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The evaluation of Mycobacterium tuberculosis complex species-specific antigens and purified protein derivatives for the detection of bovine tuberculosis using the interferon-gamma release assay

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The evaluation of *Mycobacterium tuberculosis* complex species-specific antigens and purified protein derivatives for the detection of bovine tuberculosis using the interferon-gamma release assay

by

**Kristin Elaine Bass**

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Ames, Iowa

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

*Mycobacterium bovis* is the causative agent of tuberculosis in cattle. *M. bovis* is a member of the *Mycobacterium tuberculosis* complex (Mtbc) which also includes *M. tuberculosis, M. caprae, M. microti, M. africanum, M. canetti, M. pinnipedii*, and *M. bovis* Bacillus Calmette Guerin (BCG) (28). Unlike most of the organisms in this group, *M. bovis* has a broad host range including; cattle, cervids, badgers, humans, and many other animals (52).

Currently, *M. bovis* is the most common organism isolated from cattle with tuberculosis (TB). *M. tuberculosis* can also infect cattle; however, it is not as virulent as *M. bovis* in cattle (36, 75). The infective dose of *M. bovis* is low with studies demonstrating less than 10 viable bacilli are sufficient to cause infection (32); however, multiple factors influence transmission such as frequency of excretion, period of communicability, and route, just to name a few. (49).

The broad host range, low infective dose, and presence of wildlife reservoirs make eradication of bovine TB difficult. However, several countries, including the United States, have TB eradication and control programs in place to monitor and manage these reservoirs as well as the cattle (31). Current bovine TB eradication and control programs generally rely on slaughter surveillance as well as test and cull strategies (75). However, improved ante mortem diagnostic tests are needed.
Thesis Organization

This thesis includes one manuscript. The manuscript is preceded by a general introduction chapter and followed by a general conclusions chapter. The manuscript in this thesis is an evaluation of *Mycobacterium* antigen-specific peptide cocktails and purified protein derivatives for their use in the Interferon-gamma (IFN-\(\gamma\)) release assay (Bovigam) using experimentally challenged animals and naturally infected animals with *M. bovis*. The general introduction chapter includes a literature review of the biology of *M. bovis*, the cell-mediated immune response to *M. bovis*, current ante-mortem assays including skin tests and the IFN-\(\gamma\) release assay, improvements of ante-mortem tests through identification and use of Mtbc-specific proteins, and incorporation of Mtbc-specific antigens and evaluation of PPDs in the IFN-\(\gamma\) release assay. The general conclusions chapter includes a general discussion regarding the components of this thesis and suggestions for future research. References to cited papers within each chapter are included at the end of each chapter.

Literature Review

**Biology of Mycobacterium bovis**

Organisms from the genus *Mycobacterium* are slow growing, acid-fast bacteria that are rod shaped or slightly curved (77). They were first identified as pathogenic bacteria with Koch’s discovery of *M. tuberculosis* bacilli in 1882 as the cause of TB in humans (90). Most bacteria in the genus *Mycobacterium* are saprophytic organisms
commonly found in the environment with relatively few causing diseases in animals or immunosuppressed individuals (52, 77).

Pathogenic species of *Mycobacterium* can be classified based on their ability to cause tuberculous infections. These can be categorized as Mtbc or non-tuberculous Mycobacteria spp (NTMs). An example of an NTM is *M. avium* subsp. *paratuberculosis*, the causative agent of Johne’s disease in cattle which is characterized by a chronic wasting in cattle. Other, potentially pathogenic NTMs include *M. kansasii, M. marinum* and *M. avium* complex species (52). These are known to cause disease in some cases, such as immunosuppressed individuals; however, they are generally opportunistic and less pathogenic than those in the Mtbc. Human-to-human transmission of NTM species is not known to occur and they can be found growing in the environment (76). NTMs are important in diagnostic tests for TB due to the conservation of antigens within the *Mycobacterium* genus which can interfere with test results due to cross reactivity. *Mycobacterium spp* in the Mtbc cause infections that are debilitating or even life threatening and have been classified as obligate pathogens (52). Mtbc species are generally not found free growing in the environment and usually require a host to multiply. They can be transmitted directly via aerosol, feces, sputum, body tissues, urine, semen, and milk and indirectly via exposure to contaminated feed bunks, water troughs, equipment, and other items that are shared by cattle within a herd (49)/

*Mycobacterium bovis* was first discovered in 1898 by isolation of tubercle bacilli from bovine sputum, which was inoculated into rabbits. Upon examination, it was observed to be more virulent than human tubercle bacilli in rabbits based on pathological findings (99).
Today, less than 2% of TB cases in humans are caused by *M. bovis*; however, it is more common in certain geographic regions (106). An example of this is at the U.S-Mexico border where over the last decade 7% of all TB cases resulted from infection with *M. bovis* (61). Most patients presented with extra-pulmonary disease and infection was likely due to consumption of unpasteurized, TB contaminated milk (61). Common disease sequelae resulting from *M. bovis* infections in humans include: lymphadenitis (particularly in children), extra-pulmonary disease, and pulmonary disease (e.g. from inhalation of droplets from *M. bovis* infected cattle or dissemination from other sites) (29) *M. bovis* can also be transmitted from cattle to humans. This makes *M. bovis* both an occupational hazard as well as a zoonotic hazard (42, 49).

Experimental studies with *M. bovis* in animals have demonstrated that the immune response and disease severity can vary based on the route of infection as well as the dose administered (71, 112, 116). Such routes that have been examined are intratracheal, oral, intravenous, intraperitoneal, and aerosol (116). With most hosts, the most common route of transmission is via aerosol (69). Oral ingestion is also possible through feed or water contaminated with mucous, feces, or urine from an infected animal. It can also be transmitted to offspring via TB contaminated milk while nursing (44). It is also important to note that the location of the lesion within the host can also affect how it is shed (43). Transcutaneous infection has also been seen as a result of infected animal bites. This has been documented in domestic cats, ferrets, and European badgers (84, 47). These animals are considered to be reservoirs of *M. bovis*.

White-tailed deer (i.e. in northern Michigan) are a known reservoir for *M. bovis* in the United States (74). Animals such as the European badger in the United Kingdom as
well as brushtail possums in New Zealand are also reservoirs of bovine TB due to their close contact with not only one another, but with confined animals while foraging for food (23, 19). Disease that is seen in reservoir animals is similar to that seen in cattle with *M. bovis* and humans with TB infection. With that said, subtle differences in the immune response and pathological progression of disease to *M. bovis* infection of possums, badgers, deer, and other hosts of *M. bovis* are seen.

With the development of genome sequencing, differences between species can be examined. *M. bovis* and *M. tuberculosis* were found to be 99.95% identical; however, *M. bovis* has lost several genes when compared to *M. tuberculosis* (46). For example, one point mutation in *M. bovis* causes it to be resistant to pyrazinamide, while *M. tuberculosis* is not susceptible. Other deletions may affect host adaptation (12). A number of differentiating features between *M. bovis* and *M. tuberculosis* have been observed through bacterial culture as well. A study by Konno and colleagues discovered *M. tuberculosis* produces nicotinic acid whereas *M. bovis* does not (59). A later study by Virtanen showed *M. bovis* does not produce nitrate reductase, which is an enzyme produced by *M. tuberculosis* and other mycobacterium species (109). It has also been demonstrated that glycerol can inhibit *M. bovis* growth, but not *M. tuberculosis*. Numerous distinguishing biochemical and metabolic features have been previously reviewed (46, 86). Although there are differentiating features, the genome similarity of *M. bovis* and *M. tuberculosis* has some advantages. Foremost was the development of tuberculin as a diagnostic tool and BCG as a possible vaccine for both *M. bovis* infection in cattle and *M. tuberculosis* infection of humans. These discoveries highlight the “One
Health” aspect of TB research and benefits of co-discovery for veterinary and medical applications (119).

Tuberculin was developed in the 1890s by Robert Koch and is a mixture of proteins that have been isolated from a Mycobacterial culture (67). Investigating veterinarians in Russia, Denmark, the United Kingdom, and the United States noticed that when an animal was injected with a preparation of glycerin extract of tubercle bacilli, Koch’s ‘Old Tuberculin’, and temperature was monitored for 24 hours, infected animals would show an increase in temperature compared to non-infected animals (64, 94, 118). This is the same tuberculin that was first used for TB skin testing in humans. The currently used tuberculins are referred to as purified protein derivatives (PPD) (67). These crude protein extracts have been through a purification process in which proteins, and other components such as lipids, that resulted in non-specific reactions have been removed (127). The purification process has resulted in a current PPD with a higher sensitivity and specificity (94, 127). However, PPDs are unable to distinguish BCG vaccination status from infection.

