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CHARACTERIZATION OF THE SERO-REACTIVITY OF PROTEINS MAP1152 AND MAP1156 FROM *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

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by

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Mycobacterium avium subsp. *paratuberculosis* (MAP) causes Johne's disease (JD) in ruminants. Development of genetic tools and completion of the MAP genome sequencing project expanded opportunities for antigen discovery. In this thesis, I review the current trends in diagnosis and disease control of JD and present the results of the studies on the seroreactivity of two proteins encoded for by the MAP1152-MAP1156 gene cluster. MAP1152 encodes for a PPE protein and MAP1156 encodes a diacylglycerol acyltransferase involved in triglyceride metabolism and classified in the uncharacterized protein family UPF0089. Maltose-binding protein (MBP) tagged recombinant MAP proteins were purified from *Escherichia coli*. Western immunoblotting analysis indicated that both MAP1152 and MAP1156 displayed reactivity against sera of immunized mice and rabbits, and naturally infected cattle. MAP1156 yielded a stronger positive signal than MAP1152 against sera from cattle with JD. An enzyme linked immunosorbent assay (ELISA) for the recombinant proteins was developed and used to test pre-classified positive and negative serum samples from naturally infected and non-infected cattle. Samples, with one exception, displayed no seroreactivity against MBP-

LacZ ($P > 0.05$), the negative control antigen. MAP1152 displayed seroreactivity against all positive sera, but no seroreactivity to the negative sera ($P < 0.01$). MAP1156 displayed stronger and more variable reactivity than MAP1152, but significant differences were observed between non- infected and infected cattle ($P < 0.05$). Otherwise, degrees of reactivity followed the same trend as the positive reference antigen. In conclusion, MAP infected cattle mount a humoral response to both MAP1152 and MAP1156. These findings have potential applications to diagnostics, vaccine production, and elucidation of the immuno-pathogenesis of JD.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Johne's disease (JD) is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (40, 68). JD is an incurable, chronic, contagious, malabsorptive diarrheal condition of cattle, sheep, other ruminants, and some wildlife (25, 40, 68). The diarrhea leads to malnutrition, progressive weight loss, debilitation, and eventually death. Thus, producers suffer economic loss through premature culling of clinical or sub-clinical animals (73). Dairy producers suffer additional loss from repeated testing to determine infectious status of their herds, culling false positive test animals, lost milk production, and lost cow longevity due to MAP infection. The annual economic impact to the diary industry is estimated at $276 per cow per lactation for infected cows on a dairy (131). While less well studied and characterized for beef cattle producers, JD still causes loss to the industry. Beef cattle producers suffer economic loss from lower cow fertility and longer time open during breeding and gestation periods, lost weight and weight maintenance, lower calf birth-weight, and lower calf weaning weight due to infection (53, 54). The annual economic impact to the beef industry is more difficult to estimate than in the dairy industry. However, producers report added value if their herds are deemed disease free (54, 79). Managing a herd to treat or prevent JD is difficult due to the hidden transmission by subclinically infected cattle that often appear healthy (153). Sub-clinical animals are difficult to detect because of the in-accuracy of diagnostic techniques for

testing individual animals (15, 43, 157, 160, 180). The inaccuracy that affects sensitivity of diagnostic tests for JD lies in the inability to detect the infection until the animal is well into the infectious cycle. The inaccuracy affecting specificity of JD diagnostic test lies cross reactive indicators of the disease. These inaccuracies are discussed further as I break down the diagnostic techniques available to veterinarians and producers. Vaccination is not recommended for several reasons including the potential to render animals crossreactive with the *Mycobacterium bovis* (Bovine tuberculosis) skin test, and the lack of efficacy of currently available vaccines (14, 24, 75, 149, 167). Understanding the transmission of JD and developing a herd testing strategy can help producers maintain herd biosecurity and biocontainment (43, 65-67). Here we review transmission trends in Johne's disease, diagnostic testing strategies, measures producers can take to avoid or reduce Johne's disease within their herds, and describe MAP pathogenesis and genetics as they relate to diagnostic test development and strategy.

