Development of an improved production method, determination of protein composition, and potency characterization of *Mycobacterium avium* subsp. *paratuberculosis* purified protein derivative

by

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CHAPTER 1

GENERAL INTRODUCTION

Statement of the problem

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne’s disease in cattle and wild ruminants. Johne’s disease is a chronic progressive fatal disease that presents difficulty in establishing an accurate diagnosis, especially in the early stages of disease. The lack of accurate and early detection of Johne’s disease has hindered efforts to control or eliminate the disease from US dairy herds. Unfortunately, according to the most recent NAHMS survey (NAHMS, 2008), Johne’s disease prevalence has increased to over 90% in US dairy herds. The use of cell-mediated immune (CMI) response testing methods is the best option for early disease detection. Test methods measuring CMI responses in the early stages of disease have been shown to provide better detection rates when compared to serological based test methods (Gwozdz, 2000). The use of MAP purified protein derivative (PPD) in either the intradermal skin test or gamma-interferon (IFN-γ) test provide an indicator of the early cell-mediated immune response to MAP infection.

The lack of consistent PPD production methods leads to variances in potency between production lots which contributes to problems in specificity during skin test procedures as well as in the IFN-γ assay. Until recently, research related to the characterization of MAP PPD protein components had been lacking. In 2005, the United States Animal Health Association Committee on Johne’s Disease addressed this concern with the approval of a resolution tasking the National Veterinary Services Laboratories
(NVSL) of the United States Department of Agriculture (USDA) with the development of a systematic protocol for the production and characterization of a consistent MAP PPD.

This dissertation research project is directed toward addressing this need through the improvement of the current MAP PPD production process, and to characterize current MAP PPD production lots, in an effort to arrive at a well characterized and consistent PPD that can be reliably used in both intradermal skin test procedures and the IFN-γ assay for early detection of MAP infections.

Development of an improved MAP PPD production process

MAP PPD is a crudely prepared protein suspension that is precipitated by trichloroacetic acid from autoclaved MAP culture supernatants. Studies have shown that *Mycobacterium bovis* and *Mycobacterium avium* infected animals can cross react with MAP PPD (Jungersen et al., 2002; McDonald et al., 1999). These non-specific reactions lend to diminished support for PPD use in skin testing animals, or for use in the IFN-γ assay. PPD production methods have remained the same since the early 1940s and production improvements should be re-examined in light of newer technologies. Outdated production processes require extensive incubation times for sufficient culture growth. The lack of formal production protocols detailing incubation times and specific MAP strain for use contributes to inconsistencies between PPD suspensions (Wynne et al., 2012). Previous work was successfully conducted at the USDA to produce a reference MAP PPD by using current production methods and demonstrated acceptable biological activity in MAP sensitized guinea pigs (Steadham et al., 2002). Improvements
in production methods and the consistent protein composition of MAP PPDs are imperative to allow for improved and consistent *in-vivo* and *in-vitro* testing procedures.

**Characterization of MAP PPD composition**

Until recently, interest in defining the components of MAP PPD was not a point of emphasis. Two questions have followed PPD production over the years. The first is the mycobacterial strain used for production processes. Originally, an isolate incorrectly referenced as MAP Strain 18 was used for MAP PPD production and was later characterized to be *Mycobacterium avium* subsp. *avium* and not a true MAP isolate (Chiodini, 1990; Merkal, 1979). This culture confirmation work led to a proposal to discontinue referencing Strain 18 as MAP and to discontinue its use in MAP PPD production processes (Chiodini, 1993). MAP ATCC 19698, a confirmed MAP cattle isolate, was proposed as the MAP neotype strain (Merkal, 1979). MAP ATCC 19698 has been used at the NVSL since the late 1990s for MAP PPD production processes.

Genetic analysis of mycobacterial culture strains used for PPD production has continued and revealed considerable genomic differences exist between isolates used for reagent production (Semret et al., 2006a). The acceptance of MAP ATCC 19698 as a production strain in many laboratories accompanied by further genetic analysis has allowed for consistent use of a specific well-characterized strain for PPD production.

A second question complicating the use of mycobacterial PPDs in general is the lack of information related to the protein composition of preparations. This process is complicated due to the breakdown of protein components due to autoclaving during the PPD production process. Proteomic analysis of mycobacterial PPD suspensions has been
examined in recent years (Cho et al., 2012; Prasad et al., 2013; Wynne et al., 2012). The greatest number of proteins identified in any of these studies was 265 from a *Mycobacterium tuberculosis* PPD (Prasad et al., 2013) and 134 proteins in a MAP PPD (Wynne et al., 2012) Identification of all protein components in PPD could lead to an increased ability to screen for immunologically significant components contributing to improved and more consistent PPD preparations.

**Evaluation of MAP PPD**

At the NVSL, PPD preparations are evaluated for sterility, protein concentration, phenol content, and potency prior to release for use in the field. There are no specific protein composition evaluations currently conducted by various laboratories producing PPD to ensure consistency of PPD composition between production lots. This inconsistency can lead to differences in potency and varying specificity levels. Induction of IFN-γ responses can vary significantly depending upon the PPD preparation used (de Lisle et al., 1980; Robbe-Austerman et al., 2006a; Robbe-Austerman et al., 2006d). These differences can lead to inconsistency when utilizing the intradermal skin test and IFN-γ assay. The addition of methodologies allowing for identification and measurement of specific protein components may contribute to increased consistency between production batches. However, this type of approach can only be implemented with improvements to production methods allowing for production of a well-characterized PPD.
Evaluation of MAP recombinant proteins

While PPD proteins have been identified through proteomic analysis, there is still a major knowledge gap regarding which of the 4300-plus proteins that may be useful in producing an immunologically potent PPD. Interest in the identified proteins has primarily been focused upon their use in antibody based serological assays. However, in the early stages of infection the immune response promotes a T-helper type 1 (Th1) polarization of the immune response which is accompanied by MAP-specific IFN-γ production (Stabel, 2006; Stabel and Whitlock, 2001). Evaluation of MAP recombinant proteins that will provide specific and potent IFN-γ stimulation could provide for improved diagnostics for earlier detection of MAP infected cattle.

Evaluation of recombinant proteins, emphasizing usefulness in cell-mediated immune response detection protocols, would also include guinea pig potency testing as an in-vivo model in addition to use as mitogens in an IFN-γ assay as an in-vitro model. An understanding of possible differences of biological activity in an in-vivo versus in-vitro model will be important to determine possible recombinant protein selection that can provide for use in both the field and laboratory settings.

Research objectives

The overall goal of this research was to provide a foundation for production of a more consistent, potent, and well-characterized MAP PPD that can be used for testing of CMI responses to MAP infection. The first step to address this objective was to evaluate the current production method and subsequently utilize this information to investigate
possible adaptations which would allow for development of an improved and reproducible production process (Chapter 3). The next step was to evaluate the potency of the various MAP PPD suspensions to determine if varying production methods may have an effect upon biological activity of the product and to characterize the specific protein composition of PPD (Chapter 4). A final step in the research was to evaluate and identify specific recombinant proteins as candidates for use in skin test procedures and as mitogens for in-vitro gamma-interferon (IFN-γ) testing (Chapter 4). Results of this work identified a number of proteins that are reactive in skin testing using sensitized guinea pigs and as mitogens in an IFN-γ assay. These results can contribute to improvements toward a well-defined and consistent reagent for use in in-vivo and in-vitro test procedures.

**Dissertation organization**

The dissertation is composed of a general introduction, a literature review chapter, and two additional individual chapters containing research data in a format in which they will be submitted for publication. Chapter 2 is the literature review and has the citations listed at the end of the chapter. Chapter 3 is entitled “Development of an improved production method for *Mycobacterium avium* subsp. *paratuberculosis* purified protein derivative” and provides research findings involving development of an improved PPD production method. Chapter 4 is entitled “Composition and potency characterization of *Mycobacterium avium* subsp. *paratuberculosis* purified protein derivatives and protein components” and provides research findings involving potency testing and proteomic analysis of PPD preparations. Further information is provided regarding results using
recombinant proteins, identified from mass spectrometry analysis of PPD, in the guinea pig potency test and as antigens in the IFN-γ test. Chapter 5 is a general conclusions section that summarizes the research presented in the dissertation and provides suggestions on future research.

References


CHAPTER 2: LITERATURE REVIEW

Johne’s disease discovery

The discovery of Johne’s disease was initially described by Johne and Frothingham in 1895 and was thought be bovine tuberculosis (Johne V.A., 1895). Description of the clinical signs that include emaciation, inflammation, and a thickened intestine resemble what are now the known indicators of clinical Johne’s disease. Later in 1906, the disease was recognized as being distinct from bovine tuberculosis and the disease name was changed to Johne’s disease or pseudotuberculosis (Bang, 1906). In the years following these early findings, the acid-fast bacilli attributed to these infections were isolated, characterized, and subsequently used to reproduce the disease in calves and laboratory animals (Marek, 1910; Twort, 1914; Twort, 1912). A report in the early 1900s indicated that the use of M. avium tuberculin resulted in a positive skin test response in MAP infected cattle, whereas Mycobacterium bovis (M. bovis) tuberculin did not elicit a response (Bang, 1909). The ability to isolate and grow the organism as well as the finding that M. avium tuberculin elicited a skin test response were important steps for testing the presence of Johne’s disease. The organism was originally known as Mycobacterium paratuberculosis and has been associated with Johne’s disease since 1923. (Bergey, 1923).

Johne’s disease is a chronic and progressive wasting disease caused by Mycobacterium avium subsp. paratuberculosis (MAP). Infection is normally established in young, nursing animals through the fecal-oral transmission route. A variety of animals are susceptible to infection including domestic and wild hoofstock (Council, 2003).

The overall susceptibility to infection appears to wane as an animal ages. Studies in dairy cattle have supported this theory even though the specific biological aspect of this susceptibility is not precisely understood (Larsen et al., 1975). If as adult cattle there is exposure to large or
reoccurring doses of MAP, infection may occur in spite of reduced susceptibility (Payne, 1961; Rankin, 1961, 1962).

In the United States, the prevalence of MAP infection in dairy cattle has continued to increase. Two surveys conducted by the USDA have indicated there is increased knowledge among producers about Johne’s disease; yet, the incidence of infected animals continues to increase. In the 2007 survey, results indicated that cattle in 68 percent of U.S. dairy operations were infected with MAP (NAHMS, 2008).

Because of the slow progression of Johne’s disease, the ability to detect disease in herds can be problematic. Four distinct stages of disease progression have been described as silent infection, subclinical disease, clinical disease, and advanced clinical disease (Whitlock and Buergelt, 1996). As the name implies, the silent infection stage shows no obvious disease signs and this early stage would usually be found in young animals. Subclinical disease would again offer no obvious signs of disease, but tests for cell mediated immune responses may detect animals at this stage of infection. At this stage, identification of infected animals may infrequently be detected by isolation of MAP from fecal cultures. The last two stages of disease often develop after one or more years following infection with MAP. Typical signs are weight loss, diarrhea, milk production decreases, lethargy, and increased weakness. Difficulties can be encountered during clinical disease because of challenges in obtaining an accurate diagnosis of Johne’s disease (Whittington and Sergeant, 2001).

The economic impact of Johne’s disease is estimated to reach into the millions of dollars annually. In New England, there was an estimated loss of $15.4 million dollars annually (Chiodini et al., 1984) while in Pennsylvania annual losses were estimated near $5.4 million dollars (Whitlock, 1985). A USDA study estimated a loss of approximately $200 per cow each
year with an overall economic loss of between $200 million to $250 million dollars annually to the U.S. dairy industry (Garry, 1999). The economic toll of this disease on the dairy industry is the primary motivation for efforts at disease control.

**Johnin and PPD development – growth and production**

Shortly following the successful isolation and characterization of *Mycobacterium paratuberculosis* (MP) an antigen referenced as Johnin was successfully produced from growth of MP on a beef-broth based liquid medium (M'lFadyean J., 1916). Organisms in the growth media were filtered out of the suspension, and the media was then heat concentrated. This original work records testing conducted on experimentally and naturally infected cattle, goats, and sheep over a five-year period. Testing was conducted utilizing an intradermal route, as well as intravenous and conjunctival routes. A majority of animals infected with MP did respond to Johnin as evidenced by a thickening of the skin at the injection site, but the reactions were weaker than those observed in *M. bovis* infected animals tested with *M. bovis* tuberculin and when *M. avium* tuberculin was used to test *M. avium* infected cattle. The report also indicates that the Johnin-elicited responses in both *M. bovis* and *M. avium* infected animals gave concern related to possible cross-reactions to animals infected with other mycobacteria. While there was disappointment in the sensitivity and specificity of this initial Johnin preparation, it was the first step in production of such antigen.

Further work indicated no differences in detection of infected animals with the use of either Johnin or avian tuberculin, though there were indications that differences existed between production batches (Hagan, 1928). Advances in making Johnin with organisms grown on synthetic media containing dead *M. phlei* organisms as a supplement occurred in 1932.
(Broerman, 1932). Continued production trial modifications to media formulations provided
greater support for the use of synthetic media due to the elimination of reactive animal proteins
from the media (Dunkin, 1933; Watson, 1935). The removal of *M. phlei* from culture media in
1933 indicated that Johnin could still be produced with a satisfactory potency using this media
recipe (Dunkin, 1933). These improvements in Johnin production processes, including using
synthetic media for growth, later led to successful field use of Johnin produced in this manner
(Minnett, 1935; Watson, 1935). Investigations into better standardization and use of guinea pigs
and other laboratory animals to test potency occurred in subsequent years (Glover, 1941a, b;
Johnson, 1944; Johnson, 1942; Konst, 1943). Potency, or measurement of biological activity,
was determined by the skin test responses of the various Johnin products in the sensitized guinea
pigs and allowed researchers improved information for determining optimal concentration of
Johnin. Johnson also demonstrated the use of sensitized cattle for potency testing of Johnin
products (Johnson, 1944). An additional study involved cattle sensitized against various
mycobacterial species and compared the performance of Johnin versus PPDs produced from *M.
avium, M. bovis, M. tuberculosis* var. *hominus*, and *M. phlei* (Johnson et al., 1949).

During the early developmental stages of Johnin production heat concentrating culture
filtrates through an evaporative process was the standard production method. This procedure
consisted of exposing liquid culture flask suspensions to flowing steam or by placement within a
water bath for a period of a few hours allowing for reduction of culture volume through a heat
evaporation process. The remaining contents were then filtered to remove clumps of
mycobacteria, resulting in a culture filtrate referred to as Johnin. In 1943 McIntosh and Konst
demonstrated the successful production of a purified protein derivative (PPD) by adding a
protein precipitation step to the process (McIntosh and Konst, 1943). After obtaining the final
culture filtrate one volume of trichloroacetic acid (TCA) was added to every nine parts of culture filtrate and allowed to stand overnight. The supernatant was then removed and the protein precipitate washed with 1% TCA, centrifuged, and the supernatant discarded. The precipitate was then washed multiple times with anhydrous acetone followed by multiple washes with ether. The PPD was then dried and the resulting powder stored for later reconstitution and use. This was an important advancement as purified protein derivatives were standardized based on protein concentration. They were shown to be superior to previous heat concentrated culture filtrates (Larsen et al., 1955).

