

# Polymorphisms in the PE35 and PPE68 antigens in *Mycobacterium tuberculosis* strains may affect strain virulence and reflect ongoing immune evasion

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**Abstract.** Previous studies have demonstrated that the Pro-Glu/Pro-Pro-Glu (PE/PPE) genes in strains of *Mycobacterium tuberculosis* exhibit high sequence variation and may be involved in antigenic variation and immune evasion. Region of Difference 1 (RD1), encoding genes from Rv3871 to Rv3879, was observed to be lost during the original derivation of Bacillus Calmette-Guérin between 1908 and 1921. It has been previously demonstrated that two PE/PPE proteins, PE35 (Rv3872) and PPE68 (Rv3873), are encoded by RD1 and exhibit immunodominance. To explore the genetic diversity of PE35 and PPE68, and to evaluate the impact of sequence variation on the immune recognition of these proteins, 161 clinical *M. tuberculosis* strains were selected from China and comparative sequence analysis of PE35 and PPE68 was performed. The results indicated that polymorphisms in PE35 and PPE68 may lead to alterations in the function of these proteins, which may potentially affect strain virulence. In addition, the human T-cell epitopes of PE35 and PPE68 were highly variable, suggesting that the two antigens may be involved in diversifying selection to evade

host immunity. The prevalence of strains with PE35 mutations in the non-Beijing family was significantly greater compared with the Beijing family, indicating that Beijing strains may be more conservative than non-Beijing strains in this gene.

## Introduction

Comparative genomics has identified numerous genetic regions in *Mycobacterium tuberculosis* and *M. bovis* that are deleted in *M. bovis* Bacillus Calmette-Guérin (BCG), such as region of difference 1 (RD1) and RD2 (1). RD1 was lost during the original derivation of BCG between 1908 and 1921 (2). Proteins encoded in these regions have the potential to form the basis of novel specific T-cell-based blood tests that do not cross-react with BCG. Among these antigens, early secretory antigenic target 6 (ESAT-6; ESXA, Rv3875), ESAT-6-like protein esxB (CFP10; ESXB, Rv3874), Pro-Pro-Glu 68 (PPE68; Rv3873) and Pro-Glu 35 (PE35; Rv3872) are immunodominant (3-5). The former two antigens (ESAT-6 and CFP10) have been investigated in detail in humans and are known to be predominant virulence factors (6,7) and in addition are good candidates for the diagnosis of tuberculosis (TB) (8). The latter two, PE35 and PPE68, are members of the PE/PPE family and exhibit immunodominance (9). The PE/PPE proteins are secreted or associated with the mycobacterial cell envelope, and mediate interactions at the host-pathogen interface (10-12). PE35 and PPE68 have been demonstrated to be associated with cellular immune responses to mycobacterial infections (9).

Numerous previous studies have demonstrated high sequence variation of PE/PPE genes in *M. tuberculosis* strains (13,14), an observation that suggests involvement in antigenic variation. To improve the understanding of the genetic diversity of PE35 and PPE68 belonging to the PE-PPE genes in the RD1 region, and to explore the effect of immune recognition on the sequence variation of these two genes, the current study selected 161 clinical *M. tuberculosis* isolates in China, amplified the PE35 and PPE68 genes and compared

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the sequences. The effect of the polymorphisms in PE35 and PPE68 were investigated at the protein level to identify whether alterations in the function of these proteins occurs as this may potentially affect strain virulence. In addition, the variation in the human T-cell epitopes of PE35 and PPE68 were investigated to explore whether the two antigens are involved in diversifying selection to evade host immunity.

## Materials and methods

**Ethics statement.** The study obtained approval from the Ethics Committee of National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention (Beijing, China). The patients with TB included in the present study were provided with a subject information sheet and written informed consent was obtained.