BCG is the designation given to an attenuated strain of *M. bovis*, the first one having been discovered by Calmette and Guérin in the early 1900s through in vitro passage of a virulent bovine isolate on potato slices (16, 40). They further examined it as a possible vaccine due to its retention of core antigens. They demonstrated that animals that received BCG and were later challenged with *M. bovis*, showed no signs of infection (64). Today several BCG vaccines have been created using various strains, processes, and number of passages of virulent *M. bovis* that results in differences at the genome level between BCG strains (10). BCG’s protective immunity is thought to depend on the
production of IFN-γ upon CD4+ T cell activation that in turn activates macrophages to kill *M. bovis* (2, 53). However, the effectiveness of BCG in preventing TB in humans and animals is controversial; it can prevent TB in children, but does not prevent reactivation of a preexisting infection in older people (22). BCG vaccination field trials have shown mixed results, ranging from 0 to 80% efficacy (22). Despite these findings, a majority of studies report that most people vaccinated with BCG will switch from skin test negative to positive (37). The United States does not BCG vaccinate due to its ability to render the skin test ineffective as a means of primary diagnosis. However, it has been shown that this positive skin test due to vaccination wanes over time (37, 101). Variations in efficacy of the vaccine as well as conversion to skin test positive have also been seen in cattle studies involving BCG vaccination (14). Overall, the failure of the BCG vaccine to give high protection rates indicate an improved vaccine is needed (39), as well as improved diagnostic assays to differentiate *M. bovis* infection from vaccination. Current diagnostic assays to detect bovine TB rely on the measurement of cell-mediated immune (CMI) responses to *M. bovis*.

**CMI response to *M. bovis***.

*M. bovis* is spread from animal to animal via both direct contact (e.g. aerosol droplets from infected animals) and indirect contact (e.g. contaminated feed, feed-bunks, equipment, etc). *M. bovis* infection begins most commonly with the inhalation of the mycobacteria where they gain passage into the pulmonary alveoli (71, 78). Alveolar macrophages recognize *M. bovis* by their pattern recognition and toll-like receptors (TLR). Currently, it is known that TLR2 can recognize 99 lipoproteins,
Phosphatidylmyo-inositol mannosides, and lipoarabinomannan that are present on the surface of *M. bovis* (9). Following binding there is internalization of the bacteria into a vesicle. In *M. tuberculosis* challenged mice it was found that once inside the macrophage TLR9 senses mycobacterial DNA and signals cytokine production in macrophages and dendritic cells (7).

Once the mycobacteria are internalized, they inhibit the macrophages from completing the phagolysosome fusion that prevents the vesicle from acidifying (102, 105). Infected macrophages experience a reduction in oxidative burst and a reduction in IL-12 production. IL-12 is necessary for stimulation of the T helper 1 (Th1) responses critical for IFN-γ production (24). The bacteria multiply within the phagosome and when the infected macrophage undergoes apoptosis, the mycobacteria are able to infect other nearby macrophages (27, 30, 105). As more macrophages become infected, the cytokine and chemokine levels increase resulting in an influx of neutrophils, inactivated macrophages, fibroblasts, and dendritic cells to the area (27, 103). This is followed by initiation of the cell mediated immune (CMI) response. This is seen by the presence of CD4+ and CD8+ T cells as well as B cells at the site of infection (41).

CD8+ T cells are important to contain the infection. They kill infected cells that have failed to destroy the bacteria (41). The importance of the CD8+ T cell response has been demonstrated by Sousa and colleagues who determined that mice lacking B2-microglobulin or CD8+ T cells are more susceptible to TB than are normal mice (100).

The CD4 T cells can be broken down into two different classes based on their lymphokine profiles (91). The Th1 cells produce interleukin-2 and IFN-γ. Th1 cells stimulate macrophage activation and are necessary for controlling *M. bovis* infections
(87). IFN-γ mediates mycobacterial killing through enhancement of phagosome maturation (65,128). The type 2 helper cells (Th2) produce interleukin 4, 13, and 10, which is not as helpful as IFN-γ in clearing infection (72). However, the role of Th2 and antibody is still unclear.

Thorn and Morris demonstrated that the immune response to *M. bovis* was primarily cell-mediated rather than humoral (107). Therefore, the development of assays measuring components of the CMI response became extensively studied. Responses by CD4 cells in controlling *M. bovis* infection have been incorporated for use in current diagnostic assays. Such responses include production of IFN-γ (122) as well as the development of memory T cells to *M. bovis* (26). IFN-γ stimulates activation of macrophages, which leads to the activation of the oxidative burst within the macrophage (3, 87). It also mediates mycobacterial killing enhancing phagosome maturation (65).

Knocking out IFN-γ production in mice resulted in increased susceptibility to TB infections (25). Studies in mice and humans have shown that IFN-γ levels correlate with disease progression (2, 34). It is also produced in high quantities in vitro, which can then be measured by an enzyme immunoassay (EIA) and is not readily consumed during short term culturing (122, 125). The importance in IFN-γ production to control infection makes it a reliable indicator of bovine TB. In addition to IFN-γ production, the memory response can be established by infection by mycobacterium, non-pathogenic environmental mycobacteria, or BCG vaccination (4). The development of memory T cells sensitized to proteins and antigens of *M. bovis* the basis of the ante-mortem tuberculin-skin test.
Current ante-mortem diagnostic tests

*M. bovis* infection elicits a robust CMI response in its host, which is predominantly T lymphocyte dependent (31). This makes ante-mortem tests detecting markers of CMI responses important in controlling the spread of bovine TB since they can identify infected animals early (66). Current ante-mortem diagnosis is dependent on the delayed type hypersensitivity (DTH) response elicited by skin testing, similar to that used in humans, and IFN-γ release assays.

Skin Test

In the United States, there are two types of procedures for skin testing in animals; the caudal fold test (CFT) and the comparative cervical skin test (CCT) (70, 129). The caudal fold skin test (CFT) is used as a primary test in herds where TB status of the animals is unknown (129) and retesting of is generally by CCT (70). A veterinarian performs the CFT by injecting PPD, which is comprised of water-soluble fractions of heat–treated products from *M. bovis* (PPDb), into the caudal tail fold (i.e., two folds of skin located under the base of the tail (5, 31, 129).

Antigen presenting cells ingest the proteins, break them down, and present parts of them on their surface (54). Circulating CD4 memory T cells will bind and activate if there has been prior exposure, or sensitization, to the antigens (107). Sensitization can occur by active or clinically resolved TB infection, cross-reactivity to mycobacterium conserved antigens, or BCG vaccination (4). Upon activation the T cells release cytokines, such as TNF-alpha (TNF-α), which signals for recruitment of monocytes, macrophages, and additional T cells to the area (54).
TNF-α activates the vascular endothelium in local blood vessels resulting in inflammatory effects. These inflammatory effects include the loosening of the tight junction between endothelial cells. This allows fluid to leak out of blood vessels causing swelling at the injection site (54). The activated endothelium also begins to express adhesion molecules, which allows phagocytes to migrate out of the blood vessels and to the injection site (54).

Leakage of fibronectin results in the deposition of fibrin at the site and causes a hardened, raised area referred to as an induration. This induration occurs 24 to 48 hours after injection, which gives the term delayed type hypersensitivity (54). The induration reaches its greatest intensity at 48-72 hours post-injection and regresses rapidly after (45, 82). The skin thickness at the site of injection is measured immediately before injection of PPDb and 72 hours post-injection (6). An animal with any measurable increase in skin thickness is considered a reactor and a secondary test is needed. If the animal has no prior exposure to *M. bovis* antigens there is no local inflammatory response and the antigens are degraded (31). This DTH responses can develop as early as one week post challenge or as late as 9 weeks after challenge (42, 31). The CFT has been demonstrated to have a sensitivity and specificity of 80.4-93.0% and 89.2-95.2% respectively (38).

