NIH Public Access

Author Manuscript

FEMS Immunol Med Microbiol. Author manuscript; available in PMC 2013 December 01.

Published in final edited form as:
FEMS Immunol Med Microbiol. 2012 December ; 66(3): 273–280. doi:10.1111/j.1574-695X.2012.01002.x.

Purified Protein Derivatives of Tuberculin - Past, Present, and Future

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Abstract

The tuberculin skin test, which involves monitoring the immune reaction to an injection of Purified Protein Derivative (PPD), has been the most widely used method for detecting infection with *Mycobacterium tuberculosis* since its development in 1930s. Until recently, the molecular composition of PPD was unknown. This thwarted the discovery of improved skin testing reagents and drastically hindered efforts to define the mechanism of action. Proteomic evaluation of PPD combined with a detailed analysis in the guinea pig model of tuberculosis led to further definition of the molecular composition of PPD. This communication reviews the history and current status of PPD, in addition to describing candidate next-generation PPD reagents, based on the use of an individual protein or protein cocktails.

Keywords

Tuberculosis; Purified Protein Derivative; Tuberculin skin test; Diagnosis

Introduction

Tuberculosis (TB) is a devastating infectious disease, responsible for an estimated 1.2 – 1.5 million deaths and 8.5 – 9.2 million cases in 2010, with most of these tragic events occuring in developing nations (WHO, 2011). Its severity is compounded by the ability of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, to reside as a persistent, asymptomatic infection, referred to as latent tuberculosis infection (LTBI). For almost a century, individuals infected with *Mtb* have been identified using the tuberculin skin test (TST). In 1890, Robert Koch proposed that a glycerin extract of tubercle bacilli would both cure and prevent tuberculosis. Although Koch's "Old Tuberculin" (OT) ultimately failed as a therapy, his findings were the catalyst for the development of the modern TST, the most important tool for identifying potential TB cases to date (Shingadia & Novelli, 2008).

The TST is also known as the Mantoux test, after the French physician Charles Mantoux (1877–1947) who established the diagnostic criteria for reading a TST. The Mantoux method, which is endorsed by the American Thoracic Society and Centers for Disease Control and Prevention (CDC), is the currently the gold-standard for determining whether an individual is infected with *Mtb.* This immunological test is comprised of two parts. First, the purified protein derivative (PPD) reagent is injected intradermally into the forearm. Second,

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Competing interests: None declared.

Ethical approval: Not required.

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the delayed-type hypersensitivity (DTH) response is monitored 48 to 72 hours post-injection by measuring the diameter of induration (swelling due to inflammation) in millimeters at the site of injection. A common occurrence is the visualization of erythema (redness) within the first 24 hours after administration of PPD; this should not be measured, as it is not indicative of infection. The administration and reading of this test must be performed by trained health professionals who can interpret the risk factors along with the measurement in determining a positive reaction (Mackin, 1998).

In addition to its role as an indicator of *Mtb* infection, the TST has also been used as an epidemiologic tool to evaluate the prevalence of latent TB infection (LTBI). The projection that one-third of the global population is infected with *Mtb* is partly based on the frequency of a positive TST (Dye, *et al.*, 1999).

In the current review, we provide an overview of the history, development and current uses of PPD. Moreover, research focused on defining the key molecular components of PPD and its biological activities will be also reviewed.

Past and present use of PPD

The first cutaneous tuberculin test was introduced in 1907 by Von Pirquet (1874–1929), an Austrian scientist and pediatrician (Turk, 1987). In his study, Koch's OT, a heated broth composed of a crude, undefined mixture of proteins and other macromolecules derived from the tubercle bacillus was used. Koch's OT was prepared from a concentrated glycerol peptone broth filtrate in which *Mtb* had grown for 6–8 weeks. Koch's OT and similar products are not used as TST reagents in US, due to the lack of purity, variation in potency and specificity, as well as inadequate standardization.

In 1930, an alternative formulation known as MA-100, was produced from *Mtb* culture filtrate as a polysaccharide-free formulation (Masucci & McAlpine, 1930). MA-100 was found to be significantly more potent than Koch's OT; however, its use as a standard diagnostic reagent was limited - mainly due to the sensitizing effect observed upon repeated injection in to the skin.