During this same period of time, the Bureau of Animal Industry had a strong interest in further evaluation of products for the diagnosis of Johne’s disease, and a statistician derived and published two methods which could be used for comparing allergens, now known as PPD, in cattle (Wadley, 1948, 1949). These evaluation methods were used in many of the Johnin studies that were conducted by Larsen and Johnson in the mid and late 1940s.

Improved production methods utilizing more highly purified preparations and comparing the specificities and potency of such products occurred in the early 1950s (Annau, 1959; Baisden, 1950; Baisden, 1952). Fractionation procedures with the use of alcohol, phenol, and acid allowed greater purification of proteins and showed overall promising advancements to existing production methods (Jones, 1953a, b, c). These fractionation studies involved further product concentration utilizing phenol followed by an additional fractionation step using alcohol. This additional alcohol step was added to remove the PPD active material from the phenol extract product. This additional alcohol fractionation was thought to result in a PPD with increased specificity.
The addition of one part of 40% TCA to nine parts of culture filtrate (vol:vol) is still used in the current MAP PPD production method; but the use of the phenol and alcohol extraction processes is no longer included. Following this work in the 1950’s there was a period of about 50 years where little new information was published on PPD production processes.

While the production method was now established, a more defined starting culture was needed. During the advances in Johnin and PPD production there still existed concerns over the specific cultures used in these procedures. Beach and Hastings originally expressed concern that in less advanced cases of Johne’s disease the use of an avian tuberculin resulted in no positive identification of infected animals. This work indicated the importance of selection of the true causative agent as well as media formulations when producing Johnin products (Beach and Hastings, 1922). A laboratory-adapted *M. avium* strain which was subsequently known as United States Department of Agriculture strain 18 had been commonly used for PPD production and was placed within the American Type Culture Collection (ATCC) (Merkal, 1979). Merkal later recognized culture Strain 18 growth requirement differences from other confirmed MP isolates. It was demonstrated that Strain 18 grew relatively rapidly without the addition of mycobactin, but true MP isolates required this iron chelating agent for growth and also had longer incubation times for satisfactory growth. Accompanying the culture growth characteristics, Strain 18 was found to be nonpathogenic for calves. In 1968 Merkal requested that ATCC remove Strain 18, because it was no longer believed to be MP, and replaced it with a field isolate that was ultimately identified as ATCC 19698 (Merkal, 1979). Continued research related to Strain 18 confirmed it was an *M. avium* isolate (Chiodini, 1990; Collins et al., 1990; Kunze et al., 1992). However, reference to Strain 18 continued in the literature during this time and in 1993 it was recommended that journal publications disallow reference of Strain 18 as a
MAP isolate and to ensure it is correctly identified as an *M. avium* isolate (Chiodini, 1993). During this period of time advancements were ongoing to improve the characterization and classification of MP. A numerical taxonomy analysis of 38 MP strains was conducted using 22 various tests and from this work it was determined that MP should be reclassified as MAP (Thorel et al., 1990). The refined classification divided subspecies of the various bacterial strains corresponding to pathogenicity and host range in addition to the molecular evaluations conducted.

In 2002, a reference MAP PPD was produced and results from potency testing indicated this particular PPD was comparable in regards to potency to a previous Johnin product made from *M. avium* strain 18 and could serve as a MAP PPD reference (Steadham et al., 2002). This was an important step in having a reference MAP PPD available since previous work showed that PPD preparations produced from *M. avium* Strain 18 led to confusion and concerns that this strain was not representative of MAP isolates from cattle.

**Intradermal skin testing for Johne’s disease**

In the US the first use of a Johnin product occurred in Wisconsin on a known infected dairy herd. The test utilized an intravenous administration for a majority of the cattle, and five additional animals were injected subcutaneously (Hastings EG, 1918). An intravenous inoculation route of Johnin for cattle testing was subsequently reported involving two other herds (Miller, 1928; Turner, 1929).

Even though the intravenous inoculation route was the accepted method for cattle testing, work was being conducted on intradermal injections for evaluation of a delayed type hypersensitivity (DTH) response. Dunkin conducted a study involving double intradermal
injections approximately 24-48 hours apart followed by evaluation of the subsequent DTH responses on the skin at the injection site (Dunkin, 1928). Non-specific skin reactions were observed in animals considered to be uninfected and Dunkin’s conclusion was that such responses could be distinguished from exposed animals based upon the size and diffuse nature of skin swellings (Dunkin, 1928). A 1929 report also using a double intradermal skin test provided additional information related to skin swelling characteristics, but emphasized the diffuse nature and temperature of the swellings rather than swelling size (Isherwood, 1929). However, swelling size would ultimately become the metric used to determine exposure to MAP.

A double intradermic skin test study utilizing multiple tests on animals in a herd over a 3-4 month time period was conducted using both Johnin, *M. bovis* and *M. avium* tuberculin in animals (Minnett, 1935). An important observation from this study was that the researchers recognized variability in skin responses over a period of time. Another report utilizing the double intradermal skin test concluded that use of this testing method was satisfactory for detecting animals that may be in the preclinical stages of disease (McConnell, 1935). These two reports involved herds with known positive animals and varying degrees of skin test responses. An initial indication of the specificity of Johnin was reported involving the use of Johnin in a herd with no history of Johne’s disease. The study was conducted over a three-year period (Johnson and Pratt, 1944). Initial testing of the herd resulted in seven skin test positive animals and over the study period these specific animals were tested approximately every three months. No clinical signs had developed or were detected in these animals over the seven years it had taken to build the herd. This study also tracked milk production with no decreased milk production volumes observed. It was concluded, that although animals may be infected or simply exposed, the level of infection was insufficient to produce clinical disease, yet was
detectable by skin testing. Another possibility was that the animals may be infected by other mycobacterial agents that shared antigenic determinants and cross-reacted to Johnin.

Transition from use of the caudal fold area to the neck region resulted from studies conducted to evaluate intradermal injection site locations on cattle (Baisden, 1951; Larsen, 1950; Sikes and Groth, 1950). Data from these studies revealed that there was a significant difference between skin test responses in the neck region compared to regions such as the back, side, and caudal fold region. Sensitivity of the neck region was two to three times greater than other test regions on cattle. In support of the use of a cell-mediated immune response test it was shown that transfer of sera from MAP sensitized guinea pigs into MAP negative guinea pigs did not elicit a positive skin test response (Howell, 1957). An accompanying finding by Howell in this study was that if cells from the peritoneal cavity of MAP sensitized animals were transferred into MAP negative guinea pigs that a positive skin test resulted. This finding confirmed the DTH response observed in cattle was the result of a cellular immune response. Since these early studies the use of the intradermal skin test has remained a constant over the years when conducting field trials on dairy cattle.

**Difficulties associated with Johne’s skin testing**

Studies addressing concerns of anergy due to repeated testing were conducted around 1950. Larsen reported a 55% response rate one week after initial testing of MAP-sensitized cattle followed by 87% responding four weeks after initial testing (Larsen, 1949). A follow-up study by Sikes using both naturally and artificially infected cattle indicated that a loss of skin test responsiveness was more likely to be observed in naturally infected animals than the experimentally infected animals (Sikes et al., 1951). Further elucidation of possible anergy
continued in 1953 when Sikes used naturally infected cattle and nine different intradermal injection sites over a 20-week period (Sikes, 1953). Intradermal testing was conducted at the nine various injection sites at 14, 16, 18, and 20 weeks after the initial intradermal skin test. Findings in this study were similar to the previous studies in that animals were more likely to have decreased response rates at earlier repeated test time periods following the initial skin test. This study also found there were issues related to local injection site desensitization. Sikes concluded that if repeated intradermal injections were to be conducted at the same site that a period of 20 weeks should be allowed to pass before repeated testing. Non-specific reactions can occur with skin testing.

**Serological testing for Johne’s disease (Paratuberculosis) using PPD**

Identification of Johne’s positive animals utilizing serological testing methods continues to be a struggling area of diagnostics. Early studies examined comparisons between the complement fixation test (CFT) and PPD skin testing primarily due to concerns that the two tests may interfere with each other. The CFT was initially used by early investigators and the polysaccharide antigen used provided strong antigenicity (Annau, 1958; de Lisle et al., 1980). A group in Canada conducted a comparative study and the results indicated that the use of PPD for skin testing prior to complement fixation testing did not interfere with performance of the CFT (Rice and Konst, 1959). Rice and coworkers also found that infected animals were more likely to respond to skin testing than to the CFT, but they used *M. avium* antigens in that study (Rice et al., 1958). Two studies in Denmark indicated similar results in that there was a higher response rate to skin testing than the CFT (Jorgensen, 1964; Ringdal, 1964). The two herds involved in these studies were identified as infected with avian tuberculosis and so the results may not be
specifically comparable to MAP-infected herds. Around this same time there were testing recommendations distributed by the British Veterinary Medical Association in which the use of the PPD skin test was the best test option for identifying herds with animals that may be subclinical, and that the CFT along with acid fast staining were satisfactory tests for animals exhibiting clinical disease (BVA, 1962).

**PPD use in gamma interferon testing**

During the time of ELISA development in the early to mid-1990s, significant work was concurrently exploring the potential use of MAP PPD in interferon gamma (IFN-γ) assays based upon the assay performance for detecting bovine tuberculosis. Proposals for such use began in the early and mid-1990’s with studies comparing ELISA’s with the IFN-γ assay (Billman-Jacobe, 1992; Vizard et al., 1992; Wood, 1989). An additional study used data from both clinical and subclinical animals, along with negative controls, and the data indicated the IFN-γ values were substantially higher in subclinical animals when compared to the negative control group (Stabel, 1996). The ability to detect subclinical infections in calves less than 15 months of age was shown to be problematic due to induction of non-specific IFN-γ production in natural killer cells (Jungersen et al., 2002). A two-year study comparing the IFN-γ assay and serum and milk ELISAs proved the IFN-γ assay has a greater sensitivity than the available ELISAs, but difficulties were encountered when used for testing young animals one to two years of age (Huda et al., 2004).

A possible improvement to the IFN-γ assay by addition of interleukin-12 as a stabilizer to blood samples indicated this step could enhance IFN-γ levels, but at the same time increased non-specific IFN-γ production (Jungersen, 2005). Additional work by Jungersen indicated that
the IFN-\(\gamma\) assay should be used as a herd based testing protocol to evaluate the level of exposure to MAP rather than indicating the infectious status or future disease progression of individual animals (Jungersen et al., 2012). The evaluation of 14 recombinant antigens for use in the IFN-\(\gamma\) assay resulted in findings that MAP PPD had higher levels of responses from samples of both infected and non-infected animals in contrast to the higher specificity observed for these novel antigens in serological assays (Mikkelsen et al., 2011). Another important aspect of the Mikkelsen et al. study is that three antigens had no homologue in the \textit{M. avium} nor \textit{M. bovis} genomes.

Development of the IFN-\(\gamma\) assay allowed researchers an additional tool for examining the bovine immune response to MAP infections. Along with these advances the importance of the antigenic characteristics of MAP PPD proved a vital component for successful IFN-\(\gamma\) assay development and performance. Continued evaluation of MAP PPD antigenic components may reveal possible improvements to the IFN-\(\gamma\) assay.

**Field trials related to PPD use**

Many early studies evaluated the effectiveness of Johnin in naturally infected dairy cattle in herds of substantial size. Difficulties with the specificity and sensitivity of the Johnin product continued to cause concern, but also indicated the importance of skin testing as a means to identify positive animals in the early stages of infection (Merkal, 1968; Sikes, 1953; Sikes et al., 1951). The 1968 study by Merkal involving the comparison of multiple test methods began a transition to fecal culture as the gold standard test in Johne’s diagnosis from actively shedding animals. However, the use of the skin test was found to still be of value in identifying positive animals.
A heavily infected dairy herd was tested utilizing MAP PPD approximately every three months over a period of time exceeding two years (Johnson, 1950). Even with frequent skin testing followed by the removal of positive animals the prevalence of infection continued to be maintained. Eventually it was concluded that the rate of infection was high enough that testing and removal of positive animals at three month intervals was not sufficient to prevent the spread of infection. However, an important aspect of this study was that there were no false positive animals identified as long as close attention was given to the degree of induration measured during skin testing.

Studies in Canada reported successful use of PPD in 8 herds to eradicate Johne’s disease in half of the enrolled herds (Konst and McIntosh, 1958a, b). This successful conclusion was based solely on there being no further reoccurrence of clinical signs of Johne’s disease in the herds. There were continued concerns related to the use of PPD in heavily infected herds or during particular stages of infection. A five-year study in Alabama was conducted using one-time PPD skin testing and examination of tissue smears collected at slaughter (Larsen et al., 1963). Skin test results from this study reached 51% sensitivity and 55% specificity. The quantity of infected animals in this herd was high yet the results supported earlier studies that the sensitivity of the MAP PPD skin test may not be optimal in such situations where advanced stages of infection were evident in a herd. However, an important advancement from this study was that an induration cutoff of 3 mm was established and anything short of that may have an effect on the sensitivity of the skin test results.

A study in Canada suggested that the skin test was useful for identifying subclinical or preclinical animals, but was not optimal for identifying clinical animals (de Lisle et al., 1980). Animals were tested using both skin test and complement fixation and any positive animals to
both tests were culled from the herd. Skin testing was conducted using two measurement criteria in which animals with indurations of both greater than and less than 3 mm were recorded. When test sensitivity was calculated using a cut off with greater than 3 mm induration, the overall skin test sensitivity was only 5% with a 94% specificity. If data for all animals with positive skin reactions was considered, the test sensitivity increased to 48% but showed only a 32% specificity. Neither set of test criteria contributed confidence for overall use of the MAP PPD skin test.

A study in India compared testing for cattle using the skin test to selected available serological tests (Paliwal, 1987). Results were similar to previous studies in which the skin test performed well for identifying preclinical diseased animals, but once the animals transitioned to expression of clinical symptoms, the skin test did not perform as well as the serological assays.

A Netherlands study conducted intradermal skin testing on paratuberculosis vaccinated animals between the ages of five and 14 months (Wentink et al., 1993). Similar to previous studies, negative skin test results occurred significantly more often in animals found positive post mortem by histology or culture. The combined data from these studies supported the use of a serological assay for detecting shedding or clinical stage animals, and began the emphasis toward pursuit of serological assays in place of the PPD skin test. The results of these studies were further confirmed as the immunology of MAP infections in cattle became more understood. The early stages of infection involve T helper type 1 (Th1) polarization of the immune response along with MAP-specific IFN-γ production (Stabel, 2006; Stabel and Whitlock, 2001). This finding correlates with the ability of PPD skin tests to detect infected animals in the preclinical or subclinical stages of infection prior to fecal shedding. As MAP infection progresses and fecal shedding and clinical signs appear, there is a transition to a T helper type 2 (Th2) immune
response with the production of MAP-specific antibodies (Stabel, 2006). During this transitional stage accurate diagnosis can be difficult and the use of multiple testing strategies proves most useful (Koets et al., 2002; Nielsen and Toft, 2008). Collectively, these studies support the earlier observations where PPD skin test sensitivity decreased or was non-existent for animals in the clinical stages of infection.