**Strains and DNA preparation.** A total of 161 strains were selected from 2,346 *M. tuberculosis* complex (MTBC) strains isolated in Beijing Municipality and 12 provinces and autonomous regions in China (Table I), which were genotyped by spoligotyping as described previously (15-18). Strains belonging to all major and rare spoligotypes in China were included. Considering the predominance of the Beijing family strains in China, approximately half of the Beijing family strains (82 strains) and half non-Beijing family strains (79 strains) were selected. The 82 Beijing family strains were randomly selected from the 1,738 Beijing strains among the total 2,346 strains. The remaining 79 strains were selected from 608 non-Beijing family isolates. Furthermore, it was attempted to include strains representing different spoligotypes that were isolated from different locations. Table I presents the numbers of strains used in the present study that were obtained from different provinces in China. The spoligotype patterns of the 161 strains are presented in Table II.

A total of 2,346 *M. tuberculosis* isolates were randomly collected between 2005 and 2007 from 2,346 patients at 13 different provincial tuberculosis hospitals across China (16). Subsequently, 161 strains were selected from those 2,346 isolates. Sputum specimens were collected from the TB patients and used to inoculate Löwenstein-Jensen slants. The strains were cultured using a standard Löwenstein-Jensen medium (Baso Diagnostics, Inc., Zhuhai, China) method (15), heat inactivated and then used directly in polymerase chain reactions (PCRs).

**Primers.** The nucleotide sequences of the primers used in the present study were designed with DNASTAR software (version 7.0; DNASTAR, Inc., Madison, WI, USA) according to the *M. tuberculosis* H37Rv genomic sequence and were as follows: PE35, forward 5'-GTAATCGAGTTCGGGCAA TG-3' and reverse 5'-AGGCTTCTCCCAGAGAGTT-3'; PPE68, forward 5'-GACATTGGCACGCAAGTGAG-3' and reverse 5'-TAGCGGCATCGGTCTTCATC-3'.

**PCR.** The PCRs were performed in a total volume of 20  $\mu$ l. The PCR mix contained 10  $\mu$ l PCR buffer (Tiagen Biotech (Beijing) Co., Ltd., Beijing, China), 100 nM primer, 200  $\mu$ M each of the four dNTPs (Tiagen (Beijing) Co., Ltd.) and

Table I. Number of strains in different locations in China.

Location	Number of isolates
Anhui	11
Shanxi	16
Beijing	11
Fujian	24
Gansu	12
Guangxi Zhuang Autonomous Region	29
Sichuan	1
Henan	12
Hunan	7
Xizang (Tibet) Autonomous Region,	4
Xinjiang Uygur Autonomous Region	11
Jilin	12
Zhejiang	11

Table II. Number of strains of each spoligotype pattern.

Spoligotype	Number of strains
Beijing	82
T	12
U	27
MANU	10
Haarlem	4
EAI	2
LAM	2
S	1
CAS	3
New	18

MANU, a new family from India; LAM, Latin-American-Mediterranean lineage; EAI, East African-Indian lineage; CAS, Central-Asian lineage; S, Sicily and Sardinia family; U, Ural region; T, modern tuberculosis strains.

0.5 units DNA Taq Polymerase (Takara Bio, Inc., Otsu, Japan). An initial denaturation of 5 min at 94°C was followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 62°C for 45 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min in a Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA).

Negative controls (reagents only, no DNA) were included each time the PCR was performed. The positive control was 500 pg DNA from the *M. tuberculosis* reference strain H37Rv. The presence and size of each PCR product was determined by electrophoresis on 2% agarose gel in Tris/boric acid/ethylenediaminetetraacetic acid buffer (Tiagen Biotech (Beijing) Co., Ltd.) followed by staining with ethidium bromide (SBS Genetech Co., Ltd., Beijing, China).

The PCRs were conducted a minimum of two times to validate the reproducibility. The variants were confirmed by the sequencing of the new PCR products.

