAse amino acid sequence. The mapping was determined according to the localization of the substitutions within each of the 20 domains of the protein secondary structure: 4 alpha helices ($\alpha1$–$\alpha4$), 6 beta strands ($\beta1$–$\beta6$) and 10 loops (L1–L10). Given the importance of the AS and the MCS for the PZAse function, mutations in these regions were especially recognized. The AS region is included approximately within the secondary structure domains L2 (residues 8–25) and L7 (residues 132–139) and the MCS is included approximately within L4 domain (residues 49–89) as proposed by Du et al. (2001).

To compare the distributions of amino acid substitutions across the secondary structure domains between the Peruvian and the globally reported samples, the frequency of substitutions in each secondary structure domain was estimated by normalizing the number of mutations found in the domain with the total number of mutations reported in the PZAse.

The difference of mutation frequencies between matched secondary structure domains (Peruvian versus globally reported samples) was tested with the signed-rank test against the null hypothesis that the difference equals zero. The non-parametric Spearman correlation was used to test the independency of the Peruvian and global lists of frequencies.

3. Results

The 210 worldwide and the 29 Peruvian PZAse amino acid substitutions associated to PZA-resistant MTB clinical isolates were non-uniformly distributed across the protein sequence. The two distributions appeared qualitatively very similar showing an identical clustering pattern (Fig. 1). In both the Peruvian and the globally reported samples, the highest proportion of mutations was located in the loop region between the $\beta2$ and the $\beta3$ strands, which comprises the MCS region. Similarly, a high concentration of mutations occurs in the regions $\beta1$–$\alpha1$ and $\beta4$–$\alpha3$, which comprise the AS region.

Confirming the visual similarity of the distributions of PZAse mutations across the secondary structure domains between the Peruvian and the worldwide samples, statistical tests showed that they were not significantly different. The Signed-rank non-parametric test did not reject the similarity of the paired normalized frequencies ($P = 0.50$); and the Spearman's correlation test rejected the null hypothesis that the two lists of frequencies are independent ($P < 0.0001$).

4. Discussion

The distributions of PZAse mutations associated to PZA-resistance from the Peruvian and the globally reported samples were remarkably similar. The distribution of mutations from the Peruvian strains confirms previous descriptions of the existence of *pncA* hot-spot regions associated with PZA-resistance in MTB (Scorpio et al., 1997; Lemaitre et al., 1999; Du et al., 2001). Interestingly, these hot-spot regions are close to the AS and the MCS supporting the hypothesis that PZA-resistance is caused by an impairment of the PZAse function mainly due to physical–chemical alterations of the catalytic site (Lemaitre et al., 1999).

In a previous study we reported based on a univariate analysis that only 27.3% of the variability of PZA-resistance was explained by the PZAse function. Therefore, other potential PZA-resistance mechanisms, like alterations of the *pncA* gene expression level or alterations in the POA efflux pump, may be occurring and confounding the previous figure, as confirmed by the existence of PZA-resistant isolates with *pncA* mutations that retain PZAse activity (Sheen et al., 2009a). It is important to remark that most mutations analyzed in this study are from clinical strains, thus might cumulate other yet undetermined resistance mechanisms. However, despite this potential bias, the remarkable similarity between the Peruvian and the worldwide distribution of PZAse mutations and the presence of identical hot-spot regions, suggest the existence of a conserved pattern of *pncA* mutations associated to PZA-resistance that could cause PZAse impairment by mutations preferentially in the catalytic center and most frequently in the metal coordination site region. The mutations examined in this study are specifically acquired from PZA-resistant MTB strains that have likely evolved around a single pressure of the