**Current PPD production methods at the National Veterinary Services Laboratory**

The National Veterinary Services Laboratories (NVSL) produces PPD products from *M. bovis*, *M. avium* subsp. *avium*, and MAP. The greatest emphasis is for *M. bovis* and *M. avium* subsp. *avium* PPD products in support of the USDA Bovine Tuberculosis Eradication Program.

PPD production protocols are similar for the three different products, with the primary differences being in media composition used for culture propagation and the chemical used for protein precipitation. MAP PPD production is conducted by using MAP 19698 grown in Reid’s Media supplemented with Mycobactin J. Production cultures are incubated for approximately 10-12 weeks at 37°C and are examined regularly for quality of a floating pellicle and contamination. Following incubation, production flasks are autoclaved at 121°C for 30 minutes and allowed to cool overnight. Cells are then removed by coarse filtration through sterile cheesecloth within a funnel to produce a culture filtrate (CF). Proteins within the CF are precipitated with the addition of one part of 40% TCA to nine parts of CF and mixed thoroughly during addition. This suspension is then allowed to set overnight at room temperature and the supernatant is removed the next day by aspiration and centrifugation. The resulting protein precipitate is washed twice with 1% TCA followed by multiple washes with 10% sodium chloride solution to remove the TCA. The final protein suspension is then dissolved in a sodium
phosphate chloride buffer and adjusted to a final pH of 7.0 ± 0.1 prior to final filter sterilization. The concentrate is then stored at a refrigerated temperature during protein determination testing using the micro-Kjeldahl method. The final PPD product is then adjusted to 1.0 mg/ml of protein and phenol is added to a final concentration of 0.5% prior to final sterility testing.

Overall the NVSL distributes over one million doses of *M. bovis* caudal fold tuberculin annually. Distribution of MAP PPD domestically is minor in comparison and is used in the field on a very limited basis. Contributing to the reduced demand for MAP PPD is the requirement for producers wishing to enroll in the USDA Voluntary Johne’s Disease Control Program to use either serological assays or fecal culture, or a combination of both, for determination of a herd’s eligibility for the program, which is no longer funded by the USDA. With the emphasis on culture and serological testing as the approved testing methods, the number of herds utilizing skin testing has decreased. In contrast to domestic requests the NVSL has had increased requests for MAP PPD from India and the United Kingdom. The ability to produce a consistent and well-characterized MAP PPD may allow for expanded use of the INF-γ assay and increase demands in the future.

**Advances in PPD protein proteomic analysis**

Current PPD production processes are time consuming and laborious accompanied with difficulties related to lack of product reproducibility. The slow growth requires approximately three months prior to being able to initially harvest flasks containing appropriate growth. When adding in downstream processing, sterility testing, and potency testing, a production timeline can have an additional two to three months added on to final product release. The five to six month
production timeline can be impactful should a production lot not pass all quality control testing, causing a long-term setback to providing additional product for use.

Because of difficulties associated with PPD production and the lack of new PPD research, it was decided to pursue improved PPD reagent production that is more efficient and reproducible while maintaining required potency characteristics. As a first step toward this goal, studies were conducted to determine the PPD composition and what components may be used to replace the current crude suspensions.

Proteomic analysis of PPD preparations has taken on more emphasis in recent years primarily due to improvements in proteomic techniques, but it is still a relatively new area of investigation with only a few published studies. Interest in *Mycobacterium tuberculosis* (MTb) PPD has led to the discovery of a number of conserved proteins between MTb PPD, *M. bovis* PPD, and *M. avium* PPD (Cho et al., 2012; Prasad et al., 2013).

A study comparing proteomic protein findings between MAP PPD, *M. bovis* PPD, and *M. avium* PPD identified 156, 132, and 95 proteins, respectively from each of the three PPD preparations (Santema et al., 2009). However, an interesting finding of this study was that three of the proteins selected for further immunological study showed that the proteins were non-reactive in lymphocyte proliferative assays (cellular immune responses), but yet resulted in very good serum antibody responses (humoral immune responses). Another research group conducted proteomic characterization of five in-house MAP PPD preparations along with eight additional MAP PPD preparations, one *M. avium* PPD, and one *M. bovis* PPD. One of these MAP PPDs from the NVSL was found to have insufficient protein quantity for further analysis with the remaining 13 preparations having between 76 and 134 proteins identified within each PPD (Wynne et al., 2012). Resulting protein composition differences between preparations were
most likely due to MAP strain differences and the culture age when harvested for protein preparation.

Continued genetic investigation of MAP strains used for PPD production and various antigen production processes has resulted in the identification of specific strain and isolate differences that may influence protein expression and immunogenicity of antigenic preparations (Bannantine et al., 2007; Gumber et al., 2009; Gumber and Whittington, 2009; Radosevich et al., 2007; Semret et al., 2006; Weigoldt et al., 2011).

Monoclonal antibodies to the 35 kilodalton (kDa) major membrane protein were purified and characterized in 2007 by immunoblotting, epitope mapping, and immunofluorescence microscopy (Bannantine et al., 2007). Cross reactivity observed in immunoblot experiments with the use of various mycobacterial species indicated that the 35 kDa was not species specific and there existed shared immunological determinants between mycobacteria compared in the evaluation. Many of these were members of the *M. avium* complex (MAC), though the comparison also included fast growing mycobacteria in which such cross reactivity may not have been initially anticipated.

Changes in protein levels expressed in response to varying environments was revealed by exposure of MAP isolates to varying incubation temperatures, fluctuations of temperature, hypoxia, and nutrient availability (Gumber et al., 2009; Gumber and Whittington, 2009). Evidence that protein expression variations exist due to changing environmental conditions offered insight into possible important interactions of MAP within the host. Host cell and immunological responses may provide similar environmental stressors affecting protein expression during various stages of infection. This information would play an important role in determining specific proteins with improved diagnostic potential.
Comparison of MAP strain K-10 and a field isolate from a cow exhibiting clinical signs of Johne’s disease revealed there were marked differences in protein expression when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and mass spectrometry (Radosevich et al., 2007). Differences in protein expression were related to differences in passage level of the two isolates. What was not clear from the study is how the specific protein differences may affect MAP virulence or the specific role the various proteins play in cell-mediated immune responses. This information could be important in future improvements to PPD production if it is determined that low passage cultures provide optimal protein expression related to cell mediated immune response recognition.

By using DNA microarray analysis Semret and colleagues identified 14 large sequence polymorphisms that distinguish *M. avium* from MAP (Semret et al., 2004). The authors speculated that these sequence polymorphisms may be related to MAP mycobactin dependence. Further work with various MAC isolates involved in PPD production identified a large sequence polymorphism (referred to as LSPjen) that was absent in seven MAP strains (Semret et al., 2006). Deletion of this particular locus is thought to have occurred due to continual laboratory passage as it was shown to be intact in field isolates. Two studies involving ovine Johne’s diagnostics indicated increased test sensitivity when using PPD produced from isolates with the locus intact (Robbe-Austerman et al., 2006a; Robbe-Austerman et al., 2006b). Continued work to establish improved production processes including the use of well characterized MAP strains is an important aspect to be considered from these studies.

Additional information related to differential expression of specific MAP protein components within the host was provided in a study using MAP isolates from three cows with clinical Johne’s disease (Weigoldt et al., 2011). A large number of membrane-associated
proteins and culture-derived membranes were identified using mass spectrometry. Four proteins were identified as being present only in the membrane-associated preparations. This information could prove valuable when determining possible proteins to be included in a well characterized PPD since such membrane-associated proteins would offer the best possibility as the first immunologically recognized MAP components during establishment in the host.

Additional work related to the specific immunological responses to the various identified proteins combined with the differences in conditions related to protein expression could help decipher specific changes to production processes and aid in development of improved PPD preparations.

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CHAPTER 3: DEVELOPMENT OF AN IMPROVED PRODUCTION METHOD FOR MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS PURIFIED PROTEIN DERIVATIVE

Chapter was reviewed and approved by the USDA/APHIS National Veterinary Services Laboratories manuscript review committee
Abstract

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) purified protein derivatives (PPDs) are an immunologic reagent prepared from the cultured filtrate of MAP ATCC strain 19698. PPD was produced using MAP ATCC Strain 19698 in Povitsky bottles and Erlenmeyer flasks. Traditional production consisted of floating culture incubation at 37°C, live organism inactivation by autoclaving 30 minutes at 121°C, and coarse filtration to remove cellular debris. An alternative production method was developed involving removal of the autoclaving step during the production process. Proteins in both production methods were subsequently precipitated by adding trichloroacetic acid to a final 4% concentration. Culture production in Erlenmeyer flasks was superior to that of traditionally used Povitsky bottles as determined by floating culture mat density and time required for growth. Defined protein bands on SDS-PAGE gels were masked by smearing of the gel tracks containing PPD produced by the traditional method whereas protein bands were evident in the alternate PPD preparations. Results indicate that the current production procedure and an alternate production method produced PPD equivalent to the reference NVSL PPD Lot 9801. PPD preparations will be used for further analysis to define protein components and identification of possible antigenic components.

**Key Words:** bovine, paratuberculosis, purified protein derivative, Johne’s disease, protein precipitation, culture filtrate.
Introduction

Production of purified protein derivative (PPD) from MAP, has historically been problematic because of unique culture requirements of floating cultures and maintaining floating mats during the extended incubation period. This particular method of floating cultures is used to allow for expression of surface proteins and proteins which may be secreted and available within the growth media. This important reagent is used for skin testing (Robbe-Austerman et al., 2006; Wren, 1998) and has been used as a stimulating antigen in research involving the gamma interferon test for Johne’s disease (Billman-Jacobe, 1992; Gwozdz, 2000; Kalis et al., 2003; Robbe-Austerman et al., 2007; Stabel, 1996; Stabel and Whitlock, 2001). Attempts at improvements of production methods can be traced back to the early 1900s with modification of growth media to a synthetic media to eliminate possible reactive animal protein from the media preparation (Dunkin, 1933; Watson, 1935). In addition, the production methods and time required for cultures to meet acceptable yield characteristics can lend to difficulties associated with efficiently producing a quality PPD. Early production methods using heat concentrated culture media were later modified to production of PPDs utilizing protein precipitation methods (Glover, 1941; Johnson, 1944; McIntosh and Konst, 1943; Watson, 1935). Complicating matters further, until recently the defined immunogenic components of PPD preparations had yet to be characterized. In recent years, two laboratories conducted proteomic analysis of PPD preparations (Santema et al., 2009; Wynne et al., 2012) providing information on some of the PPD protein components that may contribute to improvements in the specificity of the product. Development of a consistent and defined reagent with a high degree of specificity in early disease detection similar to that observed for bovine tuberculosis was the goal in early studies of PPD production (Johnson and Pratt, 1944).
Over time, various *Mycobacterium avium* complex (MAC) strains served as the source of antigen preparations used in PPD production and it was subsequently discovered that in some cases multiple strains were used in those PPD preparations (Dunkin, 1928; Watson, 1935). Concern over possible variations in strain differences has led to closer scrutiny of strain characteristics, and advances in analysis of culture stocks has found that in some cases *Mycobacterium avium* subsp. *avium* has been used in production of MAP PPD (Semret et al., 2006).

The objective of this investigation was to produce PPD by the traditional method and by an alternative method and to characterize protein composition and biological activity. The traditional production method of heat-killing MAP cultures and subsequent protein precipitation from the culture filtrate was used for the production of four different PPD lots. These four lots were assessed and evaluated through culture growth characteristics, PPD protein concentration, SDS-PAGE analysis, and reactivity against rabbit antiserum raised against an established PPD lot currently used in the field. In addition, two PPD lots were produced using an alternate production method and were assessed and evaluated through culture growth characteristics, PPD protein concentration, and SDS-PAGE analysis. This study was undertaken as a first step to evaluate current production methods for obtaining consistent PPDs, and provide an initial PPD from an alternative production method toward the goal of improving the PPD, which is better defined for *in-vivo* and *in-vitro* diagnostic purposes.

**Materials and Methods**

**Bacterial cultures.** *M. paratuberculosis* strain ATCC 19698 and two bovine field isolates from Iowa were selected for use in the PPD production process. MAP 19698 inoculum was
transferred from a starter culture grown on Herrold’s Egg Yolk plus Mycobactin J (HEY+MJ) agar slants and Reid’s media. The two bovine field isolates, #392 and #455, were subcultured from TREK para-JEM culture bottles (Thermo Scientific) onto three different media: 1) Herrold’s Egg Yolk Agar with Mycobactin J (HEY+MJ), 2) 15 ml of Middlebrook 7H9 broth containing oleic acid supplement and glycerol supplemented with Mycobactin J (OADC broth) and, 3) modified Reid’s media supplemented with Mycobactin J (NVSL Media Recipe 41020). Neither field isolate grew well upon subculture within any of the three media, and thus were unsatisfactory at the time for PPD production.

Three to four loopfuls of ATCC 19698 pellicle from a starter culture were subcultured into modified Reid’s media in either Povitsky bottles or Erlenmeyer flasks. Transferred pellicle material was floated onto the surface of the Reid’s media. The inoculated bottles and flasks were incubated at 37°C for 10 to 12 weeks for use in future PPD production lots produced in 2008. Any bottles or flasks in which the floating mat would sink during incubation were discarded and not used in the PPD production process. Such individual culture containers were not used in further processing because of the inability of adequately predicting protein expression within the culture filtrate during the decreased growth period and decreased cell growth.

**PPD production, traditional.** The reference MAP PPD (NVSL 9801 without phenol) used in this investigation was previously produced at the NVSL (Steadham et al., 2002). Four PPD lots (0801A, 0801B, 0802, 0803A) were produced sequentially in 2008 as previously described (Haagsma and Angus, 1995; Steadham et al., 2002). Following incubation, bottles or flasks were autoclaved at 121°C for 30 minutes and allowed to cool at room temperature overnight. Cells were removed from the medium by straining the suspensions through sterile cotton mesh lining a sterile funnel, and culture filtrate (CF) was collected in a sterile flask.
Proteins in the culture filtrate were precipitated with the addition of 1 part 40% trichloroacetic acid (TCA) to nine parts of CF. The suspension was mixed well and allowed to incubate overnight without stirring at room temperature. The next day a large portion of the supernatant was removed by aspiration and discarded. The remaining supernatant was mixed well with the precipitated proteins and transferred to centrifuge bottles. The bottles were centrifuged at 2500 x g for 15 minutes to pellet precipitated proteins. The supernatant was again discarded, and the remaining pellets were washed with 1% TCA two times, and then with 10% sodium chloride as the final wash. The precipitate was dissolved in a phosphate chloride buffer, and the final pH was adjusted to 7.0 ± 0.1 with 1 N sodium hydroxide. The final suspension was sterile filtered through a 0.22 micron cartridge filter (Pall Supor DCF) and stored at 4°C until further use. Purity testing was conducted in accordance with the Code of Federal Regulations, 9CFR part 113.26.