Table III. Comparison of the *Mycobacterium tuberculosis* strains containing mutations in the Beijing family and the non-Beijing family.

Strain	Beijing family	Non-Beijing family
Strains with PE35 mutation	4	19 <sup>a</sup>
Strains with PPE68 mutation	2	6 <sup>b</sup>
All	82	79

<sup>a</sup>P=0.001; Pearson  $\chi^2$  test, Beijing strains vs. non-Beijing strains. <sup>b</sup>P=0.253; Pearson  $\chi^2$  test, Beijing strains vs. non-Beijing strains. PE35, Pro-Glu 35; PPE68, Pro-Pro-Glu 68.

Strain	1	50	
H37Rv	MEKMSHDPIAADIGTQVSDNALHGVTAGSTALTSVTGLVPAGADEVSAQA		
GX06045	MEKMSHDPIAADIGTQVSDNALHGVTAGSTALTSVTGLVPAGADEVSAQA		
JL06018	MEKMSHDPIAADIGTQVSDNALHGVTAGSTALTSVTGLVPAGADEVSAQA		
GX06043	MEKCHMIRSLPTLARK-----		
GX06047	MEKCHMIRSLPTLARK-----		
GX06117	MEKCHMIRSLPTLARK-----		
GX06130	MEKCHMIRSLPTLARK-----		
HuN06004	MEKCHMIRSLPTLARK-----		
HuN06026	MEKCHMIRSLPTLARK-----		
HuN06101	MEKCHMIRSLPTLARK-----		
XJ06116	MEKCHMIRSLPTLARK-----		
XJ06183	MEKCHMIRSLPTLARK-----		
JL06009	MEKCHMIRSLPTLARK-----		delete an A
ZJ06098	MEKCHMIRSLPTLARK-----		
ShanX05290	MEKCHMIRSLPTLARK-----		
AH03009	MEKCHMIRSLPTLARK-----		
FJ05395	MEKCHMIRSLPTLARK-----		
FJ05413	MEKCHMIRSLPTLARK-----		
FJ05484	MEKCHMIRSLPTLARK-----		
GX06002	MEKCHMIRSLPTLARK-----		
GX06040	MEKCHMIRSLPTLARK-----		
GX06162	MEKCHMIRSLPTLARK-----		
GX06203	MEKCHMIRSLPTLARK-----		
GX06204	MEKCHMIRSLPTLARK-----		

  

Strain	51	99		Spoligotyping
H37Rv	ATAFTSEGIQLLASNASAQDQLHRAGEAVQDVARTYSQIDDDGAAGVFAE			
GX06045	ATAFTSEGIQLLPNSASAQDQLHRAGEAVQDVARTYSQIDDDGAAGVFAE		GCT-CCT	Beijing
JL06018	ATAFTSEGIQLLASNASAQDQLHRAGEAAQDVARTYSQIDDDGAAGVFAE		GTC-GCC	Beijing
GX06043	-----			U
GX06047	-----			Beijing
GX06117	-----			U
GX06130	-----			U
HuN06004	-----			MANU
HuN06026	-----			T
HuN06101	-----			new
XJ06116	-----			U
XJ06183	-----			U
JL06009	-----			Beijing
ZJ06098	-----			U
ShanX05290	-----			U
AH03009	-----			T
FJ05395	-----			T
FJ05413	-----			U
FJ05484	-----			U
GX06002	-----			U
GX06040	-----			U
GX06162	-----			U
GX06203	-----			U
GX06204	-----			new

Figure 1. Sequence alignment of the PE35 antigen of the 8 strains of *Mycobacterium tuberculosis* exhibiting alterations. Of the total 161 strains, 23 strains exhibited alterations at the gene level whilst the remaining 138 strains were identical to the H37Rv strain. The T-cell epitope areas are boxed in H37Rv. Shading indicates the location of amino acid alterations. PE35, Pro-Glu 35. MANU, a new family from India; U, Ural region.

