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Detection and identification of antigens from *M.bovis* culture filtrate with immune sera from *M. bovis* sensitized or infected cattle.

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Detection and identification of antigens from *M. bovis* culture filtrate with immune sera from *M. bovis* sensitized or infected cattle.

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Abstract

Bovine tuberculosis, caused by *Mycobacterium bovis,* infects approximately 50 million cattle worldwide and is diagnosed by the tuberculin skin test (TST). The purpose of this thesis was to characterise the culture filtrate proteins (CFP) of *M. bovis* PPD tuberculin and to compare the antibody response of *M. bovis* infected *versus M. bovis* sensitized cattle. Sterile filtered PPD tuberculin (SF-PPD) resolved into approximately 200 discrete spots using two-dimensional PAGE. While 2D Western blot analysis of both *M. bovis* sensitized and *M. bovis* infected cattle sera demonstrated an antibody boost following comparative intradermal TSTs, *M. bovis* sensitized cattle responded with greater intensity to additional SF-PPD antigens as compared to *M. bovis* infected cattle at seven weeks post infection/sensitization. In conclusion *M. bovis* sensitized cattle generated a more intense antibody response and recognized additional SF-PPD antigens as compared to *M. bovis* infected cattle within the first two months post infection/sensitization.

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List of Abbreviations

100-02-2CNS - Canadian national standard reference *M. bovis* PPD tuberculin
2D - two dimension
2DE - two dimension polyacrylamide gel electrophoresis
AN5 - strain of *M. bovis* used by the CFIA for the production of *M. bovis* PPD tuberculin
APC - antigen presenting cell
BCIP/NBT - 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium
BL-III - bio-containment level III
CBB - Coomassie brilliant blue
CFIA - Canadian Food Inspection Agency
CFP - culture filtrate protein
CFP10 - culture filtrate protein 10
CFU - colony forming units
CITST - comparative intradermal tuberculin skin test
DC - dendritic cell
dpi - dots per inch
DTH - delayed type hypersensitivity
ELISA - enzyme-linked immunosorbant assay
ESAT-6 - early secretory antigenic target - 6
HCSM - heat concentrated synthetic medium tuberculin
HK-PPD - heat-killed (autoclaved) research PPD tuberculin 100-06-1R
hpi - hours post injection
IEF - isoelectric focussing
IFN-γ - interferon gamma
IL - interleukin
kDa - kilodalton
LJ - Lowenstein Jensen culture medium
MHC - major histocompatibility complex
MTC - *Mycobacterium tuberculosis* complex
NVL - no visible lesions
OIE - Office International Epizootic
PAGE - polyacrylamide gel electrophoresis
PBMC - peripheral blood mononuclear cells
pI - isoelectric point

PPD - purified protein derivative

RD1 - region of difference 1

rRNA - ribosomal ribonucleic acid

SDS - sodium dodecyl sulphate

SF-PPD - sterile filtered *M. bovis* PPD tuberculin

SIT - single intradermal test

Tb - tuberculosis

TBST - tris buffered saline with 0.3% Tween 80

TGFβ - transforming growth factor beta

TFF - tangential flow filtration

TNFα - tumour necrosis factor beta

TST - tuberculin skin test

$WC1^+$ T cells - workshop cluster 1 (class of bovine specific gamma-delta T cells)

1. Literature review

The host immune response to bovine tuberculosis and recognition of *M. bovis* culture filtrate antigens.

1.1 *Introduction: the persistence and prevalence of bovine tuberculosis.*

Bovine tuberculosis caused by *Mycobacterium bovis* infection is a major global health threat, with approximately 50 million cattle currently infected worldwide (89). Although the majority of infected cattle reside in third world countries, bovine tuberculosis also persists in developed countries despite strict test and slaughter control strategies (89). For example, the herd prevalence of bovine tuberculosis in Great Britain is reported to be 2.8% (40) with a herd incident increase of 18% per annum (116). Difficulties regarding the eradication of bovine tuberculosis are largely due to the zoonotic tendency of *M. bovis* organisms and the persistence of wildlife reservoirs which serve to transmit the bacteria back into the agricultural sector (104). Attempts to reduce the incidence of bovine tuberculosis by controlling *M. bovis* in wildlife is in itself a complicated challenge. For example, in randomized badger cull trials of Great Britain only a moderate reduction in the overall incidence of bovine tuberculosis was achieved despite the application of a resource intense program (68). Canada currently boasts a low prevalence of bovine tuberculosis with 6 and 8 cases of *M. bovis* diagnosed during the calendar years 2004 and 2005 respectively (86). However, Wood Buffalo National Park located in northern Alberta and in the adjacent Northwest Territories as well as Riding Mountain National Park located in southwestern Manitoba are known to harbour bovine tuberculosis in their respective wild ungulate populations and have been shown to be

associated with bovine tuberculosis outbreaks on farms in the surrounding areas (104).

Apparently, cattle rarely develop tuberculosis following exposure to *Mycobacterium tuberculosis (M. tb)* (169) which is reported to latently infect 1.8 billion humans (32). Conversely, *M. bovis* is believed to have accounted for approximately 20 - 40% of human tuberculosis cases prior to the implementation of milk pasteurisation (97). It has also been speculated that *M. bovis* may account for as many as 10 - 15% of the estimated 7 - 10 million new cases of human tuberculosis occurring in developing countries (115, 126). Furthermore, human to human transmission of *M. bovis* is believed to be comparable to that of *M. tb* (84) therefore, control and eradication of this disease in cattle has the potential to directly benefit human health.

The primary method used to diagnose tuberculosis in cattle is the single intradermal test (SIT). The SIT consists of an intradermal injection of *M. bovis* purified protein derivative (PPD) tuberculin which stimulates a cell-mediated delayed-type hypersensitivity (DTH) reaction in *M. bovis* exposed cattle (16). While the sensitivity and specificity of the SIT may be influenced by disease progression and the predictive values of the test may be affected by the local prevalence of bovine tuberculosis; de la Rua-Domenech et al., 2006 summarized the results of several field trials and reported the median SIT sensitivity and specificity to be 83.9% and 96.8% respectively (125).

Another method used to diagnose bovine tuberculosis involves measuring the gamma interferon (IFN-γ) production by peripheral blood mononuclear cells (PBMC) *in vitro* after they are stimulated by either PPD or recombinant mycobacterial proteins. A major weakness of the IFN-γ-based *in vitro* test methods is that blood must be processed

within 12 - 16 hours of collection in order to optimise test performance (46, 144). Furthermore, the overall high cost and laboratory infrastructure required for effective IFN-γ-based testing renders this approach unsuitable for use in developing countries which harbour the majority of *M. bovis* infections (125, 175).

Although the SIT is the most widely used diagnostic test to detect *M. bovis* infections in cattle, little is known about the quality, relative quantity and identity of the proteins which make up PPD tuberculin. Analysis of *M. bovis* culture filtrate proteins with two dimensional polyacrylamide gel electrophoresis (2DE) has indicated that tuberculin is manufactured from a multitude of tuberculo-proteins (36, 95, 102, 141). In addition, tuberculin manufacturing methods include heating and chemical treatment of the tuberculo-proteins which may also alter their structure and antigenicity in field issue PPD tuberculin. In accordance with international standards (173) the relative antigenicity of each new batch of PPD tuberculin is measured in animals artificially sensitized with an intramuscular injection of heat killed *M. bovis* cells. However, little is known concerning the immunological differences between the bovine immune response to heat killed *M. bovis* cells *versus* that of a live infection. The following text describes the current knowledge of the bovine immune response to *M. bovis* infection as it relates to the recognition of *M. bovis* culture filtrate antigens.

1.2 *Cellular characteristics of M. bovis.*

Mycobacterium bovis are non-motile, rod shaped, obligate aerobe bacteria with gram positive cell wall type (130) that belong to the family *Mycobacteriaceae* within the order *Actinomycetes* (143). A predominant feature of mycobacterial species is their

complex cell wall structure which consists of a rich layer of long-branched mycolic acids and arabinogalactan-lipid complexes in addition to the peptidoglycan layer typical of most other Gram positive bacteria (130). The unusually thick, waxy capsule is impervious to common staining techniques, such as the Gram stain, and it also endows the bacteria with the ability to bind fushin dye when the bacteria are boiled in an acid-alcohol solution (172). Mycobacteria are therefore known as acid-fast bacteria.

M. bovis grow slowly in culture doubling only every 18 - 24 hours, therefore 6 - 8 weeks at 37°C on selective culture medium is typically required to produce visible culture growth (127). Bacteria are 0.2 - 0.7 μm wide and 1.0 to 10.0 μm in length with a non-pigmented, rough, buff colony phenotype (130).

There are more than 40 recognized species of *Mycobacterium spp.* (109) and those which cause tuberculosis in their respective hosts are grouped into the *Mycobacterium tuberculosis* complex (MTC) which currently consists of *M. tuberculosis*, *M. bovis*, *M. canettii*, *M. africanum*, *M. microti*, *M. pinnipedii*, and *M. caprae* (33). While MTC species are believed to have evolved from a common ancestor, their host range is variable, for example *M. tuberculosis*, *M. africanum* and *M canettii* are primarily human pathogens while *M microtti*, *M. pinnipedii* and *M. caprae* are associated with rodents, seal and goats respectively (18). Unlike the other members of the MTC, *M. bovis* is considered to have the most zoonotic potential of this genus and is known to commonly infect a variety of mammals including, cattle, deer, pigs, possums, badgers, cats, dogs and humans (47, 140).

Considering their varied host ranges it is remarkable that the MTC species share

99.9% genetic homology with identical 16S rRNA sequences (33). Indeed, the cattle-adapted *M. bovis* and the human-adapted *M. tuberculosis* have a sequence divergence of less then 0.05%, which is noteworthy considering that two randomly chosen humans have a genetic sequence divergence of approximately double that (140). However, the high degree of homology between MTC species is balanced by the extremely low genetic recombination rates and little evidence of horizontal gene transfer between MTC strains (156). The highly clonal characteristic of the MTC strains has thus facilitated the successful application of genetic strain identification tools such as restriction fragment length polymorphism typing, spoligiotyping and variable number tandem repeat analysis (72, 138, 149).

While it was previously believed that human tuberculosis evolved from the bovine form of the disease, comparison of the complete *M. bovis* genome sequence (40) to that of *M. tuberculosis* (24) has revealed that *M. bovis* has in fact undergone only genetic deletions relative to *M. tuberculosis* (18, 95). Parallel comparative hybridization microarrays were used to identify 14 genomic regions of difference (RD 1-14) which are absent from BCG Pasteur relative to *M. tuberculosis* H37Rv (14, 18). As such, the current theory dictates *M. bovis* to have evolved from an ancestral strain of *M. tb* (18, 24) which has undergone a process termed reductive evolution (118, 153). *M. bovis* cell wall components were shown to incorporate the highest proportion of genetic deletions, however, it is believed to be the protein expression differences between strains which accounts for host preference and virulence determinants for each MTC species (23).

1.3 *Pathogenesis of bovine tuberculosis.*

M. bovis is principally a respiratory pathogen which causes a slow and progressive sub-clinical disease in cattle associated with the development of classical tuberculosis lesions, or tubercules, are often difficult to locate even during an exhaustive post mortem examination (125). Although *M. bovis* organisms are not known to produce toxins, their virulence is associated with a low infective dose, wide host spectrum, aerosol route of transmission, the ability to circumvent the hosts immune system and the ability to persist sub-clinically for decades.

Depending on the age, immunological status and genetic predisposition of the infected animal, as well as the strain and total burden of the organism, tuberculosis may assume radically different pathological courses (112). Primary tuberculosis occurs with the incident infection of the bovine host which results in either direct progression to active disease or, prevention of sustained bacterial colonization due to host's innate immune system. Studies have shown that only 30% of *M. tb* exposed humans sustain a tuberculosis infection and that 60-90% of the infected individuals are capable of clearing the infection without progression to overt disease (94). *M. bovis* infection in cattle is thought to have a similar pathogenesis to human tuberculosis because it typically presents with few tuberculin reactor animals and appears to have a somewhat limited disease transmission on farms despite the close approximation of cattle (7, 19).

The inability of the bovine innate immune system to clear the infection leads to the activation of the cellular immune response and the formation of tuberculous granulomas or tubercules. Tuberculous granulomas typically have a semisolid core of caseous material surrounded by epitheloid macrophages, Langerhans giant cells,

lymphocytes and peripheral fibrous tissue that calcifies over time (127). During the time that the bacteria are effectively encapsulated within the granuloma they survive by entering a dormant or latent state triggered in part by the microaerophilic and low nutrient environment (130). As such, the mycobacteria can survive within the hostile granuloma environment for several years, perhaps the entire lifetime of the host. While it is accepted that 90% of human tuberculosis patients are able to drive the disease into a sub-clinical or latent state, there is little direct evidence for the occurrence of latent bovine tuberculosis. Nevertheless, current opinion maintains that the pathogenesis of human and bovine tuberculosis are very similar because a high degree of structural similarity has been observed between human and bovine tuberculosis granulomas (19, 112).

Secondary tuberculosis typically results due to a reactivation of latent tuberculosis prompted by a decrease in the host's immuno-competency and/or the subsequent liquification and cavitation of tuberculous granulomas (127). Humans with latent tuberculosis maintain a 10-15% lifetime chance of reactivation (130). Secondary tuberculosis is characterized as having a more rapid onset of active/symptomatic disease and is highly transmissible (127).

M. bovis can be transmitted through blood, milk and faeces however the majority of cow-cow transmission occurs via the aerosol route as evidenced by the predisposition of tuberculous lesions to form in the lungs of naturally infected cattle (19, 112). While the principle vehicle for transmission of *M. bovis* bacteria to humans is un-pasturized milk, this is more likely a reflection of the most common means by which humans are exposed to *M. bovis* as opposed to a preferential route of infection for the organism (115).

A study by Dean *et al.* (2005) concluded that the minimum infective dose of *M. bovis* in cattle may be as low as one colony forming unit (CFU) - a dose which was used to successfully infected 50% (3/6) of the cattle inoculated (28) Furthermore, cattle infected with 1 CFU *M. bovis* exhibited necropsy findings which were indistinguishable from cattle infected with 1000 CFUs *M. bovis* over the same time period suggesting that disease severity may not be as heavily influenced by infective dose (70) as was previously believed (103).

Clinical ante-mortem signs of active bovine tuberculosis include: respiratory distress, coughing, weight loss and emaciation (7). However, infected cattle can maintain an *M. bovis* infection for a substantial period of time before they exhibit any clinical signs and these symptoms are often not suspected to be associated with tuberculosis infection in areas with a low prevalence of bovine tuberculosis (125).

1.4 *Bovine immune response to M. bovis infection.*

1.4.1 *Bovine innate immune response to M. bovis infection.*

Bovine tuberculosis is predominantly a respiratory infection, acquiring access to the alveolar spaces following inhalation of an aerosol nuclei containing *M. bovis*. As such, the alveolar macrophage plays a pivotal role as the initial contact of the bacterium with cells of the host immune system. Although the principal role of the alveolar macrophage is to recognise, ingest and destroy pathogens, *M. bovis* has evolved the ability to survive and multiply within the naive macrophage. Furthermore, the slow growth of the bacteria and suppression of cytokine signalling by the host macrophage may permit a quiet onset of disease (82).

Virulent mycobacteria stimulate alveolar macrophage phagocytosis by engaging cell surface mannose receptors, complement receptor 3 and 4 (CR3, CR4), Fc receptors and Toll-like receptors 2 and 4 (TLR2, TLR4) (45, 167) which recognise bacterial surface moieties. Pursuant to phagocytosis, the bacterium survives within the phagosome principally by inhibiting phagosomal acidification but also by initially depressing the macrophage's Interleukin (IL) -12 production and ability to generate oxidative bursts (82). Phagosomal acidification typically occurs due to the fusion of the phagosome with lysosomes as well as the loss of vacuolar NA^+K^+ATPase pump function. *M. bovis* have been shown to inhibit phagosome-lysosome fusion with the expression of a eukaryotic-like serine/threonine protein kinase G (92) and to counteract phagosomal acidification by producing ammonia (127). The bovine immune response to dead mycobacteria and/or *M. bovis* culture filtrate proteins is therefore believed to vary substantially from that of *M. bovis* infection because active modulation of phagocytosis and cytokine expression have only been shown to occur when live *M. bovis* interact with a macrophage (4, 25).

Initially, the infected macrophage's ability to stimulate a strong immune response is inhibited by the slow bacterial growth and depression of cytokine signalling which permits the infection to progress unabated (130). However, as the bacteria propagate within the host cell, the secretion of mycobacterial antigens along with cell wall mycolic acids, trigger the expression of macrophage cytokines including TNFα and IL-12 (130). As a result, additional non-activated macrophages, dendritic cells (DC) and other innate immune cells are chemotactically drawn to the to the lesion and may serve as potential host cells for the propagating bacteria (108). Microscopic tuberculosis lesions consisting

of peripheral γδT cells, natural killer cells (NK cells), macrophages, neutrophils and DCs have been observed as early as 7 days post infection (96). While infected immune cells may remain at the inflammatory foci, others, especially DCs, may enter afferent lymphatics and thereby disseminate the bacteria to the paracortical region of lymph nodes (65, 167).

Two additional cell types which are present in early granuloma formation and involved in the innate bovine immune response to *M. bovis* infection are the Workshop Cluster 1 ($WC1^+$) T cells and natural killer cells (NK cells). $WC1^+$ T cells are a major subset of bovine γδT cells which constitute between 10 and 50% of bovine peripheral blood lymphocytes (83). While the concentration of $WC1^+$ T cells present in the bovine tuberculosis granuloma is currently contended (83, 108, 155), they have been identified as a potential source of INFγ and they have been shown to kill mycobacteria-infected macrophages in vitro (19). $WC1^+$ T may also facilitate the recruitment of αβT cells to the mycobacterial lesion through the expression of leucocyte adhesion molecules thereby linking the innate and adaptive immune systems (30, 74).

NK cells have been shown to proliferate and produce INFγ in response to secreted mycobacterial proteins (106) and to kill mycobacteria infected bovine macrophages in vitro (19). NK cells may also assist with the modulation and initiation of the adaptive bovine CMI response by regulating $CD8^+$ T cell effector function (106, 150).

1.4.2 *Bovine cell mediated immune response to M. bovis infection.*

The critical component of the host's defence against mycobacterial infections is the cell mediated immune response due to the intracellular persistence of *M. bovis* within

non-activated immune cells. While the generation of a Th1- type T cell response is known to be crucial for the control of bovine tuberculosis (4, 112, 113, 130, 145, 166), recent studies on cytokine expression and immune cell types involved in the control of bovine tuberculosis imply that the magnitude and complexity of the anti-mycobacterial immune response have been historically underestimated (132).

Due to their typical intra-phagosomal location, the majority of *M. bovis* antigens are degraded by proteases within the phagosome and delivered to the macrophage or antigen presenting cell (APC) surface by major histocompatability type (MHC) class II molecules where they can be recognized by $CD4^+$ T cells (67). Interestingly, the inability of the non-activated macrophage to destroy *M. bovis*, was not shown to affect the MHC presentation of mycobacterial antigens as the presentation of mycobacterial antigens was shown to be independant of the site of intercellular residence inside antigen presenting cells (92).

Factors currently believed to influence the differentiation of proliferating $CD4^+$ T cells include the intensity of MHC class II - TCR binding between the APC and $CD4^+$ T cell (67) and these factors are also heavily dependant on the cytokines expressed by the APC and other cells within the microenvironment (176). CD4 T cells tend to differentiate into Th1 - type cells in response to IL-12 and they are characterized by secreting high levels of INFγ. INFγ is known to be a key effector cytokine in tuberculosis which activates macrophages cells and contributes to granuloma formation, and these INFγ induced functions are the major reasons that the Th1 type immune response is considered crucial for the immunological control of tuberculosis (67, 176).

Conversely, Th2 -type T cells develop in response to IL-4 and they secrete IL-4 and IL-5 which promotes B cell activation (166). Furthermore, the propagation of Th1 and Th2 cells are inhibited by IL-4 and INFγ respectively. Therefore, the historical Th1/Th2 hypothesis postulates that these two reciprocally inhibitory lymphocyte responses contend with one another to the point that an intracellular pathogen, such as *M. bovis,* will evoke a dominant Th1, cell based immune response whereas the Th2, antibody mediated immune response is effectively suppressed in an immunocompetent host (151).

Although the Th1 - Th2 paradigm has provided a framework to explain the cell mediated or humoral direction of the bovine immune response, the recent identification of a novel subset of effector $CD4^+$ T cells - the Th17 type T cells have resulted in a re-evaluation of the understanding of the cell mediated immune response. Th17 cells propagate in response to IL-6 and IL-23, produce an inflammatory response by the expression of IL-17 and TNFα, and they have been shown to be inhibited by IL-4, INFγ and IL-27 (75, 176). Th17 cells have been shown to contribute to the anti-mycobacterial immune response by the promotion and regulation of the inflamatory response, by the recruitment of both polymorphic and mononuclear cells and by possible maintenance of granuloma integrity in advanced tuberculosis (75). $CD25^+$ regulatory T cells (Treg) also have a role in the bovine response to tuberculosis by suppressing the proliferation of effector T cells with the secretion of IL-10 in response to TGFβ. Treg cells have been observed in the outer boundary of bovine tubercules and are thought to limit granuloma advancement (83). Therefore the expression of mycobacterial peptide by the MCH class II molecules along with an ever expanding number of identified cytokines are currently

believed to instigate and regulate an intricate, four-way balance between Th1, Th2, Th17 and T reg type cells for optimal granuloma formation and anti-mycobacterial immunity (176).

The role of CD8 T cells in the immune response to bovine tuberculosis represents yet another branch of the cellular immune response which may play a more important role then was previously thought (83). While the expression of peptides by MHC class I molecules has typically been associated with viral antigens derived from the cytosol, Liebana et al, 2007 demonstrated substantial concentration of $CD8^+$ T cells in the mantle zones of human tuberculosis granulomas with a $CD4^+$: $CD8^+$ ratio of 1 :1 in discrete lesions (83). *M. bovis* antigens may gain access to the MHC class I pathway by a cross-presentation pathway in which peptides are actively retro-translocated across the phagosome lumen and into the cytoplasm (64). Mycobacterial antigens may also be presented to $CD8^+$ T cells by CD1 molecules which are MHC class I-like molecules associated with β2- microglobulin. While CD1 molecules are restricted to professional antigen presenting cells they are abundantly expressed on DCs (140).

The main function of $CD8^+$ T cells is to effect cytolytic activity on *M. bovis* infected phagocytes which are unable to destroy the intracellular mycobacteria independently, however they also have been shown to secrete INFγ and TNFα in response to mycobacterial antigens (67, 137). Effective cytolytic activity can either directly kill the bacteria or return it to the intercellular space where it is vulnerable to activated macrophages and/or Ab mediated immune responses (112, 140).

1.4.3 *Bovine humoral immune response to M. bovis infection.*

For several decades, traditional dogma contended that the humoral immune system played a very small role in the host defence of tuberculosis (43). Several reasons account for this belief including the view that the immune response to intracellular bacteria was mediated by a Th1 - type response and that intracellular pathogens were inaccessible to antibodies (37, 79). Furthermore, it was believed that increased antibody response was typically associated with advanced pathology of bovine tuberculosis along with a waning cell mediated response (112) and studies by Plackett *et al.* (1989); Ritacco *et al.* (1991) and Fifis *et al.* (1992) reported that a high proportion of *M. bovis* infected cattle possessed few or no detectable antibodies to mycobacterial antigens in the early, subclinical stages of infection (37, 111, 119).

In the last decade however, studies have shown that while a balance between immune response type does exist with respect to bovine tuberculosis, its complexity may have been underestimated. Many secreted *M. bovis* CFPs have been shown to elicit both cellular and humoral immune responses in the early stages of bovine tuberculosis and novel diagnostic tests based on antibody recognition of *M. bovis* CFPs have been developed (158, 160). Furthermore, monoclonal antibodies targeting *M. bovis* have been shown to affect the course of tuberculosis to the benefit of the host by increased host survival times and reduced bacterial dissemination (44). In addition, mycobacteria found in the extracellular space prior to phagocytosis were shown to be vulnerable to antibody mediated defences (44). In fact, IgG molecules have also been shown to mediate an effect on phagocytosed bacteria from within immune cells (44).

Several *M. bovis* antigens are now known to evoke both the humoral and cellular

immune responses (160), however, B and T cells often do not respond to identical epitopes (67). T cells recognise linear epitopes presented by MHC molecules on APCs however, B cells can recognise free antigens in the extracellular space and can therefore also produce antibodies to conformational and discontinuous epitopes specific for hydrophilic moieties extending from antigens (67). Therefore, heat or chemical treatment of *M. bovis* antigens may induce conformational changes and reduce *M. bovis* antigen recognition by the humoral immune system.

1.5 *M. bovis tuberculin production.*

Tuberculin is a crude and complex mixture of tuberculo-proteins which has changed little since its conception and original application by Dr. Robert Koch in 1890 (78). The original tuberculin, Koch's old tuberculin (KOT), was prepared from a heat sterilized culture medium containing 8 - 12 week old *M. tuberculosis* cultures concentrated to one-tenth the original volume by evaporation (78). Since the time of Koch, there have been two significant changes to tuberculin manufacture. The first was the replacement of Koch's glycerol - meat broth with synthetic culture medium (31) with the resulting product known as heat concentrated synthetic medium tuberculin (HCSM tuberculin) or old tuberculin (OT). The second change involved the precipitation of the tuberculo-proteins from the heat sterilized synthetic culture medium (133) and this form of tuberculin is now commonly referred to as purified protein derivative of tuberculin (PPD tuberculin). Over time the changes to tuberculin production methods have resulted in an overall increased tuberculin test specificity for detecting tuberculosis in cattle (173), yet altogether, today's tuberculin remains quite similar to that which was produced 100

years ago.

Canadian *M. bovis* PPD tuberculin is produced from the *M. bovis AN5* strain which was originally isolated from a field case in England in 1948 and this *M. bovis* strain is also used for the production of *M. bovis* PPD tuberculin in many countries including the UK, USA and Mexico (125). Current methodology for the production of Canadian *M. bovis* PPD tuberculin was adopted from the tuberculin production protocol described by Angus in 1978 (10). This protocol describes the switch from HCSM to PPD tuberculin and the implementation of tuberculin concentration and dialysis by molecular filtration methods. In Canada, 63 day old *M. bovis* cultures are autoclaved, and filtered to remove cellular material, concentrated by tangential flow filtration (TFF), precipitated with ammonium sulfate and resuspended and re-concentrated with TFF. The PPD tuberculin is maintained a as concentrate (bulk tuberculin) in phenolized phosphate buffer until it is diluted to 1.0 mg/mL protein and issued for field use.

Following protein concentration standardization, the potency or antigenicity of each batch of bulk tuberculin is compared to a national standard reference tuberculin using the OIE prescribed comparative intradermal tuberculin skin test (CITST) protocol which measures the DTH response in *M. bovis* sensitized animals. Although the OIE guidelines recommend that a national standard reference *M. bovis* tuberculin must be compared to the OIE (World Organisation for Animal Health) International Standard Reference *M. bovis* tuberculin in *M. bovis* infected cattle, for practical reasons the potency of each new PPD tuberculin batch is usually standardized in guinea pigs artificially sensitized with an intramuscular injection of heat killed *M. bovis* cells.

The antigenicity of tuberculins are typically tested on guinea pigs due to their low cost, easy handling and easy maintenance. Due to immunological species differences between cattle and guinea pigs (29), CITST protocols have been modified for the animal species in which it is applied. Thus, the guinea pig CITST typically consists of 0.1 mL injections which contain 5.0, 1.0 and 0.2 μg of PPD tuberculin while the bovine CITST consists of 0.1 mL injections which contain 100.0 and 20.0 μg of PPD tuberculin. Also, the guinea pig reaction to tuberculin is typically read at 48 hours post injection (hpi) and recorded as the area of the erythema while in the bovine tuberculin skin test (TST) the increase in skin thickness is measured at 72 hours post CITST.

Although animals artificially sensitized to *M. bovis* with an intramuscular injection of heat killed *M. bovis* cells have been used to ascertain the relative potency of tuberculins for more then 70 years (13), the immunological response of a sensitized animal *versus* that of a *M. bovis* infected animal has not been fully characterized. It is likely that artificially sensitized animals may be exposed to relatively small amounts of secreted antigens and to large amounts of cell wall antigens as compared to naturally infected animal which have been shown to predominantly respond to secreted antigens (9, 11, 21, 29, 48, 58, 102). Not only are immunological differences between animals infected with *M. bovis* and those sensitized with heat killed cells poorly understood, but also the reagents used (sensitin) to artificially sensitize the animals have not been standardized between laboratories. For example, Nagai *et al.* (1991) reported an antigenic response to the secreted protein MPB64 in cattle artificially sensitized with heat killed *M. bovis* cells, while Haslov *et al.* (1995) detected no such response to MPB64 in

his sensitized cattle. Haslov *et al.* (1995) suggested that the observed differences between laboratories may be due to procedural differences in sensitin preparation, for example, cell washing post heat kill, which could alter the concentration of culture filtrate proteins like MPB64 in the sensitin (58). For these reasons, the validity of the CITST in *M. bovis* sensitized guinea has been questioned (8, 11, 21, 26, 29, 48, 50, 58, 102) however this method has apparently been shown to provide a suitable measure of relative PPD tuberculin antigenicity for routine, *M. bovis* PPD tuberculin batch quality control (173).

1.6 *Antigenic M. bovis culture filtrate proteins.*

While it has long been recognised that tuberculin is composed of *M. bovis* derived protein components, early efforts to accurately characterize the antigenic components of PPD tuberculin (101) were met with difficulty. In retrospect, interpretation of early findings were likely further complicated by protein denaturing effects associated with the heat and pressure of the autoclave and the absence of effective separation and characterization techniques. Fractionation of tuberculin into 3 - 14 antigenic fractions by alcohol fractionation (3, 134), column chromatography (12, 21, 77), and crossed immunoelectrophoresis (51) has resulted in the description of a variety of tuberculo-protein fractions with poorly defined structural characteristics and/or biological activity such as the Antigen "L" (21) and PPD tuberculin fractions A, B and C (134).

With the advancement of molecular separation techniques and the PAGE examination of non-heated *M. tuberculosis* (102) and *M. bovis* (36, 102) culture broths over 800 tuberculo-proteins are currently described in the literature. Consequently, many

of the tuberculin fractions previously described as homogenous entities actually contain multiple types of mycobacterial proteins. The current accepted terminology for this complex mixture of tuberculo-proteins is culture filtrate proteins (CFPs) and this includes secreted proteins, exported proteins and non-secreted, somatic components which are released into the culture medium due to autolysis, replication and bacterial leakage (95, 141). The protein profile of a CFP set is dependent on many factors including cultivation time, temperature, growth medium and culture agitation (9, 141).