A supplementary test that can be used in conjunction with the CFT or on its own is the comparative cervical skin test (CCT). The CCT is mainly used as an ancillary serial test for CFT reactors or inconclusive reactors within the United States (6, 31). Other countries, such as the United Kingdom, use the CCT as a primary test. The CCT uses two types of tuberculin, *M. avium* derived PPD (PPDa) and *M. bovis* derived PPD (PPDb). PPDs are injected separately into the cervical region of the neck (129). Skin thickness at
both injection sites are measured before injection and after 72 hours. Responses to the
two PPDs are plotted on a scattergram and interpreted as positive, negative, or suspect
(89). The CCT test when used in conjunction with the CFT test has a sensitivity and
specificity of 74.4-88.4% and 97.3-98.6% respectively (38). However, the sensitivity and
specificity of each PPD from the same manufacturer can differ from herd to herd (51).

Current concerns with skin testing include observation-bias depending on the
visiting veterinarian, false positive reactions due to conserved mycobacterial antigens,
and time delay between consecutive skin tests (31). False positives in the CFT may occur
due to exposure to non-pathogenic mycobacterium species encountered in the
environment, which results in cross-reaction due to conserved antigens (31, 103). It has
also been demonstrated that repeated testing of a herd can lead to an increase in the
number of M. bovis infected animals that do not respond to the skin test (48, 58, 67). To
overcome this effect, skin testing protocols require an interval of at least 42 days (60 days
after the initial CFT) between skin tests (6). The IFN-γ release assay addresses these
concerns and only requires one visit to the farm, provides rapid results, and can be
performed a second time without delay if necessary (122). Pollock and Neill determined
that infected animals could be detected using the IFN-γ assay even before a DTH
response developed (81).

**IFN-γ release assay**

The IFN-γ release assay has been approved as a supplemental test to the skin test for
the TB eradication and control program in the United States. It was first developed in
1985 in which whole blood was incubated with PPD and plasma supernatants taken after
24 hours for examination of IFN-γ production (122). IFN-γ was chosen because it is known to be a robust indicator of a CMI response, particularly to Mycobacterial infection. The IFN-γ release assay was first used in large scale field trials in Australia’s TB Eradication Program in 1989 and 1990 (122, 123, 113). In diagnosing bovine TB, the IFN-γ assay was determined to be significantly more sensitive than the single intradermal tuberculin test (SIDT) (123). Wood and colleagues found that out of 125 animals that tested culture positive, the IFN-γ assay was able to detect 93.6% of animals whereas the SIDT only detected 65.6% of infected animals (123). An increase in sensitivity to 95.2% was seen when both tests were used in parallel (123). In 1991 the IFN-γ test was implemented as an official diagnostic test for bovine TB in Australia (124).

The IFN-γ release assay uses PPDa and PPDb (independently in different wells) to stimulate lymphocytes within whole blood to produce IFN-γ. Quantification of IFN-γ production is measured using an by ELISA after incubation overnight with mycobacterium specific antigens. Responses are deemed positive when IFN-γ from PPDb stimulation is greater than that of PPDa stimulation (e.g., an optical density (OD) higher than 0.1 (PPDb-PPDa) at a wavelength of 450 nanometers once the nil antigen (i.e. control) is subtracted). The EIA can also measure false positive reactions, which are indicated by a greater production of IFN-γ to PPDa than to PPDb (55). When used in parallel with the intradermal skin test, the IFN-γ release assay can increase overall disease detection sensitivity (83). With the variety of IFN-γ assay protocols, the accuracy of the tests has a sensitivity and specificity of 80.9-100% and 87.7-99.2% respectively (93). IFN-γ responses can be detected as early as 14 days after challenge with *M. bovis*
It has also been reported that the IFN-γ assay can identify infection 90 to 150 days earlier than the skin test (60, 69).

Although it is costly to perform, the IFN-γ release assay results in overall cost savings by removing the necessity of holding of cattle for testing, veterinary fees, and time delays due to retesting (125). In addition, unlike the CFT and CCT, the IFN-γ response is not reduced by multiple tuberculin skin tests thereby reducing time between testing (33, 85).

The skin test and the IFN-γ release assays are very important in the detection of bovine TB and improvement of their diagnostic capabilities is needed. Using only PPDs in both the skin test and IFN-γ assay is not effective in differentiating BCG vaccination from active infection due to response elicited to conserved antigens (14, 114). With the advent of genome sequencing, it was found that *M. bovis* BCG is missing a region of genomic DNA, referred to as region of difference 1 (RD1), which is present in *M. bovis* and most other Mtbc species (73). This has led to the identification and isolation of proteins encoded within the RD1 as a means to differentiate BCG vaccination from *M. bovis* infection (114).

**Improvements of ante-mortem tests through identification and use of Mtbc-specific proteins**

In bovine TB control and eradication, the ideal ante-mortem test would have the ability to differentiate vaccinated and infected cattle (DIVA) (114). This would enable routine vaccination as a means of protection while allowing the detection of infection without false test results due to responses to conserved antigens. Target antigens for
DIVA tests are those that are present in *M. bovis*, but absent in BCG or other species of environmental mycobacteria (10, 50, 114). Vordermeier and colleagues have separated current potential DIVA reagents into two categories; antigens encoded on gene regions that were deleted from the BCG genome and antigens discovered through comparative transcriptome analysis (114).

Currently, the two main antigenic targets for both cattle and humans most commonly used in TB diagnostic assays are Early Secretory Target Antigen 6 (ESAT-6, Rv3875) and Culture Filtrate Protein 10 (CFP-10, Rv3874) (1, 11, 79). Both ESAT-6 and CFP-10 were identified using direct genome comparisons of *M. bovis* BCG and virulent *M. bovis* strains (11, 79). Genes encoding both ESAT-6 and CFP-10 are found in the RD1 region (46, 63) which enables differentiation of BCG vaccination status from active TB in both humans and cattle (68, 111, 114, 116). ESAT-6 and CFP-10 are co-secreted proteins that play a role in escape from the phagolysosome and produced during the early phase of infection (117). This allows for the early detection of immune responses by diagnostic assays (108). ESAT-6 is often used as a fusion protein or in a peptide cocktail with CFP-10 to increase test sensitivity and specificity of the IFN-γ assay (80, 111, 113, 115). Pollock and colleagues demonstrated that the fusion protein of ESAT-6 and CFP-10 could increase the sensitivity and specificity of the IFN-γ assay to 76.3% and 99.2% respectively (80). Additionally, in naturally infected cattle, it was found that peptide cocktails containing a mixture of peptides derived from the amino acid codes of ESAT-6 and CFP-10 could be used in place of recombinant proteins with comparable efficacy (111). Vordermeier and colleagues further demonstrated that BCG vaccinated animals
did not respond to the peptide cocktail of ESAT-6 and CFP-10 whereas 70% responded to bovine tuberculin using the IFN-γ assay (111).

The ability to identify peptide pools by genetic comparison and analysis has allowed researchers to combine peptides from various regions of virulent *M. bovis* genomes into cocktails to improve diagnostic sensitivity (20, 21, 56). Cockle and colleagues evaluated 28 potential antigens encoded on the RDs absent from BCG Pasteur, (RD1, RD2, and RD14), but present in virulent *M. bovis* for immunogenicity (20). They further compared synthesized peptide pools of two proteins from RD1, Rv3873 and Rv3879c, and two ESAT-6 like proteins, Rv0288 (TB10.4), and Rv2019c based on their ability to elicit a robust IFN-γ response (21, 98). They then selected the sequences from the peptide pools with the most robust IFN-γ responses to incorporate into a peptide cocktail with ESAT-6 and CFP-10 (21). Although they did not investigate DIVA potential, they did find that the addition of these peptide sequences to a peptide cocktail with ESAT-6 and CFP-10 enhanced the sensitivity of the assay to diagnose infection in animals missed by skin testing (21, 114).