**PPD production, alternate.** Two additional PPD lots (0901 and 0902) were sequentially produced in 2009 removing the autoclaving step and utilizing sterile filtration to ensure the removal of bacterial cells and sterility of final product. In brief, the revised protocol was conducted by doing a coarse filtration of the live MAP cells from the growth suspension and collecting the subsequent CF into a sterile flask. This CF was then sterile filtered through an in-line 0.5 μm cartridge filter (Pall Preflow DCF) followed by an in-line 0.22 μm cartridge filter (Pall Supor DCF). The culture filtrate was then processed as described for the traditional production method. The first lot produced through the alternative method had the final suspension sterile filtered through a 0.22 micron cartridge filter, and stored at 4°C until further use. The second lot produced through the alternative method had the final 0.22 micron filtration step removed from the workflow. Purity testing was conducted in accordance with the Code of
Federal Regulations, 9CFR part 113.26 for both lots. Lot 0902 was initially split into two production processes to designate respective bulks retained from the two different bacterial harvest start dates. These lots are indicated as 0902A and 0902B when differentiation is required.

**Protein concentration assay.** Protein measurements of the final PPD preparations were conducted utilizing the Pierce microplate BCA protein assay procedure according to the manufacturer’s directions. Assay plates were read on a VersaMax microplate reader at a wavelength of 562 nm. Protein concentrations were calculated using SoftMax Pro software from a standard curve of bovine albumin protein standards.

**SDS-PAGE.** Pre-cast 4-12% Novex Bis-Tris gels (Invitrogen, 12 well, 1 mm thickness) or precast 10% Novex Bis-Tris gels (Invitrogen, 12 well, 1 mm thickness) were used for SDS-PAGE separation of PPD preparations. MAP PPDs, *Mycobacterium bovis* (*M. bovis*) PPD, and *Mycobacterium avium* subsp. *avium* (*M. avium*) PPD were standardized to approximately 1.0 mg/ml before loading 15 µg/well onto SDS-PAGE gels. The PPDs were prepared by the addition of 5 µl of sample buffer (Invitrogen NuPage LDS Sample Buffer) to 15 µl of PPD and then heated at 70°C for ten minutes before loading the full 20 µl of sample.

Electrophoresis was conducted in an Invitrogen XCell SureLock Mini-Cell system at a constant current of 125 mA for 35 minutes. Invitrogen SeeBlue Plus2 prestained molecular weight standards served as markers for molecular weight determination. Gels were stained with Invitrogen Novex Colloidal Blue Stain according to the manufacturer’s directions.

The 4-12% Novex Bis-Tris gels (Invitrogen, 1 well, 1 mm thickness) were used for immunoblot analysis. MAP PPDs were prepared as previously described by the addition of 175 µl of sample buffer (Invitrogen NuPage LDS Sample Buffer) to 225 µl of PPD for each 500 µl of
preparation. Designated wells were loaded with 500 μl of reduced sample. Electrophoresis was conducted in an Invitrogen XCell SureLock Mini-Cell system at a constant current of 125 mA for 35 minutes.

**Rabbit immunizations.** PPD Lot 9801 was combined with TiterMax Gold adjuvant and administered subcutaneously at dosage volumes of either 0.5 ml or 1.0 ml. Inoculations were administered on days 0, 14, 28 and 56. Blood collections were taken on days 0, 14, 28, 56, and 77. Rabbits were euthanized and exsanguinated on day 86 for the final blood collection. One rabbit (7436) was euthanized partway through the study and serum samples previously collected from this rabbit were not included in further work.

**Cattle sera.** Sera were obtained from cattle housed at the NVSL and also from naturally infected cattle from private herds. Naturally infected cattle were confirmed serologically positive on both the Prionics Paracheck® and IDEXX MAP ELISA assays. Positive cattle were also confirmed culture positive by fecal culture on HEY-M. Negative cattle samples were from negative control source animals within the NVSL and from MAP negative confirmed production herds outside the NVSL (Table 1). Negative animals were confirmed by culture and serology by the same procedures as the positive cattle.

**Immunoblot analysis.** Electrophoretic transfer of proteins onto nitrocellulose was performed using the Invitrogen XCell II Blot Module and Invitrogen NuPAGE transfer buffer at 160 mA for one hour. After transfer completion, membranes were blocked with Sigma Western Blocker solution at room temperature for one to two hours with rocking. Antisera from immunized rabbits were diluted 1:500 in blocker solution and incubated with the membranes at room temperature with rocking for approximately 60 minutes. Membranes were washed three times with Trizma-buffered saline plus 0.5% Tween 20 (TBS-T). The membrane was incubated for
approximately 60 minutes at room temperature on a rocker with peroxidase-labeled goat anti-rabbit IgG (Jackson Immunoresearch Affini-Pure) as the secondary antibody diluted 1:2000 in blocker. The membrane was then washed three times with TBS-T and developed utilizing Sigma TMB Substrate according to the manufacturer’s directions.

Evaluation of the PPDs from the two different production methods was also conducted using 16 ELISA positive and two ELISA negative cattle sera. Two individual membranes were prepared in parallel, with one membrane developed with a TMB substrate and the second membrane for chemiluminescent detection. The cattle sera were diluted 1:20 in blocker solution for both blot preparations and incubated with the membranes at room temperature with rocking for approximately 60 minutes. Membranes were washed three times with TBS-T. At this point, one set of membranes were incubated for approximately 60 minutes at room temperature on a rocker with peroxidase labeled rabbit anti-ovine IgG (Jackson Immunoresearch Affini-Pure) diluted 1:2000 in blocker. Membranes were then washed three times with TBS-T. Membranes were developed in Sigma TMB Substrate according to the manufacturer’s directions. The second set of membranes were incubated with HRP conjugated Protein A as a secondary antibody, diluted 1:20,000 in blocker, for approximately 60 minutes at room temperature on a rocker. Membranes were then washed three times with TBS-T. Membranes were developed with SuperSignal Chemiluminescent substrate for 5 minutes.

Results

Bacterial culture characteristics. *Mycobacterium avium* subsp. *paratuberculosis* strain ATCC 19698 grew well in Reid’s media in both the Povitsky bottles and Erlenmeyer flasks (Figures 1
and 2). Although historically at the NVSL, cultures for PPD production were grown in Povitsky bottles, growth rates were slightly faster in the Erlenmeyer flasks, taking approximately 10 weeks to produce a thickened, quality pellicle or floating mat suitable for harvest. The Povitsky bottle cultures also produced good floating mats, but typically were not ready for harvest until 12 weeks post-inoculation. In addition, the Povitsky bottle cultures had a greater tendency to have mats begin to fall in small sections near the end of the incubation period. Overall the Povitsky bottles produced 0.027 grams of wet weight cells per ml of media versus Erlenmeyer flask production of 0.032 grams of wet weight cells per ml of media at 12 and 10 weeks, respectively (Table 2).

The two bovine MAP field isolates, subcultured onto HEY+MJ, OADC broth, and modified Reid’s media, did not yield productive growth within any of the three different media types. Eventually growth was detected on HEY+MJ, but the colony formation was not favorable for transfer into Reid’s media for attempting to produce a floating culture. These field isolates were simply not laboratory adapted for this procedure. Therefore, no additional work was conducted with these two isolates.

**PD production and protein concentration.** The four lots of PPD (0801A, 0801B, 0802, 0803A) produced by the traditional production process all yielded acceptable protein concentrations at a minimum of 1 mg/ml. Protein concentrations ranged between 1.1 to 2.1 mg/ml and suspension characteristics were typical of previous PPDs, yielding a light brown to tan final suspension. Sterility testing of each lot did not yield growth of any contaminants and thus final QC evaluations were deemed acceptable.
PPD Lot 0901, processed through an alternative method, was not acceptable for additional analysis after the final filtration step. During the final sterile filtration, multiple filters were required to complete the process resulting in an unsatisfactory loss of protein content.

PPD Lot 0902A and 0902B, also processed through an alternative method, yielded a final suspension of a dark tan coloration and overall acceptable appearance. The final protein concentration was approximately 1.2 mg/ml, and this preparation was confirmed sterile.

**SDS-PAGE.** SDS-PAGE analysis of PPD Lot 9801 yielded results showing obvious protein smearing and the lack of any discernable protein banding patterns (Figures 3 and 4). This result is similar to that observed in other laboratories (Cho et al., 2012; Rennie et al., 2010). Lots 0801A, 0801B, 0802, and 0803A yielded products with less protein smearing, but smearing was still significant masking the presence of definable protein bands (Figures 3 and 4). Similar protein smearing characteristics have also been noted in earlier work during examination of both *M. bovis* and *M. avium* PPD preparations at the NVSL. The appearance of these “dirty” gel tracks suggested the gels might be overloaded with protein. Additional dilutions of PPD preparations were analyzed in an attempt to reduce the presence of protein smearing and resolve protein bands. With further dilution of the PPD preparations the “background” did decrease, and lightly stained bands initially obscured by the protein smearing could be detected for Lot 0801A, 0801B, 0802 whereas 0803A still showed the same characteristic smearing as an undiluted product (Figure 4). In contrast, PPD Lot 0902 yielded eight distinct protein bands common between the two different SDS-PAGE gels and showed little to no smearing (Figures 3 and 4). Common identifiable protein bands within the two gels corresponded to sizes of approximately 145 kDa, 50 kDa, 40 kDa, 34 kDa, 25 kDa, 20 kDa, 18 kDa, and 12 kDa. The 4-12% gel showed two additional protein bands at approximately 30 kDa and 15 kDa, while the 10% gel
had an additional band with an approximate size of 48 kDa. In addition, similar protein bands between the MAP PPD and an *M. bovis* PPD, were detected at approximately 25 kDa and 12 kDa (Figures 3 and 4). The *M. bovis* PPD protein bands were more diffuse and matched most closely to those particular protein bands in the MAP PPD preparations. No specific protein bands were observable in the *M. avium* PPD preparation.

**Immunoblot analysis.** Protein smearing, initially evident in the colloidal blue staining of the SDS-PAGE gels, contributed to problems with obtaining proper sera dilutions for detection of any specific protein reactions. Sera reactions to the smearing evident throughout each lane did indicate moderate to strong immune response by a majority of the rabbits. Five rabbit sera (rabbits 7429, 7430, 7432, 7437, and 7438) were evaluated against PPD Lot 0801A, 0801B, 0802, and 0803. Results for all five rabbit sera did produce light bands that were partially obscured at approximately 34 kDa, 18 kDa and 15 kDa. This indicated a more specific reactivity to the four new production lots in comparison to reactivity against PPD Lot 9801. Immunoblot results of rabbit antisera showed moderate to strong immunological reactivity when evaluated against PPD Lot 0902, adding support that removing the autoclaving step from the production process was not detrimental to the immunoreactivity. Serum from rabbit 7430 did result in a band at approximately 48 kDa that could be observed within the blots regardless of the detection method used. No other protein bands could be differentiated using anti-Lot 9801 PPD antibody against PPD Lot 0902 antigen by immunoblot. However, all inoculated rabbits did show reactivity against PPD Lot 0902. Sera from rabbits 7429, 7432, 7433, 7435, 7437, and 7438 exhibited moderate reactivity and sera from rabbits 7430, 7431, and 7434 exhibited strong reactivity. One rabbit (7434) exhibited high reactivity with the strongest response toward higher molecular weight proteins within the PPD suspensions (Figure 5).
Immunoblots utilizing cattle sera yielded comparable results for Lot 9801 and Lot 0902. One difference noted was that blots of lot 0902 antigen resulted in specific protein bands being identified in the reactions (Figures 6 and 7). Protein bands were often more evident on blots developed in the TMB substrate whereas the same bands were not as evident utilizing Supersignal. These results further suggest that removing the autoclaving step in the production process is not detrimental to the immunological characteristics of the MAP PPD.

**Discussion**

The primary objective of this study was to test the effect of heat treatment on PPD preparations by examining certain performance characteristics of PPD preparations. To achieve this objective, we measured protein concentrations and analyzed preparations by SDS-PAGE and immunoblot analysis. We found that the autoclaving step may not be necessary in producing PPD; but it is not known if this affects the potency of the PPD. In addition, the effect of autoclaving perhaps leads to increased protein fragmentation, which contributes to the smearing observed during SDS-PAGE analysis and was not a productive method to define differences between these preparations. Further work must be done to define the protein components and measure delayed-type hypersensitivity potency within these modified preparations.

With additional research in identifying the proteomic profiles of MAP, the need to identify specific protein components of the PPD has increased in recent years. Current production methods require the establishment of a floating mat or pellicle for PPD production. This particular method allows for expression of surface proteins and proteins which may be secreted and available within the growth media. This method should allow for a majority of the proteins present to be surface proteins, rather than cytosolic proteins. Because surface proteins
would be the first antigenic components encountered in an infected animal, this should allow for more specific reactions as well as possibly earlier detection. Studies involving secreted antigens have recognized the importance of such components in paratuberculosis diagnosis (Cho and Collins, 2006; Willemsen et al., 2006). The interest in secreted antigens has primarily involved their significance for use in enzyme-linked immunosorbent assays (Facciuolo et al., 2013; Shin et al., 2008). However, the significance of these culture filtrate proteins had not been examined more closely in regards to components within PPD preparations nor their significance in cell mediated immune responses.

Current culture production methods worked equally well in either Povitsky bottles or Erlenmeyer flasks. These data would suggest that the specific growth containers should not have an impact on final product quality, but should allow for sufficient surface area for the formation of a floating mat. The adequate formation of a stable and thick floating mat of cells is likely to be the greatest influence on the quality of final product.

SDS-PAGE analysis of different PPD preparations produced by traditional methods has shown little to no specific protein banding characteristics. It was thought this may be a result of the autoclaving step involved in the production process. Results indicate that removing the autoclaving step within the procedure results in a final PPD product with better defined protein bands in SDS-PAGE analysis. While some proteins could be visualized to a limited extent within PPD preparations that were autoclaved in the production method, these proteins were obscured and difficult to visualize. The modification of the current production method may allow for a better defined PPD product. Further analysis of specific proteins observed in SDS-PAGE gels from Lot #0902 should be conducted to identify possible immunogenic proteins and support possible changes to production methods.
Immunoblot analysis was able to identify three antigenic proteins against rabbit antisera raised against PPD lot 9801. One of these proteins (34 kDa) was also identified readily on SDS-PAGE analysis of the different PPD preparations, except for PPD Lot 9801. This result would indicate a possible dominant protein which may be contributing to the identification of Johne’s positive animals through the use of PPDs in field testing. However, how these particular three reactive proteins may relate to a cell mediated immune response, versus a humoral immune response, is not yet known. It is not initially known why no specific protein bands could be visualized within the PPD Lot 9801 immunoblot preparation compared to the recent PPD preparations. This may relate to the increased number of protein fragments which result from the traditional production method. However, sera from the inoculated rabbits did indicate reactivity as having protein smearing similar to what is observed within gels, but the exact immunogenic properties of these proteins or protein components is still unknown.