Today most laboratories use non-heated *M. bovis* culture filtrates rather than tuberculin for the identification of specific *M. bovis* antigens for use as diagnostic and vaccine candidates. Separation and characterization of non-heated *M. bovis* CFPs using molecular techniques such as 2D PAGE, MASS-SPEC analysis and *invitro* antigenicity assays has lead to the identification of several, highly antigenic *M. bovis* proteins, however the antigenic activity of these proteins and their conservation in *M. bovis* PPD tuberculin remains largely unknown.

CFPs harvested from 2 -5 week old cultures are of particular interest for application to both diagnostic and vaccine development since secreted and exported mycobacterial proteins from early mycobacterial cultures have been shown to play important roles in both disease pathogenesis and the stimulation of protective immunity (141). To this end, 2D PAGE has been used in several studies to characterize the early, immunologically active CFPs of *M. tuberculosis* (71, 93, 95, 102, 121, 136, 141) *M. avium* (105), and *M. bovis* BCG (52, 71, 95). While a similar 2D-PAGE analysis of *M. bovis* AN5 CFPs has not been published, analysis of *M. tuberculosis* H37Rv culture

filtrate proteins reports approximately 800 resolved protein spots (71), of which more than 381 CFPs have since been identified by MASS-SPEC analysis (http://www.mpiib-berlin.mpg.de/2D-PAGE (95). Interestingly, 2D-PAGE of *M. bovis* BCG shows deleted, as well as additional protein spots when compared to the CFP profile of *M. tuberculosis* although the *M. tuberculosis* genome possess a complete repertoire of *M. bovis* genes (105). Therefore as the presence of genes does not always accurately predict protein expression, a 2D-PAGE analysis of CFPs harvested from 9 week old *M. bovis* AN5 cultures followed by MASS-SPEC analysis would be a preferable approach to identify and characterize the protein components of *M. bovis* PPD tuberculin.

The simultaneous publication and naming of *M. bovis* CFP antigens by different researchers has created some confusion with respect to nomenclature and as a result, many homologous antigens bear multiple names. CFPs can also be referred to by their annotated genes or by the gene's location along the bacterial genome. The website "BoviList" (http://genolist.pasteur.fr/BoviList/ , Pasteur Institute, France) is a *M. bovis* database which lists DNA and protein sequences derived from *M. bovis* AF2122/97 (40), and offers links to relevant annotations and functional assignments. A detailed literature review of all antigenic *M. bovis* CFPs is beyond the scope of this thesis, therefore only the *M. bovis* CFP antigens relevant to this study and currently described in the literature will be reviewed. All of the following *M. bovis* CFPs share 100% sequence homology with *M. tuberculosis* and have been shown both to stimulate both cellular and humoral immune responses in *M. bovis* infected cattle.

MBP70 (101), MBP80 (52) and MBP83 (54) have been shown to be a highly

homologous, prominent set of *M. bovis* CFPs which have been shown to be active components of *M. bovis* PPD tuberculin. The biological activity for this set of proteins is currently not known however, it has been suggested that they have different functions due to their different locations with respect to the mycobacterial cell wall (56). MPB70, MPB80 and MPB83 resolve as distinct ~22 kDa spots in 2D SDS-PAGE and possess isoelectric points (pI) of 4.8, 4.6 and 4.5 respectively (52) however MPB70 and MPB80 are recognized by the same monoclonal antibodies and at this time appear to have the same antigenic properties. It is believed that the isoelectric variation is due to post-synthetic changes occurring after the release of a homologous protein (54). MPB70 and MPB83 have also demonstrated cross-reactive properties (61) and have been shown to be 61% homologous at the amino acid level (98). While MPB70 is strictly a soluble, secreted protein, MPB83 is a 26 kDa exported, cell wall lipoprotein which also occurs in a non-lipoylated 23 kDa from in the culture filtrate (20, 56, 61, 171).

MPB70 and MPB83 are expressed at high levels in all virulent *M. bovis* strains studied and in the BCG strains which lack RD2, and they are expressed, but at low levels in *M. tuberculosis* and other BCG strains (1, 61). Positive regulation of the expression of both MPB70 and MPB83 is attributed to *M. bovis* sigma factor K (SigK), therefore, the lack of a functional SigK repressor (Anti-SigK) in *M. bovis* is believed be the basis for this observed phenotypic difference (128).

MPB70 and MPB83 have been shown to account for approximately 10% of the protein present in 5 week old *M. bovis* (BCG Tokyo) culture filtrate as shown by SDS-PAGE (101). The high concentration of MPB70 and MPB83 in culture filtrate is not only

attributed to the considerable quantities secreted by *M. bovis* during growth but it is also likely due to the structural stability of this protein set. Two main factors which account for this structural stability are the presence of identical 133aa disulfide loops as well as the formation of stable aggregates in the culture fluid (57, 171). The heat-stability of MPB70 and MPB83 is also demonstrated by the observation that they can be detected by SDS-PAGE following autoclaving as a major component of *M. bovis* PPD tuberculin. These proteins may therefore play a major role in the antigenic activity of *M. bovis* PPD tuberculins (101).

Although both MPB70 and MPB83 are serodominant antigens, MPB83 is reported to be more commonly recognised by the humoral immune system of *M. bovis* infected cattle than MPB70 (61). MPB83 is also reported to be the first antigen to be recognized by IgG following *M. bovis* infection in cattle (61). Both MPB70 and MPB83 have also been shown to elicit IFN and IL2 production from PBMCs of *M. bovis* exposed cattle (22, 117).

Another major secreted antigen of *M. bovis* is the immunogenic protein MPB64 (52) which has an approximate molecular weight of 26 kDa, and a pI of 4.6 (123). Although the exact function of MPB64 has not been described (123), it elicits a strong DTH reaction in *M. bovis* exposed animals and, unlike MPB70 and/or MPB83, it is equally expressed by both *M. bovis* and *M. tuberculosis* (52). In 4 day-old *M. tuberculosis* culture filtrate MPB64 has been shown to account for approximately 8% of the total protein present (9). Unlike MPB70 and/or MPB83, MPB64 is heat labile and is found in decreased amounts in seven week-old *M. bovis* culture filtrate (102).

The antigen 85 (Ag85) complex is a set of immunogenic proteins which have been selected as both diagnostic and vaccine candidates for both human and bovine tuberculosis (89, 64, 100). The Ag85 complex consists of four structurally related, outer cell wall proteins Ag85a, Ag85b, Ag85c and MPT51 (102, 105), which are believed to detach from the cell wall during growth (105). These proteins have been shown to account for up to 15% of the total protein in *M. bovis* culture filtrate (170). The Ag85 complex antigens possess enzymatic mycolyl transferase activity required for the biosynthesis of trehalose-dimycolate (cord factor) and as such they are responsible for the high affinity of mycobacteria species to fibronectin (2). This set of proteins is therefore not restricted to the MTC since Ag85 homologues have also been identified in culture filtrates of environmental mycobacteria, for example: *M. avium, M. avium paratuberculosis, M. scrofulaceum, M. gordonae, M. szulgai and M. kansasii* (170). Ag85a, Ag85b, Ag85c and MPB51 are encoded by four paralogous genes, *fbpA, fbpB fpbC* and *fbpD* respectively, which have been shown to be located at distinct regions of the *M. bovis* genome (122). The *M. bovis* Ag85 complex proteins were also found to share over 99% amino acid sequence identity with the Ag85 proteins secreted by *M. avium* (124).

As shown by 2D SDS-PAGE, Ag85a Ag85b and Ag85c have molecular weights of 31, 30 and 31 kDa respectively and pIs of 5.4, 5.1 and 5.2 while MPB51 has a molecular weight of 27 kDa and a pI of 5.6 (102). The Ag85a shares 79% and 67% sequence homology with Ag85b and Ag85c respectively (123) and MPT51 is also known to exhibit 40% identity with the Ag85b (122). The extensive structural homology of

these proteins was also shown to correspond with shared and/or cross-reactive epitopes observed in immunoblot and ELISA experiments (102).

The early secretory antigenic target - 6 protein (ESAT-6) (140) and 10 kDa culture filtrate protein (CFP10) (15) are two highly immunogenic, co-transcribed, co-exported proteins of virulent *M. bovis* strains which have been shown to be both virulence factors and protective antigens (17). The ESAT-6-CFP10 dimer is expressed early in *M. bovis* infection and it has been shown to be essential for the bacteria to survive and spread *in vivo* (17, 38). However, these proteins have also been shown to initiate protective cellular and humoral immunogenic responses and are currently included in many experimental bovine tuberculosis vaccines (23, 89, 96, 100, 154). ESAT-6 and CFP10 are also optimal antigens to target for novel diagnostic tests as they are encoded by *esxA* and *esxB* respectively which are sequentially located within the RD1 genomic region of *M. bovis.* Because the majority of environmental mycobacterial and BCG lack RD1, ESAT-6 and CFP10 therefore have the potential to differentiate infected cattle from those vaccinated with BCG and/or exposed to environmental mycobacteria (1, 62, 87, 89, 129, 148, 157, 174).

The function of the ESAT-6:CFP10 dimer is a current topic of interest because the absence or altered secretion of these proteins has been shown to result in strain attenuation (17). The ESAT-6:CFP10 dimer has been implicated in the signal recruitment of macrophages through the induction of TNFα expression by the host cell which can facilitate the aggregation of additional target cells for the bacteria and may promote cell-to-cell spread of the bacteria (33). Conversely, ESAT-6:CFP10 have also

been shown to inhibit the production pro-inflamatory cytokines for example, TNFα, IL-12 and IFNγ by the downregulation of nuclear factor-κB-dependant gene expression by the host macrophage (39).

ESAT-6 and CFP 10 were shown to have an observed mass of 8 and 12 kDa and observed isoelectric points of 4.4 and 4.6 respectively on 2DE analysis (123). As the current *M. bovis* PPD tuberculin protocol in our laboratory includes the dialysis of culture filtrates to remove molecules less than 10 kDa, the presence of ESAT-6 and CFP10 in our PPD tuberculin has not been determined. However, the intradermal application of PPD tuberculin has been shown to boost the Ab response of ESAT-6 in *M. bovis* infected cattle (69, 89, 107).

MPB32 (120) is secreted in abundance in the 2-3 week old culture filtrate of *M. bovis* (88, 89, 90, 100) and, as with MPB64, the concentration of this heat liable protein (120) was reported as being considerably diminished following seven weeks culture growth (102). MPB32 localises as a double row of spots in 2D-PAGE of non-heated mycobacterial culture filtrate with approximate molecular weights of 45/47 kDa and an approximate isoelectric point 4.7 (123, 141, 165). While the precise function of this protein is unknown, it has been shown to be highly homologous to a fibronectin-binding protein of *M. leprae* and it may therefore be involved in mediating bacterial attachment to host cells (2). Sera from BCG infected guinea pigs were shown to recognise MPB32 by immunoblot while the sera from animals sensitized to *M. bovis* with heat killed BCG did not, indicating that this protein might be used to differentiate between *M. bovis* sensitized *versus* infected cattle (120).

1.7 *The bovine immune response to the tuberculin skin test.*

The tuberculin skin test (TST) is the prototypic delayed-type hypersensitivity (DTH) reaction, also known as a type IV hypersensitivity reaction, which is mediated by antigen specific T cells (67). Following the intradermal application of tuberculin in a *M. bovis* infected/sensitized animal, a local T - cell mediated inflammatory reaction evolves over 24 - 72 hours as effector T cells recognise *M. bovis* peptide presented on Langerhan's cells by MHC class II molecules. In tuberculous cattle this reaction presents as an intradermal swelling at the injection site which peaks at approximately 72 hpi and then resolves gradually over the next several days. Repeat tuberculin skin testing is not believed to compromise TST diagnostic capability (146), however cattle which test positive with the TST have been shown to become briefly desensitized to tuberculin if re-tested within 2- 4 weeks of the original TST; a phenomenon likely influenced by Treg cells (99, 152). Therefore, a period of sixty days is typically left between repeat TSTs to permit an adequate return of responsiveness.

Prior to the development of the classical DTH tuberculin reaction, in the 6 to 24 hpi - phase of the tuberculin response, an influx of $WC1^+$ cells and neutrophils have been shown to localise at the reaction site, possibly in response to a localized Arthus - type reaction (110). In the Arthus reaction, or type III hypersensitivity reaction, circulating IgG molecules specific for soluble *M. bovis* antigens may form localized immune complexes with the *M. bovis* tuberculin antigens (67). Immune complexes bind Fc receptors on leukocytes and activate the complement cascade which creates a local inflammatory response (67). As with the DTH response, this early immune reaction

presents as a thickening of the skin and it is typically not observed in *M. bovis* negative animals (41).

While the intradermal injection of tuberculin is therefore not considered immunogenic, application of the TST in *M. bovis* infected/sensitized cattle has been shown to enhance the mycobacterial antibody response and it has also been shown to intensify peripheral blood mononuclear cells (PBMC) responsiveness to *M. bovis* antigen *in vitro* (146, 159). Interestingly, injections of live *M. bovis* BCG failed to demonstrate a comparable antibody boosting effect in BCG vaccinated cattle, possibly because the BCG cells were phagocytosed by macrophages and not as available for processing by B cells as compared to the soluble tuberculin antigens (152). While the application of tuberculin prior to blood collection may therefore increase the sensitivity of *invitro* based diagnostic testing for bovine tuberculosis, the precise mechanics of this boosting effect remains to be elucidated (152).

2 Statement of Purpose

This research project was initiated to further characterize the antigenic constituents of *M. bovis* PPD tuberculin and to determine the immunological differences between *M. bovis* infected and *M. bovis* sensitized cattle. Previous work in our laboratory showed that cattle artificially sensitized to *M. bovis* with an injection of heat killed *M. bovis* cells mounted a 30% greater DTH response to the intradermal application of *M. bovis* PPD tuberculin as compared to cattle experimentally infected with *M. bovis* (unpublished data). Although it is known that comparative tuberculin potency testing against a reference standard tuberculin in *M. bovis* infected cattle offers the most accurate determination of the relative antigenicity of a tuberculin (173), the overall expense and complexity of this animal model limits its practicality. As many of the antigenic *M. bovis* CFP have been shown to elicit both cellular and humoral responses in cattle (89, 107, 161), it was therefore hypothesized that a comparison between the *M. bovis* infected and *M. bovis* sensitized antibody responses to *M. bovis* CFPs may identify immunological differences between these respective cattle models. The experiments which comprise this thesis were therefore designed to answer the following questions.

1. What are the PAGE and 2DE characteristics of heat denatured *versus* sterile filtered *M. bovis* CFPs?
2. What is the relative antigenicity of sterile filtered *M. bovis* CFPs as compared to heat denatured *M. bovis* PPD tuberculin in guinea pigs and cattle which have been sensitized to *M. bovis* with heat killed *M. bovis* cells?

3. Is the immunogenicity of sterile filtered *M. bovis* CFPs equivalent to that of heat killed *M. bovis* cells?

4. How does the antibody response to *M. bovis* CFPs differ between experimentally infected with cattle as compared to cattle sensitized with heat killed *M. bovis* cells?

3 Materials and Methods

3.1 *Production of M. bovis PPD tuberculin HK-PPD.*

M. bovis was cultured within the Biologics Production Unit, Bio-containment Level III laboratory (BLIII)(CFIA, Ottawa, Ontario). Following culture sterilization, research PPD tuberculin 100-06-1R (HK-PPD) was manufactured according to the Canadian Food Inspection Agency (CFIA) standard operating procedure MY-PR039; The Production of Purified Protein Derivative (PPD) of *M. bovis* Tuberculin. Due to the proprietary nature of the CFIA *M. bovis* production procedure only limited information pertinent to this thesis may be described herin. Two hundred production flasks containing synthetic, liquid medium, were inoculated with *M. bovis* AN5 seed cultures. Production cultures were incubated aerobically at 37 ± 2°C for 63 days following which the production cultures were autoclaved at 121°C with approximately 16 psi for 45 min. Adequate autoclave conditions for effective heat kill of *M. bovis* cultures was confirmed by Prospore® biological indicator (Raven Labs, USA) and sterility of *M. bovis* culture supernatant was confirmed by the Mycobacterial Diagnostic Unit (CFIA). Culture supernatant was filtered to remove gross debris and it was concentrated with 10 kDa tangential flow filtration (TFF) cassettes (Pellicon Cassette, Millipore Corporation, USA). Tuberculo-proteins were precipitated, pelleted by centrifugation, re-suspended in a phenolized phosphate buffer and de-salted/concentrated with 10 kDa TFF.

3.2 *Production of sterile filtered M. bovis PPD tuberculin.*

Sterile filtered *M. bovis* PPD tuberculin (SF-PPD) was produced as per HK-PPD (Chapter 3.1) with the exception that the 2 month-old *M. bovis* AN5 production cultures

were not autoclaved. In place of heat kill, the culture supernatant was separated from the live *M. bovis* cells by filtration through a 3 μm and a 0.8/0.2 μm capsule filters (Pall Corporation, USA) arranged in series. Culture supernatant was maintained for 10 weeks at 4°C while sterility was confirmed by the Mycobacterial Diagnostic Unit (CFIA). Upon confirmation of sterility, the culture fluid was removed from the BL-III laboratory and filtered once more through a 0.22 μm filter (Pall Corporation, USA).

3.3 *Polyacrylamide gel electrophoresis.*

Protein separation of SF-PPD and HK-PPD was performed using one and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Buffer salts and phenol were dialysed out of both SF-PPD and HK-PPD prior to SDS-PAGE with 5 kDa TFF (Minimate™ TFF System, Pall Corporation, USA). Protein concentrations were determined using a protein assay kit (*DC*™ protein assay Bio-Rad Laboratories, Mississauga, Ontario)(85). As per manufacturers instructions, sample proteins were serially diluted with de-ionized water and 5 μL of each dilution was aliquotted into 6 wells of a 96-well microtiter plate. A known protein standard was also serially diluted to 1.50, 1.25, 1.00, 0.75, 0.50, 0.25 μg/mL and 5 μL of each dilution was aliquotted into 4 wells. 5 μL of de-ionized water was also added to 4 wells to act as blanks. The two Bio-Rad *DC*™ protein solutions were added to all wells and the plate was incubated for 15 minutes with agitation. The plate was read at 650 nm and a linear confidence interval of 95% or greater for the serial protein standard dilutions was deemed to be acceptable.

One dimensional, vertical, SDS-PAGE was performed as per Lamelli (1970), with

the following specifications (76). A 6% acrylamide, pH 6.8 stacking gel and a 12% acrylamide, non-linear, separation gel with a pH of 8.8 were used. The acrylamide-bis ratio was 29:1 and gels were cast in either 16 x 20 cm or 8.3 x 7.3 cm sizes with thicknesses of either 0.75, 1.00 or 1.50 mm depending on the application. Electrophoresis chemicals and molecular weight standards were purchased from Bio-Rad Laboratories, Mississauga, Ontario. *M. bovis* protein samples were placed 1:4 into a reducing sample buffer which contained 2.0% (wt/vol) SDS, 5.0% (vol/vol) 2-mercaptoethanol (2-ME), 10% (vol/vol) glycerol, 0.0625 M tris base (pH 6.8), and 0.005% (wt/vol) bromophenol blue. Samples were boiled at 100°C for 5 minutes and centrifuged for 5 minutes at 14 000 xg prior to loading. The amount of protein applied to each lane varied with the application and size of the gel. Electrophoresis was performed using either the PROTEAN® II or mini-PROTEAN® III cells (Bio-Rad) at 20 mA constant current for single gels or at 40 mA constant current when two gels were processed simultaneously. Electrophoresis was manually arrested when the bromophenol blue tracking dye reached the bottom of the separating gel.

M. bovis protein samples separated by two-dimensional SDS-PAGE (2DE) were first subjected to iso-electric focusing (IEF) along an acrylamide strip, followed by SDS-PAGE, molecular weight separation in a direction 90 degrees from the IEF. The precast, 17cm IEF strips were manufactured with an immobilized pH range of 3 -10 (ReadyStrip™; Bio-Rad Laboratories). Isoelectric focusing was carried out using a Multiphor™ II Electrophoresis Unit (Amersham Pharmacia Biotech) and a PowerPac™ HV power supply (Bio-Rad Laboratories, Mississauga, Ontario). IEF strips were re-

hydrated and loaded with 150 µL of a *M. bovis* protein sample prepared in 150 µL of ReadyPrep™ Squential Extraction Kit Reagent 3 (Bio-Rad Laboratories) with 3 µL of tributyl phosphine and 0.0003% (w/v) bromophenol blue. Protein samples were diluted in de-ionized water according to application. IEF strips used for silver stains or Western blot applications were loaded with 10 - 50 or 200 µg of protein respectively. Strips were focussed for ~98,000 volt hours and immediately frozen at -80°C or prepared for the second dimension.

Prior to the second dimension, IEF strips were equilibrated sequentially for 15 min. at 37°C in first a reduction buffer (0.05M Tris, pH 6.8; 8M urea; 35% glycerol; 2% (w/v) dithiothreitol; 0.3% SDS) followed by an alkylation buffer (0.05M Tris, pH 6.8; 8M urea; 35% glycerol; 2.5% (w/v) iodoacetamide; 0.3% SDS). Following alkylation, the IEF strips were loaded into 12% SDS polyacrylamide gels with the pH 3 end of the strip towards the molecular weight marker and the strips were overlaid with 0.5% (w/v) low melting agarose containing 0.0001% (w/v) bromophenol blue. Second dimension electrophoresis was performed using the PROTEAN® II cell (Bio-Rad) at 20 mA constant current for single gels or at 40 mA constant current when two gels were processed simultaneously. Electrophoresis was manually arrested when the bromophenol blue tracking dye reached the bottom of the separating gel. Electrophoresis chemicals and molecular weight standards were purchased from BIO-RAD Laboratories, Mississauga, Ontario.

2DE of HK-PPD and SF-PPD were also digitized in 8 bit greyscale at 300 dpi with a ScanMaker *i*900 scanner (Microtek) and enumerated by PDQuest 7.1.0 2D

Analysis Software (Bio-Rad). Following automated enumeration, erroneous and duplicate spots were manually deleted.

3.4 *Polyacrylamide gel staining procedures.*

Gels subjected to Comassie Brilliant Blue (CBB) stain were rocked overnight in 0.2% (w/v) CBB R-250 stain (Bio-Rad Laboratories, Mississauga, Ontario) which contained 50% methanol and 10% acetic acid. Gels were destained for 1 hour in three changes of de-stain solution I (50% methanol, 10% acetic acid) and subsequently rocked in de-stain solution II (5% methanol, 7% acetic acid) until sufficiently de-stained based on visual evaluation.

Gels subjected to silver stain were processed using a non fixing silver stain method described by Shevchenko et al. (1996), with the following specifications (135). In brief, gels were fixed overnight in 50% ethanol, 5% acetic acid. Gels were subsequently washed in a 50% ethanol solution and sensitized with 0.02% sodium thiosulphate. Gels were stained with 0.1% silver nitrate and developed using a 2% sodium carbonate solution with 0.04% formalin. Development was stopped with 5% acetic acid based on visual evaluation of staining intensity.

3.5 *Analysis of 2DE SF-PPD spots by MASS-SPEC.*

Nine SF-PPD spots were excised from a CBB stained 2DE gel of SF-PPD (200 μg; 50 μL of 4 μg/μL). All work was carried out under a laminar flow hood, and each spot was cored and collected using individual, sterile, 5 mL pipettes. The excised spots were placed in sterile vials and maintained at -80 °C prior to submission to the Ottawa Institute for Systems Biology (University of Ottawa) for MASS-SPEC analysis.

3.6 *One and two dimensional Western blot.*

The *M. bovis* sensitized and infected cattle sera used for Western blots were collected at regular intervals during a 2004 comparative intradermal tuberculin skin test (CITST) study conducted by the Biologics Production Unit (CFIA)(Figure 1). Sera was maintained at -80°C. Cattle were screened with an *invitro* blood based assay (Bovigam™; Pfizer, Australia) prior to infection/sensitization and based on the manufacturer's cut-off values were determined to be negative for bovine tuberculosis. The Bovigam™ test is a monoclonal antibody-based sandwich ELISA which measures subsequent INF-γ production from lymphocytes that have been stimulated with either *M. bovis* or *M. avium* PPD tuberculins. Test animals are interpreted to be negative for *M. bovis* exposure when the *M. bovis* PPD absorbance values are less than those of the *M. avium* PPD for a given blood sample.

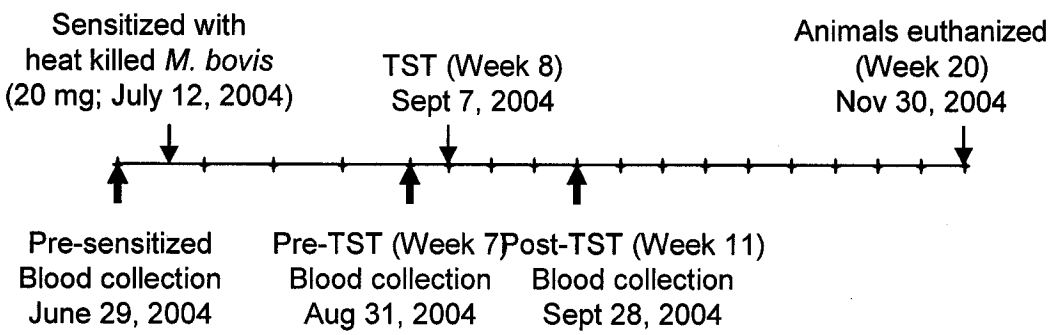
In the 2004 studies, twelve cattle received a 1.0 mL intra-tracheal injection containing 1500 CFU of virulent *M. bovis* (field strain 02/1007; CFIA designation) and another twelve cattle received a 20 mg intramuscular injection of heat killed *M. bovis* cells (field strain 02/1007, CFIA designation) suspended in 50% mineral oil, 25% lanoline and 25% saline. *M. bovis* infection status was confirmed in all cattle inoculated with live *M. bovis* by lesions observed on necropsy, by histological identification of acid fast bacteria with typical histopathology of mycobacteriosis and by isolation of *M. bovis* on culture. Likewise, all artificially sensitized cattle were confirmed negative for *M. bovis* infection by the same tests.

CITSTs were applied to both the sensitized and infected cattle using the same *M.*

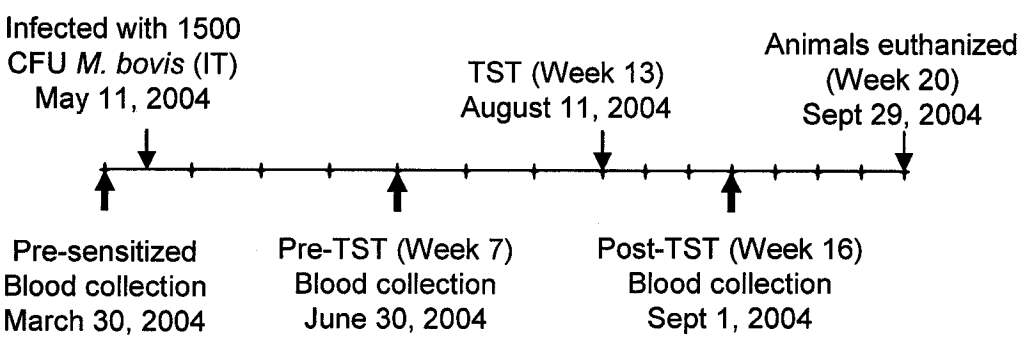
Figure 1. Serum collection points from the 2004 CITST study.

In 2004, CITSTs were conducted on twelve *M. bovis* sensitized and twelve *M. bovis* infected cattle (For details please refer to section 3.6 *One and two dimensional Western blot.*). The marks along the respective timelines represent blood collection time-points. Wide arrows indicate the blood collection time-points used in the 2D Western blot analysis component of this study.

M. bovis Sensitized Cattle



M. bovis Infected Cattle



bovis PPD tuberculins (Canadian National Standard reference PPD tuberculins (100-89CNS and 100-02-2CNS) and the International Standard *M. bovis* PPD tuberculin (World Health Organization, Weybridge, England)) at 7 and 13 weeks post sensitization/infection respectively (Figure 1). The main objective of the 2004 studies was to qualify a new Canadian National Standard reference tuberculin (100-02-2CNS) against that of the International Standard *M. bovis* PPD tuberculin.

Western immunoblotting of *M. bovis* sensitized/infected cattle sera onto either HK-PPD or SF-PPD was performed as per Towbin et al, (1979) with the following specifications (147). Electrophoretically separated HK-PPD and SF-PPD was transferred onto nitrocellulose membranes (0.45 μm pore size, Bio-Rad Laboratories) using a tank style blotting system (Transphor Electrophoresis unit, Hoefer Inc., USA) with the transfer buffer cooled to 5°C in a recirculating water bath. Transfers were performed at 100 volts for 1 hour. Nitrocellulose used for one-dimensional Western blots were cut into strips according to lane number and the strips were blotted individually using channelled incubation trays. Nitrocellulose used for 2D Western blots were marked according to gel boundaries and incubated in individual trays. The molecular weight markers were removed from Western blots in which the Precision Plus, (Bio-Rad) pre-stained molecular weight markers were used as this type of molecular weight marker was found to fade considerably following Western blotting.

Following transfer, nitrocellulose Nitrocellulose was subsequently blocked for 1 hour at 37°C in 7.4 pH, Tris buffered saline (TBST) containing 0.02 M Tris, 0.8 % (w/v) sodium chloride and 0.02% (w/v) potassium chloride and 0.3 (v/v) Tween 20.

Nitrocellulose was incubated overnight (~16 hours) at room temperature in bovine sera (primary antibody) diluted 1:200 in TBST. The following day, nitrocellulose was washed with TBST, incubated for 2 hours at room temperature with alkaline phosphatase conjugated rabbit anti-bovine IgG (Sigma-Aldrich Canada Ltd., Oakville, Ontario) diluted 1:5000 in TBST and washed again in TBST. Phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) (Mandel Scientific Company Inc., Guelph, Ontario) was applied to the nitrocellulose for 10-15 minutes at which time the reaction was stopped with the addition of de-ionized water. Western blots were examined visually and were also digitized in 8 bit greyscale at 300 dpi with a ScanMaker *i*900 scanner (Microtek).