In 1934, a more stable and consistent preparation was developed by Florence B. Seibert (1897–1991), a biochemist at the Henry Phipps Institute at the University of Pennsylvania (Seibert, 1934). Originally designated for the manner it was produced, SOTT, an acronym for "synthetic medium old tuberculin trichloroacetic acid precipitate", this product was later referred to as purified protein derivative or PPD. It was produced by steaming cultures of *Mtb* in an Arnold sterilizer and purifying the proteins by repeating precipitation with ammonium sulfate (Seibert & Glen, 1941). Compared to previous tuberculin reagents, this method of PPD preparation was drastically reduced in polysaccharides, nucleic acid, and lipid content and thus was a protein-rich reagent. In 1944, a large lot of this improved PPD (lot 49608), renamed PPD-S (PPD-Standard) was provided as the reference product in the United States. PPD-S was comprised of approximately 92.9% protein, 5.9% polysaccharide, and 1.2% nucleic acid (Seibert & Glen, 1941). Because of its enhanced purity and potency, PPD-S was adopted as the international standard of tuberculin by the World Health Organization (WHO) in 1952 (Guld, *et al.*, 1958). As of 1978, the Food and Drug Administration (FDA) required that all lots of PPD be qualified by biological assay and must show a potency equivalent to that of PPD-S (Sbarbaro, 1978). The international unit (IU) for PPD was defined as part of this effect; one IU is equal to the biological activity contained in 0.028 μg of PPD-S (0.02 μg PPD with 0.008 μg of salts). However, in the U.S and Canada, the potency of PPD is expressed as a tuberculin unit (TU) rather than IU. One TU is defined as 0.02 μg of PPD-S (Edwards & Edwads, 1960). Five TU is the standard

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dose for intradermal diagnostic use, as determined from epidemiological studies (Bothamley, *et al.*, 1999).

PPD-S2, the current U.S standard for PPD tuberculin, was developed in anticipation of the eventual depletion of PPD-S (Villarino, *et al.*, 2000). Presently, Aplisol® (JHP Pharmaceuticals, Inc, Rochester, MI) and Tubersol® (Sanofi Pasteur Limited, Swiftwater, PA) are two widely-used, commercially available PPD-S2 products (Jensen, *et al.*, 2005). The results of skin testing with Aplisol® and Tubersol® are quite comparable with that of the original PPD standard, PPD-S (Villarino, *et al.*, 1999). However, shifting use from Tubersol® to Aplisol®, or vice-versa, has resulted in skin test aberrations, although the exact reason is still unclear (Gillenwater, *et al.*, 2006, Mehta, *et al.*, 2009).

Besides PPD-S, there are several other PPD formulations in use outside of the US and Canada (Li, *et al.*, 2008). Several of these tuberculin products, including PPD RT23, are produced by the Statens Serum Institut (SSI) (Comstock, *et al.*, 1964). Currently, 2 TU of PPD-RT23 with Tween 80 is recommended by the WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD). RT23 is the most widely used PPD product globally (Rangel-Frausto, *et al.*, 2001). Several studies throughout the world have used PPD RT23 to estimate the prevalence of infection with the tubercle bacillus, including India (Rao, *et al.*, 2008), (Vashishtha & John, 2010), Ghana (Addo, *et al.* 2010), Yemen (Al-Absi, *et al.*, 2009), South Africa (Kritzinger, *et al.*, 2009) (Hanifa, *et al.*, 2009), Nepal (Shrestha, *et al.*, 2008), Brazil(Lopes, *et al.*, 2008) and Indonesia (Bachtiar, *et al.*, 2008). In addition, it has also been used to evaluate large-scale tuberculosis contacts in Netherlands (Borgen, *et al.*, 2008).

In addition to the aforementioned PPD products, other variations of PPD are also used, such as PPD RT23 Mexico (Laboratorio Nacional de Salud, Secretaria de Salud, Mexico City, Mexico), a PPD product used in Latin America (Rangel-Frausto, *et al.*, 2001), and the Japanese product PPD-s. (Kimura, *et al.,* 2005*).* The numerous PPD products currently in use are summarized in Table 1.