Overall, the current traditional production method should be further examined to provide a final product which can more easily identify proteins produced within culture supernatants that may serve as dominant cell mediated immune response components. The development of the alternative production method provided a PPD which was more conducive to analysis by SDS-PAGE and immunoblot assays; however, more work needs to be done to determine the effect on potency. Nonetheless, this new method will more readily enable comparisons in protein expression between production lots. A first step in additional characterization will be an evaluation utilizing the guinea pig potency test to determine if a change in production method will yield a product that is as potent as the current PPD Lot 9801 used in the field. Potency testing also assists in determining protein biological activity relating to a cell mediated immune response versus a humoral immune response, which will be an important aspect to be considered.
to ensure a continued quality product for animal skin testing procedures. In addition, if specific proteins can be determined as dominant components of PPD preparations, the information will lend support to establishment of a better characterized reference product for both *in-vivo* and *in-vitro* testing procedures.

**References**


Dunkin, G., 1933. The preparation of johnin from a synthetic medium without the addition of *B. phlei*. *J Comp Pathol* 46, 159-164.


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Table 2: Mycobacterium avium subsp. paratuberculosis (MAP) cells (wet weight) obtained from production flasks/bottles. Wet weight of MAP cells was consistent between culture vessels.

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Figure 2: Mycobacterium avium subsp. paratuberculosis 19698 floating culture for PPD production in Povitsky Bottle. Culture is at 4 weeks incubation at 37°C.

Figure 3: Results of a Coomassie blue stained 4-12% Novex Bis-Tris gel showing 7 PPD preparations. Note the striking differences in PPD produced using the traditional method involving autoclaving (lanes 2, 4, and 5 for example) in comparison to PPD produced using an alternative production method (lanes 6 and 7). Lane assignments include: 1- protein standards; 2-Lot #9801 PPD; 3-Lot 9801 1:10 dilution; 4-Lot 0802; 5-Lot 0803A; 6-Lot 0902A; 7-Lot 0902B; 8-M. bovis PPD; 9 – M. avium PPD; 10- protein standards.

Figure 4: Results of a Coomassie blue stained 10% Novex Bis-Tris gel showing 8 different PPDs. Observed the protein smearing of PPD produced by the traditional method involving autoclaving (Lanes 2, 6, 8, and 9). Dilution of PPD clarified protein smearing but did not resolve visibility of protein components. Distinct protein banding is observed in PPD produced by the alternative production method (Lane 9). Lane assignments include: 1- protein standards; Lane 2-Lot #9801 PPD; Lane 3-Lot 0801A (1:5 dilution); Lane 4-Lot 0801B (1:5 dilution); Lane 5-Lot 0802 (1:5 dilution); Lane 6-Lot 0803A; Lane 7-Lot 0902A; Lane 8- M. bovis PPD; Lane 9 – M. avium PPD; Lane 10- protein standards.

Figure 5: Results of humoral immune response by immunoblot using sera from rabbits 7429, 7430, 7431, 7432, 7433, 7434, 7435, 7437, and 7438 diluted 1:500 and PPD 0902. Odd numbered lanes represent day 0 (pre-inoculation) sera and even numbered lanes represent 86d post-inoculation samples. Lane assignments: 1-2: 7429, 3-4: 7430, 5-6: 7431, 7-8: 7432, 9-10: 7433, 11-12: 7434, 13-14: 7435, 15-16: 7437, 17-18: 7438. An individual band is visible at approximately the mid-way point of lane 4 (Rabbit 7430) indicating reactivity to a specific protein component. Overall responses were weak among a majority of rabbits.

Figure 6: Results of cattle sera immunoblots using PPD 0902 produced by an alternative production method on the left and PPD 9801 produced by the traditional method on the right. Both membranes contained identical serum samples loaded into independent slots. Note that distinct immunodominant bands emerge in the alternate PPD preparation. Lanes 1 – 15 cattle positive for Johne’s disease, Lanes 16 & 17 cattle negative for Johne’s disease. Lane assignments correspond to cattle listed in Table 1.
<table>
<thead>
<tr>
<th>Blot Lane</th>
<th>Animal ID</th>
<th>Serological Status&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IDEXX ELISA OD Value</th>
<th>Prionics ELISA OD Value</th>
<th>Fecal Culture Status&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>1</td>
<td>Lindenhoff 90 (PA)</td>
<td>Pos</td>
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<td>2</td>
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<td>0.465</td>
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<sup>a</sup> Serological status determined by IDEXX and Prionics ELISA test results. IDEXX ELISA OD values read at 450 nm; Prionics ELISA OD values read at 450 nm.

<sup>b</sup> 1-10 Colony forming units/gram (CFU/g) feces = low shedder; 11-50 CFU/g feces = moderate shedder; >50 CFU/g feces = high shedder.
Table 2: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cells (wet weight) obtained from production flasks/bottles. Wet weight of MAP cells was consistent between culture vessels.

<table>
<thead>
<tr>
<th>PPD Lot #</th>
<th>Media Volume (ml)</th>
<th>Cell Wet Weight (gr)</th>
<th>Grams Cells/ml Media</th>
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<tbody>
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<td>0801A (Povitsky Bottles)</td>
<td>6500</td>
<td>212.8</td>
<td>0.033</td>
</tr>
<tr>
<td>0801B (E-Flasks)</td>
<td>5800</td>
<td>218.8</td>
<td>0.038</td>
</tr>
<tr>
<td>0802 (E-Flasks)</td>
<td>6000</td>
<td>152.0</td>
<td>0.025</td>
</tr>
<tr>
<td>0803 (Povitsky Bottles)</td>
<td>10,000</td>
<td>210.0</td>
<td>0.021</td>
</tr>
</tbody>
</table>
Figure 1: *Mycobacterium avium* subsp. *paratuberculosis* 19698 floating culture for PPD production in Erlenmeyer flask. Culture is at 8 weeks incubation at 37°C.
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CHAPTER 4: COMPOSITION AND POTENCY CHARACTERIZATION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS PURIFIED PROTEIN DERIVATIVES AND PROTEIN COMPONENTS

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Chapter was reviewed and approved by the USDA/ARS National Animal Disease Center and the USDA/APHIS National Veterinary Services Laboratories manuscript review committees

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Abstract

Purified protein derivatives (PPDs) previously prepared by two different methods from the cultured filtrate of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) ATCC Strain 19698 were further characterized and compared. The traditional production method, which incorporates autoclaving prior to filtration of the culture media, was compared to a modified method that removed autoclaving during the process. PPD preparations were characterized utilizing mass spectrometry and compared for skin test responses using the guinea pig potency assay. Mass spectrometry identified 131 MAP proteins among the three PPD preparations, ten which were present in all preparations. Guinea pig potency testing between PPD preparations resulted in significant difference between production lots. The overall potency of each tested lot was acceptable based upon the measured reactivity. Selected proteins identified by mass spectrometry were recombinantly expressed and purified from *E. coli* and evaluated by the guinea pig potency assay. Seven recombinant proteins showed greater erythema as compared to the reference PPD lot 9801 in paired guinea pigs and stimulated interferon gamma production from Johne’s positive animals. These results suggest that autoclaving culture suspensions is not a necessary step in PPD production and specific proteins could supplant the PPD antigen for the intradermal skin test and for use as *in-vitro* assay reagents.

**Key Words:** bovine, paratuberculosis, proteomics, purified protein derivative, Johne’s disease, skin test, delayed-type-hypersensitivity.
Introduction

The use of cell-mediated immunity detection methods remains the optimal tool for identifying animals in the early stages of Johne’s disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Testing protocols utilizing purified protein derivatives (PPD) in either intradermal skin testing or interferon-gamma (IFN-γ) assays provide for such detection methods. In the early stages of infection the immune response involves a T helper type 1 (Th1) polarization of the immune response along with MAP-specific IFN-γ production (Stabel, 2006; Stabel and Whitlock, 2001). As disease progresses, there is a transition to a T helper type 2 (Th2) immune response with the production of MAP-specific antibodies and a decrease in IFN-γ production. During this transitional stage accurate diagnosis can be difficult and the use of multiple testing strategies proves most useful (Koets et al., 2002; Nielsen and Toft, 2008).

The importance of having a reliable and well characterized PPD as part of an intradermal skin testing program or as a mitogen in an IFN-γ assay is critical to providing improved test protocols during preclinical stages of infection (Coussens, 2001; Stabel, 1996). Various studies have reported issues related to sensitivity and specificity of PPD preparations when used for such test methods (de Lisle and Duncan, 1981; de Lisle et al., 1980; Jungersen et al., 2002; Jungersen et al., 2012; Larsen et al., 1963).

Factors including a consistent production protocol utilizing a well-defined MAP isolate can influence protein expression. Comparison of MAP strain K-10 and a field isolate from a cow exhibiting clinical signs of Johne’s disease revealed there were marked differences in protein expression when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and mass spectrometry (Radosevich et al., 2007). Differences in protein expression were related to differences in passage level of the two isolates compared. What was
not clear from the study is how the specific protein differences may affect MAP virulence or the specific role the various proteins play in cell-mediated immune responses.

Additional information contributed from work with various MAC isolates used for PPD production identified a large sequence polymorphism (referred to as LSP<sup>jn</sup>) as being absent in seven such MAP strains (Semret et al., 2006). Deletion of this particular locus is thought to have occurred due to continual laboratory passage as it was shown to be intact in field isolates.

Mass spectrometry analysis of various PPD products has begun to reveal variations in PPD composition. Proteomic analysis of *Mycobacterium bovis* (MB), *Mycobacterium avium* subspecies *avium* (MAA), and MAP derived PPD suspensions was able to identify a large number of proteins present in each preparation (Santema et al., 2009). Three MAP proteins were further analyzed in a serum antibody ELISA with MAP1138c being the best recognized. Poor reactivity in a lymphocyte proliferative assay did not support use of the selected proteins as being viable for consideration in cell-mediated immune response based assays.

An analysis of various PPD products from various laboratories and facilities revealed that the proteomic composition was highly similar and that variability identified was due to MAP strains selected for production protocols as well as the age of the MAP cultures (Wynne et al., 2012). The abundance of the various proteins in each PPD preparation varied greatly, which may be related to production processes or the specific MAP isolate used. A critical finding in this study was that MAP PPD produced at the United States Department of Agriculture (USDA) National Veterinary Services Laboratories (NVSL) contained only six detectable proteins. This result seemed in contrast to protein quantity indicated from previous potency testing results against MAP 19698 sensitized guinea pigs in which such testing revealed acceptable potency results.
In our current work, previously prepared MAP PPD suspensions were selected for characterization of the specific protein components. Characterization was conducted by mass spectrometry for initial identification of protein components. Guinea pig potency testing was conducted to compare PPD preparations and to also provide information of cell-mediated responses of specific individual proteins identified from the mass spectrometry data. Information from this study can be used toward improved production methods for a well characterized and consistent PPD. Such an improved reagent would provide higher confidence in the use of cell-mediated immune response test protocols allowing for earlier detection of MAP infected animals.

**Materials and Methods**

**Bacteria, PPD preparation and source.** MAP strain 19698 was used in all the MAP PPD production processes. *E. coli* DH5-α served as the host strain for recombinant protein expression. PPD lots produced at the NVSL in 1998 (9801), and ten years later in 2008 (0802, 0803A) were produced by traditional production methods involving autoclaving of production flasks containing floating cultures with subsequent protein precipitation using trichloroacetic acid (TCA) as described previously (Haagsma and Angus, 1995; Steadham et al., 2002). Lot 9801 currently serves as a reference lot and has been used in the field for cattle skin testing purposes since its production in 1998. Lot 0902 was produced by an alternative production method involving sterile filtration in place of autoclaving followed by protein precipitation using TCA. Lots 0902A and 0902B, which comprised the larger PPD 0902 bulk preparation, were harvested on different days from the same culture inoculum. In production of large PPD batches (lots), multiple concentrated harvest bulks are commonly combined to reduce the number of
potency tests conducted and allow for larger final product release volumes. Due to the alternative production process used for PPD 0902 these two smaller bulks were held as reserve on each harvest date to evaluate possible differences in this revised procedure. Purity testing was conducted in accordance with the Code of Federal Regulations, 9CFR part 113.26.

**Cattle sera.** Serum samples were obtained from cattle housed at the NVSL and from naturally infected animals on dairy farms in the US. Sera from these animals were used in the annual nationwide NVSL Johne’s disease serology proficiency test program, and represent a well-characterized and standardized serum set. Naturally infected cattle were confirmed serologically positive on both the Prionics Paracheck® and IDEXX MAP ELISA assays (Table 1). Positive cattle were also confirmed positive by fecal culture on Herrold’s Egg Yolk with Mycobactin J (HEY-MJ). Negative sera were from negative control source animals within the NVSL and also from MAP negative confirmed production herds participating in the USDA Voluntary Bovine Johne’s Disease Control Program. Negative animals were confirmed by bacterial culture and serology using the same diagnostic tests as the positive cattle.

**Recombinant protein production.** The cloning, production, and purification of recombinant MAP proteins were performed as previously described (Bannantine and Paustian, 2006). Briefly, maltose binding protein (MBP) fusions of MAP predicted coding sequences were constructed in *Escherichia coli* DH5α using the pMAL-c2 vector (New England Biolabs, Beverly, MA). Primers designed from the reading frame of each coding sequence contained an *Xba*I site in the 5’ primer and a *Hind*III site in the 3’ primer for cloning. Ligation of the DNA fragments was followed by transformation into *E. coli* and selection on Luria-Bertani (LB) agar plates containing 100 µg/ml ampicillin. PCR screening of ampicillin resistant colonies was conducted with primers used to amplify the sequence from MAP DNA.
Each MBP fusion protein was overexpressed in *E. coli* by induction of 1-L LB broth cultures with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Chemical Company, St. Louis, MO) for 2.5 hr with shaking at 37°C. Cells were harvested by centrifugation and resuspended in column buffer for sonication. Affinity chromatography purified protein fractions were eluted and analyzed using a NanoDrop spectrophotometer at 280 nm. The most concentrated fractions were pooled and dialyzed at 4°C against 1.5 L phosphate buffered saline (PBS). Purified proteins were aliquoted and stored at -20°C for future use.

