Mapana J Sci, **12**, 4(2013), 1-8
ISSN 0975-3303 | doi:10.12723/mjs.27.1

Mutations Associated with Pyrazinamide Resistance in the Clinical Isolates of *Mycobacterium tuberculosis* from Kerala, India

AK Anilkumar*, GK Madhavilatha†, Sarojini S‡, Ajay Kumar R§ and Sathish Mundayoor**

Abstract

Pyrazinamide is one of the four first line drugs for treatment of tuberculosis. It has been widely accepted, that pyrazinamide (PZA) resistance in *Mycobacterium tuberculosis* is correlated with mutations in the *pncA* gene. In the present study, pyrazinamide susceptibility was tested in 65 clinical isolates of *Mycobacterium tuberculosis* by *pncA* gene sequencing and was then correlated with pyrazinamidase activity. 68% of the resistant isolates showed mutation in the *pncA* gene which was further correlated with an assay for pyrazinamidase activity.

Keywords: *Mycobacterium tuberculosis*, Drug resistance, Pyrazinamide, *pncA*, Pyrazinamidase acitvity

* Assistant Professor, Department of Botany, Govt. Brennen College, Thalassery, Kerala, India anilakmrg@gmail.com

† Senior Research Fellow, Mycobacterium Research Group, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India; gkmadhavi@gmail.com

‡ Assistant Professor, Department of Biotechnology, Christ University, Bangalore, Karnataka, India;suma@christuniversity.in

§ Scientis EII, Mycobacterium Research Group, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India; rakumar@rgcb.res.in

** Corresponding author; Dean and Scientist G, Mycobacterium Research Group, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India; smundayoor@rgcb.res.in

Introduction

Tuberculosis (TB) still remains one of the leading causes of morbidity and mortality in the world. At present one-third of the world's population is infected with *Mycobacterium tuberculosis,* the causative agent of TB. In 2011, 1 million HIV negative people died from the disease and 8.7 million new cases were estimated of which 13% are coinfected with HIV [1]**.** Pyrazinamide (PZA) is one of the most important drugs in both first and second-line treatment of TB. It is particularly effective against latent TB infection and allows anti-tuberculous treatment to be shortened from 9 to 6 months [2].

PZA is one of the few anti-TB drugs effective against persistent tubercle bacilli. It is only active against *M. tuberculosis* and *M. africanum* in the host, while *M. bovis* is inherently resistant to it. Since the global resurgence of drug resistant TB strains, an increasing number of PZA resistant strains have been observed. PZA is a prodrug which is converted into the active substance pyrazinoic acid (POA) by *M. tuberculosis* at low pH medium [3]. In *M. tuberculosis* PZA is converted to POA by *pncA* gene [4]**.** PZA susceptible *M. tuberculosis* possesses a functional pyrazinamidase and mutations in this gene have been reported leading to reduction or complete loss of pyrazinamidase activity, which in turn make the bacteria resistant to PZA [5,6]. Recent study has shown that mutations in the *rpsA* gene are associated with PZA resistance in clinical isolates without mutations in *pncA* [7].

PZA is active only in an acidic microenvironment (pH 5.5) and such low pH in itself inhibits the growth of the bacilli. Hence growth-based testing for PZA resistance is both difficult and unreliable. It has also been reported that even modest variations in inoculum size can alter the pH and lead to differing results [8]. The aim of the present study was to identify the mutations in the *pncA* gene associated with PZA resistance in the clinical isolates of *M. tuberculosis* from Kerala and also correlate the results with pyrazinamidase activity assay.

Materials and Methods

Bacterial Isolates

The collection of samples, biochemical testing and drug sensitivity profiling of the samples was done as reported earlier [9]. For this study well grown clinical isolates of *M. tuberculosis* were selected in random as no information was available on the prevalence of PZA resistance in our state or in South India when this study was first initiated. Pyrazinamide-LJ slants (HiMedia) were purchased and *M. tuberculosis* isolates collected from the standard LJ media were inoculated into two of them (pH 5.5) with one slant containing pyrazinamide (100mg/ml) and one without the drug (control).