**Sequence and data analysis.** The sequences of the PCR products were determined using an ABI 3730xl DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The sequences were first aligned using ClustalW software (19) with the PE35 and PPE68 gene sequences from the *M. tuberculosis* H37Rv genome to determine the PE35 and PPE68 region. Sequence comparisons and translations were



Strain	1	60	
H37Rv	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
BJ05025	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
FJ05063	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
XJ06025	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
FJ05406	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
FJ06051	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
FJ05349	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
HuN06004	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		
HeN06041	MLWHAMPPELNTARLMAGAGPAPMLAAAAGWQTLAALDAQAVELTARLNSLGEAWTGGG		5bp deletion
Strain	61	120	
H37Rv	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
BJ05025	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
FJ05063	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
XJ06025	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
FJ05406	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
FJ06051	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
FJ05349	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
HuN06004	SDKALAAATPMVVWLQTASTQAKTRAMQATAQAAAYTQAMATTPSLPEIAANHITQAVLT		
HeN06041	QGACGNCNAGGLATNRVNTGQDPCDAGDGASRGHHPGHGHDAAAGDRRQPHHPGRPYGH		
Strain	121	180	
H37Rv	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
BJ05025	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
FJ05063	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
XJ06025	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
FJ05406	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
FJ06051	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
FJ05349	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
HuN06004	ATNFFGINTIPIALTEMDYFIRMWNQAALAMEVYQAETAVNTLFEKLEPMASILDPGASQ		
HeN06041	OLLRYOHDPRVDRDGLFHPYVEPGSPGNGGLPGRDRG-----		GAT-AAT GAT-AAT
Strain	181	240	
H37Rv	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
BJ05025	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
FJ05063	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
XJ06025	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
FJ05406	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
FJ06051	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
FJ05349	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
HuN06004	STTNPIFGMPSPGSSTPVGQLPPAATQTLGQLGEMSGPMQOLTQPLQQVTSLSFQVGGTG		
HeN06041	-----		GTG-CTG GTG-CTG
Strain	241	300	
H37Rv	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
BJ05025	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
FJ05063	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
XJ06025	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
FJ05406	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
FJ06051	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
FJ05349	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
HuN06004	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
HeN06041	GGNPADEEAAQMGLLGTSPLSNHPLAGGSGPSAGAGLLRAESLPGAGGSLTRTPLMSQLI		
Strain	301	360	
H37Rv	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
BJ05025	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
FJ05063	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
XJ06025	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
FJ05406	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
FJ06051	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
FJ05349	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
HuN06004	EKPVAPSVMPAAAAGSSATGGAAPVGAGAMGQGAQSGGSTRPGLVAPAPLAQEREEDDED		
HeN06041	-----		CCG-TCG
Strain	361	368	Spoligotyping
H37Rv	DWDEEDDW		
BJ05025	DWDEEDDW		Beijing
FJ05063	DWDEEDDW		T
XJ06025	DWDEEDDW		U
FJ05406	DWDEEDDW		EAI
FJ06051	DWDEEDDW		EAI
FJ05349	DWDEEDDW		T
HuN06004	-----EDDW	18bp deletion	MANU
HeN06041	-----		Beijing

Figure 2. Sequence alignment of the PPE68 antigen in the 8 strains of *Mycobacterium tuberculosis* exhibiting alterations. Of the total 161 strains, 8 strains exhibited alterations at the gene level and the remaining 153 strains were identical to the H37Rv strain. Shading indicates locations of amino acid alterations. PPE68, Pro-Pro-Glu 68.

conducted using Bioedit software, version 7.1.3.0 (20). The Immune Epitopes Database (IEDB) (<http://www.iedb.org/>) was used and 1 human T-cell epitope in PE35 and 62 in PPE68 were found (21). In addition, SPSS software, version 14.0 (SPSS, Inc., Chicago, IL, USA) was used to conduct  $\chi^2$  analysis.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Mutation and deletion in gene sequences.** The genes encoding PE35 and PPE68 were amplified and the sequences compared.