**Mass spectrometry.** Proteomics experiments were accomplished using previously published methods (Lippolis et al., 2014). Total protein concentrations were determined using the Biorad Protein Assay Kit (BioRad, Hercules CA), along with a known bovine serum albumin (BSA) standard. Briefly, 100 μg of each sample was resuspended in 250 μl PBS, 3 μl of 10% sodium deoxycholate acid, and 1 μl of 2% sodium dodecyl sulfate (SDS). Samples were heated at 90°C for 20 minutes, then cooled on ice for 5 minutes. Next, 2 μl of 50 mM tris-(2-carboxyethyl) phosphine and 1.5 μl fresh 1M iodoacetimide were added and the samples were incubated 1 hour in the dark at room temperature. An equal volume of methanol was added to the protein sample. Proteins were then digested using 10 μg of trypsin gold (Promega) and incubated overnight at 37°C. Formic acid (10 μl) was added, the sample vortexed, and then spun (14,000 x g for 10 minutes at 4°C) to precipitate detergents. Supernatants were transferred to clean tubes and then dried in a vacuum centrifuge. Peptides were dried in one tube, and held at -80°C until use.

Samples were injected onto nano-LC chromatography using a Proxeon Easy-nLC (Thermo Fisher Scientific, West Palm Beach, FL) connected to the mass spectrometer. The chromatography used a trapping column (Proxeon Easy-Column, 2 cm, ID 100 μm, 5um, 120A,
C18) and an analytical column (Proxeon Easy-Column, 10 cm, ID 75 μm, 3um, 120A, C18).

The gradient using a mobile phase A (95% H₂O: 5% acetonitrile and 0.1% formic acid) and mobile phase B (5% H₂O: 95% acetonitrile and 0.1% formic acid). The gradient was: 0% B for 3 minutes, 0%-8% B from 3-5 minutes, 8-18% B from 5-85 minutes, 18-30% B from 85-100 minutes, 30-90% B from 100-105 minutes, and held at 90% B from 105-120 minutes at continuous flow rate throughout the gradient of 300 nl/min.

The analytical column was connected to a PicoTip Emitter (New Objectives, Woburn, MA; FS360-75-15-N-20) cut to size. The column and Emitter were attached to a LTQ OrbiTrap Velos Pro (Thermo Fisher Scientific, West Palm Beach, FL) mass spectrometer using the Proxeon Nanospray Flex Ion Source. The capillary temperature was set at 275°C and spray voltage was 2.8 kV. The mass spectrometer used a data dependent method. In MS mode the instrument was set to scan 300-2000 m/z with a resolution of 30,000 FWHM. A minimal signal of 20,000 could trigger MSMS and 10 consecutive MSMS were possible. The activation type used was HCD. The normalized collision energy was set to 35 and repeat mass exclusion was set to 120 seconds.

Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer version 1.4.0.288 (Thermo Fisher Scientific, San Jose, CA, USA; version 1.3.0.339). All MS/MS samples were analyzed using Sequest assuming digestion with trypsin. Sequest was set up to search a combined FASTA database of *Mycobacterium avium* complex (taxon #120793) and *Mycobacterium tuberculosis* complex (taxon #77643) databases generated in July of 2013. Sequest was searched with a fragment ion mass tolerance of 0.800 Da and a parent ion tolerance of 10.0 PPM.

**Database searching.** Tandem mass spectra were extracted and charge state deconvoluted by
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**Guinea pig potency assay.** Conventional guinea pigs weighing between 450 and 600 grams were obtained and acclimated to the vivarium housing conditions prior to testing. Guinea pigs were sensitized utilizing a Johne’s bacterin preparation (NVSL lot #0601) consisting of heat-killed MAP cells homogenized in light mineral oil. The bacterin consisted of 23 grams of cells homogenized in 180 ml of mineral oil. The Johne’s bacterin was diluted 1:4.55 in mineral oil and held at 45°C on a hotplate. Each guinea pig was inoculated intramuscularly with 0.1 ml of the warmed suspension. Control guinea pigs were inoculated intramuscularly with 0.1 ml of phosphate chloride buffer (34 mM Na2HPO4, 86 mM NaCl). The sensitization period after inoculation was 35 days after which PPD lot numbers 9801, 0802, 0803A, 0902A were prepared for animal inoculation. Each preparation was diluted to 10 µg/ml and 2 µg/ml in phosphate chloride buffer. Diluted PPD suspensions were bottled and labeled as sample A, sample B, sample C, and sample D.

After the sensitization period and prior to inoculation, all guinea pigs had both sides shaved in preparation for inoculations. The shaved areas were divided into two equal blocks. Each block contained four randomized injection sites resulting in eight total injections. This resulted in replicate injections of each PPD for each guinea pig. Guinea pigs were injected intradermally with a volume of 0.1 ml of the designated MAP PPDs.
Twelve guinea pigs (6 guinea pigs for each PPD concentration) were used for PPD inoculation. An additional 4 guinea pigs were used as negative non-sensitized controls. Twenty-four hours post-injection, test responses were measured to the closest square millimeter by measuring the greater and lesser dimensions of the erythema.

All animal work was conducted under the NVSL approved Institutional Animal Care and Use Committee protocols.

**Guinea pig potency assay of individual proteins.** Forty-seven MAP proteins identified by mass spectrometry were recombinantly expressed in *E. coli* for further evaluation in the guinea pig potency assay. Eighteen guinea pigs, sensitized as described previously, were used in the screening of selected proteins.

After the 35-day sensitization period and prior to inoculation, all 16 guinea pigs had both sides shaved. There was a single row of four randomized injection sites blocked on each side of each animal. This arrangement allowed for the injection of 6 proteins and two control proteins in each animal. Seven pairs of guinea pigs each received six proteins (42 proteins total) and the final pair of animals received five proteins. One guinea pig in each pair also received either PPD 9801 or PPD 0902, whereas the second guinea pig received the MBP/LacZ control peptide diluted to 10 µg/ml in phosphate chloride buffer. This configuration enabled each of the 47 recombinant proteins to be injected twice. Guinea pigs were injected intradermally with a volume of 0.1 ml of the designated antigen.

Twelve recombinant proteins were evaluated further in a second guinea pig potency test. Thirty guinea pigs were sensitized as previously described and four guinea pigs served as non-sensitized control animals. Recombinant proteins were randomized and assigned to respective guinea pig groupings. PPD 9801 served as a positive control and phosphate chloride buffer
served as a negative control. Suspensions were randomized utilizing a Microsoft Excel randomization program. Sensitized guinea pigs were assigned to four groups of five animals per group.

After the sensitization period and prior to inoculation, all guinea pigs had both sides shaved. Each side contained four randomized injection sites resulting in eight total injections for each animal. Each sensitized animal received replicate inoculations of two recombinant proteins divided between the left and right sides. Each animal also received one inoculation of lot 9801 and one inoculation of phosphate chloride buffer. Paired non-sensitized control animals received one inoculation of designated recombinant proteins as well as control suspensions. Guinea pigs were injected intradermally with a 0.1 ml of the designated MAP recombinant protein or control suspension. Guinea pigs were housed with 5 animals per cage with each of the five animals from different treatment groups within a cage. Test responses were again measured to the closest square millimeter 24 hours post-injection. Recombinant proteins were compared against the positive control within each animal.

**Gamma-interferon testing using recombinant proteins.** Evaluation of recombinant MAP proteins was conducted using six Johne’s positive cattle, two *M. bovis* sensitized cattle, and four non-infected control cattle. Approximately 40 - 50 ml of blood was collected from each animal into vacutainer blood tubes containing sodium heparin. Blood samples were held at room temperature prior to stimulation.

Six to seven hours after collection blood tubes for each individual animal were mixed well and combined into a sterile 50 ml conical tube. For each animal 1.5 ml aliquots of blood were dispensed into each well of a 24-well tissue culture plate. Blood was cultured alone as non-stimulated controls, or with pokeweed mitogen at a concentration of 10 µg/ml to serve as a
control providing information related to the viability of cells within each sample. Whole blood was also cultured with designated mitogens at 10 µg/ml. Mitogens used for evaluation were as follows: MAP PPD 9801 (PPD-J), *M. bovis* PPD (PPD-B), *M. avium* PPD (PPD-A), MAP_1997, MAP_4143, MAP_1138c, MAP_3567, MAP_3061c, MAP_2121c, and MAP_3651c. For all mitogens, 100 µl was dispensed into each designated well and each mitogen was cultured in duplicate wells.

Plates were covered and incubated at 37°C for 21-22 hours. Plates were centrifuged at 500 x g for 15 minutes and the plasma from each well was harvested and refrigerated. Plasma samples were tested over the next 72 hours. The INF-γ ELISA was conducted according to the manufacturer’s directions (Prionics - BOVIGAM®). Samples from each well were incubated in duplicate for one hour at room temperature in a 96-well plate that is pre-coated with anti-bovine INF-γ antibody. Plates were washed six times with wash solution and then incubated with an anti-bovine INF-γ-horseradish peroxidase conjugate for 30 minutes at room temperature. Plates were then washed six times with wash solution and incubated with peroxidase substrate for 30 minutes at room temperature. Enzyme stopping solution was added to each well and the plates were read at A_{450} and A_{650} nm.

**Statistical analysis.** The PPD mean skin test responses from the guinea pig potency test were compared by ANOVA. Differences were considered statistically significant at a P of < 0.05.

Results for the recombinant protein guinea pig potency testing were evaluated by calculating the treatment group mean erythema value using the measurements for each individual animal comprising the treatment group. Paired comparisons calculating the difference between the response of the treatment to the response of the positive control for each animal were completed. Results from the five injections for each respective protein were then averaged and
ranked based on the average differences. A positive difference indicates the average response from the treatment is higher than the average response from the positive control. For this difference data, a 95% confidence interval was calculated for each treatment group.

Results

PPD potency assay in guinea pigs. Potency testing of the traditional and alternate PPD production lots resulted in significant differences ($P < 0.05$) when compared against the reference standard (lot 9801; Table 2). Traditional lot 0803A and alternate lot 0902 resulted in the greatest skin test responses and all responses showed a larger erythema as PPD concentration increased. The average area of the erythema at the injection sites for lot numbers 0803A and 0902 were much greater than the average area for the reference standard (261% and 177%, respectively). Traditional PPD lot 0802 resulted in a measurement that was 79% of the average area compared to the reference standard. An acceptable potency test result for new production lots must have a response that is a minimum of 75% compared to the reference response. Using these criteria, all three PPD lots demonstrated acceptable potency, although 0802 is marginal. These data suggest that the potency was not adversely affected by removal of the autoclave step.

Analysis of PPD preparations by mass spectrometry. Mass spectrometry analysis was performed on three PPDs, including the reference (9801) and alternative lots (902A and 902B). The total number of proteins identified among all three PPDs was 194, 78% of which were unique to an individual PPD (Figure 1). All 194 proteins, including peptide matches and molecular weights are listed in Table S1. Ten proteins were detected in all three PPD suspensions (Figure 2). These 10 proteins may represent the best candidates for development of a consistent recombinant PPD and are listed at the top of Table S1. MAP_3840 had the greatest
number of peptide matches among all the PPDs, and was also one of the most common proteins observed among PPDs analyzed in the Wynne et al., 2012 and Santema et al., 2009 studies. Furthermore, it was identified as a cell surface protein using a trypsin shaving technique (He and De Buck, 2010). These results suggest that MAP_3840, which encodes a 70-kDa heat shock protein, is present in high abundance in MAP. Also present among all PPDs were MAP_1588c and MAP_1589c, which are tandemly located on the K-10 genome (Li et al., 2005) and have been examined as potential diagnostic antigens long ago (Olsen et al., 2001). Furthermore, both have been reported as a stress protein (Gumber et al., 2009) and MAP_1589c was also found to be surface located (He and De Buck, 2010).

**Potency assay of recombinant proteins in guinea pigs.** Forty-seven of the 194 proteins identified by mass spectrometry were cloned, expressed, and purified in *E. coli*. These recombinant proteins were used for potency testing to determine if they substantially contribute to the skin test response observed with PPDs. Seven proteins showed reproducible skin test responses that were stronger than or equivalent to the reference lot 9801 (Table 3, highlighted in bold). The strongest skin test response among all recombinant proteins was elicited by MAP_1997, which is annotated as an acyl-carrier protein (Table 3). This protein has not been previously described as an antigen in the literature, although it was listed among surface exposed proteins in MAP (He and De Buck, 2010). Known antigens that are among these seven proteins include the major membrane protein (Bannantine et al., 2007; Li et al., 2007) and the LrpG protein (Leite et al., 2014; Santema et al., 2009). Seventeen additional proteins produced measureable skin test response in one of two replicate intradermal inoculations. The MBP/Lac-Z control suspension showed no visible reactivity at any inoculation site.
Twelve recombinant proteins that showed the largest skin test responses listed in Table 3 were evaluated further in a second guinea pig potency test. Even the four best proteins were still not considered significantly different from the reference 9801 PPD based upon results from the ranking by difference calculations (Table 4). Those recombinant proteins were MAP_1997, MAP_4143, MAP_3567, and MAP_1138c. The only recombinant protein that elicited responses greater than the reference PPD was MAP_1997, but this difference was not significant. The eight recombinant proteins showing the greatest responses are shown in Figure 3. MAP_3061c, MAP_3651c, and MAP_2121c elicited responses significantly less than the reference PPD.

**Gamma-interferon assay using MAP recombinant proteins.** Seven recombinant proteins having the best performance in the guinea pig potency test were further evaluated as mitogens in an IFN-γ stimulation assay. The proteins were evaluated against Johne’s positive animals (#790, #2407, #2222, #8339, #1211, and #1044) based upon results of serological and fecal shedding, *M. bovis* sensitized animals (#4 and #11), and negative control animals (#1081, #212, #61, and #1392). MAP_3651c and MAP_3567 demonstrated the greatest IFN-γ stimulation within the Johne’s positive animals with responses in some animals being greater than the stimulation values from MAP PPD 9801 (Figure 4). MAP_1997, which resulted in the greatest skin test response in the guinea pig potency test, showed the least IFN-γ stimulation. Each MAP recombinant protein resulted in negative stimulation values against samples from the *M. bovis* sensitized animals (Figure 5). All negative control samples remained negative for IFN-γ stimulation using MAP recombinant proteins or PPD suspensions, indicating good specificity.

In summary, selected recombinant proteins provided comparable or more potent responses than the PPD in skin testing procedures, as determined by the guinea pig potency assay. In addition,
these recombinant proteins may serve as antigens in an INF-γ assay, resulting in interferon-gamma production comparable or greater than stimulation using MAP PPD.

**Discussion**

This study had two objectives with the overall goal of analyzing an antigen with previously limited evaluation, but of importance for use in Johne’s disease diagnosis and research. One objective was to compare the effect of autoclaving on MAP PPD preparations and measure this effect through guinea pig potency testing. The data suggest that removal of the autoclaving step may enhance immunogenicity and prevent protein degradation, but does not negatively affect the potency. A second objective was to define the components present in each preparation through a proteomic analysis and determine proteins commonly present in the different production lots. A subset of the identified PPD proteins was then selected for evaluation in a guinea pig potency test to determine skin test responsiveness in sensitized animals. In addition these proteins were further evaluated to determine their use as mitogens in IFN-γ testing. These objectives worked toward the overall goal of obtaining a more consistent and equally potent PPD that may be composed entirely of recombinant proteins. This reagent could then circumvent the problems inherent in obtaining consistently potent PPDs. Finally, a “recombinant PPD” may avoid cross reactivity issues in cattle vaccinated against *M. bovis* or MAP.

