RESEARCH ARTICLE

Variation of the expression of *Mycobacterium tuberculosis ppe44* gene among clinical isolates

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Abstract

PPE44 is a member of the *Mycobacterium tuberculosis* PPE proteins, a polymorphic family of 69 glycine-rich proteins that predictively represent a source of antigenic variation. The genetic diversity of gene *ppe44* among clinical isolates has been studied. No genomic polymorphism of *ppe44* was found by a PCR-restriction fragment length polymorphism assay using three restriction enzymes. Nucleotide sequencing of gene *ppe44* of a number of isolates, selected to represent the major phylogenetic lineages of *M. tuberculosis*, showed no nucleotide substitution, with the exception of isolates of the Beijing genotype. These findings indicate that gene *ppe44* is basically conserved among *M. tuberculosis* strains. The expression of gene *ppe44* was then determined at the transcriptional level by a real-time reverse transcriptase PCR assay. Extremely high quantitative variations in *ppe44* expression were found among the isolates; *ppe44* expression of the Beijing strains was significantly higher than the non-Beijing strains. To test whether differential expression of gene *ppe44* has the potential to provide a dynamic antigen display, antibodies to PPE44 were titered in the sera of *M. tuberculosis*-infected subjects. Variation of antibody response to PPE44 was found with regard to both antibody titers and the proportion of responding subjects. These results indicate that the differential expression of genes *ppe* could influence the host's immune responsiveness, thus having implications in the immunopathogenesis of tuberculosis.

Introduction

Sequencing of the genome of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, has revealed that *c.* 10% of the genome codes for two large unrelated families of highly acidic glycine-rich proteins, termed PE and PPE on the basis of their characteristic Pro–Glu and Pro–Pro–Glu motifs near the N-terminal domain (Cole *et al.*, 1998). The PE/PPE protein families are unique to the genus *Mycobacterium* and are strongly present in *M. tuberculosis* complex and in other mycobacterial species (Gey van Pittius *et al.*, 2006). The PPE protein family contains 69 members characterized by a conserved N-terminal domain of about 180 amino acids and C-terminal segments that vary in sequence and length. The PPE proteins are classified into four subfamilies: the first subfamily (PPE-SVP) has the well-conserved motif Gly–X–X–Ser–Val–Pro—X–X–Trp located approximately at position 350; the second constitutes the major polymorphic tandem repeats (MPTR) subfamily and is characterized by the presence of multiple tandem repeats of the motif Asn–X–Gly–X–Gly–Asn–X–Gly; the third (PPE-PPW) is characterized by a conserved region comprising Gly–Phe–X–Gly–Thr and Pro–X–X–Pro–X–X–Trp motifs; and the last PPE subfamily includes proteins that are unrelated other than having the PPE motif (Gey van Pittius *et al.*, 2006).

Although the role of PPE proteins in *M. tuberculosis* infection is largely unknown, they are considered to have an immunological significance. Subcellular fractionation and immunoelectron microscopy studies have indicated that some PPE proteins are located at the periphery of the bacterial cell and are therefore accessible to the host immune system (Sampson *et al.*, 2001; Okkels *et al.*, 2003; Demangel *et al.*, 2004; Le Moigne *et al.*, 2005); moreover, PPE41 is shown to be secreted by pathogenic mycobacteria (Abdallah *et al.*, 2006). Their importance in tuberculosis, however, is

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supported by the finding that several genes encoding PPE proteins are deleted in the genome of the vaccine strain *Mycobacterium bovis* bacille Calmette–Guérin (BCG) (Gordon *et al.*, 1999) and by the demonstration that certain PPE proteins induce strong immune responses in animals and humans infected with *M. tuberculosis* (Dillon *et al.*, 1999; Skeiky *et al.*, 2000; Choudhary *et al.*, 2003; Okkels *et al.*, 2003; Chakhaiyar *et al.*, 2004; Demangel *et al.*, 2004; Le Moigne *et al.*, 2005; Singh *et al.*, 2005). In particular, immunization by plasmid DNA expressing genes coding for two distinct PPE proteins, i.e. PPE14 and PPE18, proved to confer protective immunity against a challenge with *M. tuberculosis* in murine experimental models (Dillon *et al.*, 1999; Skeiky *et al.*, 2000). Predictively, the PPE proteins are antigenically polymorphic and are likely to be involved in the antigenic variation of *M. tuberculosis* strains or in the inhibition of antigen processing (Cole *et al.*, 1998; Cole, 1999); this might enable *M. tuberculosis* to evade the host immune system.

