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Molecular characterization of *pncA* gene mutations in *Mycobacterium tuberculosis* clinical isolates from China

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SUMMARY

A sample of 35 pyrazinamide (PZA)-resistant and 30 PZA-susceptible clinical isolates recovered from Beijing and Taiyuan City, China were characterized by SSCP and sequence analysis for mutations in the *pncA* gene that encodes the *Mycobacterium tuberculosis* PZase. The purpose of this study was to understand the molecular basis and the characteristics of *pncA* gene mutations and its relation to PZA resistance in *M. tuberculosis* strains from China. Several mutations with base changes leading to amino acid substitutions were found in the PZA-resistant isolates. No mutations were seen in the 243 PZA-susceptible isolates. Among the 35 PZA-resistant isolates, 32 isolates (91·4%) had nucleotide substitutions, insertions and deletions that resulted in amino-acid substitution; or frameshifts in some strains. Other previously uncharacterized mutations were found as follows: Asn118→Thr, CG insertion at position 501; CC insertion at nucleotide position 403; a 8 base-pair deletion at start codon; Pro54→Thr; AG insertion at 368; Tyr41→His, Ser88→stop, and A insertion at nucleotide position 301. IS*6110* subtyping revealed that each strain was unique; indicative of the epidemiologic independence of the isolates.

INTRODUCTION

The resurgence of tuberculosis coupled with the emergence of drug-resistant *Mycobacterium tuberculosis* strains seriously compromises our ability to control tuberculosis [1]. Multiple drug-resistant strains of *M. tuberculosis* defined as resistance to at least isoniazid and rifampin have stimulated a great deal of research aimed at understanding the molecular basis of drug resistance in *M. tuberculosis*. Such knowledge should eventually facilitate the rational design of new anti-tuberculosis drugs; and the development of rapid tests for proper detection of drug resistance.

Pyrazinamide (PZA); a nicotinamide analogue, has been in use for almost 50 years as a first line drug to treat tuberculosis. PZA is bactericidal to semi-dormant mycobacteria and reduces the total tuberculosis treatment time when used in combination with isoniazid and rifampin [2–9]. This has made PZA the third important drug in the list of modern therapy for tuberculosis. Although the exact biochemical basis of PZA activity *in vivo* is not known, under acidic conditions it is thought that the bacterial enzyme pyrazinamidase (PZase) is required to convert PZA to pyrazinoic acid (POA) which is toxic to *M. tuberculosis* [1, 2, 10, 11]. The finding that PZA-resistant

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strains lose PZase activity and the hypothesis that PZase is required to convert PZA to POA intracellularly led to the recent cloning and characterization of the *M. tuberculosis* gene *pncA* that encodes PZase [2, 12]. DNA sequencing of PZA-resistant clinical isolates indicated mutations in the *pncA* gene: These mutations are currently thought to confer resistance to PZA. However, the extent of geographical variation in *pncA* gene mutations deserves further studies.

The present study sought to understand the molecular basis and characteristics of PZA resistance and its relation to potential *pncA* gene mutations in *M. tuberculosis* clinical isolates from China. We analysed the DNA sequence of the *pncA* gene in samples of PZA-resistant and PZA-susceptible clinical isolates from Beijing and Taiyuan, China.