Comparative transcriptomics has allowed scientists to assess the transcriptomes of *M. bovis* BCG and virulent *M. bovis* after the infection of macrophages using genome data and DNA microarrays (114). This has allowed the quantification and comparison in the level of gene expression between the two strains (56, 95, 114). Sidders and colleagues refer to the increased expression of genes as the ‘abundant invariome’ (95). The abundant invariome consists of a population of gene products that were expressed at high levels by virulent *M. bovis*, but not *M. bovis* BCG under various culture conditions (56, 95). One member of this group was Rv3615c. Sidders and colleagues demonstrated that when
incorporated into the IFN-γ release assay, Rv3615c was able to differentiate *M. bovis-*infected cattle from BCG vaccinated cattle (96). They also noted that some animals that did not have responses to the peptide cocktail containing ESAT-6 and CFP-10 yet responded to Rv3615c which suggested the target antigens were identified through different mechanisms (56, 96). In the IFN-γ assay, incorporating Rv3615c into a peptide cocktail with ESAT-6 and CFP-10 resulted in increased sensitivity without a loss in specificity (114, 119). When used in the single comparative cervical intradermal skin test (SICCT), the response to a protein cocktail consisting of Rv3615c, ESAT-6, and CFP-10 were comparable to those elicited by bovine PPD (17). Jones and colleagues screened 119 secreted, or potentially secreted, proteins that could serve as a DIVA reagent for the IFN-γ assay (56). They found three potential pools, Rv2346c, Rv3020c, and Sec2 (which consisted of a cocktail of nine peptides derived from multiple antigens) (56). Although the Rv2346c and Rv3020c sequences were present in both the BCG and *M. bovis* genome, it is thought that the gene transcription of these two proteins may vary between BCG and virulent *M. bovis* (56, 88).

Jones and colleagues further demonstrated that the incorporation of both Rv2346c and Rv3020c into a peptide cocktail with ESAT-6, CFP-10, and Rv3615c resulted in increased sensitivity and comparable specificity as bovine PPD when used in the SICCT as well as the ability to perform as a DIVA reagent (57). Evaluations using this peptide cocktail in the IFN-γ assay were inconclusive as animals were under 6 months of age and therefore had non-specific IFN-γ responses that interfered with the ability to differentiate responses (57). Other proteins that are present in both *M. bovis* BCG and virulent *M. bovis* that have potential as DIVA reagents are *M. bovis* protein 70 (MPB70), and MPB83.
It has been demonstrated previously, using an antibody-capture ELISA, that MPB70 and MPB83 have a high specificity in detecting infection, but a low sensitivity (62, 126). When MPB70 and MPB83 proteins were tested individually and in combination with ESAT-6 and CFP-10 using the IFN-γ assay and skin test, there was a decrease in both sensitivity and specificity when compared to bovine PPD using naturally infected animals (120).

Overall, the consensus is the same; a cocktail of defined immune-dominant peptides can result in a novel diagnostic reagent that has the ability to detect \textit{M. bovis}-infected animals that were deemed negative by skin tests (21, 56, 96, 114).

\textit{Incorporation of \textit{Mtb}c specific antigens and evaluation of PPDs in the IFN-γ release assay}

Incorporation of \textit{M. bovis}-specific antigens has the potential to increase the diagnostic accuracy of the IFN-γ release assay (21) and may provide DIVA capacity (114). The IFN-γ assay is rapid to perform, has increased sensitivity compared to the skin test, and minimizes observational variability associated with assessing skin test reactions (31, 92, 113). However, BCG vaccination has been found to compromise the specificity of the tuberculin in the IFN-γ tests (13, 14,110, 114). The IFN-γ assay is an ideal means to DIVA since it can be readily modified to accommodate various antigens unlike the tuberculin skin test (114). When DIVA reagents were incorporated into the skin test, it was found to require an impractical amount of recombinant proteins (81) and the inclusion of adjuvants to induce measurable responses (119, 114). By incorporating them
into the IFN-γ assay, the need for large amounts of Mtb-specific antigens is generally not necessary (114).

The standard use of the IFN-γ assay is performed using whole blood that is stimulated for 16-24 hours with PPDa, PPDb, media alone (no stimulation), and a mitogen control for cell viability. IFN-γ produced within the stimulated samples is then measured by an ELISA. Infection status is determined by subtracting the no stimulation response from all other responses and then measuring the differential amounts of IFN-γ produced in response to PPDb versus PPDa. Currently, there are several PPDs on the market for IFN-γ release assays.

PPDs from different manufacturers and lots are known to vary in potency (92). PPDs undergo different production processes, which causes this variation (8, 15, 48). Whipple and colleagues compared Commonwealth Serum Laboratory (CSL, New Zealand) PPDs to those produced by the United States (US) Animal and Plant Health Inspection Service using both the skin test and IFN-γ assay (121). They found that both CSL and US PPDs were able to detect M. bovis-infected and non-infected animals, but US PPDs elicited a higher response (121). Similarly, Downs and colleagues reviewed data from field surveillance results of SICCT using Prionics (Lelystad) PPD and Central Veterinary Laboratory (Weybridge) PPD (2013). The compilation and analysis of the surveillance results revealed that Lelystad PPDs were superior to Weybridge PPDs in confirming infection within herds and individual animals using the SICCT (35). These studies demonstrate the differences between PPDs and the effect it can have on detecting infected cattle using ante-mortem testing. In order to improve diagnostic accuracy,
evaluation of PPDs from different manufacturers needs to be examined before incorporation into ante-mortem testing.

References:


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CHAPTER 2: Comparison of Peptide Cocktails and Purified Protein Derivatives for Use in the Bovigam™ Assay.

A paper waiting review by co-authors for submission

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Abstract

Currently the Bovigam assay is used as an official supplemental test within the bovine tuberculosis eradication program. This assay measures interferon-gamma (IFN-γ) produced by lymphocytes in response to specific antigens. The objectives of the present study were to evaluate two Mycobacterium bovis specific peptide cocktails, purified protein derivatives (PPDs) from two sources, liquid and lyophilized antigen preparations, and a second generation IFN-γ release assay (Bovigam, B2G, Prionics AG). Three strains of M. bovis were used for experimental challenge: M. bovis 95-1315, M. bovis Ravenel, and M. bovis 10-7428. Additionally, samples from tuberculosis-affected herds (i.e. natural infection) were evaluated. Robust responses to both peptide cocktails HP (PC-HP) and ESAT6/CFP10 (PC-EC), as well as PPDs were elicited as early as three weeks after challenge. Only minor differences in responses to Commonwealth Serum Laboratories (CSL) and Lelystad PPDs were detected with samples from experimentally infected
animals. For instance, responses to Lelystad *M. avium* derived PPD (PPDa) exceeded respective response to CSL PPDa in *M. bovis* Ravenel infected and control animals. However, 1:4 dilution of stimulated plasma demonstrated greater separation of PPDb from PPDa responses (i.e., PPDb – PPDa) with use of Lelystad PPDs, suggesting that Lelystad PPDs provide greater diagnostic sensitivity than CSL PPDs. With samples from tuberculosis-affected herds, responses to Lelystad PPDs generally exceeded respective responses to CSL PPDs. Responses to lyophilized and liquid antigen preparations did not differ. Responses detected with first (B1G) and second (B2G) generation IFN-γ release assay kits (Bovigam) did not differ throughout the study. In conclusion, antigens may be stored in a lyophilized state without a loss in potency; PC-HP and PC-EC are dependable biomarkers for aiding diagnosis of bovine tuberculosis, and second generation Bovigam kits have sensitivity/specificity comparable to current kits.

**Introduction**

*Mycobacterium bovis* is the primary causative agent of bovine tuberculosis in cattle. *M. bovis* is a member of the *Mycobacterium tuberculosis* complex (Mtbc), which also includes *M. tuberculosis*, *M. caprae*, *M. microti*, *M. africanum*, *M. canettii*, and *M. pinnipedii* (1). Mtbc species are similar to one another in their ability to cause tuberculosis infections; however, their host range varies as well as their virulence between hosts. *M. bovis* has the largest host range of the Mtbc species infecting wildlife as well as alternative and domestic livestock making it difficult to control (2). In many developed countries, cattle herds are monitored for bovine tuberculosis using slaughterhouse surveillance and ante-mortem testing. Ante-mortem testing is primarily based upon measures of cell-mediated immunity such as tuberculin skin test (e.g. caudal
fold test (CFT) or single intradermal comparative cervical (SICCT)) and interferon-gamma (IFN-γ) release assays (e.g., Bovigam, Prionics AG, Schlieren, Switzerland) (3).