3.7 *Analysis of two-dimensional (2D) Western blots with PDQuest software.*

The antibody response of *M. bovis* sensitized/infected cattle to select SF-PPD antigens was quantified using 2DE analysis software (PDQuest 7.1.0 2D Analysis Software, Bio-Rad) to analyse specific 2D Western blot spots. In brief, prominent, sero-reactive SF-PPD antigens spots were identified in 2D Western blots of *M. bovis* sensitized/infected cattle sera. Digitized "TIF" images of 2D Western blots, all in 8 bit greyscale at 300 dpi, were uploaded into the PDQuest program. Identical spot parameters were overlayed onto selected SF-PPD antigens in each 2D Western blot so that each spot contained identical pixel volumes (Appendix 1 Figure 1). In the PDQuest program, each pixel is assigned an intensity between 0 and 255, with 0 being white and 255 being black. Spot pixel quantity is the sum total pixel intensity of all pixels within the spot parameter. Spot parameters were manually positioned in similar locations on each 2D Western blot

according to landmark spots such as the Ag85 complex, molecular weights markers and isoelectric points. The 2D Western blots were normalized to the background pixel intensity using three additional spot boundaries which were overlayed onto the background regions of each 2D Western blot (Appendix 1, Figure 1). Spot quantities of select, SF-PPD sero-reactive antigens were calculated by PDQuest and the fold change in spot intensity between each animal's pre-infection/ sensitization and post-infection/sensitization 2D Western blots were compared.

3.8 *Animal care.*

Prior to all animal purchases, the procedural outlines pertaining to animal use in this study were approved by the Ottawa Laboratory Fallowfield CFIA Institutional Animal Care Committee. All animals received *ad-libitum* food and water and were handled and cared for in accordance with the regulations prescribed by the Canadian Council for Animal Care. Cattle were screened with an *invitro* blood based assay (Bovigam™; Pfizer, Australia) prior to sensitization as described in section 3.6.

3.9 *Guinea pig CITST of SF-PPD vs HK-PPD.*

A CITST was employed to measure the delayed-type hypersensitivity response to SF-PPD *versus* HK-PPD according to the Biologics Production Unit, CFIA, standard operating procedure MY-PR041; *Potency Testing of Purified Protein Derivative (PPD) Tuberculins by Intradermal Inoculations of Sensitized Guinea Pigs*. In brief, twelve Dunkin-Hartley guinea pigs were obtained from the CFIA's Small Animal Facility located at the Ottawa Laboratory Fallowfield. The guinea pigs were injected intramuscularly in the caudal thigh with 2.0 mg of heat killed *M. bovis* AN5 cells suspended in 0.1 mL of

50% mineral oil, 25% lanolin and 25% saline solution. Three guinea pigs served as controls and received a 0.1 mL intramuscular injection of sterile saline.

At 35 days post sensitization, 0.1 mL intradermal injections of test tuberculins (SF-PPD and HK-PPD) were administered at concentrations of 50, 10 and 2 μg/mL diluted in a 7.2 pH, phospate buffer containing 0.0005% Tween 80. A 0.1 mL intradermal injection containing 50 μg/mL ovalbumin was also administered to each guinea pig to ascertain non-specific reactivity. Test tuberculins were injected using a zig-zag pattern on the shaved lateral thorax of the guinea pigs according to a balanced, complete Latin Square design. Erythema were measured at 48 hours post injection (hpi) and recorded as the average product of two, perpendicular diameter readings. Injections which failed to elicit a visible response were recorded as NVL (no visible lesion).

3.10 *M. bovis sensitization of guinea pigs with SF-PPD.*

The delayed-type hypersensitivity response of guinea pigs to SF-PPD and HK-PPD was also used to measure the artificial sensitization of guinea pigs to *M. bovis* with either SF-PPD or heat killed *M. bovis* cells. The CITST was carried out as per the Biologics Production Unit, CFIA, standard operating procedure MY-PR041 (outlined in section 3.9) with the following adjustments. Twenty Dunkin-Hartley guinea pigs were obtained from the CFIA's Small Animal Facility located at the Ottawa Laboratory Fallowfield. Eight of the guinea pigs received a 0.1 mL intramuscular injection in the caudal thigh with 2.0 mg of SF-PPD suspended in a 7.2 pH phosphate buffer. An additional 8 guinea pigs received a 0.1 mL intramuscular injection in the caudal thigh with 2.0 mg of heat killed *M. bovis* AN5 cells suspended in a 50% mineral oil, 25%

lanolin and 25% saline solution. Two control groups, each containing 2 guinea pigs, received 0.1 mL intramuscular injections of either sterile saline or a 50% mineral oil, 25% lanolin and 25% saline solution.

All guinea pigs were intradermally skin tested at 35 days post sensitization with SF-PPD and HK-PPD according to a balanced, complete Latin Square design (outlined in section 4.9). Erythema were measured at both 20 and 48 hpi and recorded as the average product of two, perpendicular diameter readings.

3.11 *Bovine comparative intradermal tuberculin skin test of SF-PPD, HK-PPD and Canadian National Standard Reference M. bovis PPD tuberculins.*

Delayed-type hypersensitivity responses to SF-PPD, HK-PPD and the current Canadian National Standard Reference (100-02-2CNS) *M. bovis* PPD tuberculin were measured using a CITST. The 100-02-2CNS is the Canadian reference tuberculin to which all new *M. bovis* tuberculin batches are compared and it has demonstrated equivalent, relative antigenicity to the International Standard *M. bovis* PPD tuberculin (Weybridge, England). In this experiment, fourteen, approximately four month-old steers were obtained from various local farms and raised to approximately one year of age at the CFIA's Large Animal Facility located at the Ottawa Laboratory Fallowfield. Twelve steers were sensitized intramuscularly in the caudal thigh with 5.0 mg of heat killed *M. bovis* cells (field strain 02/1007, CFIA designation) suspended in 0.1 mL consisting of 50% mineral oil, 25% lanolin and 25% saline. The *M. bovis* field strain 02/1007 was originally isolated by the Mycobacterial Diagnostic Unit, CFIA, from a Canadian beef cow (86). *M. bovis* cells were propagated aerobically in Middlebrook 7H9 liquid medium

supplemented with 10% oleic acid-albumin-dextrose complex (Becton Dickinson, Mississauga, Ontario) for 69 days at 37°C and were subsequently heat killed at 121°C for 45 minutes. Bacteria were lyophilized in 100ml, pre-weighed vials and maintained at 4°C until they were resuspended to a concentration of 40 mg/mL in 50% mineral oil, 25% lanolin and 25% saline. Two steers serving as negative controls each received a similar injection minus the *M. bovis* cells.

PPD tuberculins were applied intradermally on the shaved neck area according to a balanced, complete Latin Square design at protein concentrations of 1.0 and 0.2 mg/mL, resulting in a total of 6 *M. bovis* PPD tuberculin injections per animal,15 cm apart, 3 on each side of the neck. A single 0.1 mL intradermal injection of both 0.50 mg/mL *M. avium* PPD tuberculin (Lot 101-06-1B, Biologics Production Unit, CFIA) and 1.0 mg/mL ovalbumin (Sigma Inc., Mississauga, Ontario) were also applied to each animal on the left and right side of the neck respectively. Skin thickness at each injection site was measured with calipers immediately prior to tuberculin injection and at 24, 48 and 72 hpi. The pre-tuberculin injection measurements were subtracted from the 24, 48 and 72 hpi measurements to determine actual skin reaction size.

3.12 *Statistics.*

Statistical analyses were performed using Prism 4.0 (GraphPad, San Diego, CA). The sum of average CITST measurements between two tuberculins were statistically assessed by applying a paired t-test, while multiple dilutions used in the CITST were assessed by one-way ANOVAs incorporating Bonferroni's Multiple Comparison Test treatment. The fold increase data of spot pixel quantity in 2D Western blot as detected by

PDQuest software was also statistically assessed by applying a one-way ANOVA analysis with Bonferroni's Multiple Comparison Test treatment. Linear regression analysis, performed using Prism GraphPad software, was used to assess the DTH response of individual cattle to different *M. bovis* PPD tuberculins.

4 Results

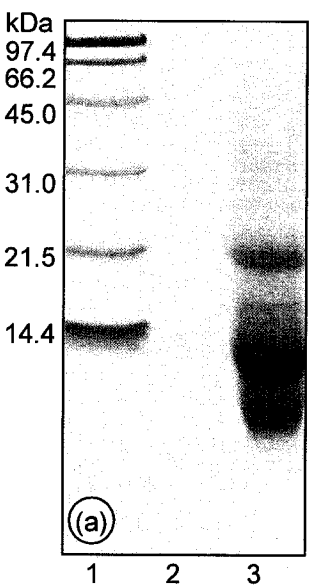
4.1 *HK-PPD and SF-PPD examination by SDS-PAGE.*

Although *M. bovis* PPD tuberculin has been used for more than a century for the diagnosis of bovine tuberculosis, the structure, antigenicity and relative quantity of the *M. bovis* CFPs which make up PPD tuberculin remain largely unknown. HK-PPD and SF-PPD were characterized by PAGE and 2DE to determine the most appropriate preparation of *M. bovis* CFPs to use for the Western blots. The components of HK-PPD and SF-PPD were separated using SDS-PAGE and stained with either Coomassie Brilliant Blue (CBB) or silver stain so that the denaturing effect of heat killing on *M. bovis* culture filtrate proteins could be visualized. Electrophoresis of HK-PPD through 15% polyacrylamide gels resulted in the appearance of four blurred bands located at approximately 23, 17, 10 and 6 kDa (Figure 2a). Lanes of HK-PPD were also dominated by the appearance of a large streak which commenced at approximately 45 kDa and increased in intensity at less than 25 kDa (Figure 2b). The 23 kDa and 10 kDa bands did not stain with equivalent, relative intensity with CBB or silver stains. The 23 kDa band stained poorly with CBB as compared to silver stained gels and the 10 kDa band which was easily visualized with CBB, was not detected with silver stain.

In comparison to HK-PPD, at least 35 bands could be visualized in SDS-PAGE of SF-PPD, many of which ranged from 23 to 80 kDa (Figure 3a, lane 3). All of the bands noted in PAGE of HK-PPD had corresponding bands of similar molecular weight in PAGE of SF-PPD however the corresponding SF-PPD bands were more discrete. Furthermore, lanes of SF-PPD lacked the streaked appearance noted in HK-PPD lanes.

Figure 2. 15% SDS-PAGE analysis of HK-PPD.

(a) Gel stained with Coomassie Brilliant Blue, R-250 (Bio-Rad). (b) Gel stained with non-fixing Silver Stain. Lanes: 1, Molecular weight standards (Silver Stain SDS-PAGE Standards Low Range, BIO-RAD). 2, Sample buffer; 20 μL. 3, HK-PPD; (a) 50 μg in 20 μL, (b) 5 μg in 20 μL.


kDa
97.4
66.2
45.0
31.0
21.5
14.4
(a)
1
2
3

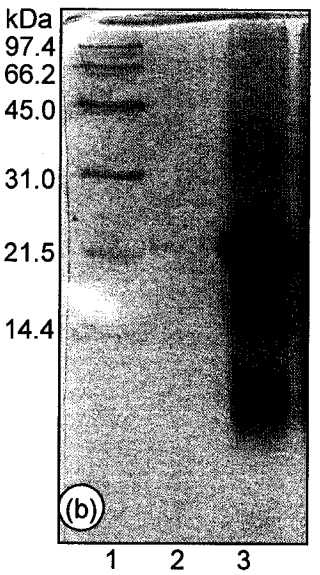
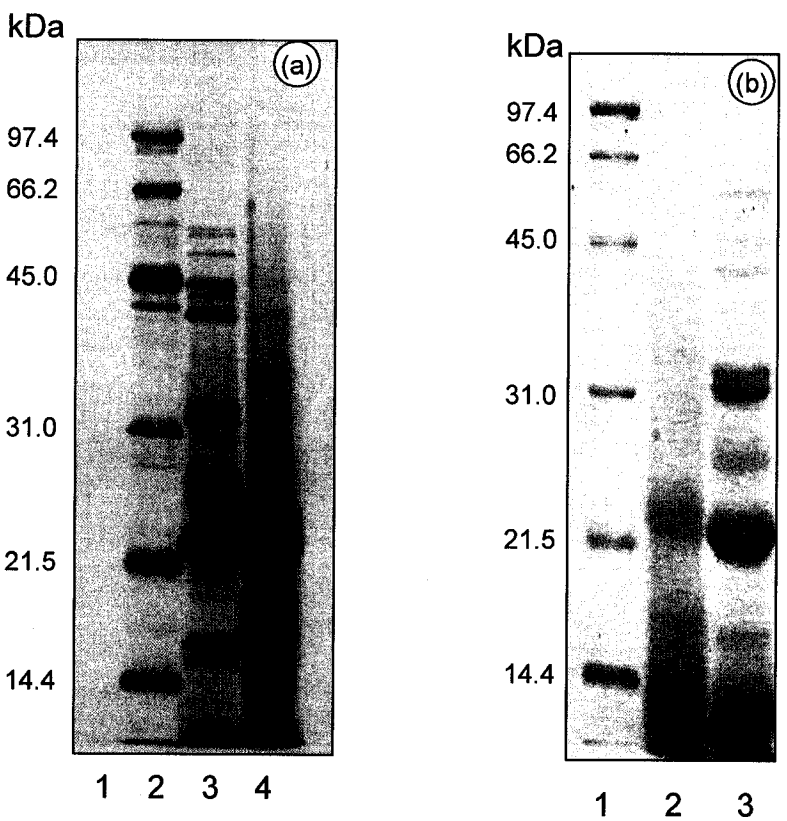

kDa
97.4
66.2
45.0
31.0
21.5
14.4
(b)
1
2
3

Figure 3. 12% SDS-PAGE analysis of SF-PPD.

(a) Gel stained with non-fixing silver stain. Lanes: 1, Sample buffer; 50 μL. 2. molecular weight standards (Silver Stain SDS-PAGE Standards Low Range, BIO-RAD). 3, SF-PPD; 12.5 μg in 50 μL. 4, HK-PPD; 12.5 μg in 50 μL. (b) Gel stained with Coomassie Brilliant Blue, R-250 (Bio-Rad). Lanes: 1, molecular weight standards (Silver Stain SDS-PAGE Standards Low Range, BIO-RAD). 2, HK-PPD; 40 μg in 40 μL. 3, SF-PPD; 40 μg in 40 μL.


kDa
(a)
97.4
66.2
45.0
31.0
21.5
14.4
1 2 3 4
kDa
(b)
97.4
66.2
45.0
31.0
21.5
14.4
1 2 3

As with HK-PPD, a major band of approximately 10 kDa appeared prominently when gels of SF-PPD were stained with CBB (Figure 3b, lane 3) but the band was not visualized in gels subjected to silver stain (Figure 3a). Conversely, the prominent SF-PPD 22 kDa band had a similar staining intensity in both CBB and silver stained gels.

Although SDS-PAGE of SF-PPD resulted in improved delineation of proteins as compared to HK-PPD, it was hypothesized that two dimensional SDS-PAGE (2DE) would offer additional protein separation and result in an improved analytical tool for use in the subsequent Western blots. 2DE of SF-PPD components incorporated isoelectric focusing as the first step followed by separation according to molecular weight with vertical SDS-PAGE (Figure 4). Approximately 200 spots were enumerated from digitized 2DE images of SF-PPD by PDQuest 2D Analysis Software (Bio-Rad), the majority of which localized within a pI range of 4 - 6 and within a molecular weight range less than 80 kDa (Figure 5).

In comparison to SF-PPD, 2D gels of HK-PPD were dominated by an indistinct smear which possessed a pI range of 3 - 5 and a molecular weight range of 0 - 80 kDa (Figure 6). The majority of the HK-PPD components appeared with molecular weights less then 25 kDa, and were blurred as compared to those of the SF-PPD gels. HK-PPD 2D gels also exposed approximately 30 spots from 5 - 15 kDa, in the 5.5 - 7.5 pI range which were not visualized in SF-PPD gels. A 50 μg to 10 μg reduction of the HK-PPD protein level applied to 2-DE reduced the intensity of the smear and facilitated the detection of additional faint spots however, fewer individual spots were enumerated by PDQuest in 2-DE of HK-PPD as compared to SF-PPD (Figure 7).

Figure 4. Silver stained 2-DE analysis of 10 μg of SF-PPD.

SF-PPD (2.0 μL of 5.0 μg/μL stock in 300 μL) was loaded onto a 17 cm pre-cast acrylamide strip with an immobilized pH range of 3 -10 (ReadyStrip™; BIO-RAD). Following isoelectric focussing, the acrylamide strip was loaded into a vertical 12% polyacrylamide gel with molecular weight standard (Precision Plus, Bio-Rad), electrophoresed and silver stained.

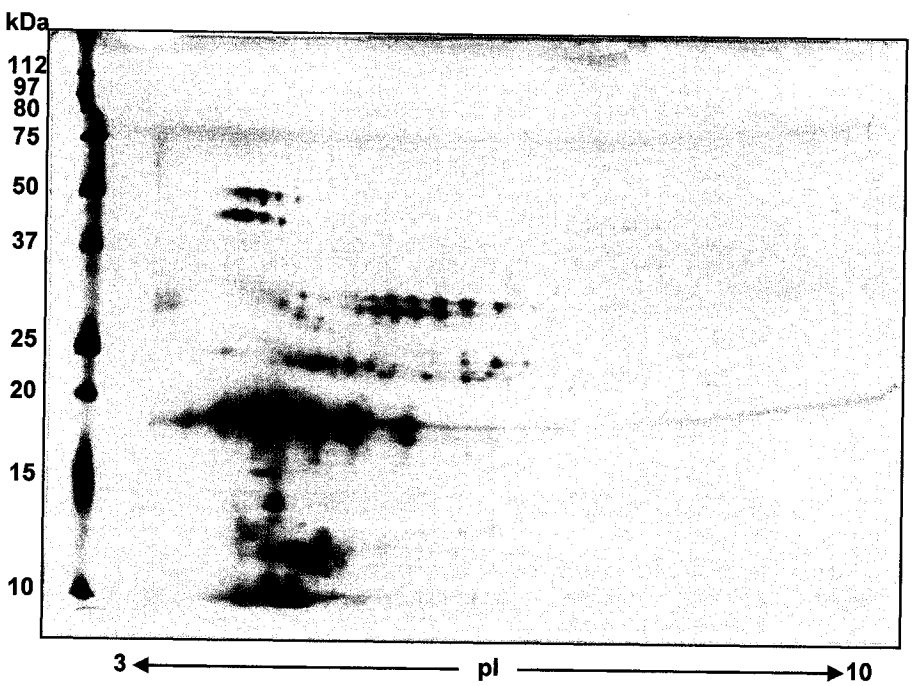

kDa
112
97
80
75
50
37
25
20
15
10
3
pI
10

Figure 5. Spot enumeration of 2-DE, silver stained SF-PPD gels.

Two dilutions of SF-PPD ((a) 10 μg: 2.0 μL of 5.0 μg/μL stock in 300 μL; (b) 50 μg: 10 μL of 5.0 μg/μL stock in 300 μL) were subjected to 2DE analysis as described in Figure 5. Gels were digitized in 8 bit greyscale at 300 dpi with a Lexmark 8000 scanner and enumerated by PDQuest 7.1.0 2D Analysis Software (Bio-Rad). Following automated enumeration, erroneous and duplicate spots were manually deleted. (a) depicts 97 spots and (b) depicts 98 spots.

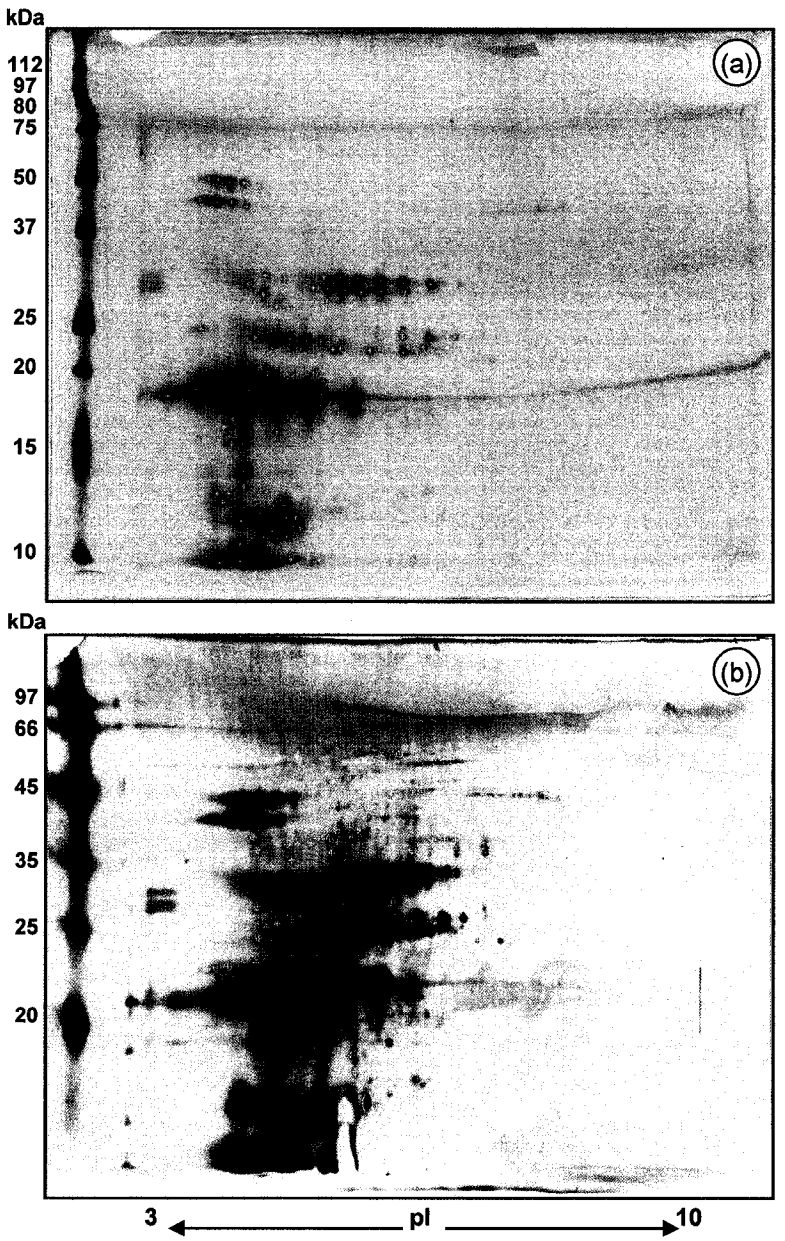

kDa
112
97
80
75
50
37
25
20
15
10
(a)
kDa
97
66
45
35
25
20
(b)
3
pI
10

Figure 6. Silver stained 2-DE analysis of HK-PPD.

HK-PPD ((a) 50 μg: 12.5 μL of 4 μg/μL stock in 300 μL; (b) 10 μg: 2.5 μL of 4 μg/μL stock in 300 μL) was loaded onto 17 cm pre-cast acrylamide strips each with an immobilized pH range of 3 -10 (ReadyStrip™; Bio-Rad). Following isoelectric focussing, the acrylamide strips were loaded into vertical 12% polyacrylamide gels with molecular weight standard ((a)Silver Stain SDS-PAGE Standards, Low Range, Bio-Rad; (b) Precision Plus, Bio-Rad), electrophoresed and silver stained.

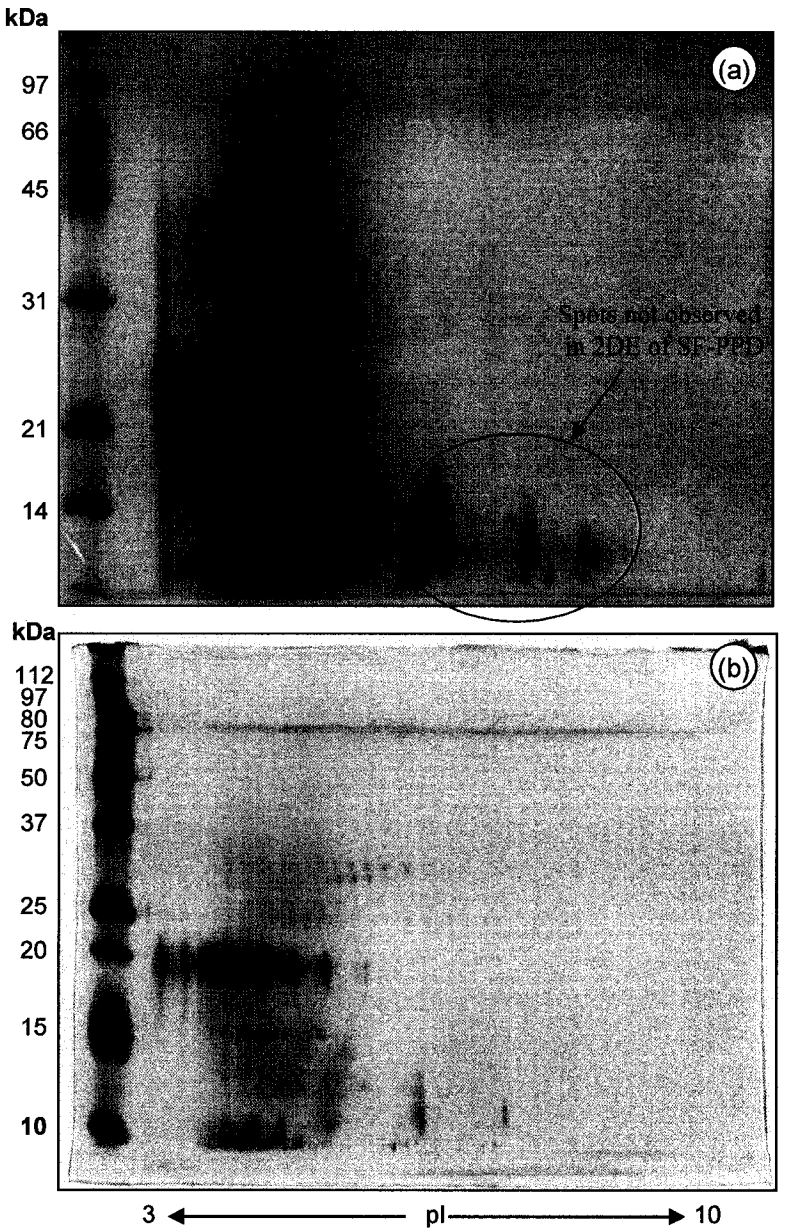
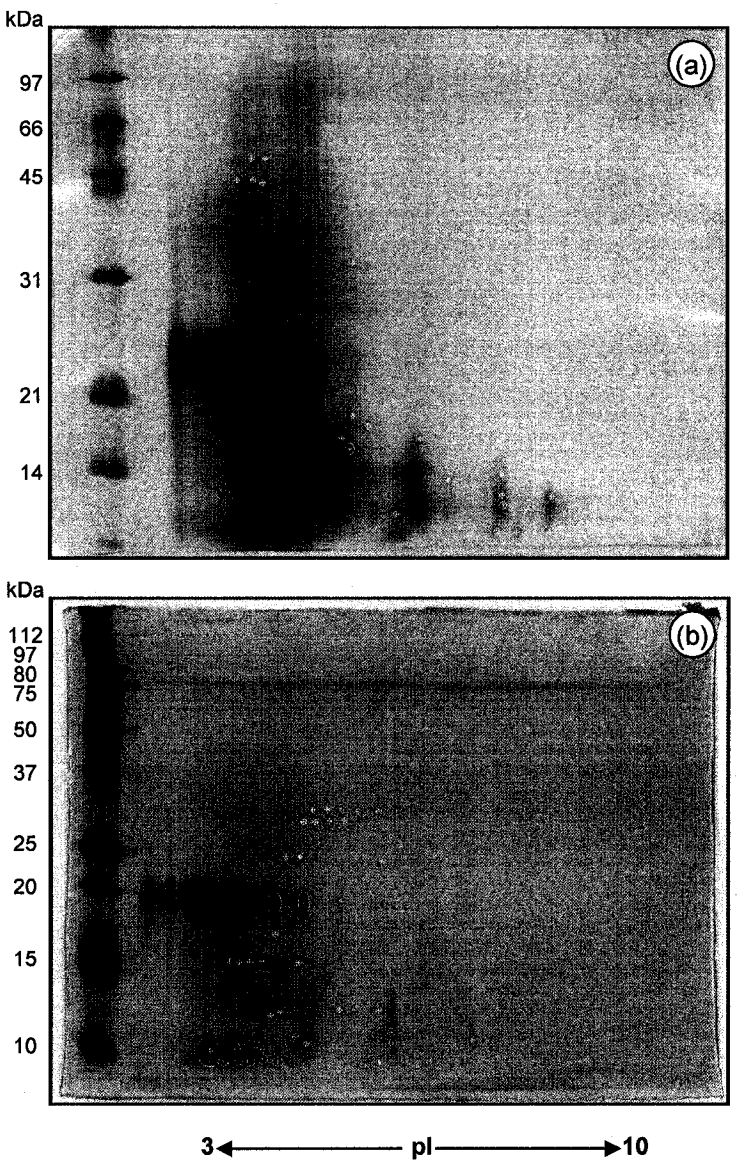

kDa
97
66
45
31
21
14
(a)
Spots not observed
in 2DE of SF-PPD
kDa
112
97
80
75
50
37
25
20
15
10
(b)
3
pI
10

Figure 7. Spot enumeration of 2-DE, silver stained HK-PPD gels.

Two dilutions of HK-PPD ((a) 50 μg: 12.5 μL of 4 μg/μL stock in 300 μL; (b) 10 μg: 2.5 μL of 4 μg/μL stock in 300 μL) were subjected to 2DE analysis as described in Figure 8. Gels were digitized in 8 bit greyscale at 300 dpi with a Lexmark 8000 scanner and enumerated by PDQuest 7.1.0 2D Analysis Software (Bio-Rad). Following automated enumeration, erroneous and duplicate spots were manually deleted. (a) depicts 23 spots and (b) depicts 46 spots.


kDa
97
66
45
31
21
14
(a)
kDa
112
97
80
75
50
37
25
20
15
10
(b)
3
pI
10

4.2 *One dimensional Western immuboblot comparison of HK-PPD and SF-PPD.*

The protein denaturing effect of the heat kill on *M. bovis* CFPs shown by PAGE and 2DE comparisons of HK-PPD *versus* SF-PPD tuberculins suggested that the antigenicity of the SF-PPD differed from that of HK-PPD. Therefore, in order to investigate this further, SF-PPD and HK-PPD were compared by Western blot analysis using serum aliquots from a positive control, *M. bovis* sensitized steer. Western blot of HK-PPD revealed that the majority of antibody binding was restricted to one, predominant 22 kDa band and to two additional faint bands of approximately 40 and 66 kDa (Figure 8, lane 4). Contrary to CBB and silver stain results (Figures 2), Western blots of HK-PPD did not detect protein components less then 20 kDa.