METHODS

Mycobacterium strains

Thirty-five PZA-resistant and 30 PZA-susceptible clinical isolates recovered from Beijing and Taiyuan City, China, over a 2-year period (1996–8) were used in this study. All patients were inhabitants of Beijing and Taiyuan City, and 33 of the patients had previously received anti-tuberculosis therapy. Treatment history was unknown for three patients. The initial anti-tuberculosis therapy included isoniazid, rifampin, and pyrazinamide. All isolates included in this study were obtained before this regimen was changed based on the respective drug susceptibility results. All strains were characterized for their IS*6110* subtypes by an internationally standardized protocol [13]. All isolates were tested for PZA susceptibility with either the BACTEC radiorespiratory method at pH 6·0 [14, 15] or by the conventional proportion method with a PZA concentration of 25 μg/ml in 7H10 agar (pH 5·5) [16]. Susceptibility was defined as MIC of no more than 100 μg/ml of PZA. Most of the strains analysed in this study were highly resistant to PZA (MIC $\geqslant$ 500 μg/ml). Re-testing for PZA susceptibility of PZA-resistant strains without *pncA* mutations was performed by both the BACTEC method and the 7H10 agar medium method at pH (5·5). Genomic DNA was prepared according to a protocol generously provided by Dr J. D. A. Van Embden [17]. All genomic DNA extraction and drug susceptibility tests were carried out at the Shanxi Medical University, Taiyuan, China; and then shipped to the University of Tsukuba, Ibaraki, Japan, for further molecular analyses. Mycobacterium culturing and DNA isolation were conducted in Biosafety level 3 laboratory.

PCR conditions

Three sets of primers were designed according to the *Mycobacterium tuberculosis pncA* sequence (558 bp) (GenBank accession number U59967) [12] (Table 1). PCR was performed as described by Dr J. D. A. Van Embden [17]. The PCR conditions were 96 °C for 3 min, followed by 30 cycles of 96 °C for 1 min, 63 °C for 1 min and 72 °C for 2 min, with a final extension of 72 °C for 6 min.

SSCP analysis

SSCP was carried out as below; Briefly, PCR products (0·5–1 μg of DNA) were mixed with 6 μl of denaturing reaction solution (Perkin–Elmer), the mixture was denatured by boiling for 10 min, and cooled on ice for 5 min. The single-strand PCR products were loaded onto a 10% polyacrylamide gel. Electrophoresis was performed at 10 V/cm for 5 h at 10 °C. Single-strand bands were visualized by the ethidium bromide staining method.

Automated DNA sequencing of the *pncA* gene

The *pncA* genes were amplified by PCR using the conditions and primers described above. PCR products from isolates showing variant banding patterns in SSCP were cut from the gel and purified with the Geneclean kit (United State Biochemicals). The purified PCR products were directly sequenced using Genetic Analyzer (Perkin–Elmer, ABI PRISM^{AM} 310). After termination dideoxy-cycle sequencing, the resulting data were assembled and edited using SeqEd v. 1.0.3 software (Applied Biosystems). The sequences were compared with published sequence of *pncA* from Genbank [12]. All mutations in the *pncA* structural gene were confirmed by re-sequencing.

RESULTS

All *Mycobacterium tuberculosis* complex clinical isolates studied had a C→T change at nucleotide 67 which has been identified as a sequence error by

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Table 1. *Oligonucleotide primers for amplification of the* pncA *gene* (558 *bp*)

Primer	Sequence	Position
Primer P1	5′-GTCGGTCATGTTCGCGATCG-3′	From −105 bp upstream
Primer P2	5′-TCGGCCAGGTAGTCGCTGAT-3	Nucleotide position 110–91
Primer P3	5′-ATCAGCGACTACCTGGCCGA-3′	Nucleotide position 91–110
Primer P4	5′-GATTGCCGACGTGTCCAGAC-3′	Nucleotide position 270–25
Primer P5	5′-CCACCGATCATTGTGTGCGC-3′	401–420
Primer P6	5′-GCTTTGCGGCGAGCGCTCCA-3′	60 bp downstream of the stop codon