The use of high pressure heat inherent in the autoclaving process results in lysis and the appearance of cytosolic proteins that may not be present when autoclaving is eliminated. The presence of the 70-kDa heat shock protein (MAP_3840) in both traditional and alternate PPDs argues against this idea since it is considered a cytosolic protein. However, because this protein
is present in high abundance in MAP, it appears at some level in all bacterial fractions including membrane and secreted fractions (Facciolo et al., 2013; He and De Buck, 2010). Since secreted or surface proteins should be the first antigenic components encountered in an infected animal, this may allow for more specific responses as well as possibly earlier detection. Multiple studies have shown that many strong antigens are also secreted proteins in MAP (Cho and Collins, 2006; Willemsen et al., 2006). The interest in secreted antigens has primarily involved their significance for use in enzyme-linked immunosorbent assays (Facciolo et al., 2013; Shin et al., 2008). In addition, the 10 proteins identified by mass spectrometry common to all three NVSL PPD preparations were also identified as part of the culture filtrate protein composition characterized by Facciolo et al (2013). In total there were 58 proteins in the NVSL PPD preparations identified by mass spectrometry that were in common with the Facciolo et al. study. Furthermore, of the seven recombinant proteins resulting in the greatest skin erythema in the guinea pig potency test five of those proteins were also identified in the culture filtrate found by Facciolo et al. (2013). These include MAP_1997, MAP_4143, and MAP_3061c, which were the most reactive in the guinea pig potency test.

Potency testing of four MAP PPD lots (9801, 0802, 08032A and 0902) was conducted to ensure that the PPD’s produced by conventional and a modified production protocol retained acceptable potency in sensitized guinea pigs. Replacing the autoclaving step with a sterile filtration process to remove cells would avoid protein degradation, but it was uncertain if this change may affect potency. Results from potency testing suggest there was no significant effect on potency regardless of whether the culture material was subjected to autoclaving or sterile filtration. These results represent the first study to examine this variable effect of heating on PPD performance in guinea pig potency testing. However, our results suggest that age of the
PPDs might have an effect on potency because the skin test responses were greater in the recently prepared PPDs (0803A and 0902) than the reference lot prepared in 1998.

Work to identify protein components of NVSL MAP PPD lot 9801 utilizing an *Escherichia coli* expression vector system resulted in limited success. The expression library screening procedure, using rabbit antiserum raised against MAP PPD 9801, identified three partial protein sequences for MAP0805c, MAP0806c, MAP0807c, and MAP2117c. The low number of proteins identified utilizing the expression library was surprising. Subsequent attempts to amplify these nucleotide sequences were unsuccessful leading to pursuing mass spectrometry for identification of individual protein components of the MAP PPD preparations.

Mass spectrometry analysis of PPD preps has revealed some of the proteins present in these complex mixtures of proteins, carbohydrates and lipids. In a separate study, proteomic analysis of *M. bovis, M. avium* subspecies *avium*, and MAP derived PPD suspensions identified 156 proteins among all PPDs (Santema et al., 2009). Three of these proteins (MAP_1718c, MAP_3515c, and MAP_1138c) were further analyzed in a serum antibody ELISA with MAP1138c (LprG) emerging as the strongest antigen in high fecal shedding cattle. MAP LprG was also previously identified from a MAP gene fusion library screening study and found to be immunogenic in bovine paratuberculosis serum samples by Western blot analysis (Dupont, 2005). Our study also identified LprG and it was among the strongest stimulators in guinea pigs (Table 3). However, LprG showed no significant reactivity in a lymphocyte proliferative assay (Santerma et al., 2009) decreasing its consideration for use in cell-mediated immune response based assays. LprG elicited IFN-γ responses in eight of nine sheep that had been vaccinated with an attenuated MAP strain in the study by Dupont et al. (2005). Using LprG as a mitogen in the IFN-γ assay 50% of Johne’s positive cows, and all *M. bovis* sensitized and MAP negative
animals were identified correctly, whereas in the Santema et al. (2009) study there was no significant difference between positive and negative animals. While results varied for use as a mitogen for in-vitro IFN-γ stimulation, its ability to elicit immunological responses in both humoral and cell-mediated assays may be indicative that MAP LprG is a highly expressed protein during all stages of Johne’s disease and could prove to be a more versatile candidate for diagnostic assay improvements.

A total of 194 proteins were identified among all preparations with 110 of these identified in lot 9801. This information greatly adds to a previous study which identified only six proteins in this same NVSL lot 9801 (Wynne et al., 2012). It is interesting to note that reference PPD 9801 had the most proteins identified by LC-MS/MS, yet was migrating as a smear in denaturing protein gels and did not appear immunogenic by Western blot analysis or as potent by guinea pig sensitization. These results show that while immune stimulating components were destroyed in the heating process, they did not affect characteristics used for MS detection. Fewer proteins were identified in the alternate PPD lots, with 67 and 74 proteins being identified in each production lot. Although a number of proteins were identified in two of the three preparations analyzed, only ten proteins were detected in all three preparations. Combining the results from the Wynne et al. (2012) and Santema et al. (2009) studies shows the number of proteins consistently appearing in PPD preparations among all three studies is three. They include MAP_4143, MAP_1595, and MAP_3840. The total number of MAP proteins identified at least once in any of the three studies was 214, which suggests a starting point for the complete PPD proteome of MAP. Although some variability may be attributed to the methodology used for protein identification, the small overlap of proteins nonetheless further suggests the
inconsistencies that can be encountered in PPD production processes and the need to improve such processes to reduce or eliminate such variability.

A subset of proteins identified from mass spectrometry data was evaluated in a well-established guinea pig potency assay. During the potency testing a Mbp/Lac-Z control was included to ensure this particular component of the recombinant protein composition would not accentuate the skin test reactions. Previous work using Mbp/Lac-Z recombinant MAP proteins in a serological assay examined the possible immunological effects of cleaving the Mbp portion from the protein structure (Gurung et al., 2013). Using serum from MAP-infected sheep Gurung found that cleaving the Mpb/Lac-Z portion of the proteins had little effect upon the immunological reactivity of the proteins. Reactivity observed in two Mpb/Lac-Z inoculation sites during the guinea pig potency testing was likely due to nearby individual protein reactions. In both instances the Mpb/Lax-Z inoculation sites were adjacent to inoculation sites of individual proteins which had large diffuse intradermal reactions. Seven proteins, including an acyl carrier protein, elongation factor protein, flavoprotein, and a hypothetical protein are among those that showed equivalent or greater skin erythema than lot 9801. Neither Mpb/Lac-Z nor the phosphate chloride buffer inoculate sites had measureable reactions during retests of those materials. These data clearly suggest that recombinant proteins are worthy of additional study and could ultimately replace the use of PPD preparations for skin testing.

Antigenicity evaluation in a serological based assay of four individual MAP proteins was recently conducted in a study with final results indicating assay performance that was similar to currently available commercial assays (Gurung et al., 2014b). Two proteins, MAP3567 and MAP2698c, evaluated in that study were also part of the protein subset screened in the guinea pig potency test as part of this study. MAP3567 induced strong skin test reactions in sensitized
guinea pigs while MAP2698c had reactivity lower than either of the PPD suspensions used as controls, but still had easily measureable cell-mediated immune responses. These results are similar to additional studies conducted by Gurung (Gurung et al., 2014a; Gurung et al., 2014c) in which those studies identified MAP2698c as a strong candidate for additional work related to either vaccine formulations or use in an IFN-γ assay. One contrasting difference found in this current study is that MAP3567 appeared to offer better cell mediated immunoreactivity and might be a better candidate toward use in a cell-mediated immune detection assay.

Santema and colleagues used MAP1138c (LprG protein) in a lymphocyte stimulation assay and enzyme linked immunosorbent assay resulting in high shedder animals having significantly higher responses than negative control animals (Santema et al., 2009). MAP LprG was also previously identified from a MAP gene fusion library screening study and found to be immunogenic in bovine paratuberculosis serum samples (Dupont, 2005). Results from these studies correlate well to the finding that MAP LprG was highly immunogenic during the guinea pig potency test screening process in this study. LprG was one of the seven highly reactive proteins identified. A surprising finding from these previous studies is that MAP LprG was immunogenic in assays based upon humoral immune responses and cell-mediated immune responses since the immune response tends to shift away from a cell-mediated immune response during clinical stages of disease. However, variation and lack of uniformity in the progression of antibody response to various antigens during Johne’s disease progression was identified in a study by Koets (Koets et al., 2001). This may be indicative that MAP LprG is highly expressed protein during all stages of Johne’s disease and could prove to be a more versatile candidate for assay improvements.
MAP_0217 and MAP_1609c that were identified by mass spectrometry in PPD lot #0902B and PPD lot #9801, respectively, were also used in a previous study evaluating a recombinant multiantigenic MAP subunit vaccine (Thakur et al., 2013). Results of this vaccination study indicated that both proteins did not produce significant cell-mediated immune responses in vaccinated cattle. This correlates to the results observed in the present study during the guinea pig potency test in which the skin test response elicited by MAP_1609c was significantly lower than the control PPD lot 9801.

MAP recombinant proteins used as antigens in an INF-γ assay showed five proteins as potential candidate antigens. MAP_3651c and MAP_3567 were the strongest mitogens with Johne’s positive samples and they correctly identified four of the six animals as positive. MAP_3061c, MAP_1138c, and MAP_4143 were comparable to each other, and also identified three of the same animals identified by MAP_3651c and MAP_3567. These results were comparable to animals identified as positive using MAP PPD 9801 with differences noted for two animals. Cow #2407 was identified as positive by MAP PPD 9801, but was negative by all individual proteins. In contrast, cow #8339 was negative using MAP 9801, but was positive by five of the seven individual proteins. These results were in contrast to the guinea pig potency test results in which MAP_3651c and MAP_3061c did not stimulate skin test responses comparable to MAP PPD 9801. Also in contrast to the strong skin test response in the guinea pig potency test, MAP_1997 was not a strong mitogen in the INF-γ stimulation assay. These contrasting differences between the two tests demonstrate that neither test is a good predictor of success in the other and may be due to the additional immunological responses ongoing at skin test sites that involve more than INF-γ alone.
Our results suggest that autoclaving during PPD preparation, though performed for decades, is an unnecessary step in PPD production. Over 100 proteins have now been identified from NVSL PPDs that have been used in field studies for decades. Results from this study have also identified a number of proteins that are reactive to skin testing in sensitized guinea pigs and as mitogens in IFN-γ assays. These proteins can contribute to improvements for a well-defined, consistent PPD for diagnostic purposes. Further research is needed to confirm the DTH responses of these proteins and examine specificity characteristics to differentiate MAP immunological responses from *M. bovis*-infected cattle.

**References**


List of Tables and Figures:

Table S1: Proteins identified by mass spectrometry from *Mycobacterium avium* subsp. *paratuberculosis* (MAP) PPD lot numbers 9801, 0902A and 0902B. Proteins are arranged according to similarity between the various PPD suspensions.

Table 1: Cattle Sera used for IFN-γ stimulation assay

Table 2: Guinea pig potency test skin test responses at 24 hours post-inoculation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) purified protein derivative (PPD).

Table 3: Positive skin test responses in a guinea pig potency test of 24 MAP recombinant proteins from 47 total proteins tested.

Table 4: MAP recombinant protein guinea pig potency testing summary of the treatments ranked by subtracted differences.

Figure 1: Number of proteins identified by LC-MS/MS for each PPD preparation analyzed. Proteins found in two or three PPD preparations are indicated by the black bars and unique proteins to each individual PPD preparation are indicated by the grey bars.

Figure 2: Venn diagram illustrating protein overlaps between PPD 9801, PPD 0902A, and PPD 0902B. The number of proteins identified for each PPD are indicated. Although the majority of proteins identified were unique to a given production lot, 10 proteins were present in all three lots analyzed (see Table S1).

Figure 3 Guinea pig potency testing of 12 MAP recombinant proteins with average skin test reaction measurements (mm²) indicated. Asterisk label indicates significantly different skin test reactions compared to the MAP PPD 9801 reference.

Figure 4 Secretion of IFN-γ using whole blood from Johne’s positive animals. Absorbance was measured at 450 nm.

Figure 5: Secretion of IFN-γ using whole blood from *Mycobacterium bovis* sensitized animals and negative animals. Absorbance was measured at 450 nm.
Table S1: Proteins identified by mass spectrometry from *Mycobacterium avium* subsp. *paratuberculosis* (MAP) PPD lot numbers 9801, 0902A and 0902B. Proteins are arranged according to similarity between the various PPD suspensions.

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<td>4</td>
<td>Neg&lt;sup&gt;d&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td>Neg</td>
</tr>
<tr>
<td>11</td>
<td>Neg&lt;sup&gt;d&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td>Neg</td>
</tr>
<tr>
<td>1081</td>
<td>Neg</td>
<td>0.036</td>
<td>N/A</td>
<td>Neg</td>
</tr>
<tr>
<td>212</td>
<td>Neg</td>
<td>0.067</td>
<td>N/A</td>
<td>Neg</td>
</tr>
<tr>
<td>61</td>
<td>Neg&lt;sup&gt;d&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td>Neg</td>
</tr>
<tr>
<td>1392</td>
<td>Neg&lt;sup&gt;d&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td>Neg</td>
</tr>
</tbody>
</table>

<sup>a</sup> Serological status determined by IDEXX and Prionics ELLISA test results.
<sup>b</sup> 1-10 CFU/gr feces = low shedder; 11-50 CFU/gr feces = moderate shedder; >50 CFU/gram of feces = high shedder
<sup>c</sup> Serum samples used in IFN-γ testing only.
<sup>d</sup> Based upon previous IFN-γ testing.

Table 2: Guinea pig potency test skin test responses at 24 hours post-inoculation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) purified protein derivative (PPD).