In comparison, Western blots of SF-PPD with the same positive control, *M. bovis* sensitized cattle sera as above recognized over 25 bands with molecular weights ranging from 20 - 90 kDa (Figure 9, lane 4). SF-PPD bands situated at less then 20 kDa were not detected by *M. bovis* sensitized sera, however, *M. bovis* sensitized sera did recognise several SF-PPD bands greater then 20 kDa which were visible in silver stained PAGE of SF-PPD (Figure 3a).

Sequential serum samples from *M. bovis* infected cattle were also analysed by one dimensional Western blots of SF-PPD and HK-PPD (Figure 8, Figure 9). While the sera was shown to recognise additional SF-PPD antigens as compared to HK-PPD, the pre-infection sera also recognised additional SF-PPD antigens at approximately 31, 33, 42, 43 and 60 kDa. These background antibody responses did not appear to vary throughout the 20 wk study. Alternatively, antibody response to a 22 kDa band was dramatically boosted

Figure 8. Western blot analysis of sequential serum samples from *M. bovis* infected bovine #107 blotted onto HK-PPD.

A 12% SDS-PAGE was performed followed by electrophoretic transfer of HK-PPD proteins to nitrocellulose. Lanes were cut into strips and individually stained or blotted. Lanes 1, 2 and 3 were loaded with 40 μL of sample buffer, molecular weight standards (Silver Stain SDS-PAGE Standards Low Range, Bio-Rad) and 10 μg (40 μL) of HK-PPD respectively and were stained with Colloidal Gold (Bio-Rad) following transfer. Lanes 4-20 were each loaded with 10 μg (40 μL) of HK-PPD and blotted with positive control sera from *M. bovis* sensitized steer #893 (Lane 4), pre-infection sera from bovine #107 (Lane 5), sequential, post-infection sera from bovine #107 (Lane 6, wk.1. Lane 7, wk. 3. Lane 8, wk. 5. Lane 9, wk. 7. Lane 10, wk. 9. Lane 11, wk. 11. Lane 12, wk. 13. Lane 13, wk. 14. Lane 14, wk. 15. Lane 15, wk. 16. Lane 16, wk. 17. Lane 17, wk. 18. Lane 18, wk. 19. Lane 19, wk. 20.). Lane 20 was blotted without sera (only TBST).

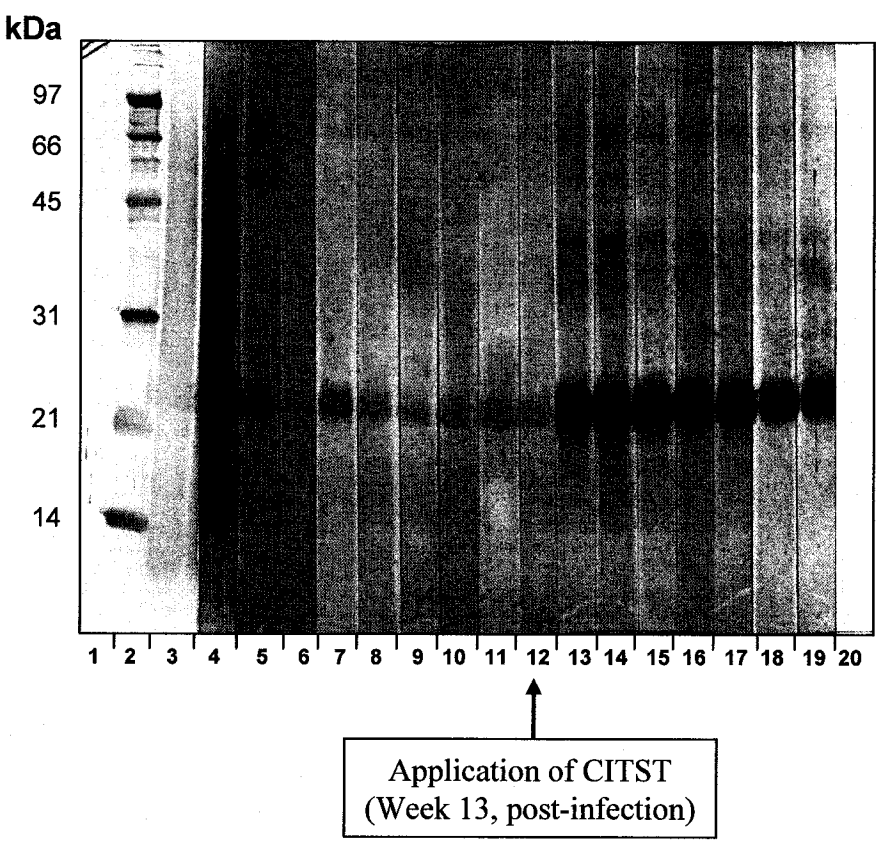
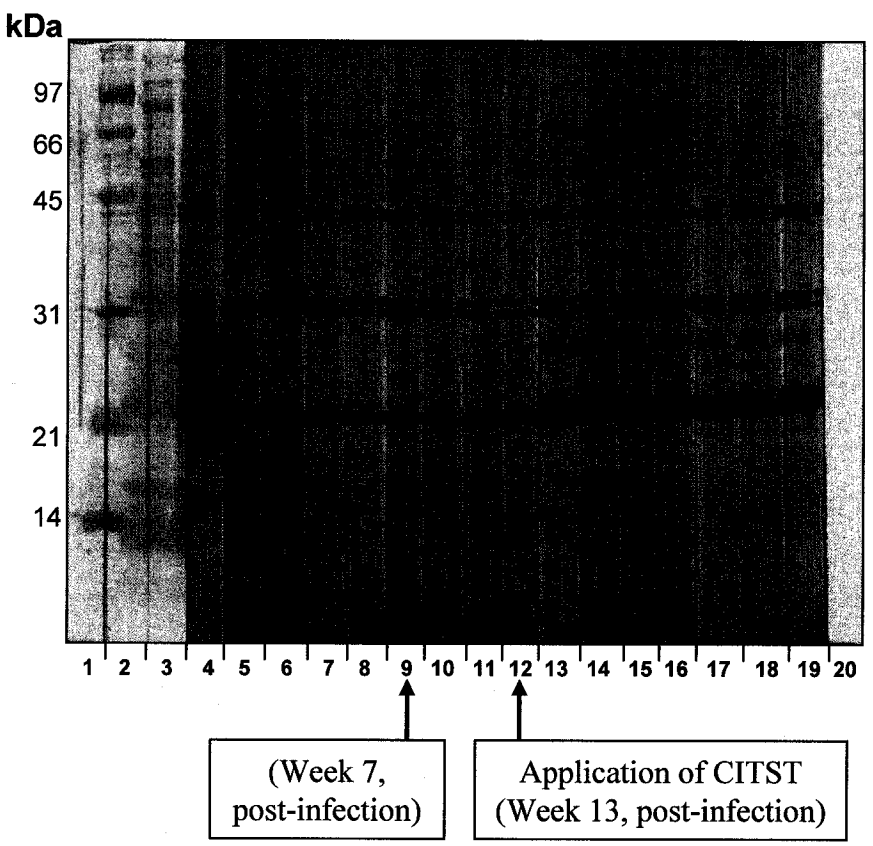

kDa
97
66
45
31
21
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Application of CITST
(Week 13, post-infection)

Figure 9. Western blot analysis of sequential serum samples from *M. bovis* infected bovine #107 blotted onto SF-PPD.

Western blot analysis was performed as per Figure 8 with the exception that SF-PPD was electrophoresed in lanes 3 – 20 in place of HK-PPD.


kDa
97
66
45
31
21
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
(Week 7, post-infection)
Application of CITST (Week 13, post-infection)

in both SF-PPD and HK-PPD Western blots following the CITST (Figure 8 and Figure 9, Lane 12).

4.3 *Guinea pig comparative intradermal tuberculin skin test of SF-PPD vs HK-PPD.*

Considerable differences were observed in the SDS-PAGE and Western blot comparisons of SF-PPD *versus* HK-PPD and it was hypothesized that the relative antigenicity of SF-PPD would also be different from that of HK-PPD. Therefore, a CITST was used to measure the relative delayed-type hypersensitivity (DTH) response of *M. bovis* sensitized guinea pigs to SF-PPD and HK-PPD at 35 days post sensitization with heat killed *M. bovis* cells. The sum of average areas for the guinea pig DTH responses to SF-PPD was shown to be 126% greater than that of HK-PPD ($P < 0.001$; paired t-test) (Table 1). However, when the DTH responses to each tuberculin dilution were analysed with a one way ANOVA, only the 50 μg/mL dilution of SF-PPD was shown to elicit a significantly larger DTH response versus that of HK-PPD at the same dilution ($P < 0.05$; one way ANOVA with Bonferroni's Multiple Comparison Test) (Figure 10). Guinea pigs sensitized with heat killed *M. bovis* cells did not produce a visible skin reaction to 50 μg of ovalbumin and the control guinea pigs injected with saline failed to produce skin reactions to intradermal injections of either SF-PPD or HK-PPD (Appendix Table 1).

4.4 *Sensitization of guinea-pigs to M. bovis using SF-PPD.*

While PAGE analysis of HK-PPD indicated that many of the *M. bovis* proteins are denatured by the heat kill process, 2DE revealed that SF-PPD proteins may exist in a more native state. Also, Western blot analyses indicated that many of the SF-PPD proteins were sero-reactive. As an *M. bovis* infected animal would be exposed to native

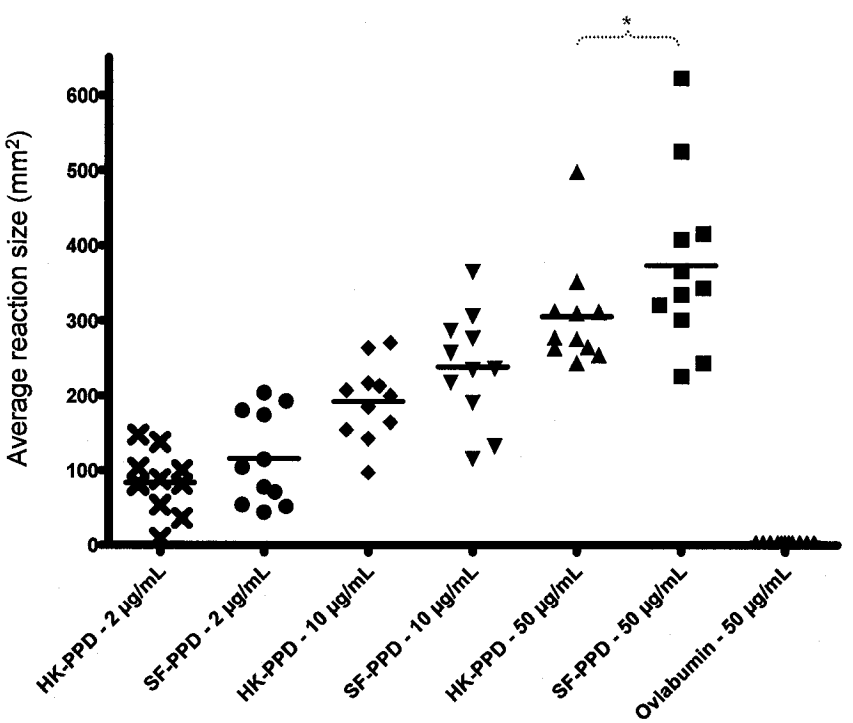
Table 1. Antigenicity of SF-PPD *versus* HK-PPD in *M. bovis* sensitized guinea pigs.

The antigenicity of SF-PPD and HK-PPD *M. bovis* PPD tuberuclins was compared using a CITST in which 12 guinea pigs were sensitized with an injection (IM) of heat killed *M. bovis* cells. Statistics were performed using Prism GraphPad software. a: one-way ANOVA with Bonferroni's Multiple Comparison; b: paired t-test.

Dilution (µg/mL)	Average area of skin reaction measurements (mm2)						SF-PPD fraction as a % of HK-PPD	
	HK-PPD	(Std. Dev.)	SF-PPD	(Std. Dev.)	Ovalbumin	(Std. Dev.)		
50	314.52	71.56	389.47	119.21	0.00	0.00	123.83%	$P< 0.05^a$
10	191.06	47.12	244.08	69.63			127.75%	$P> 0.05^a$
2	92.95	48.59	119.55	58.00			128.63%	$P> 0.05^a$
Sum of average areas	598.52		753.10				125.83%	$P< 0.001^b$

Figure 10. DTH response of SF-PPD *versus* HK-PPD in *M. bovis* sensitized guinea pigs.

Data points depict the 48 hpi guinea pig skin reaction measurements to 0.1 mL injections of 2.0, 10.0 and 50.0 mg/mL dilutions of SF-PPD and HK-PPD in 12 guinea pigs sensitized to *M. bovis* with heat killed *M. bovis* cells. Guinea pigs also received one, 0.1 mL injection of 50 mg/mL ovalbumin as negative control. Statistics were performed using a one-way ANOVA with Bonferroni's Multiple Comparison, Prism GraphPad. (*. $P < 0.05$).

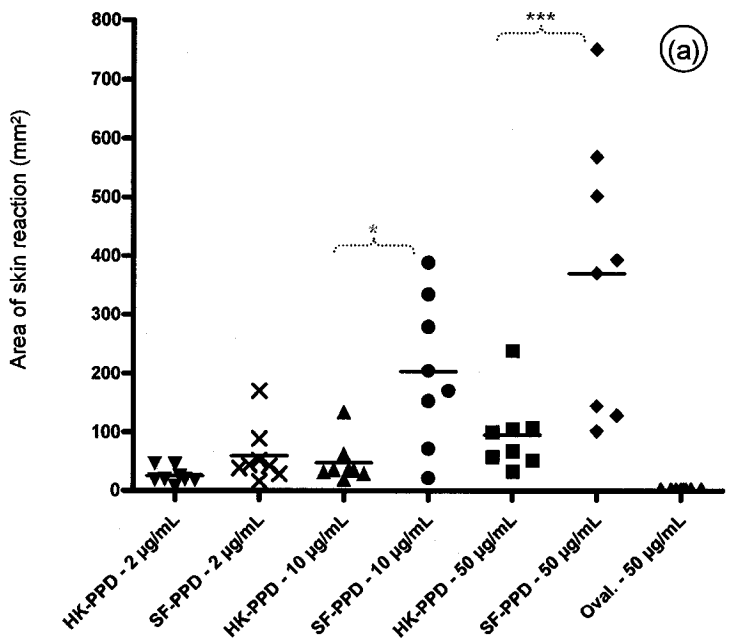

Average reaction size (mm²)
600
500
400
300
200
100
0
*
HK-PPD - 2 µg/mL
SF-PPD - 2 µg/mL
HK-PPD - 10 µg/mL
SF-PPD - 10 µg/mL
HK-PPD - 50 µg/mL
SF-PPD - 50 µg/mL
Ovlabumin - 50 µg/mL

M. bovis antigens, I hypothesized that an injection of SF-PPD would be sufficiently antigenic to effectively sensitize guinea pigs to *M. bovis*. The artificial sensitization of guinea pigs to *M. bovis* with SF-PPD *versus* heat killed *M. bovis* cells was evaluated with the application of a CITST using SF-PPD and HK-PPD tuberculins. At 24 hours post tuberculin injections (hpi), guinea pigs sensitized with SF-PPD produced greater average skin reactions to SF-PPD tuberculin than to HK-PPD tuberculin at both the 50 ($P < 0.001$) and 10 ($P < 0.05$) μg/mL dilutions (Figure 11a). The sum of average skin reaction measurement for SF-PPD tuberculin injections was also 3.75 fold greater then that of HK-PPD at 24 hpi ($P < 0.001$) in the SF-PPD sensitized guinea pigs. Although all tuberculin reactions in SF-PPD sensitized guinea pigs diminished from 24 to 48 hpi (Figure 11), SF-PPD tuberculin injections maintained a 2.4 fold greater sum of average skin reaction measurement at 48 hpi as compared to HK-PPD ($P < 0.05$). In comparison, the sum of average skin reactions of SF-PPD and HK-PPD tuberculins in guinea pigs sensitized with heat killed *M. bovis* cells demonstrated an overall equivalency of 85% and 92% (HK-PPD/SF-PPD) at 24 and 48 hpi respectively and equal concentrations of SF-PPD and HK-PPD tuberculins did not show statistical differences (Figure 12).

While the average 24 hpi skin reaction measurement for 50 μg/mL SF-PPD tuberculin was not significantly different (SF-PPD/HK-PPD = 110%) between guinea pigs sensitized with either SF-PPD or heat killed *M. bovis* cells (Figure 13), the average skin reaction measurement for SF-PPD in SF-PPD sensitized guinea pigs dropped 2.8 fold from 24 to 48 hpi (370.60 mm^2 to132.55 mm^2) ($P < 0.01$) and rose 1.1 fold (336.99 mm^2 to 374.10 mm^2) in guinea pigs sensitized with heat killed *M. bovis* cells. The red colour

Figure 11. The immunogenicity of SF-PPD in guinea pigs.

CITST skin reactions in 8 SF-PPD sensitized guinea pigs were measured at 24 (a) and 48 (b) hpi. SF-PPD, HK-PPD and ovalbumin concentrations used in the CITST are indicated along the respective "X" axis. Statistics were performed using a one-way ANOVA with Bonferroni's Multiple Comparison, Prism GraphPad. *: $P < 0.05$; ***: $P < 0.001$.


(a)
Area of skin reaction (mm²)
800
700
600
500
400
300
200
100
0
*
***
HK-PPD - 2 µg/mL
SF-PPD - 2 µg/mL
HK-PPD - 10 µg/mL
SF-PPD - 10 µg/mL
HK-PPD - 50 µg/mL
SF-PPD - 50 µg/mL
Oval. - 50 µg/mL

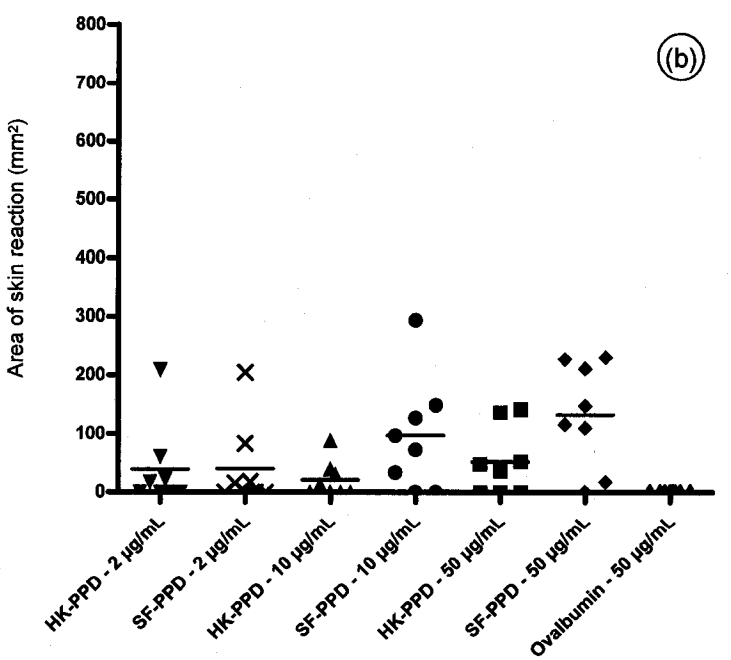
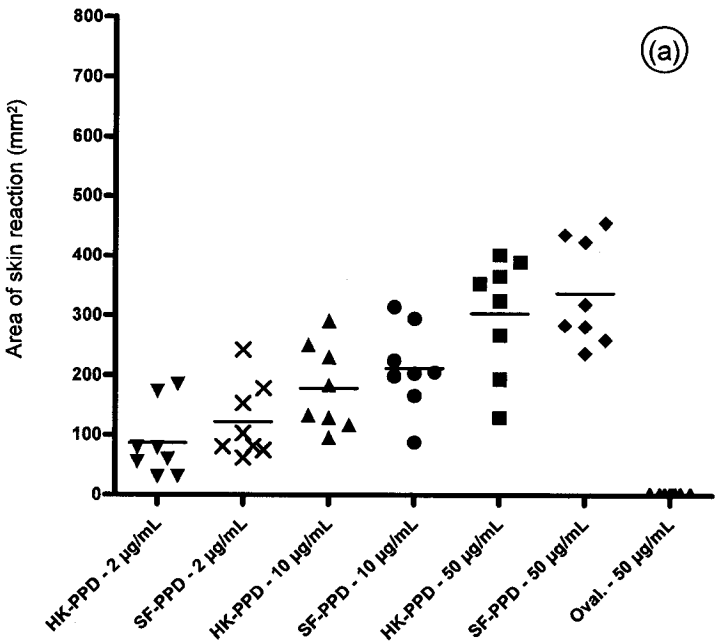

(b)
Area of skin reaction (mm²)
800
700
600
500
400
300
200
100
0
HK-PPD - 2 µg/mL
SF-PPD - 2 µg/mL
HK-PPD - 10 µg/mL
SF-PPD - 10 µg/mL
HK-PPD - 50 µg/mL
SF-PPD - 50 µg/mL
Ovalbumin - 50 µg/mL

Figure 12. Immunogenicity of heat killed *M .bovis* cells.

CITST skin responses in 8 guinea pigs sensitized with heat killed *M. bovis* cells were measured at 24 (a) and 48 (b) hpi. SF-PPD, HK-PPD and ovalbumin concentrations used in the CITST are indicated along the respective "X" axis. Statistics were performed using a one-way ANOVA with Bonferroni's Multiple Comparison, Prism GraphPad.


(a)
Area of skin reaction (mm²)
800
700
600
500
400
300
200
100
0
HK-PPD - 2 µg/mL
SF-PPD - 2 µg/mL
HK-PPD - 10 µg/mL
SF-PPD - 10 µg/mL
HK-PPD - 50 µg/mL
SF-PPD - 50 µg/mL
Oval. - 50 µg/mL

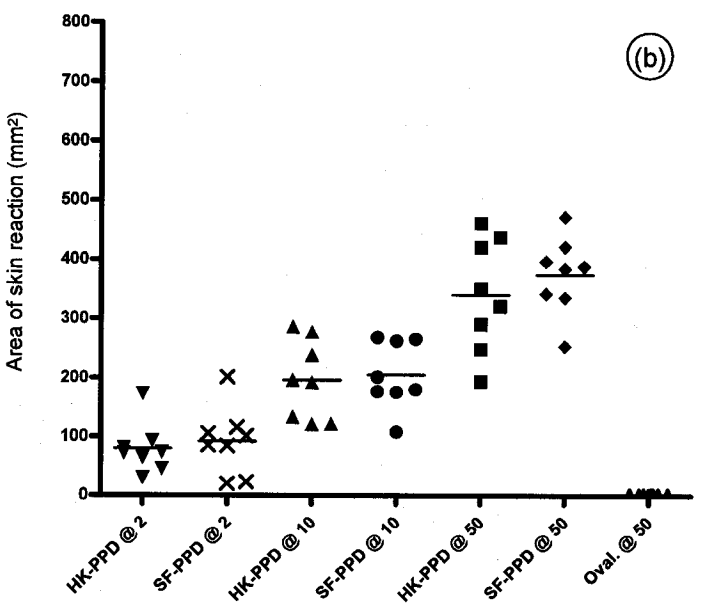
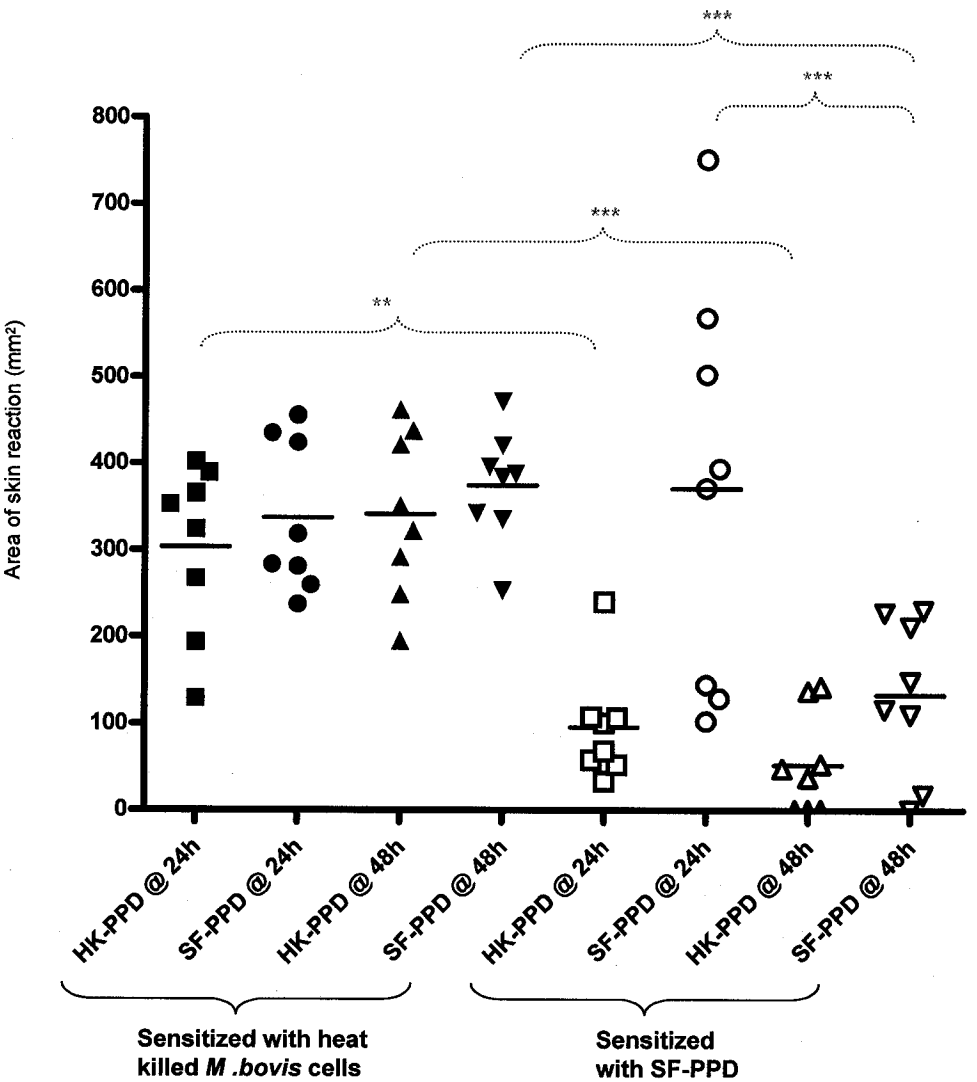

(b)
Area of skin reaction (mm²)
800
700
600
500
400
300
200
100
0
HK-PPD @ 2
SF-PPD @ 2
HK-PPD @ 10
SF-PPD @ 10
HK-PPD @ 50
SF-PPD @ 50
Oval. @ 50

Figure 13. Immunogenicity of SF-PPD *versus* heat killed *M. bovis* cells.

Skin responses to 5.0 μg of SF-PPD and to 5.0 μg of HK-PPD were measured at 24 and 48 hpi. in 8 guinea pigs sensitized with 2.0 mg heat killed *M. bovis* cells and 8 guinea pigs sensitized with 2.0 mg SF-PPD. The method of *M. bovis* sensitization, the tuberculin type and the hpi measurements are indicated along the "X" axis. Statistics were performed using a one-way ANOVA with Bonferroni's Multiple Comparison, Prism GraphPad. **: $P < 0.01$; ***: $P < 0.001$.


***
***
***
**
800
700
600
500
400
300
200
100
0
Area of skin reaction (mm²)
HK-PPD @ 24h
SF-PPD @ 24h
HK-PPD @ 48h
SF-PPD @ 48h
HK-PPD @ 24h
SF-PPD @ 24h
HK-PPD @ 48h
SF-PPD @ 48h
Sensitized with heat killed M .bovis cells
Sensitized with SF-PPD

of the erythema in the SF-PPD sensitized guinea pigs had also faded considerably and erythema boarders were not well delineated as compared to those of guinea pigs sensitized with heat killed *M. bovis* cells. As a result, the 48 hpi average skin reaction measurement for 50 μg/mL SF-PPD in SF-PPD sensitized guinea pigs was only 35.43% of that measured in guinea pigs sensitized with heat killed *M. bovis* cells ($P < 0.001$) (Figure 13). In comparison, 50 μg/mL HK-PPD average skin reactions at 24 and 48 hpi in guinea pigs sensitized with SF-PPD were 32% ($P < 0.01$) and 15% ($P < 0.001$) of those measured in guinea pigs sensitized with heat killed *M. bovis* cells (Figure 13).

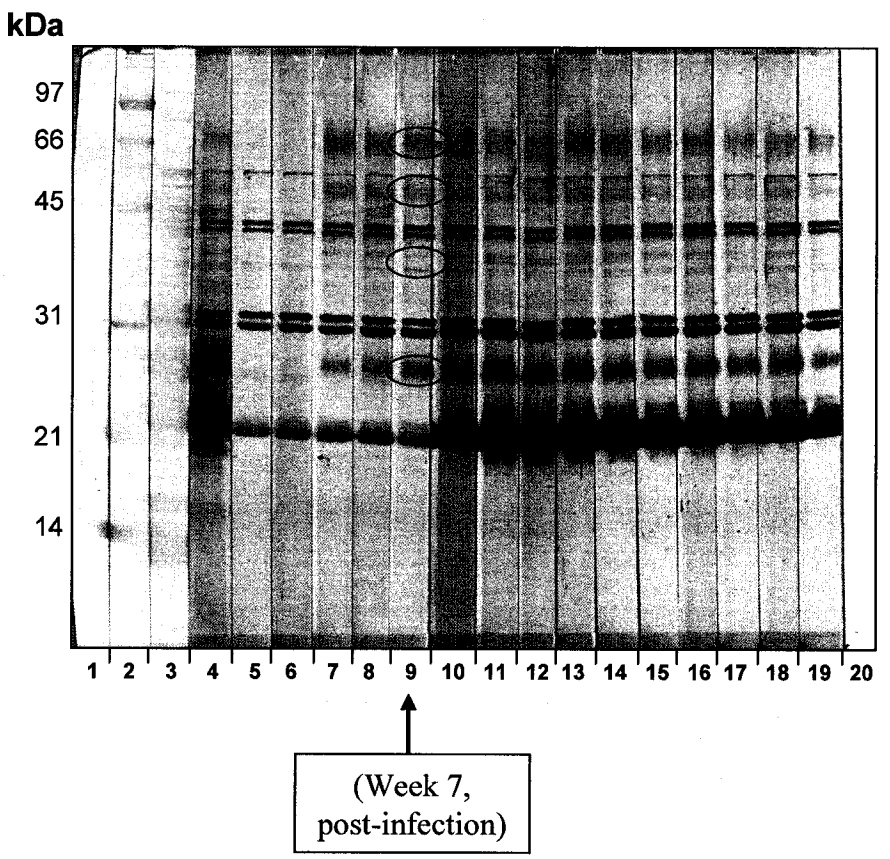
4.5 *Western immunoblot comparison of M. bovis infected/sensitized cattle sera.*

Previous work in our laboratory showed that cattle artificially sensitized to *M. bovis* with an injection of heat killed *M. bovis* cells mounted a 30% greater average DTH response to the intradermal application of *M. bovis* PPD tuberculin as compared to cattle experimentally infected with *M. bovis* (unpublished data). It was therefore hypothesized that a comparison between the *M. bovis* infected and *M. bovis* sensitized antibody responses to *M. bovis* CFPs may identify immunological differences between these respective cattle models.