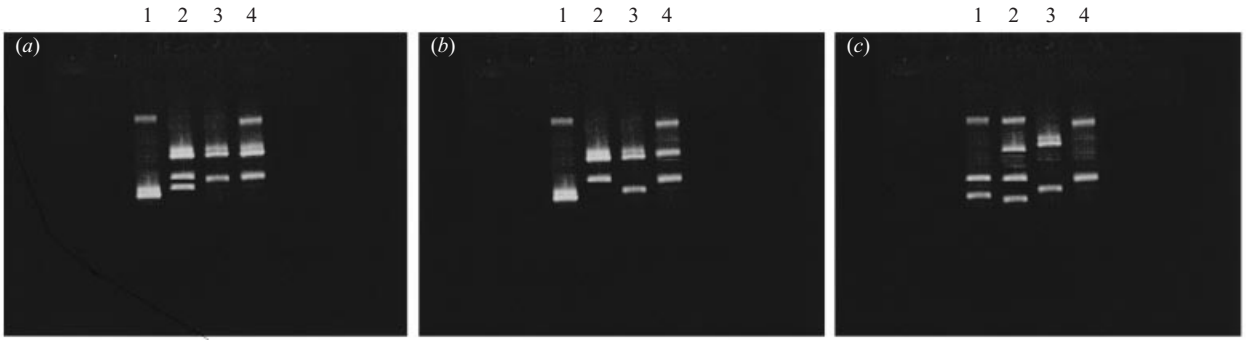

Fig. 1. PCR–SSCP analysis of *pncA* mutations in PZA-resistant *M. tuberculosis*. (A) SSCP with primer set 1. Lane 1, PZA-susceptible control strain CBS028. Lanes 2–4, PZA-resistant clinical isolates CBM04, CCT03, and CBC02, respectively. (B) SSCP analysis with primer set 2. Lane 1, PZA-susceptible control strain CBS028. Lanes 2–4, PZA-resistant clinical isolates CCT19, CBM06, and CCT01, respectively. (C) SSCP analysis with primer set 3. Lane 1, PZA-susceptible control strain CBS028. Lanes 2–4 PZA-resistant clinical isolates CBM01, CBM03, and CBM05, respectively.

Zhang and colleagues [9]: We will not consider them as mutations in this study. The results of IS*6110* subtypes revealed that each was unique, indicative of the epidemiological independence of the isolates.

pncA gene in PZA-susceptible *M. tuberculosis* clinical isolates

PZA-susceptible isolates contained the wild-type *pncA* allele; no mutations were found in these isolates. These results indicate that mutations in the *pncA* gene are essential tools for the detection of PZA resistance.

PCR–SSCP analysis

PCR–SSCP analysis was performed for the rapid detection of point mutations in the *pncA* gene. Selected PZA-resistant clinical isolates were used for the PCR–SSCP analysis. This process was rapid and very convenient (Fig. 1).

pncA mutations in PZA-resistant *M. tuberculosis* clinical isolates

Results of the direct sequence analysis of *pncA* gene from different PZA-resistant isolates are shown in Table 2. Among the 35 PZA-resistant clinical isolates, 32 isolates had mutations that included nucleotide substitutions (missense mutation), or insertions and deletions (nonsense mutation) leading to amino-acid substitutions in some cases; or frameshifts with corresponding nonsense polypeptides in some strains. The mutations found were diverse and dispersed along the entire *pncA* gene. Most of the mutations (68·8%, 22/32) are point mutations causing amino-acid substitutions (Table 2). For instance isolate CBC01 had a C to A change at nucleotide position 425, and a resulting Thr142→Lys substitution; CBC02 had A to C change at position 35, leading to an Asp12→Ala amino-acid substitution; isolate CBC03 had A to C change at nucleotide position 353 and caused a Asn118→Thr amino-acid substitution. Isolate CBC07 had a C deletion mutation at nucleotide position 28; CCT03 had C deletion at 59; CCT08 had G deletion at 71, and CCT16 had a C deletion at 28. All deletion mutations caused amino acid frameshifts. CBM01 had CG insertion mutation at position 501 and CBM02 had CC insertion mutation at 403, leading to amino-acid frameshifts. Two isolates had a mutation that created a stop codon; CBM06 had C→T change at nucleotide position 123, causing a change of Tyr41→stop. CCT19 had C to A change at