All 161 strains yielded PCR products of these two antigens. Among the 161 *M. tuberculosis* strains, 23 isolates exhibited polymorphisms in the gene sequence of PE35 (Fig. 1) and 8 strains exhibited polymorphisms in PPE68. For PE35, there were 21 strains containing an A deletion and the remaining 2 strains harbored two different nonsynonymous mutations. For PPE68, two isolates had two different deletions and six strains showed five nonsynonymous mutations (Fig. 2).

**Changes at the protein level.** Figs. 1 and 2 present the amino acid (AA) alterations and their positions in the PE35 and

Table IV. Amino acid alterations of human T-cell epitopes in the antigens, PE35 and PPE68<sup>a</sup>.

Epitope ID	Epitope peptide sequence	Rv locus	Gene	Amino acid alteration
144881	EGIQLLASNASAQ	Rv3872	PE35	GCT(A)-CCT(P); Frameshift
183	AAGSSATGGAA <b>P</b> VGAGAMGQGAQSG	Rv3873	PPE68	CCG(P)-TCG(S); Frameshift
191	AAGWQTL <b>S</b> AALDAQAVELTARLNSL	Rv3873	PPE68	Frameshift(5 bp deletion)
265	AALAMEVYQAETAVNTLF	Rv3873	PPE68	ATG(M)-ATA(I); Frameshift
2434	ALAMEVYQAETAVNTLF <b>E</b> KLEPMAS	Rv3873	PPE68	ATG(M)-ATA(I); Frameshift
2922	ALTEM <b>D</b> YFIRMWNQAALAMEVYQAE	Rv3873	PPE68	ATG(M)-ATA(I); Frameshift
3098	AMGQGAQSGGSTR <b>P</b> GLVA	Rv3873	PPE68	Frameshift
4186	ARLMAGAGPAPMLAAAAG	Rv3873	PPE68	No change
4187	ARLMAGAGPAPMLAAAAGWQTL <b>S</b> AA	Rv3873	PPE68	No change
4727	ASQSTTNPIFGMPSPG <b>S</b> STPVGQLP	Rv3873	PPE68	Frameshift
4969	ATGGAA <b>P</b> VGAGAMGQGAQ	Rv3873	PPE68	CCG(P)-TCG(S); Frameshift
5063	ATNFFGINTI <b>P</b> IAL	Rv3873	PPE68	Frameshift
11486	EEAAQMGLLGT <b>S</b> PLSNHP	Rv3873	PPE68	Frameshift
14339	ETAVNTLF <b>E</b> KLEPMASIL	Rv3873	PPE68	Frameshift
15812	FFGINTI <b>P</b> IA	Rv3873	PPE68	Frameshift
16010	FGMPSPG <b>S</b> STPVGQLPPA	Rv3873	PPE68	Frameshift
18685	GAMGQGAQSGGSTR <b>P</b> GLVAPAPLAQ	Rv3873	PPE68	Frameshift
18776	GASQSTTNPIFGMPSPG <b>S</b>	Rv3873	PPE68	Frameshift
19860	GGGSDKALAAATPMV <b>V</b> WLQTASTQA	Rv3873	PPE68	Frameshift