Therefore, in this study, sequential serum samples from *M. bovis* infected and *M. bovis* sensitized cattle were analysed by one dimensional Western blots of SF-PPD (Figure 9 and Figure 14). The antibody response to SF-PPD antigens was boosted in both the sensitized and infected cattle following the application of CITSTs (Figure 9, Lane 12; Figure 14, Lane 9). Sequential serum samples from *M. bovis* sensitized cattle were shown to respond to additional SF-PPD antigens at the same earlier time-points as compared to

Figure 14. Western blot analysis of sequential serum samples from *M. bovis* sensitized bovine #993 blotted onto SF-PPD.

A 12% SDS-PAGE was performed followed by electrophoretic transfer of SF-PPD proteins to nitrocellulose. Lanes were cut into strips and individually stained or blotted. Lanes 1, 2 and 3 were loaded with 40 μL of sample buffer, molecular weight standards (Silver Stain SDS-PAGE Standards Low Range, Bio-Rad) and 10 μg (40 μL) of HK-PPD respectively and were stained with Colloidal Gold (Bio-Rad) following transfer. Lanes 4-20 were each loaded with 10 μg (40 μL) of HK-PPD and blotted with positive control sera from *M. bovis* sensitized steer #893 (Lane 4), pre-infection sera from bovine #993 (Lane 5), sequential, post-infection sera from bovine #993 (Lane 6, wk.1. Lane 7, wk. 3. Lane 8, wk. 5. Lane 9, wk. 7. Lane 10, wk. 9. Lane 11, wk. 11. Lane 12, wk. 13. Lane 13, wk. 14. Lane 14, wk. 15. Lane 15, wk. 16. Lane 16, wk. 17. Lane 17, wk. 18. Lane 18, wk. 19. Lane 19, wk. 20). Lane 20 was blotted without sera (only TBST). Circles represent examples of bands not identified in Western blots of *M. bovis* infected cattle at 7 weeks post infection (Figure 9).


kDa
97
66
45
31
21
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
(Week 7, post-infection)

the infected cattle. For example, sera from *M. bovis* sensitized cattle collected at seven weeks post sensitization recognised SF-PPD bands at approximately 27, 40, 47 and 66 kDa while sera collected from cattle 7 week post *M. bovis* infection did not (Figure 14, Lane 9).

The antibody response to SF-PPD antigens was further characterized by a two-dimensional (2D) Western blot analysis. Following initial 2D Western blots, nine 2DE SF-PPD spots were selected for MASS-SPEC analysis based on their observed sero-reactivity with *M. bovis* infected and/or *M. bovis* sensitized cattle sera (Figure 15). While the MASS-SPEC analysis potentially identified several mycobacterial proteins co-existing at each spot (Appendix Table 1), the SF-PPD spots were annotated based on the strength of association provided by the MASS-SPEC analysis (Mascot score) and by comparison to previously published 2DE analysis of *Mycobacterium spp.* CFPs (52, 70, 91, 93, 100, 103). MPB32 was also annotated in Figure 15 based on its identical position and appearance to previously published 2DE analyses of mycobacterial CFPs (121, 139, 163)

Six *M. bovis* infected and six *M. bovis* sensitised cattle were examined by 2D Western blot analysis. Sera from each animal was blotted at three time-points: pre-infection/pre-sensitization, seven week post infection/sensitization and three week post CITST (Figure 1). Every animal's pre-infection or pre-sensitization sera generated a background antibody response to SF-PPD antigens. While the background responses varied from animal to animal, the entire Ag85 complex was consistently observed with each animal's pre-infection or pre-sensitization sera (Figure 16, Appendix Figures 2-7).

A trend observed from the 2D Western blot analysis of seven weeks post

Figure 15. Location of SF-PPD spots selected for MASS-SPEC analysis.

Spots were excised from a CBB stained 2-DE gel of SF-PPD (200 μg) and submitted to the Ottawa Institute of Systems Biology, University of Ottawa for MASS-SPEC analysis. Spots circled in red were annotated according to MASS-SPEC analysis results (Mascot score)(Appendix 1; Table 1) and by comparison to previously published 2DE analyses of *Mycobacterium spp.* CFPs (Mattow *et al.*, 2003; Jungblut *et al.*, 1999; Malen, Softeland, and Wiker, 2008; Nagai *et al.*, 1991; Rosenkrands *et al.*, 1998; Sinha *et al.*, 2005; Sonnenberg and Belisle, 1997; Ohara *et al.*, 1997; Harboe *et al.*, 1986; Weldingh, 1998). Spots circled in blue were annotated based on comparison to previously published 2DE analyses of *Mycobacterium spp.* (Sonnenberg, 1997; Rosenkrands, 2000; Weldingh, 1998).

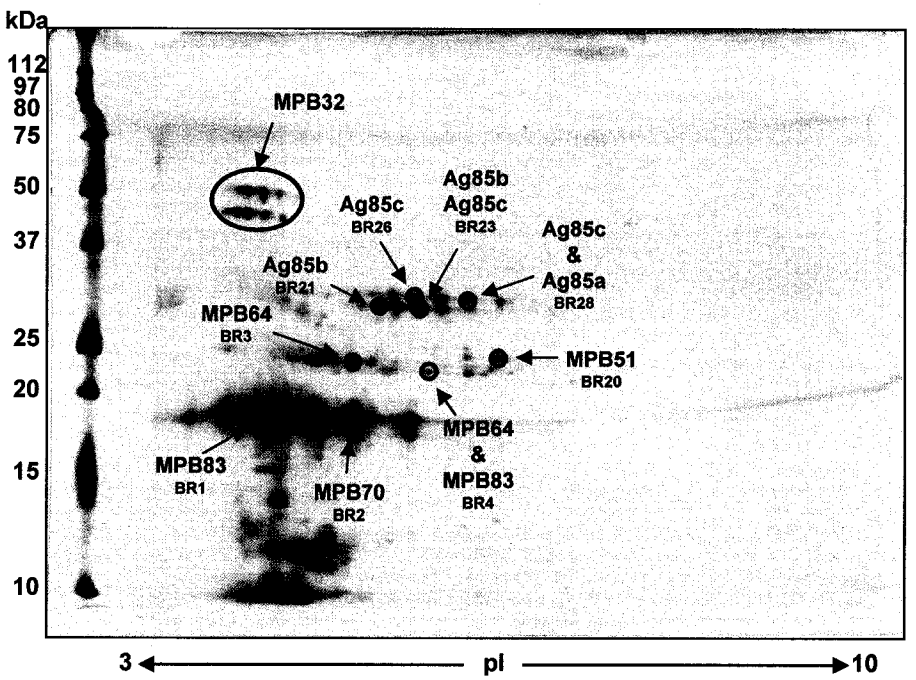
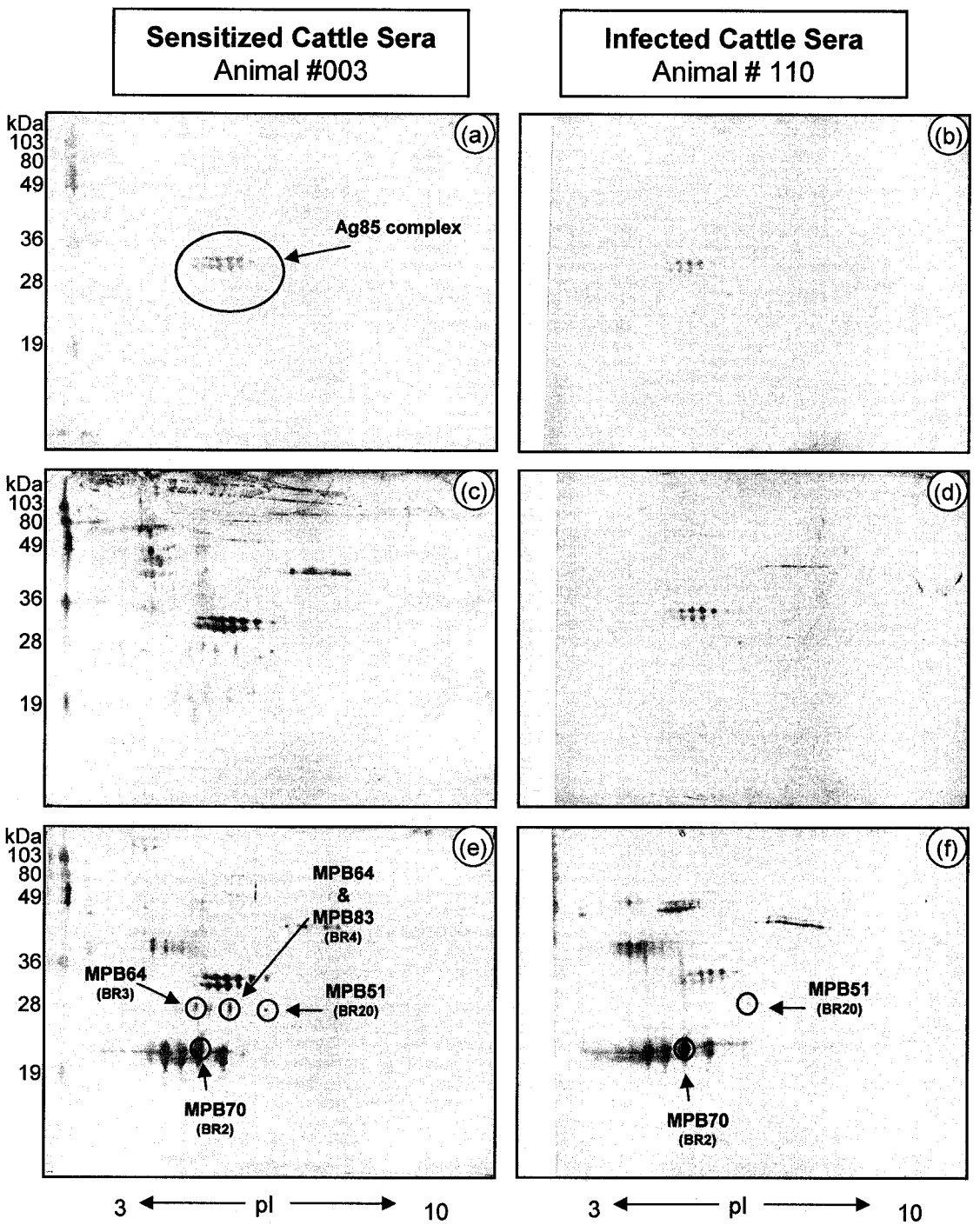

kDa
112
97
80
75
50
37
25
20
15
10
MPB32
Ag85c
BR26
Ag85b
Ag85c
BR23
Ag85c
&
Ag85a
BR28
Ag85b
BR21
MPB64
BR3
MPB51
BR20
MPB64
&
MPB83
BR4
MPB83
BR1
MPB70
BR2
3
pI
10

Figure 16. 2D Western blot analysis of the antibody response to SF-PPD proteins in *M. bovis* infected and *M. bovis* sensitized cattle.

Sera was collected prior to sensitization (a) or infection (b); seven weeks post sensitization (c) or infection (d); and three weeks post CITST (e) and (f). Western blots (a), (c) and (e) are representative blots of the *M. bovis* sensitized cattle sera ($n = 6$). Western blots (b), (d) and (f) are representative blots of the *M. bovis* infected cattle sera ($n = 6$). Background spots depicted in (a) can be seen in all frames of sensitized and infected animals. Spots circled in (e) and (f) highlight variable antibody recognition in *M. bovis* sensitized *versus M. bovis* infected cattle to select SF-PPD proteins.


Sensitized Cattle Sera
Animal #003
Infected Cattle Sera
Animal # 110
kDa
103
80
49
36
28
19
(a)
Ag85 complex
(b)
(c)
(d)
(e)
MPB64
&
MPB83
(BR4)
MPB64
(BR3)
MPB51
(BR20)
MPB70
(BR2)
(f)
MPB51
(BR20)
MPB70
(BR2)
3
pI
10

infection/sensitization sera was that the sensitized cattle appeared to respond with greater intensity and to additional SF-PPD antigens than did the infected cattle sera of the same time-point (Figure 16: c, d). Visual observations of the seven week post-infection/ sensitization 2D western blots indicated that a larger percentage of the sensitized cattle demonstrated antibody responses to MPB70, MPB64 and MPB64/MPB83 protein spots than did the *M. bovis* infected cattle (Table 2). An overall, antibody boosting effect was observed in the post-CITST time-point 2D Western blots of both the sensitized and infected cattle. While the antibody response to SF-PPD protein spots at this time-point varied considerably within the sensitized and infected cattle groups, the sensitized cattle appeared to respond more intensely to SF-PPD protein spots MPB64 and MPB64/MPB83 than did the *M. bovis* infected cattle (Figure 16, Table 2).

The intensity of the antibody response to four SF-PPD protein spots were also analysed by PDQuest 2D Analysis Software (Bio-Rad). Figure 17 shows the fold change in spot pixel quantity above the pre-infection/sensitization time-points for each of the four SF-PPD protein spots at the seven week post infection/sensitization and post CITST time-points (Figure 17). While the graphs representing the SF-PPD protein spots MPB64 and MPB64/MPB83 indicate a perceived greater antibody response to the *M. bovis* sensitized cattle sera as opposed to the infected sera, statistical analysis of the data revealed no significant differences.

4.6 *Bovine CITST of SF-PPD, HK-PPD and CNS M. bovis PPD tuberculins.*

Although SF-PPD was shown to elicit a slightly greater DTH response in *M. bovis* sensitized guinea pigs, its antigenicity in *M. bovis* sensitized cattle remained to be

Table 2. Qualitative analysis of antibody response to SF-PPD antigens on 2D Western blots.

Antibody response to specific SF-PPD antigens was assessed on 2D Western blots of *M. bovis* infected/sensitized cattle sera. Location of spots is depicted in Figure 24e.

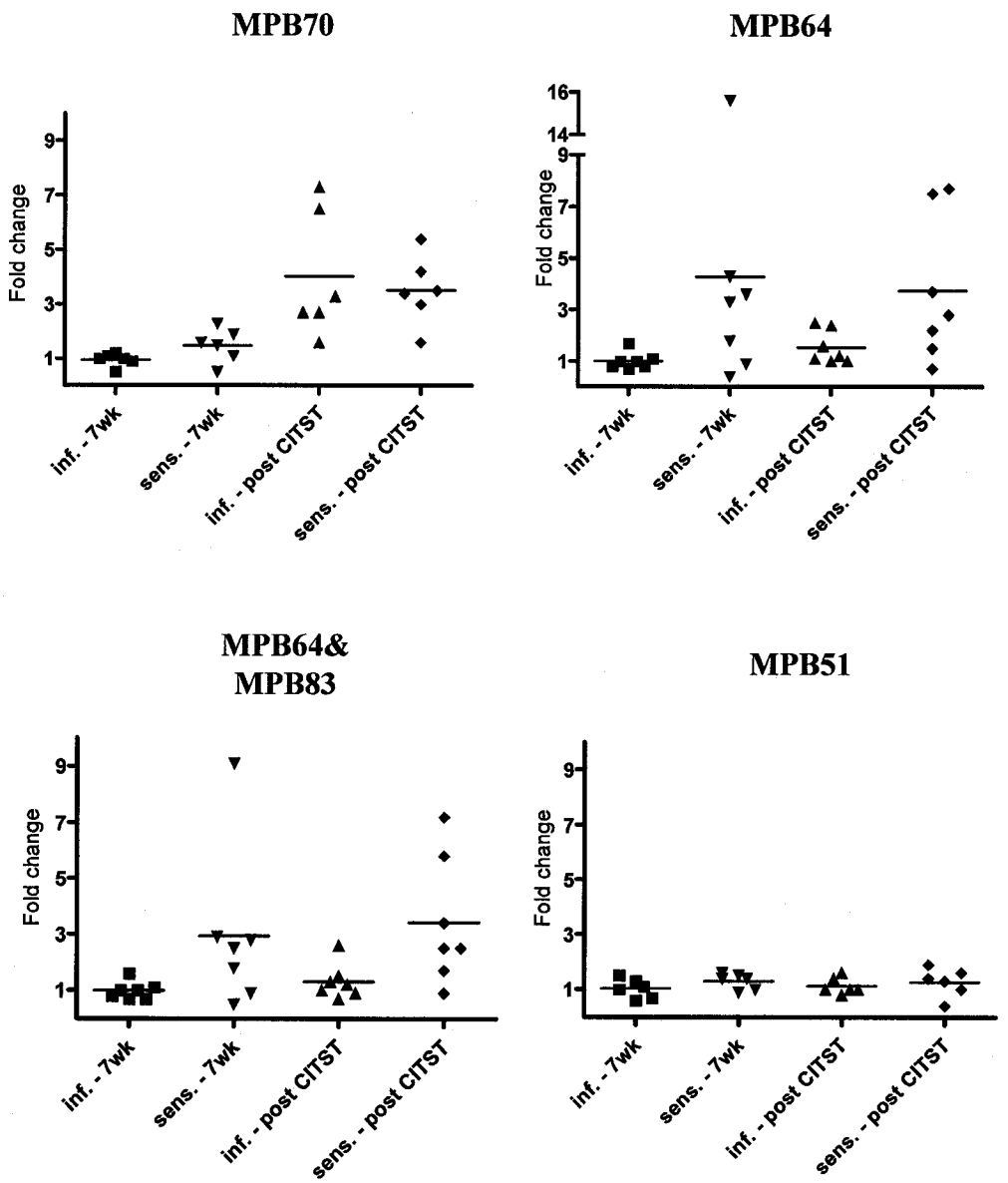
Antigen recognition in sequential Western blot analysis of sera from *M. bovis* infected and *M. bovis* sensitized cattle.

Antigen	Animal group*	Pre- inf./sens.	7 wks post inf./sens.	3 wks post CITST
MPB70 (BR2)	*M. bovis* infected (*n* = 6)	2	3	6
	M. bovis sensitized (n = 6)	3	6	6
MPB64 (BR3)	*M. bovis* infected (*n* = 6)	1	2	4
	M. bovis sensitized (n = 6)	0	5	6
MPB64&MPB83 (BR4)	*M. bovis* infected (*n* = 6)	1	1	3
	M. bovis sensitized (n = 6)	2	4	5
MPT51 (BR20)	*M. bovis* infected (*n* = 6)	6	6	6
	M. bovis sensitized (n = 6)	6	6	6

* Sera obtained from cattle described in Figure 1.

Figure 17. Antibody response to four SF-PPD antigens in *M. bovis* sensitized *versus* *M. bovis* infected cattle.

Data points in each graph represent the 2D Western blot spot pixel quantity fold change for the following SF-PPD antigenic spots: MPB70, MPB64, MPB64&MPB83 and MPB51. The 7 wk post infection (inf.) / sensitization (sens.) and the post CITST spot quantity values were thus divided by the pre-infection/sensitization spot quantity values recorded for each animal. A standard template comparing seven areas was defined from the first blot and was applied to each subsequent blot (Appendix, Figure1). Spot parameters were positioned on PDQuest (Bio-Rad) images of each 2D western according to the Ag85 complex proteins which acted as landmark spots and by comparison to positive control 2D Westerns and 2DE gels of SF-PPD.


MPB70
MPB64
MPB64&
MPB83
MPB51
Fold change
inf. - 7wk
sens. - 7wk
inf. - post CITST
sens. - post CITST

characterized. Therefore, a CITST was used to measure the delayed-type hypersensitivity response of *M. bovis* sensitized cattle to HK-PPD, SF-PPD and 100-02-2CNS *M. bovis* PPD tuberculins. The sum of the average skin reaction measurements for 1.0 and 0.2 mg/mL SF-PPD was 73.8 % that of the 100-02-2CNS ($P < 0.001$) and 78.6% of HK-PPD ($P < 0.05$) at 72 hpi (Table 3). In comparison, the sum of the average skin reaction measurements for HK-PPD was 93.8 % that of the 100-02-2CNS and they were found not to be significantly different. Skin reactions elicited by both dilutions of SF-PPD also indicated a parallel trend of reduced antigenicity as compared to the skin reactions evoked by either 100-02-2CNS or HK-PPD tuberculins (Figure 18). As well, the 1.0 mg/mL dilution of SF-PPD consistently elicited significantly smaller skin reactions than the 1.0 mg/mL dilution of 100-02-2CNS at 24, 48 and 72 hpi measurements ($P < 0.05$) (Figure 19). Furthermore, a direct comparison of the 1.0 mg/mL skin responses of individual animals demonstrated the consistency of skin reaction sizes with respect to the three PPD tuberculins as the majority of cattle elicited greater DTH responses to either HK-PPD or 100-02-2CNS than in response to SF-PPD (Figure 20). *M. avium* PPD tuberculin was found to elicit consistently smaller average skin reactions than did the 100-02-2CNS at 24 ($P < 0.05$), 48 ($P < 0.001$) and 72 hpi ($P < 0.001$). HK-PPD and SF-PPD were also shown to elicit larger average skin reactions than did *M. avium* tuberculin at 24 ($P < 0.05$) and 48 hpi ($P < 0.05$) (Figure 19).

One steer failed to produce DTH reactions to any of the tuberculins at any time-point. As the pre-sensitization and post-skin test sera from this animal were found to display a similar antibody profile, it was suspected that an error occurred and this animal

Table 3. Antigenicity of SF-PPD *versus* HK-PPD and 100-02-2CNS in *M. bovis* sensitized cattle.

The antigenicity of SF-PPD, HK-PPD and 100-02-2CNS *M. bovis* PPD tuberuclins was compared using a CITST in which 11 steers were sensitized with an injection (IM) of heat killed *M. bovis* cells. The pre-tuberculin injection measurements were subtracted from the 24, 48 and 72 hpi measurements to determine skin reaction size. Statistics were performed using Prism GraphPad software. a: One-way ANOVA with Bonferroni's Multiple Comparison; b: Paired t-test.

Average 72 hpi skin reaction measurements (mm)

Dilution (mg/mL)	100-02-2CNS		HK-PPD				SF-PPD					
	Skin reaction less 0 hpi	(Std. Dev.)	Skin reaction less 0 hpi	(Std. Dev.)	% of CNS	P value	Skin reaction less 0 hpi	(Std. Dev.)	% of CNS	P value	% of HK-PPD	P value
1.0	11.12	(5.25)	10.02	(4.06)	90.1%	$P > 0.05^a$	8.35	(2.85)	75.1%	$P < 0.05^a$	83.3%	$P > 0.05^a$
0.2	7.57	(4.14)	7.52	(3.57)	99.3%	$P > 0.05^a$	5.44	(2.60)	71.9%	$P > 0.05^a$	72.3%	$P > 0.05^a$
Sum of average areas	18.69		17.54		93.8%	$P > 0.05^b$	13.79		73.8%	$P < 0.001^b$	78.6%	$P < 0.05^b$

Figure 18. Average bovine DTH response to SF-PPD dilutions *versus* those of HK-PPD and 100-02-2CNS.

Data points depict the average skin reaction measurements for *M. bovis* PPD tuberculins (100-02-2CNS, 100-06-1R, SF-PPD) at 1.0 and 0.2 mg/mL in 11 steers sensitized with an injection (IM) of heat killed *M. bovis* cells.

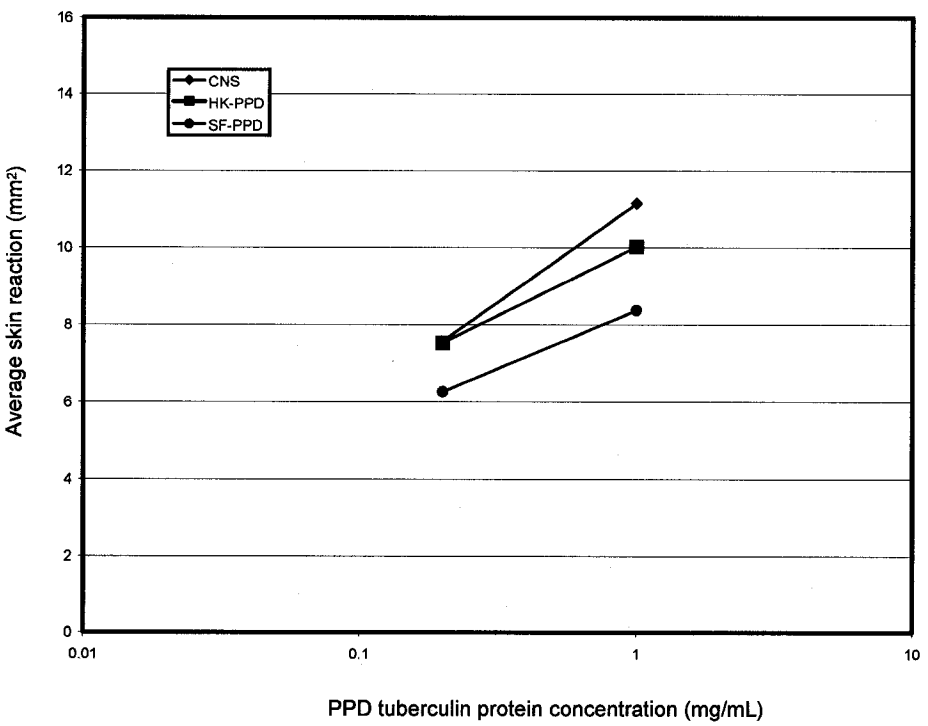
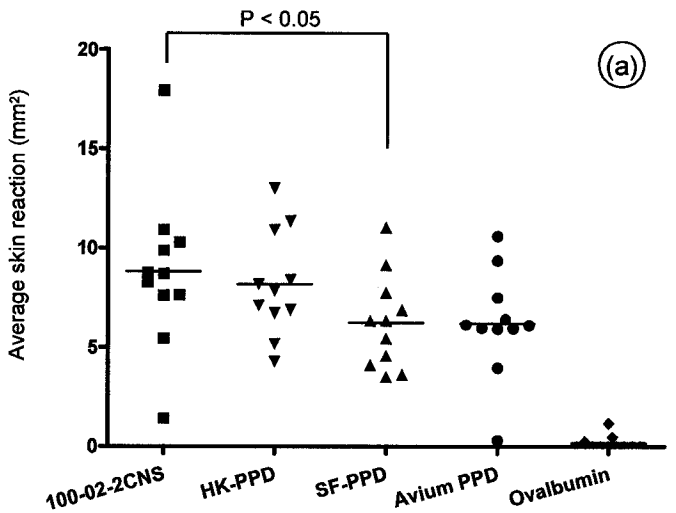
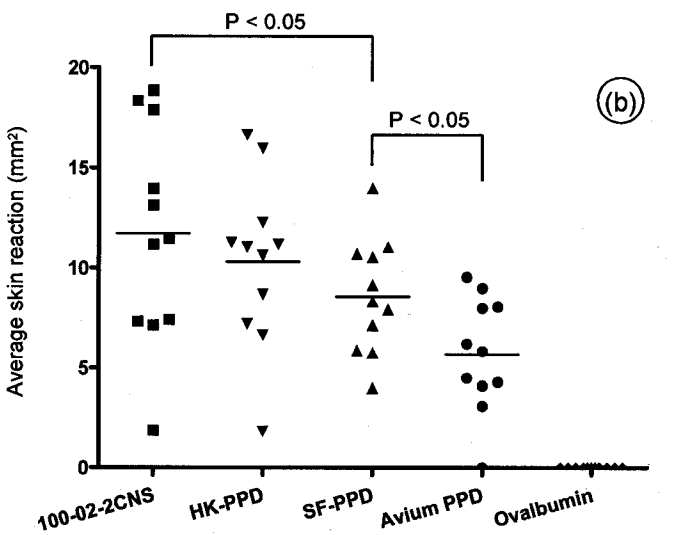

16
14
12
10
8
6
4
2
0
CNS
HK-PPD
SF-PPD
Average skin reaction (mm²)
0.01
0.1
1
10
PPD tuberculin protein concentration (mg/mL)

Figure 19. DTH response to SF-PPD, HK-PPD and 100-02-2CNS in *M. bovis* sensitized cattle at 24, 48 and 72 hpi.

Data points depict bovine skin reaction measurements to intradermal injections of 1.0 mg/mL *M. bovis* PPD tuberculins (100-02-2CNS, HK-PPD, SF-PPD), 0.5 mg/mL *M. avium* PPD tuberculin and 1.0 mg/mL ovalbumin in 11 *M. bovis* sensitized cattle at 24 (a), 48 (b) and 72(c) hpi. Statistics were performed using a one-way ANOVA with Bonferroni's Multiple Comparison, Prism GraphPad.