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Table 2. *Epidemiological data and mutations observed among clinical isolates*

Isolates	PZA MIC (μg/ml)	Base change	Amino-acid substitution	Patients' data			
				Age (yr)	Gender	History of PZA treatment	Associated drug-resistant profile*
CBC01	300	C→A at 425	Thr142→Lys	45	Male	Yes	HRS
CBC02	1500	A→C at 35	Asp 12→Ala	34	Female	Yes	HRES
CBC03	900	A→C at 353	Asn118→Thr	57	Female	Yes	S
CBC04	1500	T→C at 56	Leu19→pro	76	Male	Unknown	HR
CBC06	1000	T→C at 416	Val139→Ala	21	Male	Yes	HRS
CBC07	400	Nucleotide C deletion at 28		19	Male	Yes	HRES
CBC09	>2000	C→G at 123	Tyr41→stop	43	Male	No	HRES
CBM01	900	CG insertion at position 501		38	Female	Yes	SHE
CBM02	1000	CC insertion at 403		18	Male	Yes	RE
CBM03	500	C→A at 425	Thr142→Lys	23	Male	Yes	RHE
CBM04	1200	11-bp deletion at start		35	Female	Unknown	RHES
CBM05	600	G→A at 413	Cys138→Tyr	62	Female	Yes	RHES
CBM06	1500	C→G at 123	Tyr41→stop	57	Male	Yes	RSE
CCT01	900	A→C at 139	thr47→Pro	19	Female	Yes	SHE
CCT02	>2000	8-bp deletion at start position		39	Female	Yes	RHSE
CCT03	1800	C deletion at position 59		57	Male	Yes	RSE
CCT04	900	G→T at 288	Lys96→Asn	45	Male	No	RSHE
CCT05	600	C→A at 425	Thr142→Lys	35	Female	Yes	HS
CCT06	800	C→A at 160	Pro54→Thr	56	Male	Yes	RHES
CCT07	1200	T→G at 254	Pro69→Arg	34	Male	Unknown	HRES
CCT08	600	G deletion at 71		56	Male	Yes	RH
CCT09	900	G→T at 288	Lys96→Asn	35	Male	Yes	HRS
CCT11	1000	T→C at 416	Val139→Ala	36	Male	Yes	HS
CCT12	1000	G→C at 362	Arg121→Pro	71	Male	Yes	HRES
CCT13	1200	AG insertion at 368		68	Female	Yes	RHES
CCT14	800	T→C at 254	Leu85→Pro	34	Female	Yes	RE
CCT15	900	G→C at 415	Val139→Leu	45	Male	Yes	RHES
CCT16	1500	Nucleotide C deletion at 28		58	Female	Yes	HRS
CCT17	600	T→C at 121	Tyr41→His	76	Male	Yes	HRE
CCT18	1500	T→C at 214	Cys72→Arg	69	Female	Yes	HRES
CCT19	>2000	C→A at 263	Ser88→Stop	45	Male	Yes	HRES
CCT20	1000	A insertion at 301		67	Female	Yes	HRS

* H, isonizaid; R, rifampin; S, streptomycin; E, ethambutol.

263, leading to a change of Ser88→stop. Two isolates had deletion mutations at start codon; CCT02 had 8-bp deletion and CBM04 had 11-bp deletion. These mutations may weaken the promoter activity and thus decrease the level of PZase expression in such mutants. Overall, 26 types of mutations were found. However, a certain degree of conservation of *pncA* mutations were also observed at the following nucleotides: CCT09 isolates and CCT04 had the same mutation at codon Lys96→Asn; CBC06, CCT11 had the same mutation of Val139→Ala; and CBM03, CBC01 and CCT05 had the same mutation of Thr142→Lys.