Since there are several manufacturers of PPD, it is important to evaluate variations in potency among these PPD products. Due to the limited knowledge of the exact composition of each PPD product, it is impossible to use traditional quality control methods to compare PPD preparations. Therefore, comparisons utilizing animals infected with mycobacteria are made to assess the biological potency of PPD products. In doing so, PPD products must be administered under the same conditions in which they will be used in the clinical setting (Hansen, *et al.*, 1964).

The first published comparison of PPD potency was performed between PPD-S and PPD-RT23. The research, which was carried out in 6 populations in the US including Eskimo children, tuberculosis patients, and recruits at the USA Naval Training Centers, concluded that 2.5 TU of PPD-RT23 containing Tween 80 had similar potency to 5 TU of PPD-S (Comstock, *et al.*, 1964). More recently, a study of 69 TB patients and 1,189 low-risk subjects in the US compared PPD-S2 to PPD-S1. These two products were found to be statistically indistinguishable in the TB patients. In addition, equally high specificity was observed among the low-risk subjects. This study revealed that PPD-S2 is functionally equivalent to PPD-S1 and can readily replace it (Villarino, *et al.*, 2000). Multiple studies have compared the potency of RT23 prepared at SSI with other sources of PPD, including IC-65, and equivalent potency has been found among them (Ulea, *et al.* 2010), (Chadha, *et al.*, 2003), (Schiller, *et al.* 2010). However, a similar study in Mexico compared the potency between locally produced PPD RT23 (Mexico), Tubersol®, and PPD RT23 (SSI), and observed that of the three, RT23 (Mexico) had a much lower sensitivity (Rangel-Frausto, *et*

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al., 2001). RT23 and Merieux tuberculin (developed at Pasteur-Merieux) were also recently compared for their relative potency. Both preparations were generated from multiple strains of mycobacteria (RT23 was prepared from seven strains of *Mtb*, Merieux was produced from three strains of *Mtb,* plus *M. bovis*) and appear to have equivalent biological potency to PPD-S, which is a product of one strain. However, RT23 frequently produces a larger antigenic reaction than the Merieux preparation (Sgountzos, *et al.*, 2009). Recently, Schiller et al compared the diagnostic reliability of PPDs from different sources via and innovative approach monitoring interferon-γ responses in whole blood cultures (Schiller*, et al.* 2010). In this study whole blood samples were stimulated with several different tuberculins and IFN-γ responses monitored after 24 hrs of stimulation. Their results support that there are significant differences between PPDs from different sources and indicates a need for further standardization of PPD products. A quantitative scale referred to as RP30 (Relative potency 30), defined as the protein concentration at which a specific PPD preparation has 30% of maximal activity, was introduced. The RP30 may be used as a tool for the rapid comparison of biological potency in batches and sources of PPDs. While these reports highlight the importance of assessing the biological potency of PPD products from different resources, discrepancies in potency are difficult to explain due to the complexity and ambiguity in the molecular composition of PPD. The proteomic characterization of PPD has been described by our laboratory (Cho, *et al.*, 2012) and others (Borsuk*, et al.*, 2009), demonstrating that PPD is comprised of hundreds of distinct proteins. Additional comparative proteomic, biological, and histological analyses were used to measure the relative differences in molecular composition and biological potency between PPD-S2, RT23, and PPD-KIT (PPD from the Korean Institute of Technology) (Cho, *et al.*, 2012). This study demonstrated that while all 3 PPD preparations were indistinguishable in their capacity to induce a DTH response, histological differences and differences in the relative abundance of several proteins, including members of the Esx protein family, were apparent, suggesting a correlation between increased histopathology and the increased concentration of Esx proteins in PPD (Cho, *et al.*, 2012). Cumulatively, all these comparative reports illustrate the complexity of PPD and challenges to generating a standardized reagent.