Pyrazinamidase Activity Assay

Pyrazinamidase (PZase) activity was determined by Wayne's method [10]**.** Briefly, a heavy loopful of *M. tuberculosis* colonies was obtained from cultures that were actively growing in LJ medium and inoculated onto the surfaces of two agar butt tubes, each containing 5 ml of Wayne's medium supplemented with 100 µg/ml of PZA (Sigma-Aldrich, USA). The tubes were incubated at 37°C. Four days after incubation, 1 ml of freshly prepared 1% Ferrous Ammonium Sulphate was added to the first tube. The tube was left at room temperature for 30 minutes and examined. The assay was positive if a pink band was present on the subsurface of the agar. If the test was negative, the test was repeated with a second tube and examined after 7 days of incubation. The results were blindly read by two independent observers. *M. bovis* BCG and *M. tuberculosis* H37Rv were used as negative and positive controls, respectively.

DNA Sequencing and Analysis

DNA of all the isolates was subjected to an in house developed tetraplex PCR to confirm whether they are *M. tuberculosis* or NTM [11]. *pncA* gene was amplified and sequenced using primers pncA1F - 5'TCGGTCATGTTCGCGATCG 3' and pncA4 5'CGCTTGCGGCGAGCGCTCCA 3' – (Figure 1). The sequences were subjected to BLAST analysis in the *M. tuberculosis* DNA sequence database (http://genolist.pasteur.fr/TubercuList/) for mutation analysis.

Results and Discussion

Correlation of drug resistance with a defect in a specific drug resistant gene has been observed for several anti-TB drugs. Drug resistance phenotype and mutations in the drug resistance genes has been observed in varying proportions for drugs such as ofloxacin (*gyrA* 75–94%), Rifampicin, (*rpoB* 90–95%), Ethambutol (*embB* 47–65%) and isoniazid (*katG* 31–97% /*inhA* 8–43%) [3,12]. The correlation of PZA resistance and mutation in *pncA* has also been reported in a variety of ranges from as low as 41% in Taiwan [13] to as high as 97% in Japan [14] and S. Korea [15]. In the present study 65 *M. tuberculosis* strains with known microbiological PZA susceptibility or resistance were analyzed. They were subjected to *pncA* gene sequence analysis and out of 31 PZA resistant clinical isolates 68% (21 isolates) showed mutations in the *pncA* gene.

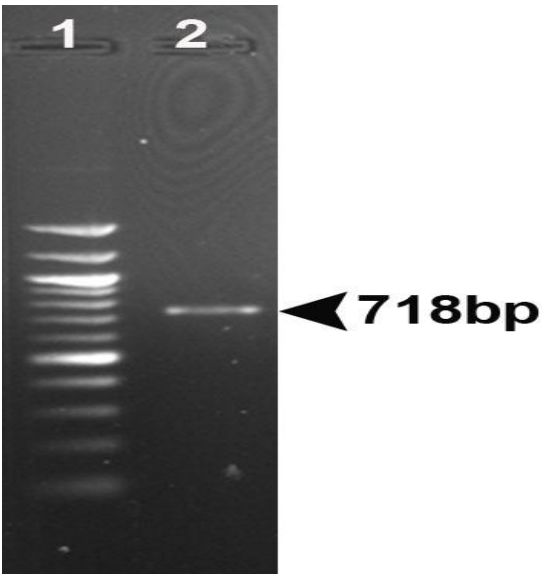

Fig 1: Amplification of *pncA* target for identification of mutations

Lane 1: 100bp Ladder (NEB); Lane 2: *pncA* amplified from an *M. tuberculosis* clinical isolate

The uniqueness in the mutations of *pncA* gene is its diversity and scattering along the whole gene though there does appear to be some degree of clustering at three regions of pncA protein (3 to 17, 61 to 85, and 132 to 142). These regions are likely to contain catalytic sites for the PZase enzyme [16]**.** Ten isolates 15.4% showed mutations which include codons 49, 65, 68, 76, 132, 139, 180. Five clinical isolates (RGTB 143, 342, 343, 431 and 502) had a mutation in codon 65 (TCC→TCT; Ser→Ser) that has been reported by other groups too [17, 18, 19, 20]. RGTB 399 had a mutation in codon 49; GAC→GAG, Asp→Glu; RGTB 416 had a mutation in codon 68; TGG→GGG, Trp→Gly. RGTB 495 had a mutation in codon 132 - GGT→GCT, Gly→Ala. We report that the mutations observed in codons 49, 68 and 132 are novel mutations that have been observed in the present study. RGTB 233 had a mutation in codon 76 - ACT→CCT, Thr→Pro that has been reported by other groups [19, 21, 22, 23]. RGTB 86 had a mutation in codon 180 - GTC→TTC, Val→Phe that has been reported by other groups [17, 19, 23]. RGTB 143 had mutations both at codon 65 and codon 139 - GTG→GCG, Val→Ala. Mutation at codon 139 has already been reported to be responsible for pyrazinamide resistance [19, 22, 24]. Thus, although this sample also has a silent mutation at codon 65, mutation at codon 139 can confer resistance to the drug. In a previous study, it has been reported that Cys-138, Ala-134, Thr-135, Trp-68, and Asp-8 in the *M. tuberculosis* PZase could be key residues for hydrolysis of PZA [25]. Hence the mutations occurring within or close to the regions containing these residues could result in conformational modifications of the active site of the PZase and consequently, in a loss of PZase activity. In this study the mutations found are in and near the suggested positions. So these can lead to the loss of PZase activity and thereby conferring resistance to the drug.