P < 0.05
(a)
Average skin reaction (mm²)
20
15
10
5
0
100-02-2CNS
HK-PPD
SF-PPD
Avium PPD
Ovalbumin


P < 0.05
P < 0.05
(b)
Average skin reaction (mm²)
20
15
10
5
0
100-02-2CNS
HK-PPD
SF-PPD
Avium PPD
Ovalbumin

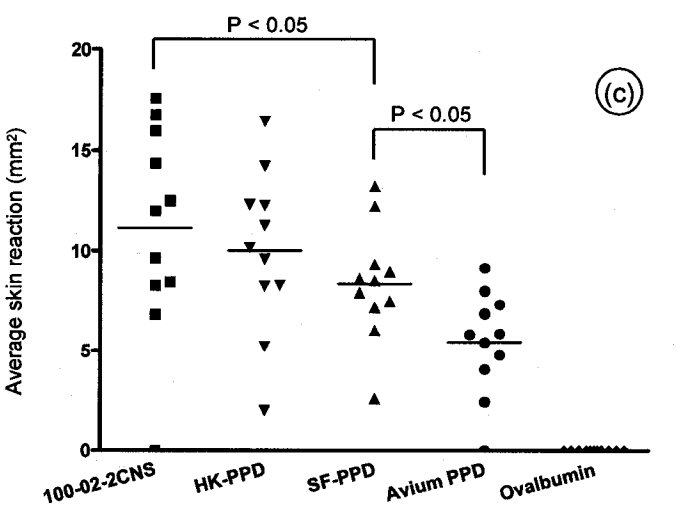
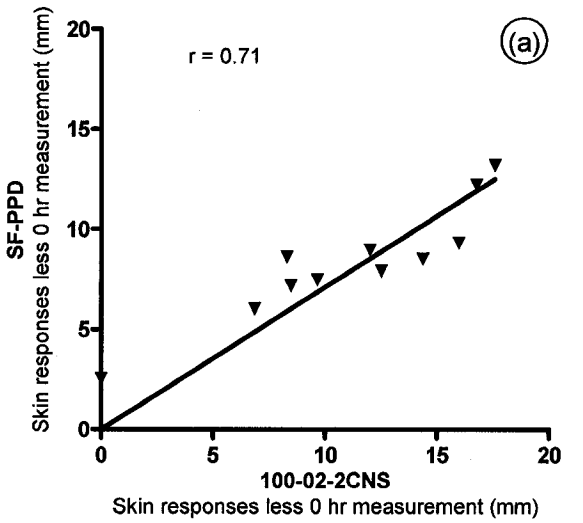
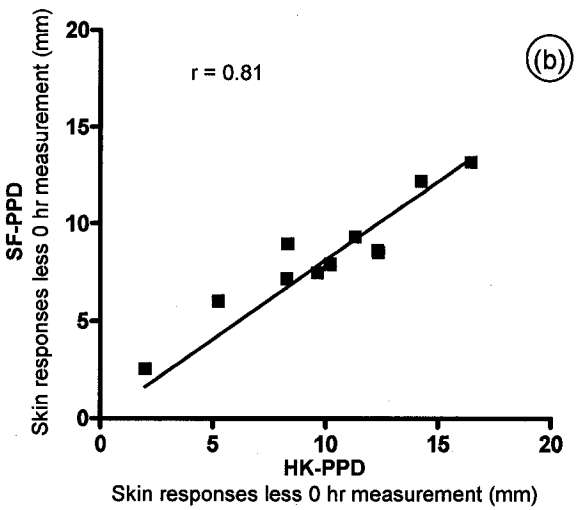
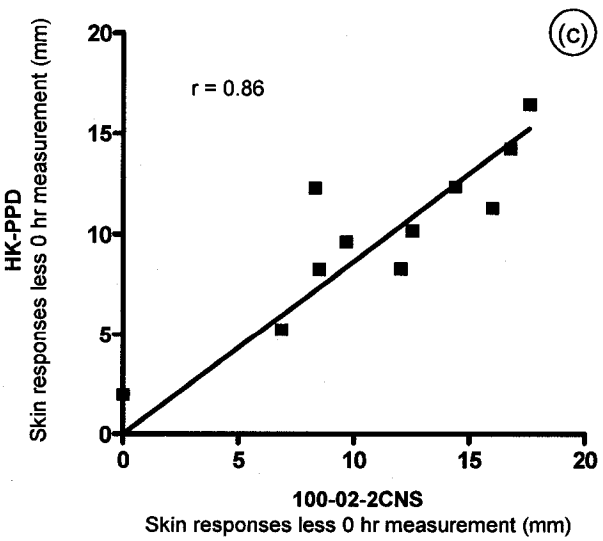

P < 0.05
P < 0.05
(c)
Average skin reaction (mm²)
20
15
10
5
0
100-02-2CNS
HK-PPD
SF-PPD
Avium PPD
Ovalbumin

Figure 20. Comparative analysis of the DTH response to SF-PPD, HK-PPD and 100-02-2CNS in individual *M. bovis* sensitized cattle.

Each data point compares the DTH measurements of two different *M. bovis* PPD tuberculins at 72 hpi. All injections consisted of 1.0 mg/mL of the respective *M. bovis* PPD tuberculins. (a) Compares SF-PPD (Y axis) to HK-PPD (X axis). (b) Compares SF-PPD (Y axis) to 100-02-2CNS (X axis). Linear regression performed using Prism GraphPad software.


(a)
r = 0.71
SF-PPD
Skin responses less 0 hr measurement (mm)
0
5
10
15
20
100-02-2CNS
Skin responses less 0 hr measurement (mm)


(b)
r = 0.81
SF-PPD
Skin responses less 0 hr measurement (mm)
0
5
10
15
20
HK-PPD
Skin responses less 0 hr measurement (mm)


(c)
r = 0.86
HK-PPD
Skin responses less 0 hr measurement (mm)
0
5
10
15
20
100-02-2CNS
Skin responses less 0 hr measurement (mm)

did not receive the 5 mg of heat killed *M. bovis* cells as was planned and it was therefore omitted from this study (Appendix Figure 8).

5 Discussion and Conclusions

The primary purpose of this thesis was to characterize the immunological differences between *M. bovis* infected and *M. bovis* sensitized cattle as they related to *M. bovis* CFP antigens. While previous studies from our laboratory indicated that, on average, *M. bovis* sensitized cattle elicited greater skin responses to the intradermal application of tuberculin than did *M. bovis* infected cattle (unpublished results), a comparison of the antibody responses to *M. bovis* CFPs in these two animal models has not been described.

5.1 *PAGE Separation of SF-PPD and HK-PPD.*

The first objective was to identify a suitable preparation of *M. bovis* CFPs which could be electrophoretically separated and be used in Western blot analysis to compare the antibody response of *M. bovis* sensitized *versus* *M. bovis* infected cattle. Although it is now recognised that the 2-4 week old, non-heated *M. bovis* culture filtrate is composed of more than 800 CFPs (95), the presence and antigenicity of these proteins in *M. bovis* PPD tuberculin remains largely unknown. In our study heat killed *M. bovis* PPD tuberculin (HK-PPD) was used as the source of the *M. bovis* CFPs, however this proved to be problematic as the HK-PPD did not resolve well on electrophoresis. Subsequent PAGE and 2DE analyses of sterile filtered *M. bovis* PPD tuberculin (SF-PPD) demonstrated improved separation of *M. bovis* CFP components as compared to HK-PPD.

Previous attempts to separate the active components of tuberculin by a variety of fractionation (3, 12, 21, 75, 134) and electrophoretic techniques (48, 51) have led to equivocal results and difficulty in interpretation, most likely due to the current realization

that heat killing during tuberculin production leads to profound structural and possibly other un-characterized changes to the mycobacterial CFPs. In this study, the effect of autoclaving on mycobacterial CFPs was readily evidenced by our observation that there was a three-fold increase in the number of spots enumerated in 2DE gels of the non-heated SF-PPD (Figure 4 *versus* Figure 6). Furthermore, the differences observed between gels of SF-PPD and HK-PPD did not appear to be restricted to protein denaturation as both SF-PPD and HK-PPD proteins were denatured to their primary structure by the SDS-PAGE protocol which uses a combination of heat, SDS and 2ME treatment prior to electrophoresis. Therefore it is the combination of heat and pressure encountered in the autoclave process that were likely responsible for the creation of the HK-PPD tuberculo-protein peptide fragments which appeared as streaks or smears on PAGE or 2DE gels. The interpretation of electrophoretic studies of autoclaved mycobacterial CFPs is likely further complicated by the presence of the less structurally stable proteins which, when reduced to peptide fragments, may obscure the visualisation of small quantities of other whole proteins.

In this study, the proteins which appeared to have the greatest resilience to the effects of autoclaving was the MPB70 and MPB83 protein group. This result was in accordance with previous studies involving autoclaved *M. bovis* culture filtrate (51), where the heat stability of the distinct but highly homologous MPB70 and MPB83 proteins was attributed to the presence of identical 133aa disulfide loops and the formation of stable aggregates in the culture fluid (57, 171). The detection of MPB32 in HK-PPD was especially interesting as this protein was described by Nagai *et al.* in 1991

as the most heat labile protein as compared to MPB64 and the Ag85 complex proteins (102). The presence of MPB32 in HK-PPD thus implies that small amounts of several other *M. bovis* CFPs may have survived the autoclave process to persist in HK-PPD and that field issue *M. bovis* PPD tuberculin may contain a greater number of whole mycobacterial proteins than was previously believed (3, 12, 51).

2D gels of HK-PPD also displayed protein spots which were not observed in 2DE of SF-PPD (Figure 6). These HK-PPD spots were located between 10 - 15 kDa and possessed approximate pIs of 6 - 8. Due to the low molecular weight of these proteins and the failure to detect these spots in 2DE SF-PPD, I suspected that these spots actually represented amalgamations of fragmented CFPs which were reduced to peptide fragments by the autoclaving process.

The majority of approximately 200 distinct protein spots observed on 2DE gels of SF-PPD were easily visualized and clearly delineated as compared to the smeared presentation of HK-PPD. Although this number falls well short of the 800 plus protein spots reported by Mattow et al. (2003) to exist in *M. bovis* culture filtrate (94), the MASS-SPEC results from our study indicated that each protein spot actually contained a mixture of multiple *M. bovis* CFPs (Appendix, Table 1). Future studies incorporating a more stringent 2DE separation of SF-PPD with the utilization of narrow pI ranges and the use of a MASS-SPEC compatible silver stain may permit improved precision in the annotation of SF-PPD proteins in 2DE gels.

One dimensional Western blots of *M. bovis* sensitized sera onto HK-PPD revealed that the majority of antibody binding to HK-PPD was largely restricted to a 22 kDa band

however, when the same sera was blotted to SF-PPD an additional 25 distinct bands were visualized. The 22 kDa band visualized in blots of HK-PPD has previously been interpreted to indicate reactivity to both MPB70 and MPB83, however, the two additional weak bands observed (apparent weights of 40 and 66 kDa) (Figure 8) may also have consisted of MPB70 and MPB83 as these proteins have previously been shown to present as dimers and trimers in Western blots of *M. bovis* CFPs (57, 171). The streaked appearance of HK-PPD Western blots was likely due to the HK-PPD peptide fragments which are suspected to have been recognized by antibodies in the *M. bovis* sensitized/infected sera. As with PAGE gels of HK-PPD, the recognition of HK-PPD peptide fragments possibly masked many of the weaker antibody responses to small quantities of whole HK-PPD proteins. Therefore, although the antibodies in *M. bovis* sensitized sera may have recognised peptide fragments originating from a multitude of HK-PPD antigens, I could not distinguish the parent proteins from whcih these *M. bovis* peptide fragments originated.

Therefore, due to the improved resolution of *M. bovis* CFPs in SF-PPD and the ability to see more spots as compared to gels of HK-PPD, and because the *M. bovis* sensitized cattle sera recognized discrete SF-PPD antigens in Western blots, I decided to use SF-PPD in the 2D Western blot comparison of *M. bovis* sensitized *versus* *M. bovis* infected cattle.

5.2 *SF-PPD DTH responses in M. bovis sensitized guinea pigs and cattle.*

Since the introduction of the tuberculin skin test (TST) by Dr. Koch in 1890 (78), tuberculin has been produced from heat-sterilized *M. bovis* culture filtrate and it remains

the principal ante-mortem diagnostic test for bovine tuberculosis worldwide. Although control programs based on test and slaughter principals have all but eradicated bovine tuberculosis from many developed countries, many of these same countries harbour a lingering, low prevalence of the disease. Apart from the transmission of *M. bovis* from either wildlife or human reservoirs to cattle (68, 97, 104, 115, 126) the low sensitivity and specificity of the TST (approximately 85%) can result in false negative results which also complicates the eradication of bovine tuberculosis (125).

The specificity of the TST is believed to be adversely affected in part by the existence of shared antigens between *M. bovis* and environmental mycobacteria (51, 88), while the sensitivity of the TST has been suggested to be in some way affected by the autoclaving of the *M. bovis* culture supernatant during production thus causing denaturation of the majority of the *M. bovis* CFPs (41, 51). In comparison, laboratory based γINF diagnostic tests which use specific, recombinant MTC antigens apply the antigens in their native conformation (63, 81, 87, 157). Consequently, the native conformation of the *M. bovis* CFPs may partially account for performance differences between these two tests as cocktails of non-heated recombinant MTC antigens have been shown to successfully elicit DTH responses in *M. bovis* sensitized or infected animals (148, 174). I therefore hypothesized that the native conformation of the SF-PPD antigens would be capable of eliciting a more intense DTH response in animals sensitized with heat killed *M. bovis* cells as compared to HK-PPD.

In agreement with my hypothesis, CITST of SF-PPD *versus* HK-PPD in *M. bovis* sensitized guinea pigs had a 1.3 fold greater sum of average areas for the DTH responses

to SF-PPD as compared to that of HK-PPD. However, the average skin thickness response for SF-PPD in a CITST using *M. bovis* sensitized cattle was only 78% that of HK-PPD and only 74 % that of 100-02-2CNS. The disparity of the relative potency of SF-PPD as compared to that of HK-PPD between the animal models may be explained in part by the differences between the bovine and guinea pig CITST procedures and also because of the inherently different immunological responses which occur in guinea pig *versus* bovine models.

Both the *M. bovis* sensitized guinea pig and bovine animal models are prescribed by the OIE as a means to evaluate the DTH response of a tuberculin against a reference standard PPD tuberculin (173). As well, accommodations to the test protocol are prescribed by the OIE according to the animal species in which it is applied. For example, guinea pigs were skin tested five weeks post sensitization with heat killed *M. bovis* cells while the CITST was performed on cattle at eight weeks post sensitization. Also, guinea pigs were skin tested with 5.0, 1.0 and 0.2 μg of tuberculin while cattle received 100 and 20 μg. Although the majority of these procedural differences presumably exist for the optimal CITST performance in the respective animal models, the respective strains of heat killed *M. bovis* used to sensitize each animal model and the genetic diversity of the animals used in each model may have influenced the respective skin responses to SF-PPD in my study.

Cattle received sensitin prepared from a heat killed culture of a virulent field isolate of *M. bovis* while the guinea pigs received sensitin prepared from heat killed *M. bovis* AN5 cells; the lab-adapted strain used for production of both SF-PPD and HK-PPD.

Therefore the cattle and guinea pigs responded to *M. bovis* sensitins which invariably differed in antigenic type and concentration.

Another difference between the guinea pig and bovine CITSTs was that the guinea pigs were obtained directly from a research facility whereas the cattle were obtained from local farms. While the importance of similar age and genetic make-up of the animals used in a CITST has been reported by Landi in 1975 (77), cattle have been shown to differ considerably in their ability to mount both cell mediated and humoral immune responses based on genetic determinants (59, 60). Therefore, the uniformity of the age and genetic make-up of the guinea pigs as compared to the cattle used in our CITSTs may be partially responsible for the lower standard deviations in the guinea pig CITST results as compared to those of the bovine CITST (Figure 19c *versus* Figure 10).

Another difference between the *M. bovis* sensitized guinea pig and cattle models was that the cattle were housed outside in a large animal facility which was freely accessible to birds and other potential sources of environmental mycobacteria, while the guinea pigs were maintained in isolation and were ventilated with filtered air. In this study and in other previous studies, cattle have demonstrated antibodies to *M. bovis* proteins prior to either artificial sensitization with heat killed *M. bovis* cells or experimental infection with *M. bovis* (6, 100). Therefore, if cattle have had previous exposure to environmental mycobacteria, it is possible that they may have developed an antibody titre to conformational and/or discontinuous epitopes of the environmental mycobacteria which could cross-react with native *M. bovis* CFPs. A high titre of antibodies specific for native SF-PPD epitopes may therefore have resulted in a greater

number of the SF-PPD antigens being targeted by antibodies in Arthus-type hypersensitivity reactions and being cleared from the reaction site by neutrophils as compared to either HK-PPD or 100-02-2CNS. As the guinea pigs were less likely to encounter environmental mycobacteria while maintained in isolation with a filtered air supply, they may not have possessed a pre-existing titre of antibodies specific for mycobacterial antigens. Therefore, relatively less SF-PPD antigen may have been cleared by Arthus - type reactions in the *M. bovis* sensitized guinea pigs as compared to the *M. bovis* sensitized cattle. To further evaluate this finding a Western blot analysis of pre-sensitized guinea pig sera with *M. bovis* CFPs could be performed or the skin test reactions of the *M. bovis* sensitized guinea pigs or cattle could be histologically examined for the presence of IgG immune complexes.

Inherent immunological differences between the *M. bovis* sensitized guinea pigs *versus* the *M. bovis* sensitized cattle may have also attributed to the disparity of the relative potency of SF-PPD as compared to that of HK-PPD between the animal models. It is well documented that CITSTs which employ *M. bovis* sensitized guinea pigs offer, at best, a rough approximation of tuberculin test performance for on-farm diagnosis of bovine tuberculosis in the field (11, 26, 50, 77). For this reason, the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals describes this method of tuberculin potency testing as a less reliable alternative as compared to live infection trials in either cattle or guinea pigs. It also states that the use of the sensitized guinea pig model should be limited to laboratories which do not have the required isolation facilities for housing live infected animals (173). There are only a few scientific studies published which

validate the use of *M. bovis* sensitized cattle in a CITST, however Radunz et al. (1985) reported that *M. bovis* sensitized cattle elicited DTH responses to *M. bovis* PPD tuberculin which were of a similar skin thickness to those provoked by *M. bovis* PPD tuberculin in tuberculous cattle (80, 114).

Disparity in bovine DTH responses between HK-PPD *versus* SF-PPD may have also been due to the fact that the sensitin contained *M.bovis* CFPs which had been denatured by autoclaving. The *M. bovis* sensitized cattle may therefore have been more responsive to the denatured conformation and/or peptide fragments of a *M. bovis* CFP found in HK-PPD as opposed to native conformation of the same protein which is likely present in SF-PPD. Therefore, *M. bovis* infected cattle, which would principally encounter native *M. bovis* proteins, might respond more intensely to SF-PPD as compared to *M. bovis* sensitized cattle. This hypothesis was in accordance with the findings of Trivedi *et al.* in 2006 who reported that non-heated *M. bovis* CFPs from three-week-old *M. bovis* cultures, provoked DTH responses in infected cattle which were similar in magnitude to those elicited by *M. bovis* PPD tuberculin (148). The reduction of *M. bovis* CFPs to peptide fragments by autoclaving may have also resulted in a greater total number of antigenic moieties per milligram of protein in HK-PPD and 100-02-2CNS as compared to SF-PPD. The greater total number of potential exposed epitopes in HK-PPD and 100-02-2CNS may therefore have increased the opportunity for effector T cell - Langerhan cell contact and thereby provoked a greater change in skin thickness as compared to SF-PPD. Furthermore, the peptide fragments present in the autoclaved tuberculins presumably required less endosomatic degradation prior to binding MHC

class II molecules than did the native SF-PPD proteins. While these theories appear to be contradicted by the *M. bovis* sensitized guinea pig CITST results, which showed that the application of SF-PPD elicited a greater average skin response as compared to HK-PPD, it was difficult to directly compare the results of the bovine and guinea pig CITSTs due to the multitude of differences between the two animal models.

In conclusion, both animal models identified SF-PPD as having a significantly different relative potency from that of HK-PPD, however SF-PPD was shown to provoke a greater skin response in *M. bovis* sensitized guinea pigs and a smaller DTH response in *M. bovis* sensitized cattle as compared to HK-PPD.

5.3 *Sensitization of guinea pigs to M. bovis with SF-PPD.*

CITSTs performed in *M. bovis* infected guinea pigs are believed to more accurately determine the relative potency of a PPD tuberculin for the diagnosis of bovine tuberculosis as compared to the use of *M. bovis* sensitized guinea pigs for this test procedure (11, 26, 50, 77). Therefore, I hypothesised that the native conformation of the *M. bovis* CFPs in SF-PPD would provide a more accurate simulation of the secreted *M. bovis* antigens to which an infected guinea pig would immunologically respond.

While the average 24 hpi skin responses to SF-PPD were approximately equivalent to those seen in guinea pigs sensitized with either heat-killed *M. bovis* cells or SF-PPD, the 48 hpi skin responses in the SF-PPD sensitized guinea pigs were considerably reduced in both area and redness of erythema. The skin response in the SF-PPD sensitized guinea pigs which showed a reduction in readings starting at 24 hpi was not characteristic of the cell mediated DTH response to tuberculin which typically

develops over the first 24 hours and persists for more than 48 hours post tuberculin application (30, 110). The skin responses in the SF-PPD sensitized guinea pigs may be explained by the different antigenic determinants to which the cell-mediated and humoral immune responses typically target. Cell-mediated determinants typically target sequence based epitopes while the humoral determinants characteristically include conformation-based, hydrophobic epitopes localised on the molecule surface (51, 67). Therefore, the SF-PPD sensitized guinea pigs may have produced a higher titre of IgG antibodies specific for conformational and discontinuous epitopes of SF-PPD antigens as compared to the guinea pigs that were sensitized with heat killed cells. The subsequent intradermal application of SF-PPD antigens presumably triggered the formation of immune complexes between the antibodies and SF-PPD antigens which may have resulted in localised Arthus - type hypersensitivity responses that typically develop within 24 hpi. While the existence of a localized Arthus - type skin reaction in response to the application of tuberculin has been contended by other studies, (42, 74) the presence of native *M. bovis* CFPs may have provoked a more intense Arthus - type response than is characteristically observed in response to the application of *M. bovis* PPD tuberculin. Since the majority of HK-PPD antigens are denatured, the IgG molecules specific for conformational epitopes of *M. bovis* CFPs may have recognized fewer HK-PPD antigens. Therefore, relatively lower recognition by the SF-PPD sensitized guinea pigs of the HK-PPD antigens as compared to SF-PPD by may partially explain the lower skin responses to HK-PPD at 24 hpi as compared to SF-PPD in the SF-PPD sensitized guinea pigs.

The minimal development of a DTH response at 48 hpi to SF-PPD in SF-PPD

sensitized guinea pigs is somewhat contradicted by previous studies (41) in which guinea pigs sensitized to native proteins elicited a DTH response in addition to an antibody mediated Arthus-type response following intradermal application of the protein. However, Gell and Benacerraf (1959) also mentioned that the inclusion of complete Freund adjuvant reduced the required amount of protein necessary to provoke the said responses (41). Therefore, although de Bruyn *et al.* (1987) demonstrated that native *M. bovis* protein P64 effectively elicited both a DTH and antibody response, I suspect that the DTH response could have been attributed to the inclusion of incomplete Freund adjuvant in the P64 sensitizing inoculum (27).

The inability to visualize a characteristic DTH response in *M. bovis* sensitized guinea pigs with the application of SF-PPD corresponded to the results shown by bovine tuberculosis vaccination trials in which either *M. bovis* (162, 164) or *M. tuberculosis* (163) CFPs were used. The vaccination trials indicated that in the absence of an effective adjuvant, vaccination with *M. bovis* CFPs elicited a predominantly humoral immune response. Therefore, if an adjuvant had been added to the sensitin containing SF-PPD a stronger DTH response to SF-PPD may have been observed in our *M. bovis* sensitized guinea pigs. In conclusion an injection of SF-PPD alone (without adjuvant) is not sufficient in guinea pigs to elicit a delayed type hypersensitivity to either *M. bovis* PPD tuberculin or to native *M. bovis* CFPs. However, it does appear that an antibody mediated Arthus type response may have occurred but this finding must be further characterized in future experiments.

5.4 *M. bovis antigen recognition in pre-exposure sera.*

The occurrence of antibody recognition to SF-PPD antigen in all twelve pre-infection/sensitization cattle sera was unexpected, especially since all twelve animals tested negative on the pre-screening INF-γ based *in vitro* test for bovine tuberculosis. Although the pre-existing antibody level to SF-PPD antigens varied between animals, all twelve cattle recognized a similar group of SF-PPD proteins which were similarly located on the Western blots to the Ag85 complex proteins as identified by MASS-SPEC analysis in 2DE of SF-PPD. Interestingly, this background antibody level did not appear to be correlated to animal age or to housing facilities. Cattle designated for the infection trial were purchased from a single farm at four months of age and maintained within a Bio-containment Level III facility while cattle designated for the *M. bovis* sensitization trial were purchased from various local farms and maintained to approximately 1.5 years of age in an open air, large animal facility prior to the initiation of the study.

Ag85 complex proteins are not specific to MTC strains but are also known to be expressed by most environmental mycobacteria (35, 170). *M. avium* and *M. bovis* Ag85 complex proteins have also been shown to share 99 % homology at the protein levels (124). Therefore it is not surprising that cross reactive epitopes have been shown between the respective Ag85 complexes (102). Both Mustafa *et al.* (2006) and Amadori *et al.* (1998) accredited a prior exposure to environmental mycobacteria as the basis for low levels of Ag85 complex antibody recognition in *M. bovis* negative cattle (6, 100). Espitia *et al.* (1992) and Al-Attiyah *et al.* (2006) also attributed environmental mycobacteria as a potential determinant for Ag85 complex antibody recognition in the sera of tuberculosis-free humans (5, 34).

Therefore, due to the consistent recognition of all four Ag85 complex members by the pre-infection and pre-sensitization cattle sera, and due to the possibility of exposure of cattle to environmental mycobacteria, I concluded that the recognition of SF-PPD antigens in the pre-sensitized/pre-infected sera was most probably caused by previous exposure to the mycobacterial Ag85 complex proteins and not as a result of epitope mimicry to non-mycobacterial proteins.

5.5 *Western immunoblot comparison of* M. bovis *infected/sensitized cattle sera.*

According to the OIE, comparative tuberculin potency testing against a reference standard in *M. bovis* infected cattle offers the most accurate determination of tuberculin performance (173), however the overall expense and complexity of this animal model limits its practicality. Currently in Canada, potency testing of tuberculin is typically carried out in *M. bovis* sensitized guinea pigs despite the known limitations of this method (9, 11, 21, 26, 29, 48, 50, 58, 102). *M. bovis* sensitized cattle are an alternative animal model which may offer a more realistic determination of the relative antigenicy of a tuberculin however, the comparability of this model *versus M. bovis* infection in cattle has not been fully characterized.

CITST studies performed in both *M. bovis* sensitized and *M. bovis* infected cattle indicated equivalent relative antigenicities between the tuberculins tested, however, on average, the sensitized animals responded with approximately 30% greater skin test thickness measurements (unpublished results). The focus of this portion of the thesis was therefore to further characterize the immune response of *M. bovis* sensitized *versus M. bovis* infected cattle by comparing their respective antibody responses to *M. bovis* CFPs.

Visual interpretation of the Western blot data of sera collected at seven weeks post *M. bovis* infection/sensitization suggested that the *M. bovis* sensitized cattle tended to recognise additional SF-PPD antigens than did the infected cattle. Also, the sensitized cattle sera appeared to possess a higher titre of the allegedly heat labile protein MPB64 as compared to the infected cattle sera. While Nagai *et al.* (1991) also reported antibody recognition of MPB64 in animals sensitized with heat killed *M. bovis* cells, it was Haslov *et al.* (1991) who suggested that the recognition of MPB64 by animals sensitized with heat killed *M. bovis* cells may be attributed to the presence of *M. bovis* CFPs in the sensitin (58, 102). As the heat killed *M. bovis* cells used to prepare the sensitin in this study were not separated from the culture fluid in which they were suspended, the *M. bovis* sensitized cattle were likely sensitized to *M. bovis* with a combination of somatic and culture filtrate proteins. The relatively weak response to SF-PPD antigens in sera taken at seven weeks post infection was also in accordance with other previous *M. bovis* infection studies in which antibody response to *M. bovis* CFP antigens was initially detected between 7 -10 weeks post infection (53, 160). Therefore I concluded that the sensitized cattle possessed a higher titre of SF-PPD specific antibodies than did the infected cattle because more *M. bovis* CFPs were available for presentation to the humoral immune system of the *M. bovis* sensitized cattle as compared to the *M. bovis* infected cattle within first two months post infection/sensitization.

There are many parameters which could be considered when trying to explain or interpret the immune response of *M. bovis* infected *versus* sensitized cattle. For example, infected cattle would have encountered *M. bovis* CFPs associated with live growing

bacteria, predominately in a native conformation, and therefore may have produced antibodies to conformational and/or discontinuous epitopes of the antigens. Cattle sensitized with heat killed cells however, would presumably have chiefly encountered denatured and/or peptide fragments of CFP antigens. As the SF-PPD proteins would have been denatured by the 2DE process, the 2D Western blot analysis was presumably restricted to antibodies which were directed to linear epitopes. Therefore, the weaker response to SF-PPD antigens by the infected cattle as compared to the sensitized cattle may be because the infected cattle had a lower antibody titre to the linear epitopes of *M. bovis* CFPs.

Application of the CITST resulted in a dramatic boosting of the antibody response to SF-PPD in both the *M. bovis* infected and *M. bovis* sensitized cattle. While several other studies have indicated a similar boosting effect caused by tuberculin for both the humoral (53, 89, 107, 146, 161) and cellular (89, 107, 168) immune responses of *M. bovis* infected cattle, the precise mechanisms by which this occurs remains to be determined (152). Harboe *et al.* in 1990, originally hypothesized that the marked increase in antibody response following skin testing in cattle was principally due to the presence of native MPB70 in *M. bovis* PPD tuberculin (53). Harboe *et al.* (1990) further postulated that a similar antibody boosting effect following a TST was typically not observed in human tuberculosis patients due to the minimal amount of MPB70 present in *M. tuberculosis* PPD tuberculin (53). This is somewhat contradictory to the results presented in this thesis which have indicated that a general increase in antibody response to many of the antigenic SF-PPD proteins did occur. The disparity of results between Harboe *et al.* (1990) and the

findings in this study may be explained, in part, by the different methods used to analyse the sera. Our use of 2D Western blots presumably provided an increased separation and sensitivity of the *M. bovis* CFPs as compared to the one dimensional Westerns performed by Harboe et al. (1990). The increased separation of *M. bovis* CFPs may have therefore permitted a more precise analysis of the antigen recognition by serum antibodies.

Our study observations also concur with the results of a *M. bovis* infection study by Lyashchenko *et al.* (2004) which indicated that the application of a TST induced an antibody boost to several other *M. bovis* CFPs proteins including MPB64 (89). Therefore, the boosting effect may not be restricted to the existence of native mycobacterial antigens in tuberculin but may also be attributed to the effect of denatured or peptide fragments of *M. bovis* CFPs which were present in the *M. bovis* PPD. While the mechanism of this boosting effect remains to be completely characterized, it is likely that peripheral B-cells are stimulated to produce antibodies to predominantly linear epitopes of the soluble PPD tuberculin proteins following cognate interactions with T-cells.