Pitfalls of PPD

While the TST has been the standard in identifying persons at risk for active TB for the past century, it has several fundamental flaws which serve as the impetus for the development of more standardized methodology and more effective tools to identify LTBI. The primary concern with the current test is the high level of false positive results, caused by the inability of the TST to distinguish between *Mtb* infection and either exposure to nontuberculosis mycobateria or vaccination with *M. bovis* Bacille Calmett-Guérin (BCG) (Huebner, *et al.*, 1993). Both cases of false positive responses are generally attributed to an immune response triggered by homologous antigens from either vaccination with BCG or from environmental mycobacteria (Harboe, 1981, Huebner, *et al.*, 1993). These assumptions were recently verified by molecular analyses of PPD demonstrating that four heat shock proteins (GroEl, GroEs, DnaK, and HspX) contribute to roughly 60% of the PPD proteomic content (Cho, *et al.*, 2012, Borsuk*, et al.*, 2009). These chaperone proteins share a high homology (upwards of 70%) and are conserved amongst most mycobacterial species (Cho, *et al.*, 2012, Borsuk*, et al.*, 2009). This complicates the use of the TST as a tool for both epidemiologic studies and identification of persons infected with *Mtb* due to the potential of cross-reactivity from BCG vaccination or infection with non-tuberculous mycobacteria. False negatives are also problematic, particularly in children and immunocompromised individuals (Farhat, *et al.*, 2006, Shingadia & Novelli, 2008). This is due to the fact that a positive PPD requires an efficient DTH response. Therefore it is likely that PPD fails as an indicator of *Mtb* infection in those populations where robust T-cell immunity is lacking. Finally, while the TST can be used detect LTBI, it fails to differentiate between this, active disease, or the convalescent

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patient. Despite these pitfalls, the TST remains the most commonly used tool to detect *Mtb* infection.

The future of PPD - discovery and development of next generation PPDs

The development of novel and more effective reagents to detect LTBI is the key to success in the fight against tuberculosis. Improved detection of the latent bacilli will lead to early intervention strategies, and likely reduce disease morbidity and break the cycle of disease transmission.

Improvement of the current TST reagents must not be overlooked as we head towards the goal of novel reagents for detection of LTBI; however, it is worth noting that the method of quantifying the immune response is highly subjective. Rather than measuring the diameter of indurations in millimeters, there are several novel methods being tested. These include: laser Doppler imaging in human cases (Harrison, *et al.*, 1993), using a hand-held spectrophotometer to measure the DTH reaction (Chambers, *et al.*, 2002), and ultrasonographic measurement in patients (Ciftci, *et al.*, 2005). These alternative methods can be applied for the objective quantification of the TST and may overcome the limitations of the conventional route of measurement; however, the use of cost-prohibitive technology in resource-limited regions must be considered.

In addition to improving the method of measurement to improve standardization of the test, the actual composition of PPD can be improved upon. Defining the molecular composition of PPD was a significant hurdle for many years. The prolonged heating of crude tuberculin to prepare PPD contributed to the denaturation, partial degradation, and aggregation of many of the protein components. Numerous studies identified PPD as mixtures of very heterogeneous proteins ranging in size from very large aggregates to very small degraded molecules (Klausen, *et al.*, 1994, Rowland, *et al.*, 1999, Ho, *et al.*, 2006). Similarly, little was known regarding which of these components in PPD was responsible for the DTH response. With the recent identification of over one hundred proteins from four different PPDs via mass spectrometry (Borsuk, *et al.*, 2009, Cho, *et al.*, 2012), novel approaches can be applied to tease out which of these components illicit the DTH response.

Nearly two decades prior to the publication of the molecular composition of PPD, numerous studies were performed on individual proteins to test their ability to induce a DTH reaction (Klausen, *et al.*, 1994). Such studies continue to be critical to optimizing PPD and understanding how it modulates the immune system. The antigens being tested as future PPD reagents have been summarized in Table 2.

In addition to proteomics, genomics has been key to the identification of *Mtb*-specific antigens. A genomic comparison between the *Mtb* strain H37Rv and several *M. bovis* vaccine strains identified 129 ORFs unique to *Mtb*, clustered in 16 regions of difference (RDs) on the chromosome. The evaluation and incorporation of proteins encoded from these regions may play a vital role in making the next generation of PPD reagents more specific to *Mtb* (Mustafa, 2001). Among these 16 RDs, the most extensively studied is RD1; the genes predicted in this DNA segment are deleted from all the vaccine strains of BCG, while they are conserved in all the laboratory and clinical isolates of *M. bovis* and *Mtb* tested thus far (Mahairas, *et al.*, 1996). Two candidates specific to the *Mtb* complex and encoded by the RD1 region are the low-molecular weight secreted proteins CFP10 and ESAT-6 (Olsen, *et al.*, 2000, van Pinxteren, *et al.*, 2000, Brusasca, *et al.*, 2001, Mustafa, 2002, Aagaard, *et al.*, 2004).