<table>
<thead>
<tr>
<th>PPD Concentration</th>
<th>PPD 9801</th>
<th>PPD 0802</th>
<th>PPD 0803A</th>
<th>PPD 0902</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/ml</td>
<td>143.22 ± 48.82</td>
<td>93.63 ± 15.59</td>
<td>264.25 ± 39.72</td>
<td>202.07 ± 39.63</td>
</tr>
<tr>
<td>2 µg/ml</td>
<td>45.52 ± 32.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.45 ± 15.49</td>
<td>228.41 ± 41.91</td>
<td>132.50 ± 67.79</td>
</tr>
</tbody>
</table>

<sup>a</sup> PPD 9801, 2 µg/ml sample data, contained one inoculation of one guinea pig which had not measurable skin reaction. This resulted in an elevated standard deviation value. All guinea pigs were sensitized with MAP strain 19698 killed cells. Values represent the average response (mm² ± standard deviation) of six guinea pigs used for evaluating each PPD concentration.
<table>
<thead>
<tr>
<th>Protein Description</th>
<th>MAP Locus Tag</th>
<th>Area Measurement (mm$^2$)</th>
<th>Standard Deviation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl carrier protein</td>
<td>1997</td>
<td>231.52</td>
<td>87.70</td>
</tr>
<tr>
<td>Elongation factor Tu</td>
<td>4143</td>
<td>209.08</td>
<td>124.13</td>
</tr>
<tr>
<td>Electron transfer flavoprotein (Beta-subunit) FixA</td>
<td>3061c</td>
<td>192.36</td>
<td>87.68</td>
</tr>
<tr>
<td>Putative uncharacterized protein</td>
<td>2694</td>
<td>171.72</td>
<td>56.83</td>
</tr>
<tr>
<td>Putative acyl-CoA dehydrogenase</td>
<td>3651c</td>
<td>166.53</td>
<td>77.51</td>
</tr>
<tr>
<td>LprG protein</td>
<td>1138c</td>
<td>161.79</td>
<td>50.09</td>
</tr>
<tr>
<td>Major membrane protein 1</td>
<td>2121c</td>
<td>128.90</td>
<td>11.11</td>
</tr>
<tr>
<td>Peroxisomal multifunctional enzyme type 2</td>
<td>3567</td>
<td>121.26</td>
<td>N/A</td>
</tr>
<tr>
<td>Chaperone protein DnaK</td>
<td>3840</td>
<td>110.91</td>
<td>N/A</td>
</tr>
<tr>
<td>Putative uncharacterized protein</td>
<td>3692c</td>
<td>74.29</td>
<td>N/A</td>
</tr>
<tr>
<td>Lipoprotein LprC</td>
<td>2497c</td>
<td>70.98</td>
<td>N/A</td>
</tr>
<tr>
<td>Superoxide dismutase (Fragment)</td>
<td>0187c</td>
<td>65.95</td>
<td>N/A</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase class-I</td>
<td>4308c</td>
<td>61.16</td>
<td>N/A</td>
</tr>
<tr>
<td>LppZ protein</td>
<td>3041</td>
<td>52.14</td>
<td>N/A</td>
</tr>
<tr>
<td>DNA-binding protein HU</td>
<td>3024c</td>
<td>34.39</td>
<td>N/A</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase</td>
<td>1693c</td>
<td>24.26</td>
<td>N/A</td>
</tr>
<tr>
<td>Fatty acid desaturase</td>
<td>2698c</td>
<td>22.13</td>
<td>N/A</td>
</tr>
<tr>
<td>Antigen 85-B</td>
<td>1609c</td>
<td>20.98</td>
<td>N/A</td>
</tr>
<tr>
<td>ATP synthase subunit alpha</td>
<td>2453c</td>
<td>20.74</td>
<td>N/A</td>
</tr>
<tr>
<td>Serine/threonine protein kinase</td>
<td>0016c</td>
<td>19.42</td>
<td>N/A</td>
</tr>
<tr>
<td>Wag31 protein</td>
<td>1889c</td>
<td>18.36</td>
<td>N/A</td>
</tr>
<tr>
<td>Putative uncharacterized protein</td>
<td>2020</td>
<td>14.79</td>
<td>N/A</td>
</tr>
<tr>
<td>Peroxiredoxin</td>
<td>1589c</td>
<td>13.06</td>
<td>N/A</td>
</tr>
<tr>
<td>6-phosphogluconolactonase</td>
<td>1174c</td>
<td>8.16</td>
<td>N/A</td>
</tr>
<tr>
<td>Mbp/Lac-Z</td>
<td>----</td>
<td>7.52</td>
<td>13.99</td>
</tr>
<tr>
<td>PPD 9801</td>
<td>----</td>
<td>74.57</td>
<td>86.33</td>
</tr>
<tr>
<td>PPD 0902</td>
<td>----</td>
<td>241.84</td>
<td>37.60</td>
</tr>
<tr>
<td>Phosphate-Chloride Buffer (negative control)</td>
<td>----</td>
<td>1.44</td>
<td>3.95</td>
</tr>
</tbody>
</table>

$^a$ For the proteins which resulted in skin test responses for both inoculation sites the values represent the average response (mm$^2$) and the standard deviation from two guinea pigs. For the 17 proteins which exhibited only one positive skin test reaction the value represents the response from the single reactive inoculation and the standard deviation is not available (N/A).
Table 4: Guinea pig potency testing of MAP recombinant proteins ranked by subtracted differences.

<table>
<thead>
<tr>
<th>MAP Protein (Locus tag)</th>
<th>Rec Protein Mean</th>
<th>MAP PPD 9801 Mean</th>
<th>Difference&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CI Upper Range&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CI Lower Range&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Guinea Pig ID's</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP_1997</td>
<td>328.93</td>
<td>248.89</td>
<td>80.04</td>
<td>212.00</td>
<td>-51.93</td>
<td>306, 312, 318, 324, 530</td>
</tr>
<tr>
<td>MAP_4143</td>
<td>199.68</td>
<td>277.11</td>
<td>-77.43</td>
<td>0.83</td>
<td>-155.69</td>
<td>304, 310, 316, 322, 528</td>
</tr>
<tr>
<td>MAP_3567</td>
<td>192.05</td>
<td>272.39</td>
<td>-80.33</td>
<td>88.62</td>
<td>-249.28</td>
<td>305, 311, 317, 323, 529</td>
</tr>
<tr>
<td>MAP_1138c</td>
<td>173.70</td>
<td>264.41</td>
<td>-90.72</td>
<td>24.25</td>
<td>-205.68</td>
<td>301, 307, 313, 319, 325</td>
</tr>
<tr>
<td>MAP_3061c</td>
<td>106.80</td>
<td>277.11</td>
<td>-170.32</td>
<td>-73.52</td>
<td>-267.12</td>
<td>304, 310, 316, 322, 528</td>
</tr>
<tr>
<td>MAP_3651c</td>
<td>72.57</td>
<td>256.51</td>
<td>-183.94</td>
<td>-132.90</td>
<td>-234.99</td>
<td>302, 308, 314, 320, 526</td>
</tr>
<tr>
<td>MAP_2121c</td>
<td>74.40</td>
<td>272.39</td>
<td>-197.98</td>
<td>-63.20</td>
<td>-332.77</td>
<td>305, 311, 317, 323, 529</td>
</tr>
<tr>
<td>MAP_2497c</td>
<td>50.25</td>
<td>248.89</td>
<td>-198.65</td>
<td>-176.95</td>
<td>-220.34</td>
<td>306, 312, 318, 324, 530</td>
</tr>
<tr>
<td>MAP_3692c</td>
<td>34.73</td>
<td>243.82</td>
<td>-209.08</td>
<td>-141.21</td>
<td>-276.95</td>
<td>303, 309, 315, 321, 527</td>
</tr>
<tr>
<td>MAP_2694</td>
<td>42.20</td>
<td>256.51</td>
<td>-214.31</td>
<td>-133.99</td>
<td>-294.64</td>
<td>302, 308, 314, 320, 526</td>
</tr>
<tr>
<td>MAP_3840</td>
<td>47.95</td>
<td>264.41</td>
<td>-216.46</td>
<td>-92.78</td>
<td>-340.14</td>
<td>301, 307, 313, 319, 325</td>
</tr>
<tr>
<td>MAP_0187c</td>
<td>15.31</td>
<td>243.82</td>
<td>-228.51</td>
<td>-184.33</td>
<td>-272.68</td>
<td>303, 309, 315, 321, 527</td>
</tr>
</tbody>
</table>

<sup>a</sup>Differences were calculated by subtracting the MAP protein skin test response from the PPD skin test response for each animal and then averaging the calculated differences for all animals in each treatment group.

<sup>b</sup>Upper and lower range values represent 95% confidence interval (CI) calculations.
Figure 1: Number of proteins identified by LC-MS/MS for each PPD preparation analyzed. Proteins found in two or three PPD preparations are indicated by the black bars and proteins unique to each individual PPD preparation are indicated by the grey bars. A total of 66 proteins were identified in PPD 0902A; 73 proteins were identified in PPD 0902B; 110 proteins were identified in PPD 9801. Ten proteins were identified as being in common within all three PPDs. PPD 0902A contained 33 unique proteins, PPD 0902B contained 45 unique proteins, PPD 9801 contained 77 unique proteins.
Figure 2: Venn diagram illustrating protein overlaps between PPD 9801, PPD 0902A, and PPD 0902B. The number of proteins identified for each PPD is indicated. Although the majority of proteins identified were unique to a given production lot, 10 proteins were present in all three lots analyzed (see Table S1).
Fig. 3  Guinea pig potency testing of 12 MAP recombinant proteins with average skin test reaction measurements (mm^2) indicated. Asterisk label indicates significantly different skin test reactions compared to the MAP PPD 9801 reference.
Figure 4: Secretion of IFN-γ using whole blood from Johne’s positive animals. Absorbance was measured at 450 nm.
Figure 5: Secretion of IFN-\(\gamma\) using whole blood from *Mycobacterium bovis* sensitized animals and negative animals. Absorbance was measured at 450 nm.
CHAPTER 5: CONCLUSIONS

General discussion

Early detection of MAP-infected animals is a key strategy to controlling Johne’s disease in herds and improving overall herd management and production. The use of PPDs for skin testing animals for Johne’s disease was the first diagnostic test used and other than moderate changes to the PPD production process there has been little advancement to improving this reagent.

In 2005, the United States Animal Health Association passed a resolution seeking improvements to MAP PPD with a goal to develop a well characterized and consistent PPD. This resolution, in combination with the USDA NVSL seeking to develop improved production techniques for various reagents, provided an opportunity to investigate more closely the PPD production process and characteristics of the PPDs produced at the NVSL.

Diagnostic specificity issues have been well known for PPD used for Johne’s disease testing. Before these issues can be addressed in current studies it is important to better understand the specific components of the PPD suspension. The ability to evaluate protein banding patterns through electrophoretic processes is hindered because of the degradation of protein components during the autoclaving step of the PPD production process. The ability to visualize protein banding patterns on a gel would offer a fairly rapid option for evaluating consistency between PPD production lots, but based upon current production protocols is not feasible.
This initial finding led to investigating modifications to develop an improved PPD production protocol as well as alternative processes for protein analysis. Recent advances in defining the MAP genome allowed greater use of proteomic methodologies for protein analysis of PPDs. Proteomic analysis of three PPDs provided an extensive list of proteins, but only a minor percentage were found to be present in all three preparations.

Further work using the guinea pig potency test with a subset of the identified proteins provided additional data regarding which of the identified proteins may be good candidates for use in cell mediated immune response assays. Data from the potency test evaluation indicates there are proteins that can be good candidates for further studies and investigations leading to production of a well characterized PPD that could be used in skin testing or IFN-γ assays.

**Development of an improved PPD production process**

Specific criteria for determining optimal incubation and harvest times for MAP cultures used for PPD production is lacking. In the past, *M. avium* Strain 18 was used at the NVSL for MAP PPD production and was then replaced by MAP 19698. This was a first step many years ago in improving MAP PPD. Consistent harvest times of culture flasks may very well play a role in improving consistency between PPD production lots. An initial analysis of PPD produced by the traditional method revealed protein smearing on gels and an inability to clearly define protein banding patterns.

Development of a modified production method involving sterile filtration rather than autoclaving with coarse filtration to inactivate and remove cells was investigated.
Degradation of proteins due to autoclaving during the traditional PPD production process resulted in difficulties discerning specific protein components through electrophoretic analysis. PPD, produced through an alternative production method, evaluated on gels showed distinct banding patterns and allowed for better comparison of protein banding patterns between production lots.

**Proteomic evaluation of PPD**

Proteomic evaluation offers a powerful tool for in-depth information on specific protein composition of a PPD. Results of proteomic evaluation of various NVSL PPDs has indicated inconsistency in protein expression, but has provided additional information of specific protein components. There were 194 total proteins identified between three various PPDs, with 131 of those being MAP-related proteins. These results were encouraging as being similar to other PPD suspensions that have been analyzed by mass spectrometry. Increased emphasis has been occurring for use of proteomic evaluation of PPD products in an effort to aid in improved diagnostic tools related to cell mediated immune response detection.

**Potency evaluation of PPD and recombinant MAP proteins**

Potency testing of MAP PPDs that were produced either by use of sterile filtration or by autoclaving during the production process revealed significant differences between biological activity in sensitized guinea pigs. These results indicate that the use of autoclaving during PPD production is not necessary and substituting sterile filtration in place of autoclaving may enhance antigenicity and prevent protein degradation. More
important is ensuring the development of the alternative production process does not negatively affect potency. The acceptable potency test results were an important finding should changes to the PPD production process be proposed in the future.

Potency testing of individual recombinant MAP proteins provided data indicating that 24 proteins were responsive in MAP sensitized guinea pigs. Seven of these proteins gave strong consistent reactions in the potency test and were individually equivalent to PPD. This pool of identified proteins provides future consideration as a well-characterized test reagent that can be reliably produced and have better quantitative parameters assigned for evaluation and release of a final product.

**Gamma-interferon testing using MAP recombinant proteins**

Use of selected MAP recombinant proteins as mitogens in the gamma-interferon assay showed that MAP_3651c and MAP_3567 had the greatest IFN-γ stimulation within the Johne’s positive cattle, and in some animals a greater response than resulting stimulation values from MAP PPD 9801. Results also indicated variations of protein reactivity in the guinea pig potency test versus IFN-γ stimulation. MAP_1997 provided the best skin test response in potency testing, but the least overall amount of IFN-γ stimulation from Johne’s positive animal blood samples. This variation between responses in an *in-vivo* testing model and an *in-vitro* assay are important parameters to balance in selection of recombinant proteins holding the greatest promise for improved diagnostics. Each MAP recombinant protein resulted in negative stimulation values from *M. bovis* sensitized animal samples as well as negative control animal samples. These
results support the conclusion that good specificity was obtained using selected recombinant proteins for use in an *in-vitro* IFN-γ stimulation assay.

**Future research considerations**

Sensitivity and specificity of MAP PPD in tests to detect MAP infection in cattle is of primary importance. Development of a well characterized PPD that can be used for both *in-vivo* and *in-vitro* testing will require evaluation in field tests comprising naturally infected animals from various regions and during varying climate conditions.

The use of specific recombinant proteins should be further characterized by conducting skin testing in Johne’s positive cattle as well as further *in-vitro* validation using paired sampling and testing from animals included in a field study. Identifying specific recombinant proteins that can offer strong stimulation characteristics in both skin tests and IFN-γ assays would allow for a well characterized suspension that could be used both in the field and laboratory.

Ultimately, production of a well characterized PPD consisting of specific protein components at an established protein concentration for each protein would provide a much improved diagnostic reagent for use in skin testing and IFN-γ procedures. This type of research would provide improved confidence in a MAP PPD and more rigorous test validation.