Application of the CITST did not appear to boost antibody responses to the SF-PPD proteins of molecular weights less than 20 kDa in either the *M. bovis* infected or sensitized cattle. This finding was in contradiction to findings by other researchers who showed that both ESAT-6 and CFP10 (which are reported to resolve at approximately 8 and 12 kDa respectively in 2DE analyses of mycobacterial CFPs (123)), have previously been shown to elicit an antibody boosting effect in *M. bovis* infected cattle following skin testing (89, 107, 160). Since ESAT-6 is known to be secreted in the early stages of *M. bovis* infection (17, 38, 55, 66), it is highly likely that at least the *M. bovis* infected cattle

would have elicited an antibody response to ESAT-6 and /or CFP10, however, this was not detected by the Western blot analyses performed in our study.

An explanation for the undetectable antibody response to either ESAT-6 or CFP10 may be that only very low ESAT-6 and CFP10 concentrations were present in the SF-PPD. The use of a very sensitive technique such as multi-antigen print immunoassay (MAPIA) which employs recombinant mycobacterial proteins may be required to detect serum antibodies to ESAT-6 and CFP-10 as has been used previously (89, 107, 161). In our study, however, the concentrations of ESAT-6 and CFP10 in SF-PPD were unknown and also the dialysis of SF-PPD at 10 kDa may have resulted in the additional loss of these two proteins from the culture filtrate. Therefore, while the presence and quantity of both ESAT-6 and CFP10 in SF-PPD may be determined by future studies using monoclonal antibodies specific for the respective proteins or another more sensitive method, the existence of an antibody response to either ESAT-6 or CFP10 from *M. bovis* infected/sensitized cattle can not be concluded from this study.

Although several trends were observed from this preliminary comparison of the *M. bovis* infected *versus* the *M. bovis* sensitized bovine antibody response to *M. bovis* CFPs, there were a number of limitations to the interpretation of these studies. Time and financial constraints restricted the 2D Western blot analysis to only 6 infected and 6 sensitized cattle and as such, repeat blots were not done. Furthermore, although the 2D Western blot analysis showed improved separation of the *M. bovis* CFPs, over SDS-PAGE, it is likely that another more accurate separation method could be used since subsequent MASS-SPEC analysis indicated the co-existence of multiple proteins per 2DE

spot of SF-PPD. Therefore, this work represents an approximate characterization of the relative bovine antibody response to either *M. bovis* infection or sensitization and as such, these preliminary findings are primarily intended to direct the focus of subsequent work.

Results of the present study have identified several methods which may be used to further characterize the immunological differences between *M. bovis* sensitized and infected cattle. Increased specificity of the 2D Western blots with the utilization of narrow pI ranges and the optimization of acrylamide concentrations would improve the separation of SF-PPD proteins and permit a more accurate 2D Western blot analysis of the cattle sera. Furthermore, the completion of the 2D Western blot analysis of the sera from the remaining six cattle of each of the *M. bovis* sensitized and infected studies may increase the statistical significance of the data. As well, recombinant *M. bovis* CFP antigens may also be used in ELISA or MAPIA assays as alternative methods to compare the antibody responses of *M. bovis* sensitized/infected cattle to specific proteins.

6 Summary

M. bovis PPD tuberculin has been used for more than a century to effectively control one of the most devastating bacterial diseases of all times however the actual antigenic constituents of *M. bovis* PPD tuberculin and the immunological events initiated by it's use have yet to be fully understood. The main purpose of this thesis was to characterize the antigenic constituents of *M. bovis* CFPs and to compare the antibody response of *M. bovis* infected cattle to that of cattle artificially sensitized to *M. bovis* by 2D Western blot analysis. The immunological differences observed between *M. bovis* infected and *M. bovis* sensitized cattle may provide a basis for the development of an *M. bovis* sensitization method which will be consistent with the immune response of an experimentally *M. bovis* infected animal. The development of an accurate, non-infectious bovine tuberculosis model would reduce the complexity and bio-containment risks associated with live *M. bovis* studies.

The principal conclusions generated from this thesis are as follows.

1. SF-PPD is superior to HK-PPD tuberculin for the resolution of *M. bovis* protein components by electrophoresis and therefore, SF-PPD is a more appropriate *M. bovis* CFP preparation to use for the antibody analysis of *M. bovis* infected/sensitized cattle sera.
2. Native *M. bovis* CFPs provoke a greater DTH response in *M. bovis* sensitized guinea pigs and a smaller DTH response in *M. bovis* sensitized cattle as compared to *M. bovis* PPD tuberculin.
3. An intramuscular injection of native *M. bovis* CFPs (without adjuvant) is not

sufficient to elicit delayed type hypersensitivity to either *M. bovis* PPD tuberculin or to native *M. bovis* CFPs.

4. The intradermal application of *M. bovis* PPD tuberculin in both *M. bovis* sensitized and infected cattle has an antibody boosting effect which varies considerably between cattle regardless of the infection or sensitization group from which the animal originated.

5. *M. bovis* sensitized cattle generate a more rapid antibody response to additional *M. bovis* antigens as compared to *M. bovis* infected animals within the first two months of *M. bovis* infection/sensitization.

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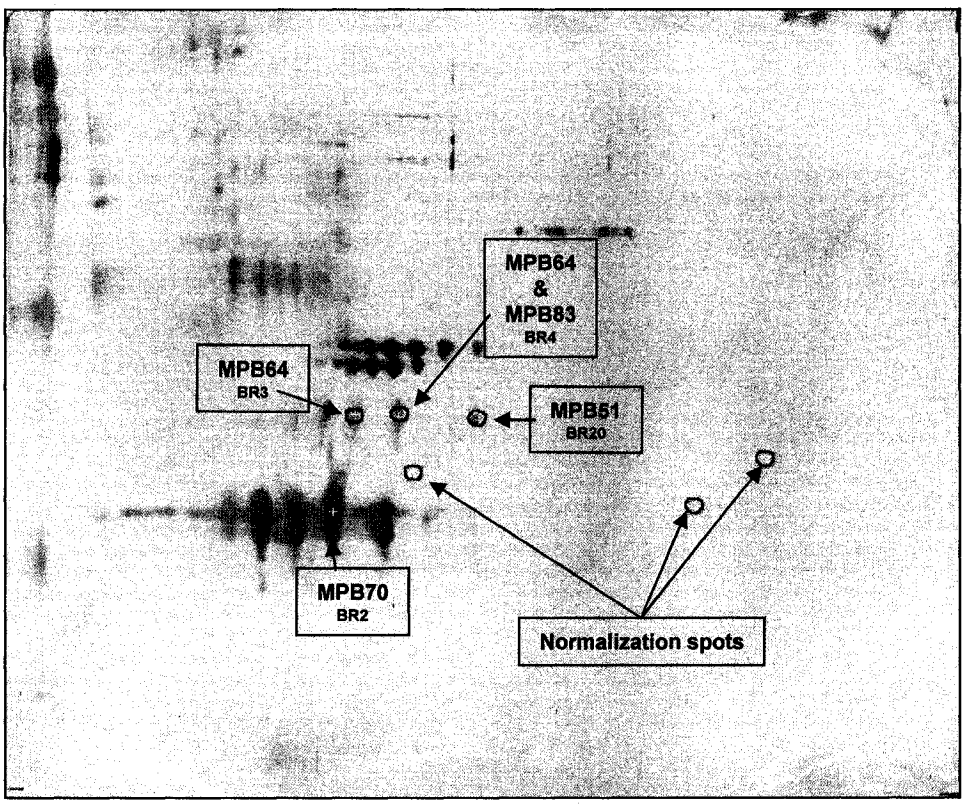
Appendix



Appendix, Figure 1. Spot parameters of 2D Western blot examined by PDQuest

The digital reproduction of this 2D Western was exported from PDQuest (Bio-Rad) to show the spot boundaries used to quantify the antibody response to SF-PPD proteins in *M. bovis* infected and *M. bovis* sensitized cattle. Note that all spot boundaries are identical regardless of actual spot size which eliminated quantification errors resulting from non-symmetrical spots. The identical spot parameters thereby allowed for the quantitative comparison of the pre-infection/sensitization Western blot spots to the post-infection/ sensitization Western blot spot.

Appendix, Table 1. Results of MASS-SPEC analysis of 9 spots excised from a Coomassie brilliant blue, stained 2DE gel of SF-PPD.
Protein spots were excised from a CBB stained 2-DE gel of SF-PPD (200 μg; 50 μL of 4 μg/μL) and submitted to the Ottawa Institute of Systems Biology, University of Ottawa for MASS-SPEC analysis. In the table, the five most probable candidate proteins as determined by Mascot search results are listed for each submitted spot. Bold text is used to identify the most likely constituent or majority constituent protein for each submitted spot. Presumptive identifications are based on data provided by the Mascot search results and and previously performed 2DE analysis of *Mycobacterium spp.* CFPs (Mattow *et al.*, 2003; Jungblut *et al.*, 1999; Malen, Softeland, and Wiker, 2008; Nagai *et al.*, 1991; Rosenkrands *et al.*, 1998; Sinha *et al.*, 2005; Sonnenberg and Belisle, 1997; Ohara *et al.*, 1997; Harboe *et al.*, 1986).

SF-PPD protein spots submitted for MASS-SPEC analysis			Mascot Search Results		
Submission designation	Approximate 2DE spot location		Protein hits		
	mass (kDa)	pI	Name	mass (kDa)	Score
BR1	22	4.2	**MPB83**	**22.2**	**471**
			CFP10	10.8	458
			MPB70	22.5	433
			MPB63	13.7	430
			ESAT-6	10.0	337
BR2	22	4.7	**MPB70**	**22.5**	**588**
			MPB83	22.2	473
			Rv1314c	20.8	196
			CFP10	10.8	177
			MPB63	16.5	47
BR3	25	4.5	**MPB64**	**25.1**	**733**
			MPB83	22.2	509
			MPB70	22.5	434
			M.tb protease	23.5	381
			lipoprotein LPPX	24.3	377
BR4	25	5.4	Adenylate kinase	20.1	504
			MPB83	**22.2**	**466**
			MPB64	**25.1**	**366**
			Rv2557	24.7	356
			Ag85b	30.8	339
BR20	27	6	**MPB51**	**31.1**	**699**
			enoyl-CoA hydratase	24.5	506
			MPB64	25.1	145
			MPB70	22.5	122
			peptide of a 24 kDa immunoprotective protein	2.1	99
BR21	31	5	**Ag85b**	**30.8**	**609**
			M.tb protein MT3693	28.2	534
			Beta-1, glucanase precursor	32.2	329
			chaperonin GroEL	31.2	289
			29 kDa Ag	28.5	210
BR23	31	5.3	**Ag85b**	**30.8**	**610**
			Ag85c	31.2	417
			Ag85a	32.7	276
			Conserved membrane protein	27.3	107
			dehydrogenase/reductase	29.9	42
BR26	32	5.3	**Ag85c**	**33.1**	**632**
			esterase	34.0	611
			Ag85b	30.8	289
			Ag85a	32.8	191
BR]28	32	5.7	**Ag85c**	**33.1**	**578**
			Ag85a	**32.8**	**351**
			Ag85b	30.8	309
			amidohydrolase	29.1	181
			hypothetical protein TB15.3	15.3	133

Appendix, Table 2. Raw DTH response of *M. bovis* sensitized guinea pigs to SF-PPD, HK-PPD and Ovalbumin.
Sensitized guinea pigs (N=12) received a 2 mg injection (IM)(0.1 mL of 20 mg/mL) of heat killed *M. bovis* cells 35 days prior to CITST. Saline sensitized guinea pigs (N=3) received a 0.1 mL injection (IM) of saline 35 days prior to CITST. DTH responses to 0.1 mL intradermal injections of SF-PPD, HK-PPD and ovalbumin were measured 48 hpi.

M. bovis Sensitized	Dilution 1 HK-PPD @ 50 ug/mL				Dilution 2 HK-PPD @ 10 ug/mL				Dilution 3 HK-PPD @ 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)
445	23.00	17.99	20.50	413.77	13.58	13.08	13.33	177.63	15.41	12.80	14.11	197.25
443	20.41	15.26	17.84	311.46	18.67	14.13	16.40	263.81	14.31	10.31	12.31	147.54
452	18.69	14.84	16.77	277.36	12.68	12.14	12.41	153.94	10.74	8.18	9.46	87.85
455	15.52	17.06	16.29	264.77	14.96	13.87	14.42	207.50	11.25	9.14	10.20	102.83
454	19.24	16.23	17.74	312.27	14.83	14.61	14.72	216.67	11.48	12.01	11.75	137.87
447	19.75	17.80	18.78	351.55	18.45	14.67	16.56	270.66	9.62	10.40	10.01	100.05
457	18.31	15.02	16.67	275.02	12.62	11.28	11.95	142.35	10.12	7.89	9.01	79.85
446	16.55	15.90	16.23	263.15	13.20	12.46	12.83	164.47	10.17	8.09	9.13	82.28
437	18.96	16.33	17.65	309.62	14.84	12.47	13.66	185.05	6.33	5.65	5.99	35.76
458	16.06	15.81	15.94	253.91	10.38	9.35	9.87	97.05	3.00	3.00	3.00	9.00
438	25.59	19.44	22.52	497.47	15.35	13.04	14.20	200.16	8.87	9.19	9.03	81.52
436	16.11	15.14	15.63	243.91	17.62	12.11	14.87	213.38	7.83	6.84	7.34	53.56
	Average		17.71	314.52	Average		13.77	191.06	Average		9.28	92.95
	Standard Deviation		1.95	71.56	Standard Deviation		1.80	47.12	andard Deviati		2.80	48.59

M. bovis Sensitized	Dilution 4 SF-PPD @ 50 ug/mL				Dilution 5 SF-PPD @ 10 ug/mL				Dilution 6 SF-PPD @ 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)
445	29.68	19.01	24.35	564.22	20.89	14.70	17.80	307.08	13.91	11.97	12.94	166.50
443	22.96	17.79	20.38	408.46	16.29	14.51	15.40	236.37	15.94	12.10	14.02	192.87
452	18.89	16.99	17.94	320.94	14.89	12.85	13.87	191.34	12.49	9.20	10.85	114.91
455	21.91	15.70	18.81	343.99	17.97	14.34	16.16	257.69	13.55	13.30	13.43	180.22
454	25.99	20.21	23.10	525.26	20.00	15.32	17.66	306.40	10.39	10.01	10.20	104.00
447	22.10	18.83	20.47	416.14	19.90	18.36	19.13	365.36	16.02	12.73	14.38	203.93
457	19.32	15.59	17.46	301.20	16.44	14.30	15.37	235.09	9.62	8.03	8.83	77.25
446	20.49	16.34	18.42	334.81	13.86	9.55	11.71	132.36	6.94	7.76	7.35	53.85
437	20.20	18.14	19.17	366.43	17.79	16.14	16.97	287.13	13.26	13.13	13.20	174.10
458	16.49	13.69	15.09	225.75	11.88	9.75	10.82	115.83	7.54	5.85	6.70	44.11
438	27.25	22.85	25.05	622.66	18.33	15.08	16.71	276.42	8.71	8.18	8.45	71.25
436	15.32	15.91	15.62	243.74	16.62	13.11	14.87	217.89	7.66	6.74	7.20	51.63
	Average		19.65	389.47	Average		15.54	244.08	Average		10.63	119.55
	Standard Deviation		3.05	119.21	Standard Deviation		2.36	69.63	andard Deviati		2.76	58.00

M. bovis Sensitized	Dilution 7 Ovlabumin @ 50 ug/mL			
	Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)
445	NVR	NVR	0.00	0.00
443	NVR	NVR	0.00	0.00
452	NVR	NVR	0.00	0.00
455	NVR	NVR	0.00	0.00
454	NVR	NVR	0.00	0.00
447	NVR	NVR	0.00	0.00
457	NVR	NVR	0.00	0.00
446	NVR	NVR	0.00	0.00
437	NVR	NVR	0.00	0.00
458	NVR	NVR	0.00	0.00
438	NVR	NVR	0.00	0.00
436	NVR	NVR	0.00	0.00
	Average		0.00	0.00
	Standard Deviation		0.00	0.00

Saline Sensitized	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7
	Reaction Size						
439	NVR	NVR	NVR	NVR	NVR	NVR	NVR
471	NVR	NVR	NVR	NVR	NVR	NVR	NVR
472	NVR	NVR	NVR	NVR	NVR	NVR	NVR

* NVR: No visible reaction

MPB70 BR2

Spot quantity total pixel density/1000					
Infected cattle #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
103	161	83	0.5	435	2.7
106	37	39	1.1	122	3.3
107	50	44	0.9	363	7.3
108	40	47	1.2	109	2.7
109	29	28	1.0	45	1.6
110	31	32	1.0	202	6.5
Averages	**58.0**	**45.5**	**0.9**	**212.7**	**4.0**

Spot quantity total pixel density/1000					
Snsitized cattle #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
003	33	50	1.5	138	4.2
112	41	44	1.1	138	3.4
207	51	99	1.9	176	3.5
211	47	76	1.6	256	5.4
457	66	34	0.5	107	1.6
993	41	96	2.3	124	3.0
Averages	**46.5**	**66.5**	**1.5**	**156.5**	**3.5**

MPB64 BR3

Animal #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
103	55	43	0.8	56	1.0
106	44	30	0.7	46	1.0
107	38	29	0.8	92	2.4
108	32	53	1.7	80	2.5
109	29	32	1.1	36	1.2
110	30	30	1.0	33	1.1
Averages	**38.0**	**36.2**	**1.0**	**57.2**	**1.6**

Animal #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
003	33	58	1.8	73	2.2
112	32	30	0.9	48	1.5
207	20	312	15.6	149	7.5
211	31	112	3.6	239	7.7
457	50	21	0.4	34	0.7
993	30	100	3.3	85	2.8
Averages	**32.7**	**105.5**	**4.3**	**104.7**	**3.7**

MPB64 & MPB83 BR4

Animal #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
103	63	51	0.8	42	0.7
106	47	32	0.7	46	1.0
107	41	30	0.7	63	1.5
108	31	49	1.6	81	2.6
109	30	33	1.1	35	1.2
110	33	32	1.0	31	0.9
Averages	**40.8**	**37.8**	**1.0**	**49.7**	**1.3**

Animal #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
003	33	58	1.8	82	2.5
112	35	33	0.9	60	1.7
207	18	164	9.1	104	5.8
211	32	90	2.8	231	7.2
457	45	23	0.5	39	0.9
993	32	79	2.5	81	2.5
Averages	**32.5**	**74.5**	**2.9**	**99.5**	**3.4**

MPB51 BR20

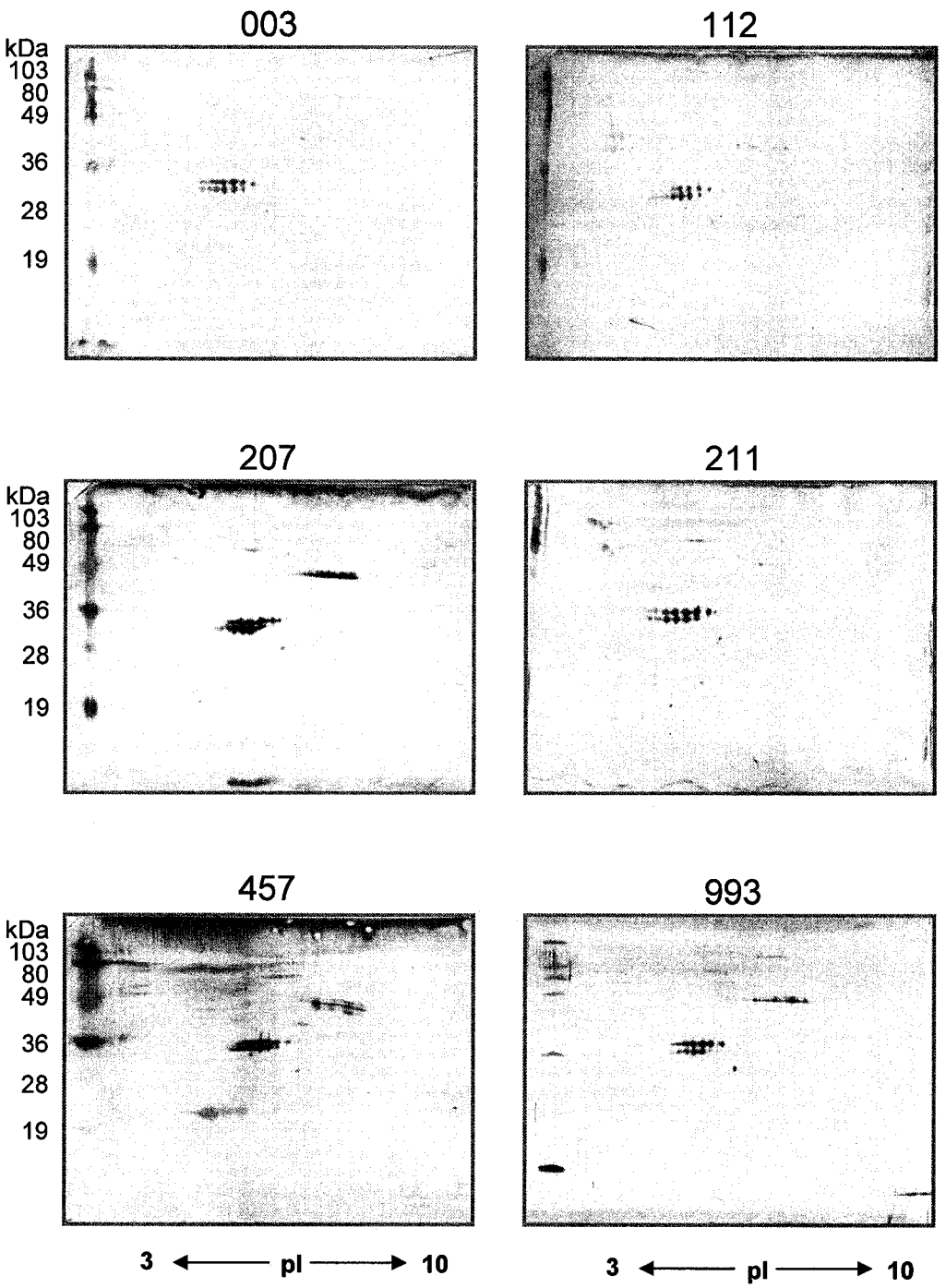
Animal #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
103	150	94	0.6	117	0.8
106	65	47	0.7	65	1.0
107	41	39	1.0	56	1.4
108	33	49	1.5	53	1.6
109	40	53	1.3	40	1.0
110	40	45	1.1	38	1.0
Averages	**61.5**	**54.5**	**1.0**	**61.5**	**1.1**

Animal #	pre-inf	pre-TST	pre TST/ pre inf	post TST	Post inf/ pre inf
003	34	51	1.5	53	1.6
112	56	50	0.9	80	1.4
207	112	156	1.4	44.9	0.4
211	54	55	1.0	105	1.9
457	55	77	1.4	72	1.3
993	93	148	1.6	94	1.0
Averages	**67.3**	**89.5**	**1.3**	**74.8**	**1.3**

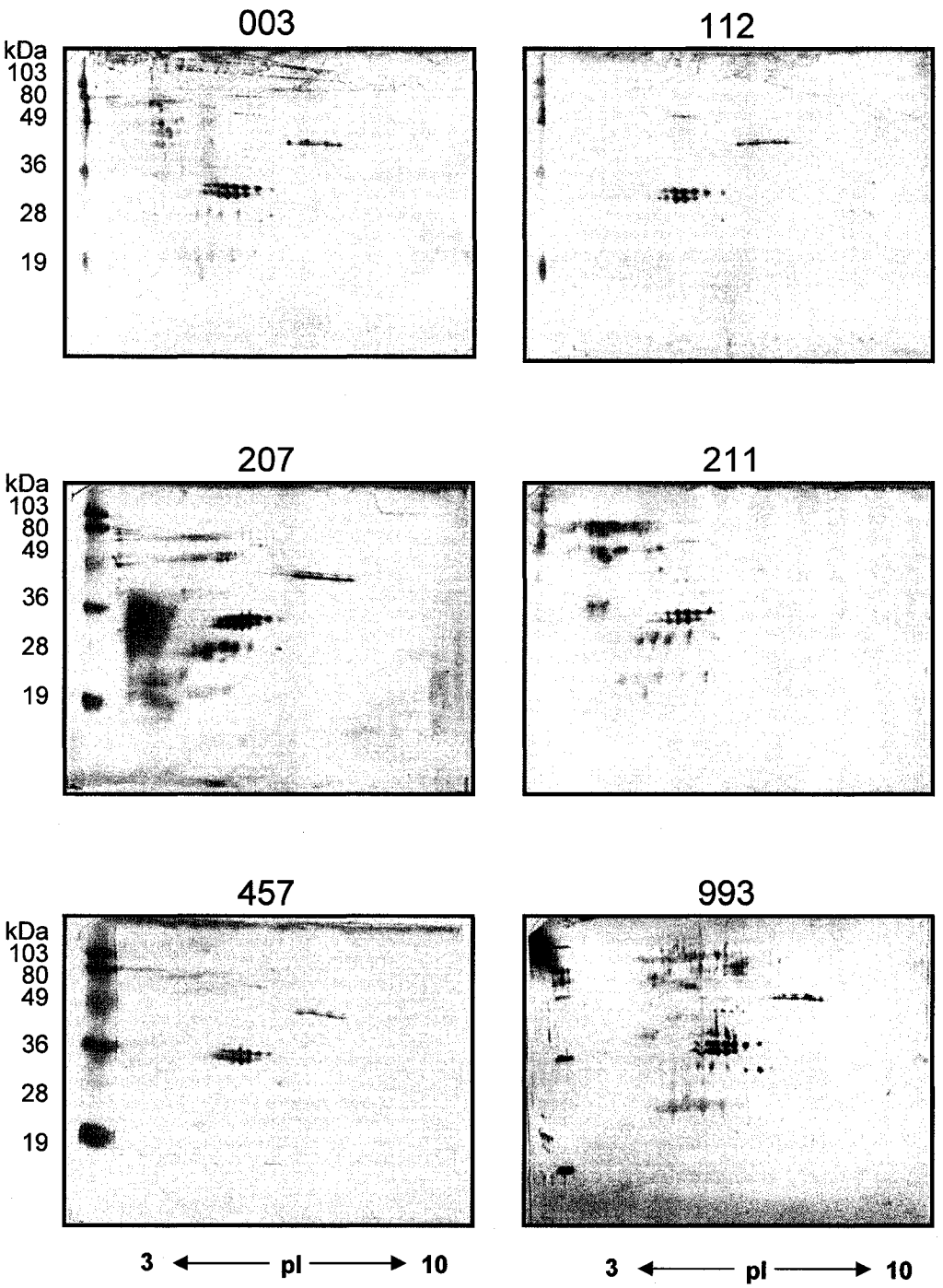
Appendix, Table 3. Pixel quantities of specific SF-PPD protein spots as determined by PDQuest.

2D Western blots were analyzed by PDQuest 2DE analysis software (Bio-Rad). All spots parameters were identically sized and were manually positioned according to the Ag85 complex proteins which acted as landmark spots and by comparison to positive control 2D Westerns and 2DE gels of SF-PPD.

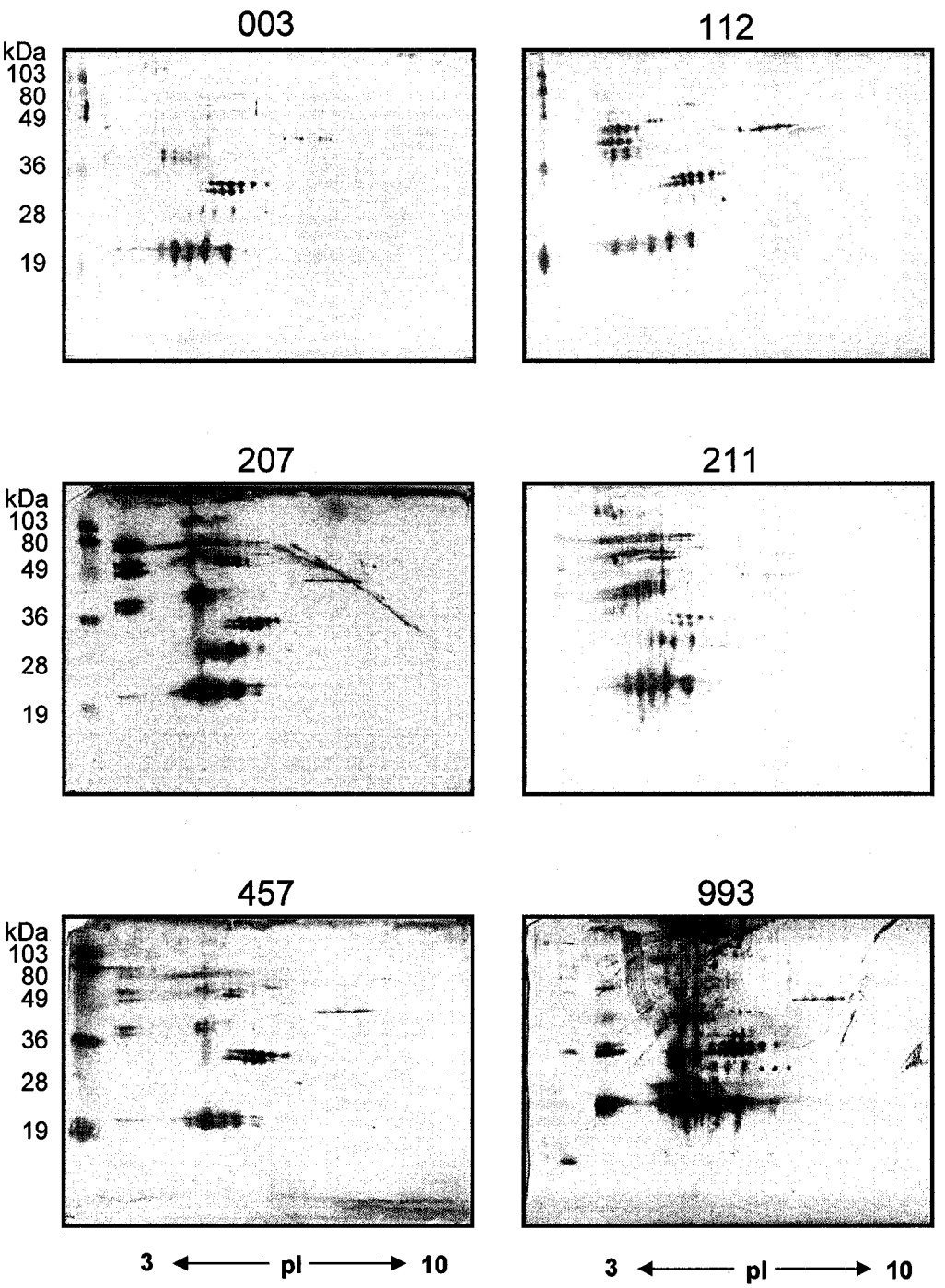
Appendix, Figure 2. Western blot analysis of the antibody response to SF-PPD proteins in cattle prior to *M. bovis* sensitization.