ESAT-6 (Rv3875) and CFP10 (Rv3874), highly studied T-cell antigens that are absent in BCG, are currently used as reagents for the diagnosis of tuberculosis via an Interferon-

gamma release assay (IGRA) (Mazurek, 2005, Chang KC & Leung CC, 2010). Recombinant ESAT-6 elicits a positive skin response in *Mtb*-infected guinea pigs and humans (Wu, *et al.*, 2008). Compared to the maximal DTH response at 72 h induced by PPD, the DTH response to ESAT-6 peaked 24 h later (Pollock*, et al.*, 2003). Interestingly, the combination of ESAT-6 and CFP10 were found to be highly sensitive and specific by DTH response (van Pinxteren*, et al.*, 2000). CFP10 functions as chaperone and binds with ESAT-6 in a tight 1:1 complex, stabilizing its folded structure (Renshaw*, et al.*, 2002). Research on a recombinant dimer ESAT-6 (rdESAT-6) overexpressed in *Lactococcus lactis* has shown that it may be a successful diagnostic, as it discriminates *Mtb* infection from BCG vaccination and toxicity profiles of rdESAT-6 in several animal models have validated rdESAT-6 as a safe TST reagent (Aggerbeck & Madsen, 2006). Recently, a double-blind randomized phase I study comparing rdESAT-6 to RT23 in humans was completed. While this study did show very promising results, with respect to dosage and safety – further studies are necessary to sufficiently demonstrate adverse effects and efficacy, as well as to address sensitization (Arend*, et al.*, 2008). The potency of ESAT-6 and CFP10 in the induction of DTH responses have also the subject of controversy, as they have been shown to induce necrotic responses (Elhay*, et al.*, 1998).

Similar studies have paired ESAT-6 with a second culture filtrate protein, MPT64 (Rv1980c). Like ESAT-6, recombinant MPT64 has been shown to elicit a DTH response in *Mtb* infected guinea pigs. Further experiments identified that the 15 residues between amino acids Gly-173 to Ala-187 are key to eliciting a DTH response (Oettinger*, et al.*, 1995). Animals subjected to the ESAT-6-MPT64 cocktail indicated that this combination has potential as a highly specific reagent (Elhay*, et al.*, 1998). It was reported in 2007 that MPT64 was under phase III clinical trials to evaluate its potential to replace PPD (Wang*, et al.*, 2007).

The *Rv0061* gene is unique to the *Mtb* complex and encodes the DPPD protein which is capable of inducing a strong DTH response in guinea pigs infected with *Mtb* (Coler*, et al.*, 2000). Follow-up studies on tuberculosis patients and clinically healthy individuals strongly suggest that DPPD is a promising alternative for PPD (Campos-Neto*, et al.*, 2001, Liu*, et al.*, 2004). A recent study confirmed the biological activity of the purified recombinant DPPD using peripheral blood mononuclear cells from PPD positive blood donors, indicating DPPD could be used as a purified antigen for the detection of tuberculosis (Kashino & Campos-Neto, 2011).

Outlook and conclusions

Despite the identification of over a dozen protein candidates for incorporation into next generation PPD reagents and promising preliminary data from animals and human studies, derivation of a new reagent – of either single or multiple antigens - to replace PPD remains challenging. A TST specific for the detection of exclusively active or latent tuberculosis disease would greatly benefit diagnostic and epidemiologic programs. Thus, new strategies need to be employed to discover more sensitive and specific skin test antigens. On the other hand, a single antigen may not effectively replace the PPD, as a cocktail of antigens or a combination of several DTH-inducing epitopes may be required for the optimal next generation of PPD reagent (Oettinger*, et al.*, 1995, Lyashchenko*, et al.*, 1998, Rhodes*, et al.*, 2000).