In countries where annual skin testing is largely absent, such as tuberculosis-free states within the United States, most infected cattle go unnoticed until a lesioned animal is detected by slaughterhouse surveillance (4). After infection is detected, the movement history is investigated to determine other contact herds and the origin of infection (3). Affected herds may be depopulated or a test and slaughter approach is applied using skin test and/or the IFN-γ release assay. Additionally, emerging serologic tests (i.e. antibody based tests) are being evaluated for use in tuberculosis control programs. Although these tests are relatively accurate, improvement of ante-mortem diagnostic methods is needed.

The IFN-γ release assay was developed to aid in the diagnosis of bovine tuberculosis and is currently used mainly as a supplemental assay to the skin test in most TB eradication/control programs. IFN-γ is produced in high quantities in vitro and is not readily consumed during short-term culture (5, 6) making it a good biomarker for use in tuberculosis diagnostic tests (7). The standard use of the IFN-γ assay is performed by stimulating whole blood for 16 to 24 hours with *M. bovis* purified protein derivative (PPDb), *M. avium* PPD (PPDa), media alone (no stimulation), and a mitogen control for cell viability (6). IFN-γ produced within the stimulated samples is then measured by ELISA. Infection status is determined by comparing differential amounts of IFN-γ produced in response to PPDb and PPDa stimulation.

When compared to the skin test, the IFN-γ assay has increased sensitivity, requires a single visit to the farm, and prevents observational variability associated with assessing skin test reactions (8, 3, 9). With the use of PPDs, the sensitivity and specificity
of the IFN-γ assay is estimated at 73-100%, and 87.7-99.2%, respectively (4,9).
Implementing TB-specific antigens into the IFN-γ assay may increase the accuracy of the test (10) and may provide differentiation of infected from vaccinated animals (DIVA) (11). Early Secretory Antigen Target 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP10) are TB-specific proteins that have been extensively studied for use in the detection of bovine tuberculosis (12, 13, 10, 14). When used as recombinant proteins or peptide cocktails, ESAT6 and CFP10 are known to elicit an immune response; both in vitro (13, 15, 14) and in vivo (16), in M. bovis infected cattle. Aagaard and colleagues (10) demonstrated decreased sensitivity (86% vs. 97%) yet increased specificity (99% vs. 94%) when comparing an ESAT6 and CFP10 peptide cocktail versus PPDs as antigens for use in the IFN-γ assay. This increased specificity elicited by the ESAT6 and CFP10 peptide cocktail is promising; however, more research is needed to determine methods to increase sensitivity (17). For instance, use of ESAT6 and CFP10 in combination with Rv3615 in the IFN-γ assay has been shown to increase sensitivity without loss of specificity (18).

Objectives of the current study were to compare IFN-γ responses elicited by Commonwealth Serum Laboratories (CSL) and Lelystad PPDs (Prionics AG), two Mtbc-specific peptide cocktails, as well as liquid and lyophilized preparations. Studies were performed using samples from three groups of cattle, each experimentally infected with a different strain of M. bovis, as well as samples from tuberculosis-affected herds (i.e. natural infection) within the United States. Strains for experimental infection included: M. bovis Ravenel (laboratory adapted strain attenuated in cattle), M. bovis 95-1315 (white-tailed deer isolate), and M. bovis 10-7428 (cattle field isolate from a Colorado
dairy herd). PPDs and peptide cocktails were also used to compare a second-generation Bovigam kit to the currently available kit.

**Materials and Methods**

*Calves, aerosol challenge, and necropsy*

For the first study, 15 nine-month old Holstein castrated male cattle were housed in a BSL-3 containment facility in Ames, IA at the National Animal Disease Center. All animal care and use procedures were reviewed and approved by the NADC Animal Care and Use Committee. Treatment groups included *M. bovis* 95-1315 inoculated cattle (10^5 cfu, n=5), *M. bovis* Ravenel (10^5 cfu, n=5, ATCC strain 35720, kind gift from John Chan, Albert Einstein College of Medicine, NY) inoculated cattle, and a non-infected control group (n=5). For the second study, 23 six month old Holstein castrated male cattle were housed as above. Treatment groups included *M. bovis* 95-1315 (5 x 10^4 cfu, n=8), *M. bovis* 10-7428 (5 x 10^4 cfu, n=8), and a non-infected control group (n=7). Strains were prepared using standard procedures (19) in Middlebrook 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% oleic acid-albumin-dextrose complex (OADC) plus 0.05% Tween 80 (0.5% Glycerol included for strain Ravenel only). Enumeration of *M. bovis* challenge inocula, necropsy procedures (~3.5 months after challenges) gross and microscopic assessment of lesions, as well as mycobacterial culture of *M. bovis* from tissues were performed as described (20, 15). The inoculation of each of the strains in both studies was via aerosol as described (20). Additionally, whole blood samples were obtained from TB-affected herds in California (2 separate herds), Colorado (1 herd), and Washington (1 herd).
**Antigens**

PPDs and peptide cocktails were provided by Prionics AG (Schlieren, Switzerland) in liquid and lyophilized forms. RPMI 1640 medium (Life technologies, Grand Island, NY) was used as a negative control and pokeweed mitogen (PWM, 5ug/ml; Sigma-Aldrich, St. Louis, MO) was included as a positive control of cell viability. The two peptide cocktails consisted of ESAT-6 and CFP10 (PC-EC, Prionics AG) and ESAT6, CFP10, Rv3615, and three other mycobacterium antigens (PC-HP, Prionics AG). Aliquots of liquid preparations of PC-HP and PC-EC were kept frozen at -20°C until the day of use. PC-HP and PC-EC were used according to insert instructions. CSL PPDa and CSL PPDb were used at a concentration of 20ug/ml and Lelystad PPDa and Lelystad PPDb at 200IU and 250IU, as recommended by the manufacturer. All antigens were diluted in RPMI 1640 medium.

**Whole blood stimulation**

For the first study, whole blood was collected in sodium-heparinized tubes two week prior to aerosol challenge and at 3, 6, 8, 11, and 12 weeks post-challenge. On the day of blood collection 250μl of blood was added to 96-well plates from each animal. Twenty-five microliters of each antigen (PPDs and peptide cocktails) and controls (e.g. RPMI 1640 and PWM) were added in duplicate to selected wells. Plates were incubated at 39°C with 5% CO₂ for 18-22 hours. Plasma was harvested from each stimulation for each animal, and placed in individual microtubes (VWR Scientific, Radnor, PA) and stored at -80°C until analyzed for detection of IFN-γ via the Bovigam assay. For the second study, blood was collected at 2 weeks pre challenge as well as 2, 3, 4, 6, 8, and 12
weeks post-challenge. Whole-blood stimulation procedures were the same as the first study.

*IFN-γ detection*

In the first study, IFN-γ assays were performed using the first generation Bovigam kit (Prionics AG). In the second study, first and second generation Bovigam kits were used. All IFN-γ release assays were performed according to kit procedures. Each plasma sample and standard was tested in duplicate. Recombinant bovine IFN-γ (Thermo Scientific, Rockford, IL) was used as a standard at 12.5 ng/ml, 6.25 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, and 0.3125 ng/ml, along with the positive and negative controls supplied by the manufacturer. Samples were read at a wavelength of 450nm using a SPECTRAmax 340 microplate reader (Molecular Devices, Sunnyvale, CA) and analyzed using SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) to calculate optical density (OD). Responses to negative control (RPMI 1640) for each animal were subtracted from all antigen and mitogen responses. Results were considered positive if responses to PPDb (OD_{450}) minus PPDa (OD_{450}) were above 0.1OD.

*Tuberculin skin test procedures*

Skin tests were performed as specified in the circular on uniform methods and rules for the eradication of bovine tuberculosis in the United States (Animal and Plant Health Inspection Service, APHIS circular 91-45-011) (21). At 10 weeks (study 1) or 12 weeks (study 2) post-challenge, skin thickness was measured with calipers immediately before intradermal injection of PPDa and PPDb. The skin thickness was measured again after 72 hours. The skin test was performed in the mid-cervical region (i.e, comparative
cervical test). Responses were recorded on a scattergram for interpretation of comparative cervical test to determine the outcome of the test (i.e., negative, suspect or reactor).