Strains of *M. tuberculosis* that are resistant to PZA are often defective in PZase activity. Isolates that showed mutations in the *pncA* gene were subjected to pyrazinamidase assay to correlate the mutation results with loss of PZase activity. Pyrazinamidase test is rapid, economical and easy to perform. PZA susceptible *M. tuberculosis* H37Rv developed a pink band on the surface after the addition of ferrous ammonium sulfate. This showed that PZase is active. But PZA resistant *M. bovis* BCG and mutated clinical isolates did not show any color development. This showed a loss of PZase

activity in them. The five clinical isolates which had silent mutation developed a pink colour showing that they were really susceptible and had PZase activity. Other samples failed to develop colour as they had lost their PZase activity. This showed that they were resistant to the drug.

In conclusion, PCR based sequencing is an effective method to identify PZA resistance and can be correlated with PZase assay because the enzymatic activity is very sensitive to sequence alterations in any region of the protein.

Acknowledgements

AK Anilkumar and GK Madhavilatha are recipients of Senior Research Fellowship from Council for Scientific and Industrial Research (CSIR), Govt. of India. We thank Laiza K Paul for excellent technical assistance and Manoj P for assistance in sequencing.

References

[1] WHO, World Health Organization. The Global tuberculosis Report 2012

[2] Sheen, P., K. Lozano, R. H. Gilman, H. J. Valencia, S. Loli, P. Fuentes, L. Grandjean, and M. Zimic. *pncA* gene expression and prediction factors on pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 93:515-22.

[3] Bhuju, S., L. D. Fonseca, A. G. Marsico, G. B. de Oliveira Vieira, L. F. Sobral, M. Stehr, M. Singh, and M. H. Saad. *Mycobacterium tuberculosis* isolates from Rio de Janeiro reveal unusually low correlation between pyrazinamide resistance and mutations in the *pncA* gene. Infect Genet Evol 19:1-6.

[4] Blanchard, J. S. 1996. Molecular mechanisms of drug resistance in *Mycobacterium tuberculosis*. Annu Rev Biochem 65:215-39.

[5] Butler, W. R., and J. O. Kilburn. 1983. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob Agents Chemother 24:600-1.

[6] McClatchy, J. K., A. Y. Tsang, and M. S. Cernich. 1981. Use of pyrazinamidase activity on *Mycobacterium tuberculosis* as a rapid method for determination of pyrazinamide susceptibility. Antimicrob Agents Chemother 20:556-7.

[7] Shi, W., X. Zhang, X. Jiang, H. Yuan, J. S. Lee, C. E. Barry, 3rd, H. Wang, W. Zhang, and Y. Zhang. Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. Science 333:1630-2.

[8] Kurbatova, E. V., J. S. Cavanaugh, T. Dalton, S. C. E, and J. P. Cegielski. Epidemiology of pyrazinamide-resistant tuberculosis in the United States, 1999-2009. Clin Infect Dis 57:1081-93.

[9] Joseph BV, Soman Smitha, Radhakrishnan I, Madhavilatha GK, Paul L, Mundayoor S and Kumar RA. 2009 Drug resistance in Mycobacterium tuberculosis isolated from TB patients in Kerala, India. The International Journal of Tuberculosis and Lung Disease 13(3):494-499.

[10] Wayne, L. G. 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis 109:147-51

[11] Anilkumar A K, G. K. Madhavilatha, Paul L, R. Indulakshmi and S. Mundayoor. 2012. Standardization and evaluation of a tetraplex PCR to detect Mycobacterium complex and nontuberculous mycobacteria a retrospective study on pulmonary TB patients. Diagn Microbiol Infect Dis. 72(3) : 239-47.