Towards this goal, the identification of the molecular composition of PPD facilitates the development of a more refined reagent. Proteomic studies identified the highly conserved chaperones GroES, GroEL2, and DnaK as three of the most dominant proteins, and may explain the positive attributes and diminished specificity of PPD (Borsuk*, et al.*, 2009, Cho*,*

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et al., 2012). Our group recently identified two novel formulations, DnaK/GroEL2/Rv0685 and DnaK/GroEL2/Rv0009, that were capable of inducing DTH responses equivalent to PPD in the guinea pig model of *Mtb* (Yang, *et al.*, 2011). A better understanding the DTH response driven by these defined proteins can contribute to the discovery of rapid and sensitive next generation skin test reagents for detection of *Mtb* infection.

Acknowledgments

This work was funded by Tuberculosis Vaccine Testing and Research Materials contract (HHSN266200400091C) from the NIH.

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Table 1

The PPD products currently used in human subjects

Name	Manufacturer	Dose	References
PPD S2 (Aplisol®)	JHP Pharmaceuticals, Inc, Rochester, MI, USA	5 TU	(Villarino, *et al.*, 1999)
PPD S2 (Tubersol®)	Sanofi Pasteur Limited, Swiftwater, PA, USA	5 TU	(Villarino, *et al.*, 1999, Teixeira, *et al.*, 2000, Rangel-Frausto, *et al.*, 2001)
PPD RT23 SSI	Statens Serum Institute, Copenhagen, Denmark	2 TU	(Maes, *et al.*, 2011, Teixeira, *et al.*, 2000)
PPD RT23 Mexico	Laboratorio Nacional de Salud, Secretaria de Salud, Mexico City, Mexico	2 TU	(Rangel-Frausto, *et al.*, 2001)
PPD RT23 (Evans PPD)	Celltech Pharma S.A., Madrid, Spain	2 TU	(Fernandez-Villar, *et al.*, 2004)
PPD-s	Nihon BCG Seizo Co., Tokyo, Japan	3 TU	(Shigeto, 1990)
PPD IC-65	Cantacuzino Institute, Bucharest, Romania	2 TU	(Ulea, *et al.* 2010)

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Table 2

Antigens currently under evaluation as next generation PPD candidates

Gene number	Antigen	Animal	Dosage (μg)	Country	Reference
Rv1980c	MPT 64	GP	0.1	Denmark	(Oettinger, *et al.*, 1995)
Rv3875/Rv1980c	ESAT-6/MPT 64	GP	1	Denmark	(Elhay, *et al.*, 1998)
Rv0652	ribosomal protein L7/L12	GP	0.2	Japan	(Kitaura, *et al.*, 1999)
Rv0061	DPPD	GP/Hu	2 (GP)	USA	(Coler, *et al.*, 2000)
Rv3874	CFP10	GP	2	USA	(Colangeli, *et al.*, 2000)
Rv3875/Rv3874	ESAT-6/CFP10	GP/Ca	1 (GP) 2 (Ca)	UK	(van Pinxteren, *et al.*, 2000)
Rv3875	ESAT-6	Ca	25–400	Denmark	(Pollock, *et al.*, 2003)
Rv0061	DPPD	Hu	0.2–5	USA	(Campos-Neto, *et al.*, 2001)
Rv0061	DPPD	GP	5	USA	(Liu, *et al.*, 2004)
Rv3875	ESAT-6	GP	0.01–1	Denmark	(Aggerbeck & Madsen, 2006)
Rv3875	ESAT-6	Hu	0.01–1	The Netherlands/Denmark	(Arend, *et al.*, 2008)
Rv3875	ESAT-6	GP/Hu	0.1–1(GP) 1 (Hu)	China	(Wu, *et al.*, 2008)
Rv0934	TPA38	GP/Hu	3–5	China	(He, *et al.*, 2007)
Rv0350	DnaK	GP	0.4	USA	(Yang, *et al.*, 2011)
Rv0685	GroEL2	GP	0.4	USA	(Yang, *et al.*, 2011)

GP: guinea pig; Hu: human; Ca: cattle