*Cattle pathogenesis studies: bacterial recovery and assessment of lesions*

All calves were euthanized ~3.5 months after challenge by intravenous administration of sodium pentobarbital. Tissues were observed for gross lesions and collected and processed for microscopic analysis and isolation of *M. bovis*. Tissues collected included: palatine tonsil; lung; liver; and mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, and mesenteric lymph nodes. Lymph nodes were sectioned at 0.5 cm intervals and examined. Each lung lobe was sectioned at 0.5 – 1.0 cm intervals and examined separately.

Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut in 5 μm sections and stained with hematoxylin and eosin. Adjacent sections from samples containing caseonecrotic granulomata suggestive of tuberculosis were excised and stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria.

Isolation of *Mycobacterium bovis* from cattle tissues were as described (22). Briefly, tissues were macerated in phenol red nutrient broth using a blender (Oster, Shelton, CT). Homogenates were decontaminated with NaOH, and then neutralized with HCl. Samples were centrifuged, supernatant removed, and pellet used to inoculate media (Middlebrook 7H10, 7H11, and 7H11 with antibiotics). Plates were incubated at 37°C and examined weekly for growth.
Statistics

Results were analyzed using Graph Pad Prism (Software, La Jolla, CA). Optical densities were converted to ng/ml using the standard curve calculated from standards observed on each plate for each date. Data were analyzed using repeated measures analysis of variances with Bonferroni multiple comparisons test and Mann-Whitney T test.

Results

Experimental infection

Ten to 12 wks after challenge, all cattle receiving *M. bovis*, regardless of strain (i.e., Ravenel, 95-1315, 10-7428), were classified as reactors based upon standard interpretation of the comparative cervical skin test. Delayed type hypersensitivity responses were not detected (i.e., classified as negative) with any of the animals within the non-infected control group. Upon necropsy, only one *M. bovis* Ravenel-inoculated calf had a single small granuloma in the left caudal lung lobe. In contrast, all *M. bovis* 95-1315- and *M. bovis* 10-7428-inoculated cattle had granulomatous lesions in lungs and lung-associated lymph nodes. *M. bovis* was isolated from 4 of the 5 cattle inoculated with *M. bovis* Ravenel and all cattle receiving *M. bovis* 95-1315- or *M. bovis* 10-7428. *M. bovis* was not isolated from cattle within the non-infected group. Findings are consistent with the observation that *M. bovis* Ravenel is attenuated in cattle (Waters / Palmer, personal observations) and *M. bovis* strains 95-1315 and 10-7428 are fully virulent.

Comparison of liquid and lyophilized antigen preparations
IFN-γ responses to liquid and lyophilized preparations were detected in *M. bovis* 95-1315 and *M. bovis* Ravenel inoculated animals as early as three weeks after challenge (Fig. 1, *P* < 0.05). When all time points were pooled, responses to liquid and lyophilized antigen preparations by *M. bovis* Ravenel and *M. bovis* 95-1315 challenged animals to PPDb, PC-HP, and PC-EC exceeded (*P* < 0.05) respective responses by control animals (Fig. 1b). Significant differences were not detected between liquid and lyophilized products for any antigens within each treatment group (Fig. 1b). As utilized for official use of the Bovigam test kit, responses to PPDb-PPDa did not differ (*P* > 0.05) for liquid and lyophilized antigen preparations at any time point within each treatment group (Fig. 1c).

IFN-γ responses to PPDb exceeded (*P* < 0.01) respective responses to PPDb for control animals (Fig. 1b), demonstrating prior sensitization to non-tuberculous mycobacteria (NTM). Responses to Mtbc-specific antigens (PC-HP and PC-EC) were not detectable prior to challenge (Fig. 2a), and subtraction of PPDb responses from PPDb (PPDb-PPDa) resulted in values less than 0.1 ΔOD (i.e., negative) for all animals prior to challenge (Fig. 1c). Thus, responses to PPDb detected prior to challenge in all groups (Fig 1a) were likely due to cross-reactive responses elicited by NTM.

*Evaluation of peptide cocktails for the detection of bovine tuberculosis*

As early as three weeks after challenge, responses to PC-HP and PC-EC by *M. bovis* Ravenel and *M. bovis* 95-1315 inoculated animals exceeded (*P* < 0.05) respective pre-challenge responses and responses by control animals (Fig. 2a). When all time points were pooled, there was no difference (*P* > 0.05) between responses to PC-HP and PC-EC within treatment groups (Fig. 2b). Similar responses were detected in the second study
using *M. bovis* 95-1315 and *M. bovis* 10-7428 challenged animals, and responses to PC-HP and PC-EC did not differ within treatment groups (Supplemental Fig 1). These findings demonstrate that responses to PC-HP and PC-EC are equivalent for the early detection of *M. bovis* infection in cattle.

*Comparison of IFN-γ responses to Lelystad and CSL PPDs in experimentally and naturally infected animals*

Lelystad and CSL PPDs are commonly used as antigens in the Bovigam assay for the detection of *M. bovis* infection in cattle (23, 24); however use of these antigens within IFN-γ release assays have yet to be directly compared. Experimental infection with *M. bovis* Ravenel, *M. bovis* 95-1315, and *M. bovis* 10-7428 elicited robust IFN-γ responses to both CSL and Lelystad PPDs (Fig. 3 and Supplemental Fig. 2). When pooled over time, responses to Lelystad PPDa exceeded (*P* < 0.05) responses to CSL PPDa within the control and *M. bovis* Ravenel treatment groups (Fig. 3a). Similarly, responses to Lelystad PPDb exceeded (*P* < 0.05) that of CSL PPDb in control animals when pooled (Fig 3a).

Experimental infection of cattle with *M. bovis* results in robust IFN-γ responses to both PPDb and PPDa (due to cross-reactivity of antigens) that are often at the maximum detection limit of the assay; thereby limiting the comparisons of PPD potencies and diagnostic potential. Thus, several dilutions of stimulated plasma were evaluated at select time points to further evaluate the kinetics of the response and potency comparison of the two PPDs (Table 1, Fig. 4). When plasma from experimentally-infected animals was diluted, it was determined that the majority of responses after challenge to PPDa were lower than the response to PPDb, giving a difference greater than 0.1 OD$_{450}$ indicating a
positive test result (Table 1, Fig 4). When diluted, responses to Lelystad PPDb-PPDa exceeded \(P < 0.05\) responses to CSL PPDb-a respectively (Figure 4). In general, diluting the plasma enabled accurate detection of \(M. bovis\) infection in experimentally-infected animals in addition to reducing the false positive test results within the control group.

To further compare CSL and Lelystad PPDs, samples were obtained from four tuberculosis affected dairy herds (California herd 1, n=710; California herd 2, n=78; Washington, n=11; Colorado, n=126) in the United States. All animals were considered tuberculosis-exposed, as cattle that were positive by skin test had been removed from each of the herds, excluding the Colorado herd that included both tuberculosis-exposed and -infected animals. In contrast to experimental infection studies in which samples were placed in culture with antigen within two hours of blood collection, samples from naturally exposed animals were shipped overnight and set up within 20 hours of blood collection. With samples from California and Washington, responses to Lelystad PPDa exceeded \(P < 0.01\) that of CSL PPDa, and responses to CSL PPDb exceeded \(P < 0.01\) that of Lelystad PPDb (Fig. 5 a). Also with PPDb-PPDa calculations, CSL exceeded \(P < 0.01\) that of Lelystad (Fig. 5 a). With samples from the Colorado dairy, responses to Lelystad PPDs exceeded \(P < 0.01\) those to CSL PPDs, including PPDb-a (Fig. 5b).

*Performance of second-generation Bovigam kits (B2G) in comparison to the currently licensed kit (B1G).*

B1G and B2G kits were compared using experimentally infected animals (Fig. 6). IFN-\(\gamma\) responses were not different \(P > 0.05\) between the two kits when time points
were pooled (Fig. 6a). B2G kits were just as effective in the differentiation of control and infected groups when PPDb-PPDa calculations were performed throughout the course of the study (Fig 6 b,c,d).

Discussion

IFN-γ is a reliable biomarker for use in the detection of *M. bovis* infection. With the current use of the Bovigam assay, antigens are provided within the kit as a liquid preparation. In this study, lyophilization had no effect on antigen performance. Responses were detected as early as three weeks post-challenge with both liquid and lyophilized antigen preparations. These findings indicate that lyophilized preparations may be implemented into the IFN-γ assay without loss in potency, which could potentially increase the shelf-life of the test kits. Additional TB-specific antigens are needed to further increase the accuracy of the IFN-γ release assay. In experimentally infected animals, responses to PC-HP and PC-EC were detectable as early as three weeks post-challenge. PC-HP and PC-EC are commercially available products that may prove useful as antigens for bovine tuberculosis test kits, both for research and diagnostic purposes.