Based on the observation that gene Rv2770c, now termed *ppe44*, of *M. tuberculosis* H37Rv is underexpressed in the attenuated strain H37Ra (Rindi *et al.*, 1999), the authors' research has been recently focused on the PPE-SVP PPE44 protein and in this context the antigenic nature of PPE44 for mice infected with *M. bovis* BCG was demonstrated (Bonanni *et al.*, 2005). To understand the immunological role of PPE44 in human infection, the study of the genetic diversity of PPE44 gene among clinical isolates might be of interest, as genetic variations could potentially account for some of the differences in the ability of the isolates to evade the host immune system. These considerations prompted investigation of the polymorphism, if any, and the expression of the gene *ppe44* among clinical isolates of *M. tuberculosis* and then testing of the antigenicity of protein PPE44 in human infection.

Materials and methods

Bacterial strains and growth conditions

A study sample of 30 clinical isolates was selected from 248 isolates collected in 2002 in Tuscany, Italy; the isolates, genotyped by the standardized spoligotying and IS*6110*-restriction fragment length polymorphism (RFLP) methods (Lari *et al.*, 2005), were selected to represent the genotypes reported in the fourth international spoligotyping database (SpolDB4) (Brudey *et al.*, 2006). As shown in Fig. 1, the isolates show distinct spoligotypes and IS*6110*-RFLP patterns. *Mycobacterium tuberculosis* strains H37Rv and H37Ra were used as controls. For some experiments, 10 additional isolates of Beijing genotype were studied. The strains were cultured using mycobacteria growth indicator tubes (MGIT), according to the standard procedures of the BD BACTEC MGIT 960 system (Becton Dickinson).

DNA extraction

Bacterial cells from 1.5 mL aliquots of MGIT cultures were suspended in 300 μL of 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% NaN_3 containing 20% Chelex 100 (Biorad). Samples were held for 30 min at 56 °C, vortexed, and then maintained at 100 °C for 12 min. After spinning down at 12 000 ***g*** for 3 min, the supernatants were collected and the samples were stored at 4 °C until subsequent analysis.

PCR-restriction fragment length polymorphism (RFLP) analysis of gene *ppe44*

Gene *ppe44* was amplified by PCR using the primers ^{142}GTCATCACGCGGCTGAGCAC^{161} and ^{1131}GGGCATAACAATCGGCTTGA^{1112}. PCR conditions were 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 1 μM primers, 0.2 mM deoxynucleoside triphosphates, 1.25 U of Taq polymerase (Dynazyme), and 10 ng DNA per 50 μL of reaction mixture. PCR amplification was performed under the following conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Ten-microliter aliquots of PCR products were analyzed by 2% agarose gel electrophoresis. The 990-bp PCR product was purified with the QIAquick gel extraction kit (Qiagen, Chatsworth, CA) and then digested with AluI, NaeI or RsaI. The digested products were separated on a 10% polyacrilamide gel and visualized by silver stain.

RNA extraction

MGIT tubes were inoculated with 0.2 mL of a log-phase culture, and $OD_{600\,nm}$ was measured daily. Total RNA was extracted when cultures reached an OD of 0.4–0.6 (log-phase growth). Bacteria were mechanically broken in a FastPrep cell disruptor (Bio101, Thermo) by six pulses of 20 s at 6.0 m s^{-1}. Between pulses, samples were maintained on ice for 2 min. RNA was then extracted with 300 μL of chloroform and, after 5 min of centrifugation at 13 000 ***g*** and 4 °C, the aqueous phase was collected and total RNA was precipitated for 1 h at −80 °C with 0.1 volume of 5 M ammonium acetate and an equal volume of isopropanol. The pellet was washed with 75% ethanol and resuspended in 30 μL of diethyl pyrocarbonate (DEPC)-treated H_2O. RNA was purified using an RNeasy kit (Qiagen) and treated with DNase (Qiagen).