Transmission of Johne's Disease

The first factor to understand in the transmission of JD is that cattle less than a year of age are the most susceptible to MAP. A recent review (179) analyzed the age susceptibility of cattle to MAP exposure. Calves less than 6 months of age are the most susceptible, 75% progress to clinical disease after exposure. Calves 6-12 months have a lower rate of infection and progression to clinical disease (50%). Cattle older than 12 months are the least susceptible; less than 20% progress to clinical signs of disease. The percentage of infection was also found dose dependant in this study. Thus, the authors suggest commingling calves with high shedding animals can expose calves of the age

groups to equivalent amounts of MAP infectious dose as used in the study, thereby lending weight to their findings of progression to infectious disease.

Calves are the most susceptible population for several possible reasons, though there is incomplete understanding of susceptibility trends. Reasons include heightened exposure to manure through nursing during the first months of development, differences in gut physiology between adult cattle and calves, MAP exposure transmitted through the milk of the dam, the calf reliance on passive immunity during the first months of development, and possible *in-utero* infection before parturition (179). Consideration of these factors guided consensus recommendations for maintaining hygienic neo-natal calf practices by limiting exposure to MAP (6, 67, 83, 95, 154, 162).

Exposure to manure through nursing, contaminated feed, and contaminated water supplies are the most common sources of exposure (148, 177). Also demonstrated is the isolation of MAP from aerosolized particles of dust and dry manure in barnyard environments (48). The threat of aerosolized MAP particles is not well characterized, but it does present an exposure/transmission route by inhalation or ingestion depending on the shed bacterial load within the herd.

Due to these exposure routes, it is recommended that calves be removed from their dams as soon as possible; within the first 24 hours of life. Feeding replacement colostrum, if possible, also may reduce the risk of MAP exposure (114, 154). However, feeding pooled colostrum and pooled milk should be avoided, or the fed colostrum and milk should be pasteurized to reduce the risk of MAP exposure (114, 142, 154). Calves

should not receive feed with possible manure contamination. Loaders and wagons used for hauling manure should not be used for transporting feed. Feed bunks and creep feeders should be cleaned daily. Water sources for calves should be separate from that of older cattle and should be checked to assure contamination is not possible due to run-off from feedlots and milking barns.

In-utero (178), blood to blood (119), respiratory secretions (35), and transmission through semen (7) can also occur, though are less common routes of exposure/transmission, and only occur when the adult cattle are in advanced stages of the disease. However, these possible routes hinder eliminating JD from a herd, and should be taken into account with measures to limit exposure of calves post-parturition. Thus, culling of any animal showing clinical signs and off-spring from that animal is highly recommended to limit the spread of JD (43, 102, 131, 133, 134). Culling in itself will reduce the amount of shed MAP in a herd and the environmental load of MAP, but it is not enough to eliminate the agent from a herd (102).

Testing and herd management strategies must be employed beyond culling to limit the transmission of JD, because the clinical signs of JD do not develop for years. This creates an iceberg effect whereby the largest population of cattle infected with MAP is undetectable. Thus, within herd transmission occurs because sub-clinically infected cattle shed the bacteria to their young and other calves. Between herd transmission occurs when healthy appearing, sub-clinically infected cattle are introduced in the herd to increase herd size, or purchase of replacement heifers and bulls.

Diagnosis

There are several common, commercially available diagnostic tests for the detection of MAP infection, the use of each is dependent upon what the producer aims to accomplish in their control of JD and whether they are seedstock producers or commercial production ranchers (32). Seedstock producers should aim at maintaining a disease free status of their herd, thus their testing strategy should be much more stringent. Tests of high specificity are indicated for seedstock producers to insure correct diagnosis. Likewise, commercial production ranchers should desire to maintain disease free status, but may be more open in their cattle replacement and marketing strategies. Commercial producers should aim at using cost effective screening tests that are sensitive, and then following up positive test results with a test of higher specificity to insure correct diagnosis. Commercial producers should also practice purchasing from test negative disease free herds, and this point will be elaborated further in the next section on disease control.