[12] Zhang, H., L. J. Bi, C. Y. Li, Z. G. Sun, J. Y. Deng, and X. E. Zhang. 2009. Mutations found in the *pncA* gene of *Mycobacterium tuberculosis* in clinical pyrazinamide-resistant isolates from a local region of China. J Int Med Res 37:1430-5.

[13] Huang, T. S., S. S. Lee, H. Z. Tu, W. K. Huang, Y. S. Chen, C. K. Huang, S. R. Wann, H. H. Lin, and Y. C. Liu. 2003. Correlation between pyrazinamide activity and *pncA* mutations in *Mycobacterium tuberculosis* isolates in Taiwan. Antimicrob Agents Chemother 47:3672-3.

[14] Hirano, K., M. Takahashi, Y. Kazumi, Y. Fukasawa, and C. Abe. 1997. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuber Lung Dis 78:117-22.

[15] Lee, K. W., J. M. Lee, and K. S. Jung. 2001. Characterization of *pncA* mutations of pyrazinamide-resistant *Mycobacterium tuberculosis* in Korea. J Korean Med Sci 16:537-43.

[16] Lemaitre, N., W. Sougakoff, C. Truffot-Pernot, and V. Jarlier. 1999. Characterization of new mutations in pyrazinamide-resistant strains of *Mycobacterium tuberculosis* and identification of conserved regions important for the catalytic activity of the pyrazinamidase PncA. Antimicrob Agents Chemother 43:1761-3.

[17] Somoskovi, A., J. Dormandy, L. M. Parsons, M. Kaswa, K. S. Goh, N. Rastogi, and M. Salfinger. 2007. Sequencing of the *pncA* gene in members of the *Mycobacterium tuberculosis* complex has important

diagnostic applications: Identification of a species-specific *pncA* mutation in "*Mycobacterium canettii*" and the reliable and rapid predictor of pyrazinamide resistance. J Clin Microbiol 45:595-9

[18] Sekiguchi, J., T. Nakamura, T. Miyoshi-Akiyama, F. Kirikae, I. Kobayashi, E. Augustynowicz-Kopec, Z. Zwolska, K. Morita, T. Suetake, H. Yoshida, S. Kato, T. Mori, and T. Kirikae. 2007. Development and evaluation of a line probe assay for rapid identification of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* strains. J Clin Microbiol 45:2802-7.

[19] McCammon, M. T., J. S. Gillette, D. P. Thomas, S. V. Ramaswamy, Rosas, II, E. A. Graviss, J. Vijg, and T. N. Quitugua. 2005. Detection by denaturing gradient gel electrophoresis of pncA mutations associated with pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from the United States-Mexico border region. Antimicrob Agents Chemother 49:2210-7.

[20] Denkin, S., D. Volokhov, V. Chizhikov, and Y. Zhang. 2005. Microarray-based *pncA* genotyping of pyrazinamide-resistant strains of *Mycobacterium tuberculosis*. J Med Microbiol 54:1127-31.

[21] Morlock, G. P., J. T. Crawford, W. R. Butler, S. E. Brim, D. Sikes, G. H. Mazurek, C. L. Woodley, and R. C. Cooksey. 2000. Phenotypic characterization of *pncA* mutants of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 44:2291-5.

[22] Sreevatsan, S., X. Pan, Y. Zhang, B. N. Kreiswirth, and J. M. Musser. 1997. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. Antimicrob Agents Chemother 41:636-40.

[23] Tracevska, T., I. Jansone, A. Nodieva, O. Marga, G. Skenders, and V. Baumanis. 2004. Characterisation of *rpsL, rrs* and *embB* mutations associated with streptomycin and ethambutol resistance in *Mycobacterium tuberculosis*. Res Microbiol 155:830-4.

[24] Lemaitre, N., I. Callebaut, F. Frenois, V. Jarlier, and W. Sougakoff. 2001. Study of the structure-activity relationships for the pyrazinamidase (PncA) from *Mycobacterium tuberculosis*. Biochem J 353:453-8.

[25] Suzuki, Y., A. Suzuki, A. Tamaru, C. Katsukawa, and H. Oda. 2002. Rapid detection of pyrazinamide-resistant *Mycobacterium tuberculosis* by a PCR-based in vitro system. J Clin Microbiol 40:501-7