Synthesis of cDNA

RNA (12 μL) was reverse transcribed with 20 pmol of specific antisense primer LC ppe44-R or LC sigA-R (Table 1) using the transcriptor first-strand cDNA synthesis kit (Roche Diagnostics), as recommended by the

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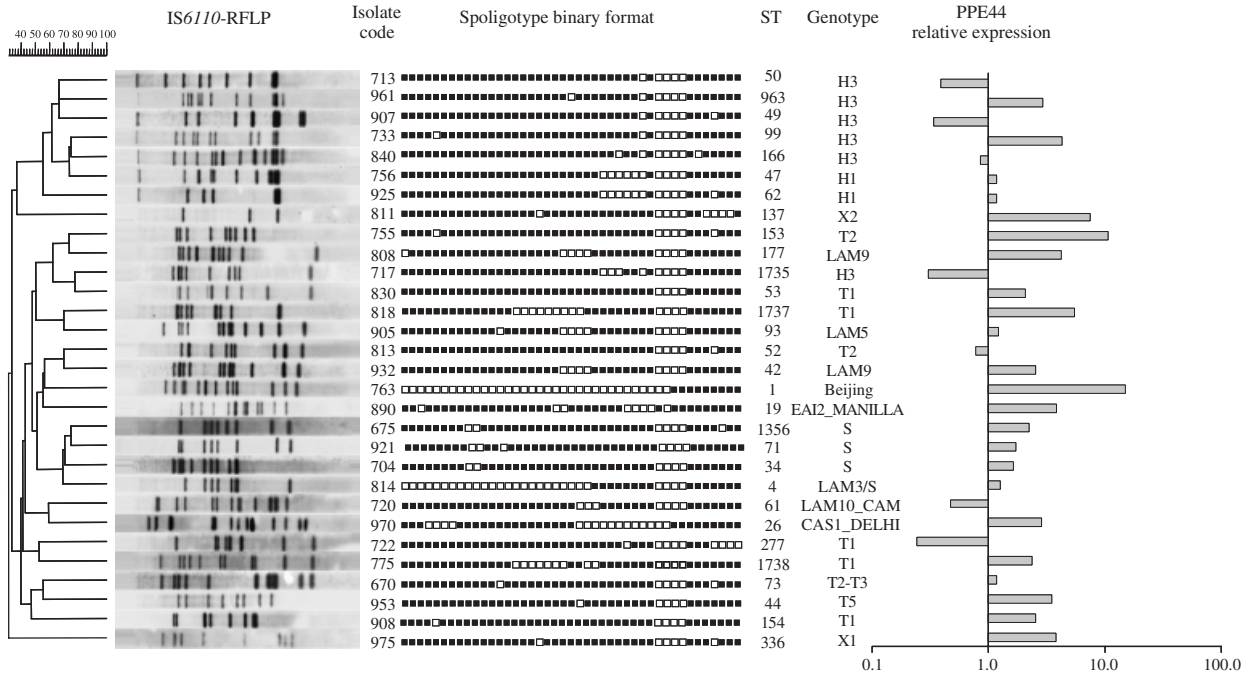

Fig. 1. Molecular characteristics and PPE44 expression in *Mycobacterium tuberculosis* clinical isolates. IS*6110*-RFLP pattern, code, spoligotype binary format, sharetype (ST), and genotype are shown for each isolate. The dendrogram on the left of the RFLP panels, showing the relatedness between the isolates, was constructed by the UPGMA clustering method using the Dice coefficient (Lari *et al.*, 2005); share types and genotypes were attributed to the isolates according to the SpolBD4 database (Brudey *et al.*, 2006). PPE44 expression of the clinical isolates is shown on the right. The horizontal bars show the ratio between the *ppe44* mRNA levels in the clinical isolates and in reference strain H37Rv, both normalized to the level of *sigA* mRNA (relative expression).

Table 1. Primers used for RT-LightCycler PCR

Gene	Primer	Sequence (5′–3′)	PCR amplicon size (bp)
ppe44	LC ppe44-F	CCGCAAGACTGAACCC	232
	LC ppe44-R	GGAACATCGAGATTGAGG	
	ppe44 EXT-F	GCTATGGCGAAATGTGG	324
	ppe44 EXT-R	CGTGAAACGCGGATTCT	
sigA	LC sigA-F	CGCCTACCTCAAACAGA	346
	LC sigA-R	GGAGAACTTGTACCCCT	
	sigA EXT-F	GTCAAGCACGCAAGGAC	429
	sigA EXT-R	TGATGGCCTGGCGAATC	

manufacturer. To exclude the possibility of DNA contamination, the RNA samples were subjected to PCR without prior RT.