PPDs from differing manufacturers and lots are known to vary in potency (8). Commonwealth Serum Laboratories (CSL) has been the producer of PPDs for the Bovigam assay since the test was first approved for use in the United States. Due to manufacturing and marketing reasons, it is possible that CSL PPDs will not be available in the near future. Thus, it is critical to validate use of different PPDs within bovine tuberculosis diagnostic tests. Whipple and colleagues performed direct comparisons between CSL PPDs and PPDs prepared in the United States (USDA, APHIS, National
Veterinary Services Laboratory) using the CFT and IFN-γ assay and found the final interpretation of the test (i.e. positive and negative animals identified by each PPD) was usually the same. However, United States PPDs elicited a higher response (25). Recently Lelystad (Prionics AG) was adopted for use in the skin test and Bovigam assay in the United Kingdom, Republic of Ireland, and various other European Union countries.

Downs and colleagues reviewed data from field surveillance results of single intradermal comparative cervical skin test (SICCT) using Lelystad and Weybridge (Central Veterinary Laboratory) PPDs in England, Scotland, and Wales from 2005 to 2009 (26). Due to financial reasons, Defra halted production of tuberculin at Weybridge and their tuberculin supply was exhausted in 2009 (26). Lelystad became the sole source of tuberculin in Great Britain; however, there was no data directly comparing the two sources of PPDs. Compilation and analysis of field surveillance results determined that Lelystad PPDs were superior to Weybridge PPDs in confirming infection within herds and individual animals using the SICCT (26). Lelystad has been used in IFN-γ release assays since 2007 (27); however, direct comparison to CSL PPDs has not been performed. In the present study, IFN-γ responses to Lelystad and CSL PPDs largely did not differ in un-diluted samples from experimentally infected cattle. (Fig. 3 and Supplemental Fig. 2). However, use of diluted sera (1:4) revealed greater differentiation of PPDb from PPDa responses (i.e., PPDb-PPDa, Fig. 4) elicited after experimental *M. bovis* infection. With samples from tuberculosis-affected herds, responses to Lelystad PPDs generally exceeded respective responses to CSL PPDs.

Experimental infection of cattle resulted in IFN-γ responses to both PPDb and PPDa that reached the maximum limit of detection resulting in a PPDb-PPDa responses
of less than 0.1 ΔOD (i.e. negative) even though these animals were clearly infected as evidenced by robust IFN-γ responses to Mtbc peptide cocktails, delayed type hypersensitivity responses to PPDb exceeding that of PPDa, and isolation of *M. bovis* from tissues at necropsy. Thus, differences between control and infected animals were not detectable using the PPDb-PPDa analysis with undiluted stimulated plasma. To address this issue, dilutions of plasma were evaluated to ascertain IFN-γ concentrations detectable within assay limits. After dilution (1:4), samples from infected animals had responses to PPDb that exceeded (*p < 0.05*) respective responses to PPDa, thus enabling an accurate diagnosis using PPDb-PPDa calculations. Dilution of plasma may be incorporated as a means to determine differences in IFN-γ responses to PPDb and PPDa. This may be particularly useful in regions where detection of bovine tuberculosis is hindered by high levels of exposure to NTM that elicit cross-reactive responses to PPDs. Additionally, evaluation of diluted samples may be useful when evaluating responses by animals with very robust responses as seen with experimentally-infected animals in this study. Indeed, use of diluted plasma revealed responses to Lelystad PPDs exceeding that of CSL PPDs with samples from experimentally-infected cattle. These findings suggest that use of Lelystad PPDs will provide increased diagnostic sensitivity than CSL PPDs.

In the current study, three strains of *M. bovis* were used to compare IFN-γ responses to PPDs and peptide cocktails elicited after experimental infection. Using the IFN-γ release assay, robust responses were detected using PPDs from the two different manufacturers as well as PC-HP and PC-EC. Strains examined were *M. bovis* 95-1315 (white-tailed deer field isolate), *M. bovis* Ravenel (laboratory adapted strain) and *M. bovis* 10-7428 (cattle field isolate from a Colorado dairy). *M. bovis* strain 95-1315 has
been used previously in experimental infection of cattle and has been shown to elicit robust immune responses as well as granulomatous lesions at necropsy (15). *M. bovis* strain Ravenel was isolated in the early 1900s and although it is virulent in mice (28), rabbits (29; 28), and guinea pigs (30; 28; 13), it does not lead to progressive disease in cattle (Waters and Palmer, unpublished observations). These findings demonstrate that *M. bovis* Ravenel is attenuated in cattle. *M. bovis* strain 10-7428 was isolated from a Holstein cow in a dairy herd in Colorado. This strain is speculated to be highly virulent given the rapid rate of progression of disease in this herd (Tolani Francisco, personal communication). In the present study, all *M. bovis* 95-1315 animals had granulomatous lesions, whereas only one *M. bovis* Ravenel animal had a small tuberculous lesion. In the second study, all animals in the *M. bovis* 95-1315 and *M. bovis* 10-7428 groups showed lesions upon necropsy as well as robust skin test responses. Thus, evaluation of IFN-γ responses to various antigen preparations were evaluated with samples from animals inoculated with 3 different strains of varying virulence.

PPDs and peptide cocktails were also used for comparison of two generations of Bovigam kits. The second generation kit has a decreased number of repeated washes and combined the conjugate and chromogen into one solution; however, stimulation and incubation times within the test did not change. Although the new kit did not decrease the performance time, it did reduce the time in work labor. Present findings support the use of the second generation Bovigam kit (B2G) for replacement of the current kit (B1G) in the detection of bovine tuberculosis.

In conclusion, lyophilized PPDs, PC-EC, and PC-HP are an effective replacement of liquid antigens for use in the Bovigam assay. PC-HP and PC-EC are reliable
biomarkers of bovine tuberculosis and second generation Bovigam kits perform similarly as first generation kits. Present findings, while not definitive, are encouraging for replacement of CSL PPDs with Lelystad PPDs for use in the Bovigam assay for the detection of bovine tuberculosis. These findings, along with future evaluation of naturally infected animals, have the potential to increase the accuracy of current ante-mortem diagnostic methods in the detection of bovine tuberculosis.

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References:


Figure 1: Comparison of IFN-γ responses to liquid and lyophilized antigen preparations from control (n=5), as well as *M. bovis* Ravenel (n=5), and *M. bovis* 95-1315-infected (n=5) animals. a) Response kinetics: PPDb minus no stimulation. b) Liquid and lyophilized antigen preparations pooled over time. c) Response kinetics: PPDb-PPDa. Responses to pokeweed mitogen (PWM) are included as a positive control for cell viability.
Figure 2: Comparison of IFN-γ responses to PC-HP and PC-EC in control (n=5) as well as, *M. bovis* Ravenel (n=5), and *M. bovis* 95-1315 infected animals. a) Responses over time b) Pooled over time.
Figure 3: IFN-γ responses upon experimental infection with *M. bovis* to CSL (Commonwealth Serum Laboratory) and Lelystad (LEL) PPDs. a) Comparison of IFN-γ responses to PPDs by control (n=5) as well as, *M. bovis* Ravenel (n=5), *M. bovis* 95-1315 infected (n=5) animals pooled over time. b) Comparison of IFN-γ responses to PPDs by control (n=7) as well as, *M. bovis* 95-1315 (n=8), and *M. bovis* 10-7428 infected (n=8), animals pooled over time. PPDa refers to *M. avium* derived PPD, PPDb refers to *M. bovis* derived PPD, and B-A indicates values for PPDb-PPDa
Table 1: Number of positive animals per group after challenge using CSL and Lelystad PPDs when samples were not diluted and diluted.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>PPD-PPDa</th>
<th>Control</th>
<th>M. bovis 95-1315 3 weeks post challenge</th>
<th>M. bovis 10-7428</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>CSL</td>
<td>1/7</td>
<td>4/8</td>
<td>5/8</td>
</tr>
<tr>
<td></td>
<td>Lelystad</td>
<td>1/7</td>
<td>4/8</td>
<td>4/8</td>
</tr>
<tr>
<td>1:4</td>
<td>CSL</td>
<td>0/7</td>
<td>7/8*</td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td>Lelystad</td>
<td>0/7</td>
<td>7/8*</td>
<td>8/8</td>
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4 weeks post-challenge