20016	GGSGPSAGAGLLRAESLP	Rv3873	PPE68	Frameshift
20048	GGTGGGNPADEEAAQMGL	Rv3873	PPE68	Frameshift
20997	GLLGT <b>S</b> PLSNHPLAGGSGPSAGAGL	Rv3873	PPE68	Frameshift
21179	GLVAPAPLAQEREED <b>DEDDWDEEDD</b>	Rv3873	PPE68	18 bp deletion; Frameshift
21707	GPMQQLTQPLQ <b>Q</b> VTSLFS	Rv3873	PPE68	GTG(V)-CTG(L); Frameshift
22351	GSGPSAGAGLLRAESLPAGGSLTR	Rv3873	PPE68	Frameshift
22531	GSSTPVGQLPPAATQTLGQLGEMSG	Rv3873	PPE68	Frameshift
22657	GTGGGNPADEEAAQMGLLGT <b>S</b> PLSN	Rv3873	PPE68	Frameshift
29846	KALAAATPMV <b>V</b> WLQTAST	Rv3873	PPE68	Frameshift
35251	LDPGASQSTTNPIFG	Rv3873	PPE68	GAT(D)-AAT(N); Frameshift
35652	LEPMASILDPGASQSTTN	Rv3873	PPE68	GAT(D)-AAT(N); Frameshift
35819	LFEKLEPMASILDPGASQSTTNPIF	Rv3873	PPE68	GAT(D)-AAT(N); Frameshift
37727	LLRAESLPAGGSLTR <b>P</b>	Rv3873	PPE68	Frameshift
38448	LPEIAANHITQAVLTATN	Rv3873	PPE68	Frameshift
38492	LPGAGGSLTRTP <b>L</b> MSQLIEKPVAPS	Rv3873	PPE68	Frameshift
39817	LTATNFFGINTI <b>P</b> IA	Rv3873	PPE68	Frameshift
41291	MDYFIRMWNQAALAMEVY	Rv3873	PPE68	ATG(M)-ATA(I); Frameshift
42095	MLWHAMPPELNTARLMAG	Rv3873	PPE68	No change
46130	NTIPIALTEM <b>D</b> YFIRMWN	Rv3873	PPE68	Frameshift
48567	PMLAAAAGWQTL <b>S</b> AALDA	Rv3873	PPE68	No change
49875	PVGQLPPAATQTLGQL <b>E</b>	Rv3873	PPE68	Frameshift
50366	QATAQAAAYTQAMATTPSLPEIAAN	Rv3873	PPE68	Frameshift
51367	QLIEKPVAPSVMPAAAAGSSATGGA	Rv3873	PPE68	Frameshift
52167	QQVTSLSFSQVGGTGGGN <b>P</b>	Rv3873	PPE68	GTG(V)-CTG(L); Frameshift
52556	QTLGQLGEMSGPMQQLTQ	Rv3873	PPE68	Frameshift
60492	SQLIEKPVAPSVMPAAA	Rv3873	PPE68	Frameshift
62250	SVMPAAAAGSSATGGA <b>P</b>	Rv3873	PPE68	CCG(P)-TCG(S); Frameshift
64822	TLGQLGEMSGPMQQLTQPLQ <b>Q</b> VTSL	Rv3873	PPE68	Frameshift
65054	TL <b>S</b> AALDAQAVELTARLN	Rv3873	PPE68	Frameshift (5 bp deletion)
65767	TPSLPEIAANHITQAVLTATNFFGI	Rv3873	PPE68	Frameshift
65912	TQPLQ <b>Q</b> VTSLFSQVGGTGGGNPADE	Rv3873	PPE68	GTG(V)-CTG(L); Frameshift
66074	TRPGLVAPAPLAQEREED	Rv3873	PPE68	Frameshift
66364	TSPLSNHPLAGGSGPSAG	Rv3873	PPE68	Frameshift

Table IV. Continued.