Quantitative real-time PCR

Oligonucleotides were designed using LIGHTCYCLER PROBE DESIGN software (Roche) and are listed in Table 1. The quantification of gene expression by LightCycler (Roche) was determined relative to a standard curve for the *ppe44* gene and the normalizing gene (*sigA*) and was included in each experiment. The template for the standard curves was generated via conventional PCR using genomic DNA from *M. tuberculosis* H37Rv and 0.2 μM of the respective primers EXT (sequences shown in Table 1). Cycling conditions were one cycle at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. A standard curve was prepared for each gene (*sigA*, *ppe44*) by purification and 10-fold serial dilution of the respective amplicons.

For quantification, LightCycler PCR was performed in 20 μL final volume in capillary tubes in a LightCycler instrument (Roche Diagnostic, Mannheim, Germany). The reaction mixtures contained 4 μL of LightCycler FastStart DNA mastermix plus for SYBR Green I (Roche Diagnostic), 0.25 μM *ppe44* primers (LC ppe44-F and -R) or 0.50 μM *sigA* primers (LC sigA-F and -R), and 5 μL of cDNA. All capillaries were sealed, centrifuged at 700 ***g*** for 5 s, and then amplified in a LightCycler instrument, with activation of polymerase (95 °C for 10 min), followed by 45 cycles of 10 s at 95 °C, 5 s at 60 °C, and 10 s at 72 °C. The temperature transition rate was 20 °C s^{-1} for all steps. Double-stranded PCR product was measured during the 72 °C extension step by detection of fluorescence associated with the binding of SYBR Green I to the product. To confirm the specificity of the PCR amplification products, melting curve analysis

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was performed under the following conditions: 95 °C for 0 s, cooling to 65 °C for 15 s, and finally a slow increase in the temperature to 98 °C at a rate of 0.1 °C s^{-1}. To further verify the specificity of the LightCycler PCR, the amplification product was analyzed by 2% agarose gel electrophoresis.

The concentration of each gene transcript was calculated by reference to the respective standard curve. *ppe44* expression level was measured by normalizing to *sigA*, and relative values were expressed as a ratio of normalized *ppe44* expression level in clinical isolates relative to that in *M. tuberculosis* H37Rv.

For each isolate, the quantitative expression assay was run in triplicates, yielding values with $< 20\%$ SD. For some representative strains, the experiments were repeated twice with high reproducibility using two different RNA samples.

Human sera and anti-PPE44 antibody assay

For the evaluation of antibodies against rPPE44, serum samples from 31 patients with active pulmonary tuberculosis, from eight healthy individuals with latent *M. tuberculosis* infection, as assessed by a QuantiFERON-TB positive test (Cellestis Limited, Carnagie, Vic., Australia) (Taggart *et al.*, 2004), and from nine healthy individuals with no immunological evidence of past infection (QuantiFERON-TB negative test) were tested by an enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates (Probind, Falcon, Italy) were coated overnight at 4 °C with 0.5 μg $well^{-1}$ of recombinant protein PPE44 (rPPE44) (Bonanni *et al.*, 2005) in carbonate buffer (pH 9.6). The plates were subsequently blocked for 1 h with phosphate-buffered saline (PBS) pH 7.4 containing 1% bovine serum albumin (PBS–BSA). The plates were then washed two times with PBS-0.05% Tween 20 (PBS–Tw) and incubated for 1 h with appropriate dilutions of human sera in PBS–Tw. The plates were washed with PBS–Tw and further incubated with either anti-human IgG-horseradish peroxidase (HRP) or anti-human IgM-HRP (Sigma). The enzyme reactions were carried out with tetramethylbenzidine (Sigma) and stopped with 0.05 M H_2SO_4; the absorbance values were measured at 450 nm. All steps were performed at room temperature (r.t.). Postcoating was performed with 150 μL $well^{-1}$; antigens, samples, conjugate, substrate, and H_2SO_4 were added in volumes of 100 μL $well^{-1}$. Anti-rPPE44 serum titer was considered as the highest dilution giving optical readings greater than a cut-off value calculated as the mean OD of six sera from tuberculin skin test-negative healthy individuals plus 3 SD at the initial serum dilution of 1 : 200.