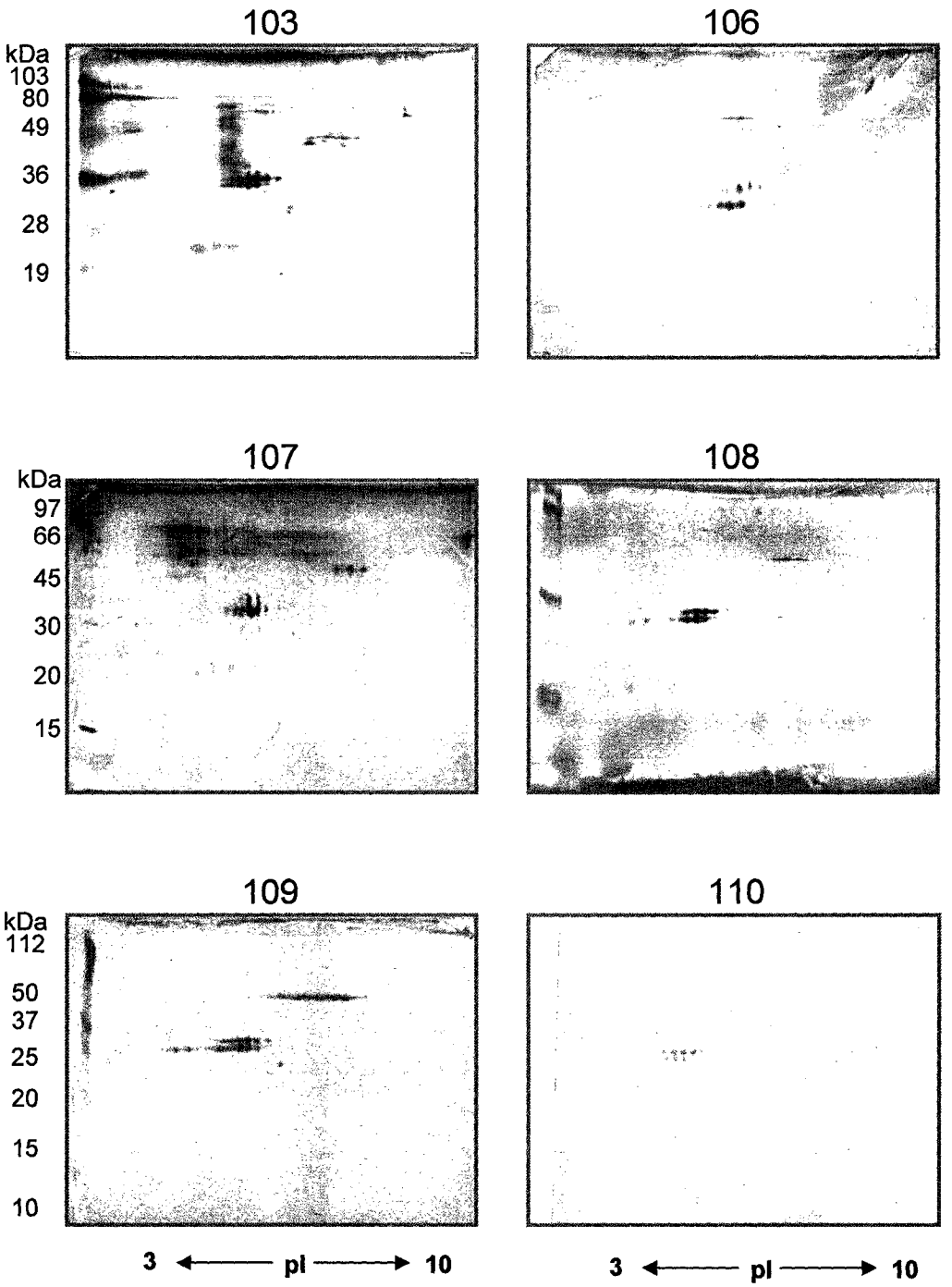
Appendix, Figure 3. Western blot analysis of the antibody response to SF-PPD proteins in cattle at seven weeks post *M. bovis* sensitization.



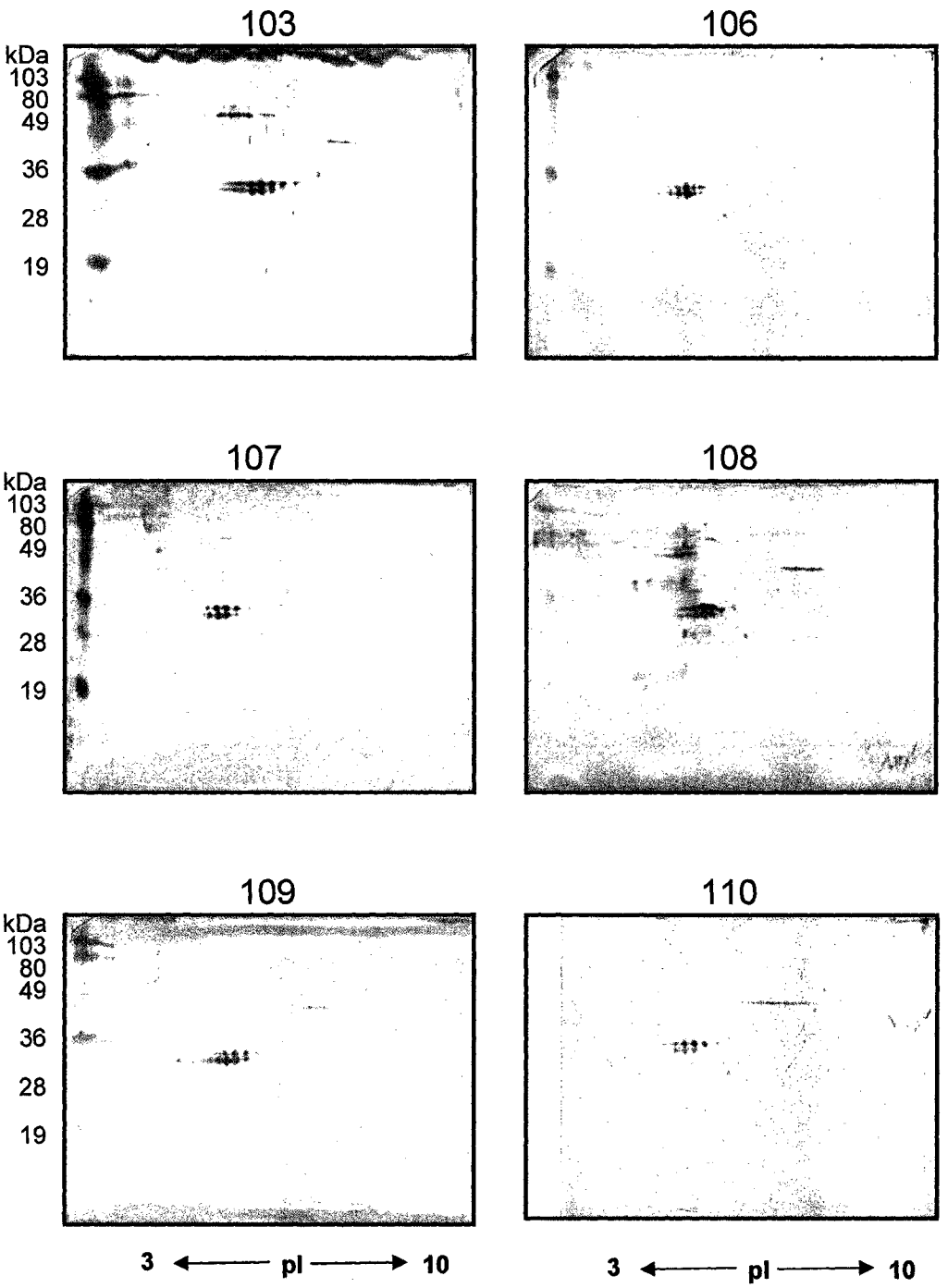
Appendix, Figure 4. Western blot analysis of the antibody response to SF-PPD proteins in *M. bovis* sensitized cattle post CITST.



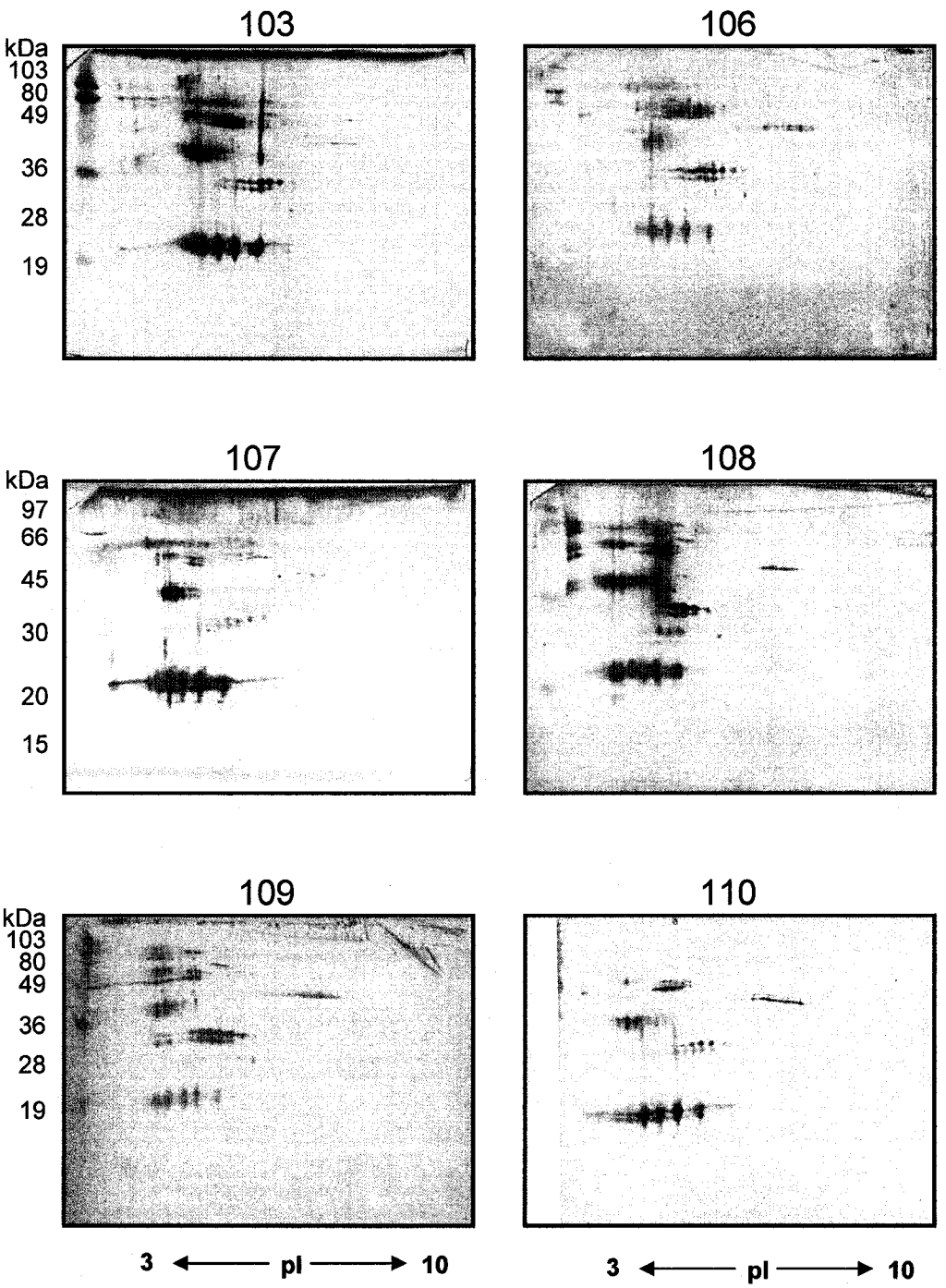
Appendix, Figure 5. Western blot analysis of the antibody response to SF-PPD proteins in cattle prior to *M. bovis* infection.

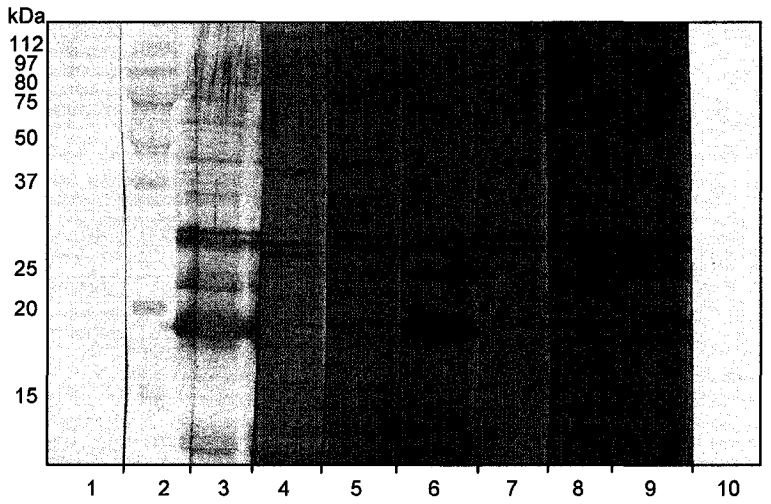


Appendix, Figure 6. Western blot analysis of the antibody response to SF-PPD proteins in cattle at seven weeks post *M. bovis* infection.



Appendix, Figure 7. Western blot analysis of the antibody response to SF-PPD proteins in *M. bovis* infected cattle post CITST.





Appendix, Figure 8. Western blot analysis of steer #A6 which failed to elicit visible DTH response to *M. bovis* PPD tuberculin.

Sera from steer #A2 was blotted onto SF-PPD to determine it's sensitivity to M. bovis CFPs. Lanes 1- 3 were loaded with 40 µL of sample buffer, molecular weight standards (Silver Stain SDS-PAGE Standards Low Range, Bio-Rad) and 10 µg (40 µL) of HK-PPD respectively and were stained with Colloidal Gold (Bio-Rad) following transfer. Lanes 4-10 were each loaded with 10 µg of 4 µg/µL SF-PPD (40 µL), cut into strips and individually blotted with sera diluted 1:200 with TBST. (Lane 4) pre-sensitized sera from steer #A1; Lane(5) pre-CITST sera from steer #A1; Lane (6) post-CITST sera from steer #A1; (Lane 7) pre-sensitized sera from steer #A6; Lane(8) pre-CITST sera from steer #A6; Lane (9) post-CITST sera from steer #A6; (Lane 10) negative control, no 1° antibody.

Appendix, Table 4a. CITST measurements of guinea pigs sensitized with heat killed *M. bovis* cells at 24 and 48 hpi.

24 hpi

HK-PPD *M. bovis* tuberculin	Dilution 1 50 ug/mL				Dilution 2 10 ug/mL				Dilution 3 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)
657	13.62	9.49	11.56	129.25	13.84	9.28	11.56	128.44	11.93	6.66	9.30	79.45
658	23.12	15.82	19.47	365.76	18.94	15.34	17.14	290.54	15.78	11.74	13.76	185.26
673	21.72	16.27	19.00	353.38	20.39	12.28	16.34	250.39	7.29	7.59	7.44	55.33
674	21.83	18.42	20.13	402.11	11.29	11.73	11.51	132.43	9.10	6.61	7.86	60.15
676	17.24	18.84	18.04	324.80	10.82	10.79	10.81	116.75	5.77	5.39	5.58	31.10
651	14.61	13.26	13.94	193.73	11.21	8.48	9.85	95.06	5.54	5.54	5.54	30.69
652	20.88	18.66	19.77	389.62	14.11	13.00	13.56	183.43	10.43	16.63	13.53	173.45
664	19.66	13.63	16.65	267.97	16.22	14.15	15.19	229.51	9.05	8.71	8.88	78.83
	Average		17.32	303.33	Average		13.24	178.32	Average		8.99	86.78
	Standard Deviation		2.89	91.95	Standard Deviation		2.55	66.85	Standard Deviation		2.97	56.20

SF-PPD *M. bovis* tuberculin	Dilution 4 50 ug/mL				Dilution 5 10 ug/mL				Dilution 6 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)
657	21.22	15.00	18.11	318.30	18.44	12.17	15.31	224.41	10.81	9.52	10.17	102.91
658	22.06	19.21	20.64	423.77	18.45	17.02	17.74	314.02	14.82	12.02	13.42	178.14
673	21.02	13.50	17.26	283.77	16.16	12.28	14.22	198.44	10.76	7.52	9.14	80.92
674	17.61	13.47	15.54	237.21	14.57	11.39	12.98	165.95	9.85	6.27	8.06	61.76
676	16.98	16.58	16.78	281.53	11.80	7.41	9.61	87.44	9.00	8.94	8.97	80.46
651	20.10	12.92	16.51	259.69	16.16	12.58	14.37	203.29	9.77	7.64	8.71	74.64
652	25.08	18.18	21.63	455.95	18.98	15.52	17.25	294.57	13.83	17.53	15.68	242.44
664	21.59	20.18	20.89	435.69	14.85	13.82	14.34	205.23	12.97	11.80	12.39	153.05
	Average		18.42	336.99	Average		14.48	211.67	Average		10.82	121.79
	Standard Deviation		2.16	81.86	Standard Deviation		2.37	66.45	Standard Deviation		2.54	59.45

48 hpi

HK-PPD *M. bovis* tuberculin	Dilution 1 50 ug/mL				Dilution 2 10 ug/mL				Dilution 3 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)
657	17.53	11.10	14.32	194.58	13.69	9.77	11.73	133.75	9.80	8.32	9.06	81.54
658	21.61	16.24	18.93	350.95	18.13	15.30	16.72	277.39	14.36	12.04	13.20	172.89
673	21.13	19.94	20.54	421.33	19.74	14.50	17.12	286.23	6.72	6.88	6.80	46.23
674	24.33	17.99	21.16	437.70	17.09	13.97	15.53	238.75	8.34	8.92	8.63	74.39
676	18.62	15.67	17.15	291.78	11.11	10.95	11.03	121.65	8.61	7.56	8.09	65.09
651	15.42	16.13	15.78	248.72	12.55	9.64	11.10	120.98	5.52	5.52	5.52	30.47
652	22.75	20.29	21.52	461.60	14.30	13.39	13.85	191.48	8.52	8.52	8.52	72.59
664	18.60	17.29	17.95	321.59	14.50	13.51	14.01	195.90	10.04	9.27	9.66	93.07
	Average		18.42	341.03	Average		13.88	195.77	Average		8.68	79.54
	Standard Deviation		2.44	88.88	Standard Deviation		2.29	62.89	Standard Deviation		2.10	39.85

SF-PPD *M. bovis* tuberculin	Dilution 4 50 ug/mL				Dilution 5 10 ug/mL				Dilution 6 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w + h) / 2 (mm)	area w x h (mm^2)
657	21.46	15.63	18.55	335.42	17.37	10.17	13.77	176.65	9.40	10.81	10.11	101.61
658	24.24	19.45	21.85	471.47	18.42	14.59	16.51	268.75	11.23	10.34	10.79	116.12
673	23.38	16.60	19.99	388.11	18.53	14.36	16.45	266.09	11.20	9.43	10.32	105.62
674	24.33	17.31	20.82	421.15	14.38	12.23	13.31	175.87	6.55	3.56	5.06	23.32
676	22.37	17.19	19.78	384.54	12.91	8.41	10.66	108.57	9.62	8.81	9.22	84.75
651	17.40	14.56	15.98	253.34	15.55	12.95	14.25	201.37	4.49	4.49	4.49	20.16
652	22.31	17.77	20.04	396.45	18.58	14.15	16.37	262.91	14.12	14.27	14.20	201.49
664	19.45	17.60	18.53	342.32	14.15	12.74	13.45	180.27	8.52	10.03	9.28	85.46
	Average		19.44	374.10	Average		14.34	205.06	Average		9.18	92.32
	Standard Deviation		1.66	60.81	Standard Deviation		1.90	53.30	Standard Deviation		2.93	53.41

Appendix, Table 4b. CITST measurements of guinea pigs sensitized with SF-PPD at 24 and 48 hpi.

24 hpi

HK-PPD *M. bovis* tuberculin	Dilution 1 50 ug/mL				Dilution 2 10 ug/mL				Dilution 3 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)
636	11.71	9.17	10.44	107.38	6.05	6.06	6.06	36.66	5.87	3.45	4.66	20.25
637	8.41	6.91	7.66	58.11	12.99	10.32	11.66	134.06	8.31	5.59	6.95	46.45
650	18.22	13.17	15.70	239.96	7.49	8.36	7.93	62.62	7.15	6.63	6.89	47.40
653	10.66	9.40	10.03	100.20	5.66	5.08	5.37	28.75	5.13	4.87	5.00	24.98
655	6.80	7.73	7.27	52.56	7.15	5.00	6.08	35.75	4.70	4.00	4.35	18.80
631	5.98	5.57	5.78	33.31	4.32	4.32	4.32	18.66	2.91	2.91	2.91	8.47
635	9.63	7.07	8.35	68.08	4.09	7.66	5.88	31.33	4.48	4.48	4.48	20.07
645	11.34	9.37	10.36	106.26	6.40	5.39	5.90	34.50	5.29	3.61	4.45	19.10
	Average		9.45	95.73	Average		6.65	47.79	Average		4.96	25.69
	Standard Deviation		2.82	60.22	Standard Deviation		2.11	34.62	Standard Deviation		1.27	13.00

SF-PPD *M. bovis* tuberculin	Dilution 4 50 ug/mL				Dilution 5 10 ug/mL				Dilution 6 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)
636	27.50	20.69	24.10	568.98	19.05	14.68	16.87	279.65	7.24	5.32	6.28	38.52
637	28.22	26.65	27.44	752.06	22.22	17.50	19.86	388.85	14.29	11.91	13.10	170.19
650	24.12	20.84	22.48	502.66	19.47	17.20	18.34	334.88	9.74	9.10	9.42	88.63
653	11.38	9.00	10.19	102.42	14.81	10.31	12.56	152.69	7.58	5.60	6.59	42.45
655	21.79	18.09	19.94	394.18	16.91	12.14	14.53	205.29	8.59	6.04	7.32	51.88
631	14.05	10.27	12.16	144.29	4.63	4.63	4.63	21.44	6.67	6.59	6.63	43.96
635	20.22	18.37	19.30	371.44	13.98	12.19	13.09	170.42	3.72	4.27	4.00	15.88
645	11.11	11.59	11.35	128.76	8.77	8.19	8.48	71.83	5.79	4.93	5.36	28.54
	Average		18.37	370.60	Average		13.54	203.13	Average		7.34	60.01
	Standard Deviation		6.02	219.23	Standard Deviation		4.76	118.10	Standard Deviation		2.62	46.09

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HK-PPD *M. bovis* tuberculin	Dilution 1 50 ug/mL				Dilution 2 10 ug/mL				Dilution 3 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)
636	12.98	10.57	11.78	137.20	6.92	5.67	6.30	39.24	0.00	0.00	0.00	0.00
637	13.17	10.79	11.98	142.10	0.00	0.00	0.00	0.00	8.05	7.61	7.83	61.26
650	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
653	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.17	4.17	4.17	17.39
655	9.05	5.83	7.44	52.76	9.79	9.00	9.40	88.11	0.00	0.00	0.00	0.00
631	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
635	6.94	6.94	6.94	48.16	5.50	5.50	5.50	30.25	16.00	13.06	14.53	208.96
645	6.13	6.13	6.13	37.58	3.55	3.55	3.55	12.60	5.63	4.09	4.86	23.03
	Average		5.53	52.23	Average		3.09	21.27	Average		3.92	38.83
	Standard Deviation		4.72	54.44	Standard Deviation		3.43	29.06	Standard Deviation		4.88	67.26

SF-PPD *M. bovis* tuberculin	Dilution 4 50 ug/mL				Dilution 5 10 ug/mL				Dilution 6 2 ug/mL			
	Reaction Size				Reaction Size				Reaction Size			
Guinea Pig ID #	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)	width w (mm)	height h (mm)	avg. dia. (w+h)/2 (mm)	area w x h (mm^2)
636	16.20	14.22	15.21	230.36	6.60	5.16	5.88	34.06	0.00	0.00	0.00	0.00
637	4.19	4.19	4.19	17.56	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
650	0.00	0.00	0.00	0.00	19.36	15.17	17.27	293.69	4.22	4.22	4.22	17.81
653	11.14	9.88	10.51	110.06	9.05	8.07	8.56	73.03	14.84	13.75	14.30	204.05
655	18.17	12.51	15.34	227.31	13.84	7.03	10.44	97.30	11.14	7.45	9.30	82.99
631	11.20	10.36	10.78	116.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
635	15.74	13.41	14.58	211.07	13.99	10.62	12.31	148.57	0.00	0.00	0.00	0.00
645	13.43	11.02	12.23	148.00	12.17	10.47	11.32	127.42	3.91	3.91	3.91	15.29
	Average		10.35	132.55	Average		8.22	96.76	Average		3.97	40.02
	Standard Deviation		5.19	84.06	Standard Deviation		5.63	90.57	Standard Deviation		4.98	67.33

Appendix, Table 5. Raw skin response measurements of *M. bovis* sensitized cattle to *M. bovis* (SF-PPD, HK-PPD and 100-02-2CNS) and *M. avium* (101-06-1B) PPD tuberculins and to Ovalbumin.

	Dil. 1 (100-02-2CNS @ 1.0mg/ml) Measurement less 0 h reading			Dil. 2 (100-02-2CNS @ 0.2mg/ml) Measurement less 0 h reading			Dil. 3 (100-06-1R @ 1.0mg/ml) Measurement less 0 h reading			Dil. 4 (100-06-1R @ 0.2mg/ml) Measurement less 0 h reading		
Steer #	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
1	10.92	13.15	12.02	5.45	8.22	6.45	7.12	8.70	8.31	6.56	8.48	6.37
3	5.46	7.17	6.86	3.03	2.26	4.09	5.19	7.25	5.23	7.02	7.45	5.26
4	17.95	18.36	17.60	11.47	13.20	13.37	11.35	16.65	16.45	12.12	16.05	13.82
5	9.88	17.90	14.37	7.68	8.93	8.24	8.19	12.29	12.33	6.05	9.35	8.49
6	NVR	NVR	NVR	NVR	NVR	NVR	NVR	NVR	NVR	NVR	NVR	NVR
7	8.27	13.98	15.99	5.67	9.54	13.56	6.72	11.05	11.29	4.78	7.84	6.95
8	8.77	18.87	16.78	6.83	13.20	13.21	8.39	15.98	14.23	4.99	12.28	14.08
9	7.61	11.50	12.52	7.66	9.06	7.31	7.87	10.63	10.19	5.88	8.43	7.92
10	10.29	11.19	9.66	4.61	5.08	5.26	13.02	11.18	9.62	7.64	8.73	5.76
121	8.71	7.38	8.48	4.98	3.75	4.14	6.90	6.67	8.27	4.38	3.66	3.19
123	1.43	1.88	0.00	1.84	0.40	1.50	4.30	1.83	1.99	2.67	2.22	3.67
124	7.63	7.45	8.30	5.55	7.38	6.10	10.92	11.28	12.29	5.96	7.29	7.26
Average	8.81	11.71	11.14	5.89	7.37	7.57	8.18	10.32	10.02	6.19	8.34	7.52
St DEV	4.00	5.44	5.20	2.56	4.14	4.14	2.65	4.19	4.06	2.39	3.71	3.57

	Dil. 5 (SF-PPD @ 1.0mg/ml) Measurement less 0 h reading			Dil. 6 (SF-PPD @ 0.2mg/ml) Measurement less 0 h reading			Avium (101-06-1B 0.5 mg/ml) Measurement less 0 h reading			Ovalbumin @ 1.0 mg/mL Measurement less 0 h reading		
Steer #	24h	48h	72h	24h	48h	72h	A- 24 h	A- 48 h	A- 72 h	A- 24 h	A- 48 h	A- 72 h
1	6.32	11.02	8.97	3.37	5.31	5.14	5.95	8.01	5.88	0.47	0.00	0.00
3	5.43	5.87	6.04	2.51	2.66	2.01	0.29	0.00	0.00	1.16	0.00	0.00
4	11.01	13.97	13.21	8.47	11.99	11.40	7.5	9.00	7.35	0.00	0.00	0.00
5	6.32	9.14	8.52	6.09	6.13	6.67	5.92	9.55	8.02	0.00	0.00	0.00
6	NVR	NVR	NVR	NVR	NVR	NVR	NVR	NVR	NVR	0.00	0.00	0.00
7	7.73	10.53	9.33	6.38	8.62	8.02	6.1	5.84	5.84	0.00	0.00	0.00
8	3.62	10.70	12.23	2.45	6.96	8.74	10.58	6.22	4.77	0.00	0.00	0.00
9	9.13	7.93	7.93	4.77	7.29	8.31	9.36	8.08	9.16	0.00	0.00	0.00
10	6.85	8.33	7.49	5.10	5.32	5.45	5.97	4.10	4.04	0.00	0.00	0.00
121	4.57	5.74	7.19	4.17	5.97	6.28	6.39	4.29	6.89	0.00	0.00	0.00
123	3.49	3.98	2.56	3.43	3.13	1.96	6.13	3.09	2.41	0.00	0.00	0.00
124	4.10	7.15	8.62	3.11	4.12	4.89	3.96	4.50	5.43	0.24	0.00	0.00
Average	6.23	8.58	8.37	4.53	6.14	6.26	6.20	5.70	5.44	0.15	0.00	0.00
St DEV	2.37	2.88	2.85	1.87	2.63	2.84	2.67	2.87	2.60	0.35	0.00	0.00

Negative controls	Dil. 1 (100-02-2CNS @ 1.0mg/ml) Measurement less 0 h reading			Dil. 2 (100-02-2CNS @ 0.2mg/ml) Measurement less 0 h reading			Dil. 3 (100-06-1R @ 1.0mg/ml) Measurement less 0 h reading			Dil. 4 (100-06-1R @ 0.2mg/ml) Measurement less 0 h reading		
115	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
117	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Average	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Negative controls	Dil. 5 (SF-PPD @ 1.0mg/ml) Measurement less 0 h reading			Dil. 6 (SF-PPD @ 0.2mg/ml) Measurement less 0 h reading			Avium (101-06-1B 0.5 mg/ml) Measurement less 0 h reading			Ovalbumin @ 1.0 mg/mL Measurement less 0 h reading		
115	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
117	0.00	0.00	0.00	0.00	0.00	0.00	3.45	3.68	3.41	0.00	0.00	0.00
Average	0.00	0.00	0.00	0.00	0.00	0.00	1.73	1.84	1.71	0.00	0.00	0.00