Epitope ID	Epitope peptide sequence	Rv locus	Gene	Amino acid alteration
68284	VELTARLNSLGEAWTGGG	Rv3873	PPE68	Frameshift(5 bp deletion)
68285	VELTARLNSLGEAWTGGGSDKALAA	Rv3873	PPE68	Frameshift (5 bp deletion)
69128	VITMLWHAMPPELNTARLMAGAGPA	Rv3873	PPE68	Frameshift
69795	VLATNFFGINTIPIALT	Rv3873	PPE68	Frameshift
69796	VLATNFFGINTIPIALTEMDYFIR	Rv3873	PPE68	Frameshift
71944	VWLQTASTQAKTRAMQAT	Rv3873	PPE68	Frameshift
71945	VWLQTASTQAKTRAMQATAQAAYT	Rv3873	PPE68	Frameshift
73833	YFIRMWNQAALAMEV	Rv3873	PPE68	ATG(M)-ATA(I); Frameshift
121011	TPMVVWLQTASTQAKTR	Rv3873	PPE68	Frameshift
144907	IALTEMDYFIRMWNQAALAMEVY	Rv3873	PPE68	ATG(M)-ATA(I); Frameshift
144964	TNFFGINTIPIALT	Rv3873	PPE68	Frameshift

<sup>a</sup>The coding sequence of PE35 and PPE68 of *Mycobacterium tuberculosis* H37Rv strain has been used as the reference sequence. Bold letters indicate locations of amino acid alterations. PE35, Pro-Glu 35; PPE68, Pro-Pro-Glu 68; ID, identification.

PPE68 antigens. All the alterations resulted in an AA change. A total of 21 strains with an A deletion in PE35 resulted in a frameshift, and therefore the premature termination of the protein, preventing its production and thereby impacting upon protein function. HeN06041 contained a 5 base pair (bp) deletion located at the fifth AA of PPE68, which additionally resulted in premature termination, and may impact upon protein function due to the deletion abolishing the production of the protein. HuN06004 contained an 18 bp deletion, which resulted in a six AA deletion in PPE68.

**Spoligotyping of variant strains.** For PE35, 23 variant strains were identified, including 4 Beijing strains, 13 U family strains, 3 T strains, 1 MANU strain and 2 new spoligotype strains. The two strains with nonsynonymous mutations were members of the Beijing family. For PPE68, 8 variant strains including 2 Beijing strains, 2 T strains, 2 EAI strains, 1 U strain and 1 MANU strain were identified. The two EAI strains, FJ06051 and FJ05406, exhibited the same mutation in 229(V-L) in the AA sequence of PPE68, which may represent a unique mutation in EAI strains. HuN06004 exhibited polymorphisms in PE35 and PPE68.

The prevalence of strains containing a PE35 mutation in the non-Beijing family is significantly greater compared with the Beijing family (Table III,  $P < 0.01$ ). The prevalence of strains with the PPE68 mutation in the non-Beijing family is greater compared with the Beijing family, however this was identified to be a significant difference.

**Alterations in T-cell epitopes.** There is 1 human T-cell epitope in PE35 and 62 in PPE68 according to the IEDB (<http://www.iedb.org/>) (21). Table IV presents the alterations in the T-cell epitopes of the two antigens. All mutations observed in PE35, except for that in JL06018, affected the T-cell epitopes. For PPE68, there were no non-epitope regions in the gene, as the 62 T-cell epitopes covered the whole gene sequence. This additionally indicates the importance of the PPE68 antigen for the development of T-cell immune responses following infection. Among all of the strains, 58/62 T-cell epitopes in PPE68

(93.5%), exhibited AA alterations resulting from nucleotide alterations (Table IV).

The 5 bp deletion in HeN06041 resulted in a frameshift in the PPE68 protein code, leading to alterations in the corresponding T-cell epitopes including IEDB\_ID 191, 65054, 68284, 68285 and further downstream epitopes. The 18 bp deletion in HuN06004 resulted in a 6 AA deletion in IEDB\_ID21179.

## Discussion

In the present study, 161 clinical *M. tuberculosis* strains in China were selected which originated from a large geographical area and exhibited different spoligotyping patterns. This strategy was selected so that the data provided would be representative of the genetic diversity that may be present within China, at least to some extent.