Results and discussion

To study the polymorphism, if any, of gene *ppe44* of *M. tuberculosis*, a PCR-RFLP analysis was performed on 30 different clinical isolates that were selected to represent the major phylogenetic lineages of the SpolDB4 database (Brudey *et al.*, 2006). *Mycobacterium tuberculosis* H37Rv was used as a reference strain. In particular, a 990-bp sequence of gene *ppe44* was amplified by PCR from DNA extracted from each isolate, the amplicon was digested with restriction enzymes AluI, NaeI, or RsaI, and the digested products were electrophoresed on a 10% polyacrilamide gel. All the clinical isolates yielded the same restriction pattern as reference strain *M. tuberculosis* H37Rv (data not shown). Similar analysis was performed for other *ppe* genes (i.e. *ppe10*, *ppe13*, *ppe16*, and *ppe21* coding for PPE-MPTR proteins; *ppe28* coding for a unique PPE protein; *ppe33* coding for a PPE-SVP protein; and *ppe37* coding for a PPE-PPW protein) and identical restriction patterns were found in all the isolates (data not shown). The absence of restriction fragments polymorphism rules out insertion and deletion events, which are the major sources of genetic diversity within the *M. tuberculosis* complex (Brosch *et al.*, 2001). However, as PCR-RFLP analysis does not rule out that single nucleotide polymorphisms may occur in *ppe* genes, the *ppe44* gene and an upstream region of 120 bp were sequenced in 10 isolates (coded in Fig. 1 as 704, 720, 755, 763, 840, 905, 908, 921, 953, and 975); isolates were selected to represent the major phylogenetic lineages. With the exception of isolate 763 of the Beijing genotype, all the isolates did not show any nucleotide substitution as compared with reference strain H37Rv. Isolate 763, and 10 additional isolates of Beijing genotype, showed a mutation at gene position 581 (TTC → TCC, Phe → Ser), thus indicating the specificity of this nucleotide substitution in the Beijing genetic lineage. On the whole, the present findings indicate that gene *ppe44* is basically conserved among the isolates of different genotypes, in agreement with the report by Musser *et al.* (2000) suggesting that a relatively small percentage of PE and PPE proteins are variable. However, evidence has been reported that the PPE gene family exhibits a higher degree of sequence polymorphism than the genome as a whole (Fleischmann *et al.*, 2002) and an extensive codon volatily (Plotkin *et al.*, 2004).

As an alternative to structural and/or sequence variations of *ppe*44 among clinical isolates, a source of variation of PPE44 might come from a differential dynamic expression of *ppe44* gene. To test this hypothesis, the expression of gene *ppe44* was determined at the transcriptional level in the clinical isolates by a quantitative real-time reverse transcriptase (RT)-PCR assay using the LightCycler Instrument. Isolates were tested under a single condition, i.e. the exponential-phase growth in liquid culture; while this does not represent the natural environment in which mycobacteria grow, it was the most efficient way to test a large number of isolates. For this purpose, total RNA was extracted from the clinical isolates and from the reference

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strains *M. tuberculosis* H37Rv and H37Ra; RNA was reverse-transcribed by antisense primers specific for *ppe44* and for *sigA*, a gene encoding the major sigma factor, which is constitutively expressed in *M. tuberculosis* (Manganelli *et al.*, 1999); and the resulting cDNAs were amplified by real-time PCR in the presence of SYBR Green I and their concentration was calculated by standard curves obtained by running samples containing a known amount of target copies. The amount of *ppe44* transcript was then normalized to *sigA* and the *ppe44* relative expression of each isolate was compared with that of the *M. tuberculosis* H37Rv reference strain. As shown in Fig. 1, where *ppe44* expression data are placed next to those relative to the molecular characteristics of the isolates, the ratio between isolate and H37Rv relative expression was close to one in seven isolates, indicating that the *ppe44* mRNA levels were basically the same as reference strain H37Rv. In five isolates, the expression of *ppe44* was lower than in H37Rv (ratio ranging from 0.47 to 0.24), with a 4.12-fold decrease in isolate 722. The ratio for *ppe44* relative expression was 0.67 in *M. tuberculosis* H37Ra, the avirulent variant of H37Rv (data not shown), confirming an under-expression of *ppe44*, in agreement with earlier findings based on mRNA differential display and RT-PCR analysis (Rindi *et al.*, 1999). In 18 isolates, the level of *ppe44* expression was 1.64–15.09-fold higher than in H37Rv; interestingly, the isolate showing the highest *ppe44* expression (isolate coded 763) belonged to the Beijing genotype, a family of *M. tuberculosis* strains that are spreading world-wide and are considered to be potentially endowed with high virulence (Glynn *et al.*, 2002; Brudey *et al.*, 2006). To evaluate whether the high expression of *ppe44* was characteristic of this phylogenetic lineage, 10 additional Beijing isolates from the authors' collection were studied; the expression of *ppe44* was found to be higher than that of H37Rv in nine isolates (range 2.01–9.38). On the whole, the *ppe44* relative expression of the Beijing strains tested (mean $\pm$ SD, 5.87 ± 3.98) was significantly higher than that of the non-Beijing strains (2.54 ± 2.32) ($P = 0.023$, t test with Welch correction), which might be suggestive of a correlation of *ppe44* with virulence.