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Other previously uncharacterized mutations were also found, and includes the following changes: Asn118→Thr, CG insertion at position 501; CC insertion at 403; an 8 bp deletion at start codon; Pro54→Thr; AG insertion at 368; Tyr41→His, and Ser88→stop and A insertion at 301. Several other mutations previously identified by Hirano and colleagues were also found in our clinical isolates (Table 2) [18].

Three out of 35 PZA-resistant isolates did not contain any detectable *pncA* mutations (CBC05, CBC08 and CTT10). One of these isolates was actually susceptible to PZA; while two of them were resistant to PZA upon re-testing by both methods [14–16].

DISCUSSION

Recent studies have indicated that mutations in the *M. tuberculosis pncA* gene are associated with PZA resistance [12]. These studies have thus promoted a more thorough examination of sequence variations in this gene in order to identify genetically distinct susceptible and resistant organisms. In this study we found that 32 out of 35 PZA-resistant clinical *M. tuberculosis* isolates had mutations in the *pncA* gene. Unlike mutation in other *M. tuberculosis* genes such as *rpoB* and *KatG* genes, the spectrum of mutations in the *pncA* gene was very broad. A large number of distinct *pncA* gene mutations were associated with PZA resistance; and the changes were distributed virtually throughout the entire length of the gene. Most of the *pncA* gene mutations had nucleotide substitutions resulting in single amino-acid replacements. This discovery extends the common observation that has emerged from studies of antimicrobial resistance *M. tuberculosis* [1].

Our data also showed nucleotide deletion (18·8%; 6/32) and nucleotides insertion mutation (12·5%; 4/32). Changes resulting in frameshift mutations may be associated with PZA resistance. The results obtained in our study are supported by several published literatures [1, 12, 19]. Hirano and colleagues found a correlation of the development of PZA resistance with mutations in the *pncA* gene. The large number of distinct *pncA* gene mutations associated with PZA resistance supports the notion that enzymatic activity against PZA can be influenced by more than one molecular mechanism [1].

Further studies are needed to assess the role of the specific mechanisms of the respective *pncA* gene mutations on PZA resistance, and the level of PZase activity alterable by the different mutations. The results of this study also indicate that regular survey of clinical isolates with a combination of PCR–SSCP analysis could help not only in epidemiological studies of *M. tuberculosis* outbreaks, but also in understanding the rate and trends in mutations; in relation to PZA resistance over time. In this study we observed a possible association between single nucleotide changes leading to frameshift mutations and PZA resistance. Similar changes were found by Srinand and colleagues [1]; the occurrence of these mutations could probably influence the survival of the organisms. For instance, in their study, Srinand and colleagues observed that deletions at nucleotide 70 conferred to the organism an ability to maintain viability in the total absence of PZase activity [1].

Obviously, additional mechanisms of PZA have been known to exist. However, such mechanisms have not been fully elucidated. The most up-to-date study on alternate mechanisms was by Raynaud and colleagues in 1999; they showed that the uptake of PZA is required for the activation of the pro-drug. Also, an ATP-dependent transport system is probably involved in the uptake of PZA. Diffusion experiments by Raynaud and colleagues also showed that PZA diffusion through membrane bilayers was faster than glycerol in liposomes; whereas the presence of ompATb, a porin-like protein of *Mycobacterium tuberculosis* in proteoliposomes slightly increased the diffusion of PZA. Their study also indicated that only naturally PZA-susceptible species exhibited both PZase activity and PZA uptake; whilst no such correlation was observed in naturally resistant species [11]. Thus, a functional PZase and a PZA transport system are necessary for the susceptibility of *Mycobacterium tuberculosis*. Additionally, the lack of *pncA* gene mutations among the PZA-susceptible isolates is a clear indication of the utility of the methods applied in this study and elsewhere for differentiating susceptible and resistant clinical isolates [1, 12, 19].

It remains to be seen however, how the PCR–SSCP method can be accessible to clinical laboratories for rapid PZA susceptibility analysis, considering the economic costs and its combined portability.

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