In previous studies, genetic approaches coupled with biochemical analyses have indicated that proteins encoded by the RD1 locus are part of a secretion system required for ESAT-6 and CFP-10 export (22-26), hereafter referred to as the ESAT-6 system-1 (ESX-1). PE35 (Rv3872) and PPE68 (Rv3873) are encoded by RD1 and exhibited immunodominance (9). PE35 is an important antigen that stimulates human peripheral blood mononuclear cells in protective Th1 cell assays, demonstrating antigen-induced proliferation and  $\gamma$ -interferon secretion (4). PPE68 is predominantly associated with the cell wall (27) and forms complexes with the RD1 locus proteins Rv3866, Rv3868, CFP-10 and ESAT-6 (28,29). A recent study (9) indicated that PE35 and PPE68 may serve a major role in RD1-associated pathogenesis, and may contribute to the establishment and maintenance of *M. tuberculosis* infection. Among the 161 strains investigated in the present study, 14.3% of strains with an A deletion in PE35 resulted in premature termination leading to a 16 AA peptide as opposed to the full length protein of 99 AA. This deletion would result in the prevention of protein production, and consequently lead to the complete loss of PE35 function. In addition, the 5 bp deletion in HeN06041 of PPE68 resulted in premature



termination, and therefore may exert an effect on protein function via the abolition of protein production. Strains carrying mutations that lead to alterations in the functions of PE35 and PPE68 may be significantly compromised with regards to their virulence. Therefore, polymorphisms in PE35 and PPE68 may result in alterations in the functions of these proteins, which may potentially affect strain virulence. Furthermore, as PE35 has been demonstrated to be essential for ESXA/B secretion and RD1-mediated virulence (30), the null mutant of Rv3872 may influence the release of the ESX-1 antigens, CFP-10 and ESAT-6. PPE68 is a gating protein that regulates the release of ESX-1 antigens (30) therefore, abolition of PPE68 protein may additionally affect the release of CFP-10 and ESAT-6. To investigate this further, virulence comparison of mutant strains and wild strains of PE35 and PPE68 should be conducted.

PE/PPE genes are known to vary and to encode cell surface-exposed proteins, which has led to the hypothesis that they may be involved in antigenic variation (31) and have been suggested to be a 'molecular mantra' to aid in the escape of host immunity. Comas *et al* (32) reported that the human T-cell epitopes of *M. tuberculosis* are evolutionarily hyperconserved and suggested that *M. tuberculosis* lacks antigenic variation and immune evasion ability, however, the study excluded PE/PPE genes. In the current study, there were 63 human T-cell epitopes identified in PE35 and PPE68 according to the IEDB (21). Among the strains, 59/63 T-cell epitopes (93.7%), exhibited AA alterations resulting from nucleotide alterations. The large number of amino acid alterations in these T-cell epitopes may reflect ongoing immune evasion. The data from the current study supports the view that certain PE/PPE genes exhibiting high sequence variation may be involved in antigenic variation induced immune evasion.

The prevalence of strains with PE35 mutations in the non-Beijing family was significantly greater compared with the Beijing family, indicating that the Beijing family strains are less changeable in the T-cell epitopes of PE35 than the non-Beijing family strains. This is supported by a previous study, which demonstrated that Beijing strains from different geographic areas exhibited a high degree of genetic conservation compared with the other *M. tuberculosis* strains (33). There is evidence that T-cell responses may contribute directly to human-to-human transmission of MTBC (34). The current study indicated that the Beijing strains were more likely to be recognized by host T-cells in PE35 than the non-Beijing strains, which may render them easier to transmit than the non-Beijing strains. Furthermore, analysis of sequences of PE35 and PPE68 in isolates of these various lineages that have been isolated from non-Chinese populations could provide interesting information and insight.

In conclusion, it has been previously reported that PE35 had potential as a serodiagnostic candidate for *M. tuberculosis* (35). The results of the current study indicate that PE35 harbors a comparatively high number of AA alterations, suggesting that strain diversity should be considered during the further development of novel serodiagnostic candidates that contain PE35.

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