The significant diversity in gene expression among *M. tuberculosis* clinical isolates was recently demonstrated regarding some categories of genes, including PE/PPE genes (Gao *et al.*, 2005). In fact, the expression of the PPE genes appears to be controlled by a variety of independent mechanisms, indicating that the differential expression of such genes has the potential to provide a dynamic antigenic profile during host infection (Voskuil *et al.*, 2004). Therefore, the present results support the concept that PPE44 gene regulation could provide an additional mechanism, other than gene mutation, for differential antigen display that, in turn, might influence immune responsiveness to the antigen. To test this hypothesis the extent of the variation of

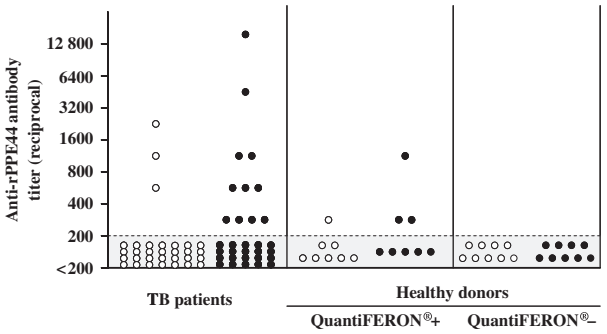

Fig. 2. IgM (open symbols) and IgG (filled symbols) antibody titers to rPPE44 in different groups of donors. Each symbol represents one donor. Antibody titer was considered as the highest dilution, giving optical readings greater than a cut-off value calculated as the mean OD of six sera from tuberculin skin test-negative healthy individuals plus 3 SDs at the initial serum dilution of 1 : 200 (horizontal dashed line).

immune response to PPE44 in *M. tuberculosis* human infection was determined by assaying IgG and IgM antibodies to rPPE44 in the sera of patients with active pulmonary tuberculosis and of healthy individuals with latent *M. tuberculosis* infection, as assessed by a QuantiFERON-TB-positive test; healthy individuals with no immunological evidence of past infection (QuantiFERON-TB negative) were used as controls. As shown in Fig. 2, significant titers of IgG anti-rPPE44 were detected in 11 of 31 (35.5%) patients with active tuberculosis and in three of eight (37.5%) healthy individuals with latent tuberculosis; anti-rPPE44 IgM antibodies were detected in three tuberculosis patients and, at a low titer, in one healthy donor with latent tuberculosis infection; as expected, none of the serum samples from negative healthy donors responded to rPPE44. These results indicate that variation of the antibody response to PPE44 does occur among patients with active tuberculosis, with regard to both antibody titers and, more importantly, to the proportion of responding patients. Although the variations of the antibody response of the tuberculosis patients cannot be directly related to the variations of gene expression of the clinical isolates, as tested sera and clinical isolates are from different donors, it is tempting to speculate that differential gene expression could provide a source of antigenic variability of *M. tuberculosis* that may have implications in the immunopathogenesis of tuberculosis.

Further clarification can be made on the role of PPE44 protein by comparing the clinical isolates of *M. tuberculosis* grown under different conditions that are more closely related to host infection, as well as *in vivo*. Moreover, studies of the association between the differential expression of the *ppe44* gene and the clinical phenotypes of the isolates will also generate useful information to understand the role of PPE44 in infection.

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