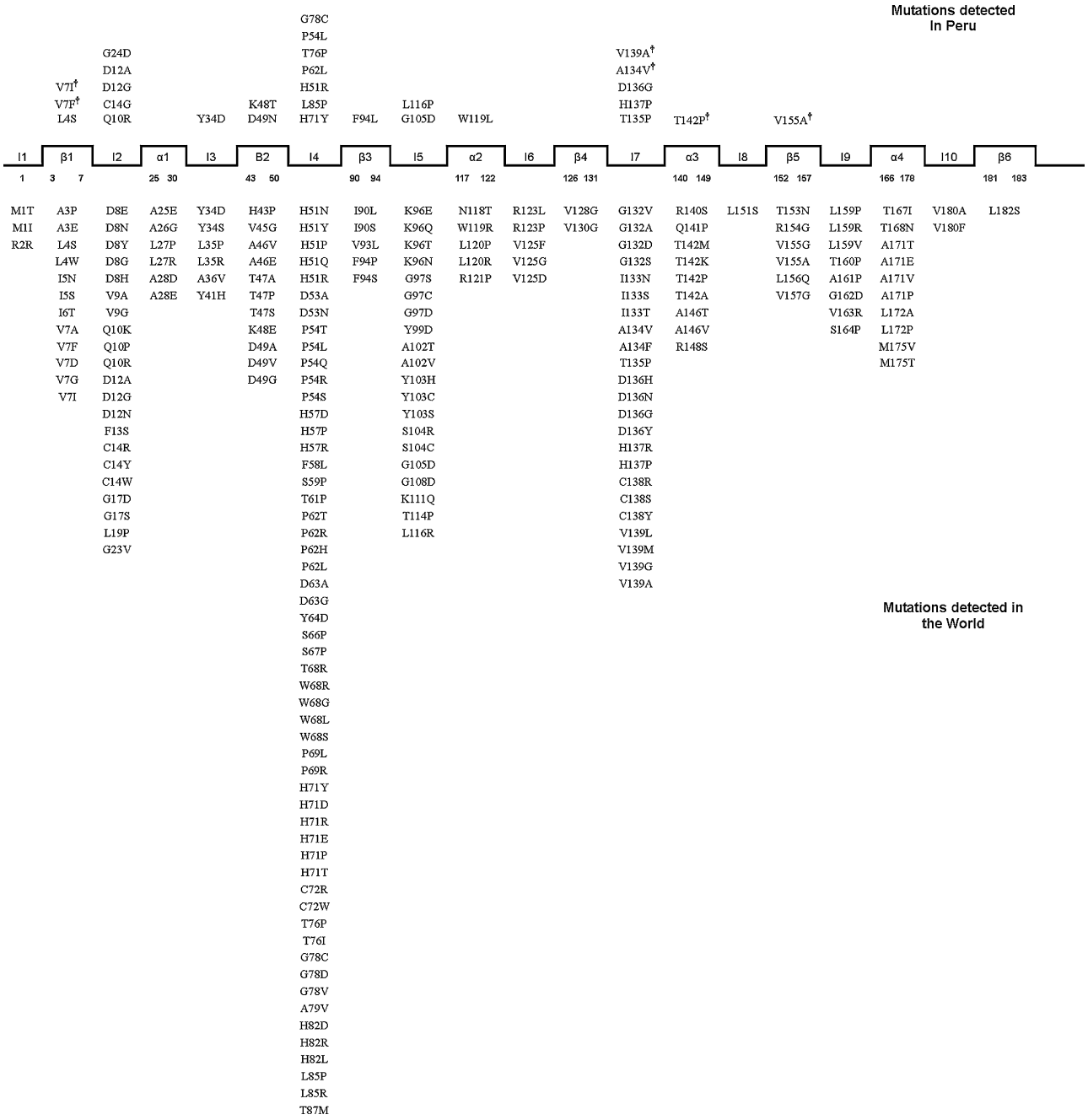

Fig. 1. Distribution of PZAse missense mutations in Peruvian and worldwide *Mycobacterium tuberculosis* PZA-resistant strains. The amino acid sequence of the PZAse and its secondary structure is considered. Four alpha helices (α1–α4), 6 beta strands (β1–β6) and 10 loops (L1–L10). The AS is included within the secondary structure domains L2 and L7, while the MCS is included within L4. (†) Peruvian mutations reported by Escalante et al.

drug, which in turn, affects a clear and known target. In our previous study, among 108 MTB strains, we did not find any *pncA* silent mutation (Sheen et al., 2009a), suggesting the presence of a strong drug selection pressure.

The existence of a conserved pattern of *pncA* mutations associated to PZA-resistance demonstrated in this study, suggests that the drug selection pressure is acting similarly and uniformly in different world regions. Further research should be done to understand the relationship between *pncA* mutations and enzymatic activity within a structure–function level.

Competing interests

No competing of interests is declared.

Author's contributions

MZ, PS and RHG conceived the general idea and participated in preparing the manuscript. AG and MQ carried out the searching of global *pncA* mutations, selected the sequences that meet the inclusion criteria and drafted the manuscript. MZ conducted the statistical analysis.

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