<table>
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<th>Dilution</th>
<th>PPD-PPDa</th>
<th>Control</th>
<th>M. bovis 95-1315 3 weeks post challenge</th>
<th>M. bovis 10-7428</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>CSL</td>
<td>1/7</td>
<td>6/8</td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td>Lelystad</td>
<td>0/7</td>
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</tr>
<tr>
<td>1:4</td>
<td>CSL</td>
<td>0/7</td>
<td>6/8*</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>Lelystad</td>
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<td>7/8*</td>
<td>8/8</td>
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6 weeks post-challenge

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<th>M. bovis 95-1315 3 weeks post challenge</th>
<th>M. bovis 10-7428</th>
</tr>
</thead>
<tbody>
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<td>6/8</td>
<td>6/8</td>
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<tr>
<td></td>
<td>Lelystad</td>
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<td>7/8</td>
</tr>
<tr>
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<td>CSL</td>
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<td>7/8*</td>
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</tr>
<tr>
<td></td>
<td>Lelystad</td>
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8 weeks post-challenge

<table>
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<th>M. bovis 95-1315 3 weeks post challenge</th>
<th>M. bovis 10-7428</th>
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<tbody>
<tr>
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<tr>
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12 weeks post-challenge

<table>
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<th>Control</th>
<th>M. bovis 95-1315 3 weeks post challenge</th>
<th>M. bovis 10-7428</th>
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<tbody>
<tr>
<td>Normal</td>
<td>CSL</td>
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<td>6/8</td>
</tr>
<tr>
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<td>Lelystad</td>
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<tr>
<td></td>
<td>Lelystad</td>
<td>0/7</td>
<td>8/8</td>
<td>8/8</td>
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</tbody>
</table>

Animals were considered positive when PPDa responses are subtracted from PPDb responses and the difference in OD$_{450}$ was greater than 0.1.

*Some animals within the experimentally infected groups were still at the maximum limit of detection making it difficult to detect differences between PPDb and PPDa.
Figure 4: Comparison of PPDs using plasma dilutions at select time points after challenge and pooled over time to detect differences. a) Control (n=7). b) *M. bovis* 95-1315 challenged animals (n=8). c) *M. bovis* 10-7428 challenged animals. Data are presented as responses to PPDb minus PPDa (B-A).
Figure 5: Evaluation of IFN-γ responses in samples obtained from dairy cattle within tuberculosis-affected herds (i.e. natural infection). Comparison of CSL (Commonwealth Serum Laboratories) and Lelystad (Prionics AG) *M. avium* derived PPD (PPDa) and *M. bovis* derived PPD (PPDb). a) Data were pooled from two outbreaks in California (n=710 and n=73), and one in Washington (n=11). All animals were considered tuberculosis-exposed, as tuberculosis-infected animals had been removed from these herds. Samples from animals with no stimulation responses exceeding responses to PPDa and PPDb were excluded (n=10). b) Data were pooled from infected and exposed animals from Colorado. Animals were categorized based on positive culture of *M. bovis* (Infected, n=56) or having been in contact with known infected animals (Exposed, n=70).
Figure 6: Comparison of current (B1G) and new (B2G) Bovigam (Prionics AG) kits using responses to individual antigens and PPDb-PPDa with samples from control (n=7) as well as, *M. bovis* 95-1315 (n=8), and *M. bovis* 10-7428 infected (n=8) animals. a) IFN-γ responses pooled over time. b) Pre-challenge CSL and Lelystad PPDb-PPDa responses c) 4 week post-challenge CSL and Lelystad PPDb-PPDa responses d) 12 weeks post-challenge CSL and Lelystad PPDb-PPDa responses.
Supplemental 1: Comparison of IFN-γ responses to PC-HP and PC-EC in control (n=7) as well as, *M. bovis* 95-1315 (n=8), and *M. bovis* 10-7428 infected (n=8) animals. a) Responses over time b) Pooled over time.
Supplemental 2: IFN-γ responses to CSL and Lelystad (LEL) PPDs. a) Responses at 3, 4, and 6 weeks post-challenge in control (n=5) as well as, *M. bovis* Ravenel (n=5), and *M. bovis* 95-1315 infected (n=5) animals. b) Responses at 3, 6 and 12 weeks post-challenge in control (n=7) as well as, *M. bovis* 95-1315 (n=8), and *M. bovis* 10-7428 infected (n=8) animals.
CHAPTER 3: GENERAL CONCLUSIONS

General Discussion

This thesis was aimed at evaluating Mtbc-specific antigens as well as PPDs from different manufacturers’ to improve the diagnostic ability of the IFN-γ assay to detect bovine TB. In this study it was determined that the two peptide cocktails tested containing Mtbc-specific antigens and the two PPDs tested were equally effective in detecting bovine TB in experimentally infected cattle using M. bovis strains that differed in virulence. Peptide cocktails were shown to detect infection as early as three weeks after M. bovis challenge. It was also of note that adding Rv3615c and three other mycobacterium-specific antigens into a peptide cocktail with ESAT-6 and CFP-10 resulted in comparable diagnostic capability as a peptide cocktail composed of only ESAT-6 and CFP-10.

PPDs were further compared using samples from cattle herds that had been in contact with an M. bovis infected animal that was detected by slaughterhouse surveillance. It was determined that although both were successful in detecting bovine TB, Lelystad PPDs classified more animals as positive then CSL PPDs respectively. This finding brings back the point that not all PPDs perform the same that has been demonstrated by previously (2, 3, 6).

These experimental studies also demonstrated that reactions to conserved mycobacterial antigens can result in misinterpretation of the IFN-γ assay when using PPDs alone. The high responses to avian PPD due to conserved antigens resulted in the failure to detect experimentally-challenged cattle once PPDb-PPDa calculations were
performed. However, this study demonstrates that dilution of the stimulated plasma is an effective means to detect *M. bovis* infected cattle when there are robust responses to conserved antigens. This is an important discovery as the presence of high numbers of environmental mycobacteria in some geographical regions greatly hinders the performance of the IFN-γ assay as a diagnostic tool.

**Recommendations for Future Research**

This thesis examined ways to improve the diagnostic capability of the IFN-γ assay using a controlled experimental-challenge model. However, further studies are needed using naturally infected animals, BCG vaccinated animals, and cattle under the age of six months as these are the areas in which improved diagnostics are needed.

The goal of the TB eradication and control programs are generally focused on improved ante-mortem testing. IFN-γ responses elicited to peptide cocktails in experimental-challenge studies may be much higher than those responses elicited by naturally infected cattle that could decrease their ability to detect infection. In this study, lesions were seen at 13 weeks post-challenge signifying the development of an immune response over time that can be measured. In natural infection, it is not known how long an animal has been infected, infectious dose, or the time it took for visible lesions to form. Therefore, animals being tested in the field may not elicit as robust as a response as seen in experimental studies.

This study tested DIVA reagents in an experimental challenge setting without BCG vaccination. It would be interesting to examine the same reagents in BCG vaccinated animals that are later challenged with *M. bovis* to determine if they are still
capable of detecting infection. These studies would help examine the potential of vaccinating all cattle as a means of prevention without affecting the performance of diagnostic assays (5). It would also be interesting to test DIVA reagents in infected animals less than six months in age. Current testing of this age group using the IFN-γ assay and PPD results in non-specific responses; however, this response wanes with age (1, 4). This makes it difficult to test herds with young animals that had contact with an infected animal using the IFN-γ assay. Responses to DIVA reagents by young animals have not been done and would further test if these reagents are capable of detecting infection at a young age.

Experimentally challenged animals elicit robust IFN-γ responses to both PPDa and PPDb due to conserved mycobacterium antigens. This study found that by diluting stimulated plasma resulted in improved ability to detect infection by bringing the responses down to a measurable level. This procedure could be incorporated into regions where there are high levels of non-pathogenic mycobacteria that obstruct detection of bovine TB using the IFN-γ assay. However, further studies are needed using animals from those regions.

References


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