The most common diagnostic tests available are: culture of the bacteria from the feces of animals, testing for DNA/RNA of the bacteria (Polymerase Chain Reaction, PCR; or Real Time Polymerase Chain Reaction, RT-PCR), serological tests (Enzyme Linked ImmunoSorbent Assay (ELISA)), and tests for cellular immunity. Each of these tests has its benefits and drawbacks, and there is a large investment in research aimed at improving the performance of each.

Culturing the bacteria from feces is very specific (estimated at 99.9%), because MAP is isolated and visually apparent, and readily accessible for IS900 PCR confirmation and

typing (175). However, bacteria must be shed in relatively high numbers, making this test low in sensitivity (estimated at 35 – 40%) in the detection of sub-clinical animals and animals in the initial stages of the disease (37, 44, 45, 173). MAP takes 2 months to grow on culture medium. The culture technique is prone to contamination and cultures can result in false positive identifications, reducing specificity (37, 89, 110), as illustrated by the concept of passive shedding (R. Whitlock, Pennsylvania State University, personal communication at the 10th International Colloquium on *Paratuberculosis*). Whitlock et. al. have demonstrated that in herds that contain animals classified as "super shedders", the environmental bacterial load can be high enough for adult cattle to graze and shed the bacteria without becoming truly infected. The absence of infection in these passive shedding cattle was confirmed by serology, and histology after necropsy. However, their data do not conclusively show how long a passive shedder can harbor MAP and shed it in high enough numbers to be cultured. Culture is also labor intensive, making it impractical for testing large numbers of cattle. Even with these limitations, culture remains the gold standard to which other diagnostic tests are compared (20, 23, 33, 47, 81, 85, 92, 107, 140, 150, 157, 168). It has been employed in environmental testing strategies (4, 18, 101, 124, 132). Culture of MAP from milk has been used as a herd testing strategy and for protection of the milk supply (8, 49, 64, 157). Culture of pooled samples has also been employed for herd level diagnosis and management (45, 87, 90, 164, 169, 172). Culture of environmental samples, milk, or pooled fecal samples can tell a producer if MAP is present within their herd. However, individual animal identification cannot be achieved by these methods. Thus, it is not recommended that

seed stock producers take these approaches without proper additional individual animal testing.

MAP DNA testing (Real Time-PCR) has become more common (21, 28, 30, 36, 50-52, 81, 116, 123, 143, 171). This test uses MAP DNA isolated based on established procedures (144) from either fecal material or milk. Attaining enough DNA can be problematic, though the detection limits are becoming better with more advanced methods. While molecular testing is faster than culture (results coming back within days) (55), it is also expensive and requires experienced personnel (21, 55, 117). However, advances have been made in using both culture and Real Time - PCR for environmental sampling (4, 34, 176).

Serological testing is widely used for herd surveillance (23, 31, 33, 39, 70, 74, 76, 77, 83, 95, 102, 108, 109, 134, 157, 163, 164, 173, 175). Serological testing is rapid (results coming back within days) and relatively in-expensive, making it possible to test large numbers of animals. However, cross-reactive antibodies can elicit false positive test results and reduce specificity of this approach (1, 10, 115, 120, 125). A negative test result is also inconclusive as the animals may have not yet seroconverted or produced sufficient antibodies for detection in ELISA tests (173, 175).

Testing for the Cell Mediated Immune (CMI) inflammatory responses requires the animal be injected with MAP Purified Protein Derivative (Johnin PPD) (60, 86, 156). A minimum of 48 hours later, the skin test is read. In another linked test of the cellular immune response, blood is drawn and a test is performed for the presence of interferon

gamma, an inflammatory cytokine secreted by immune cells upon stimulation of the immune cells by the Johnin PPD. Alternatively, detection of IL-10 in response to Mycobacterium antigens, specifically the MAP41 PPE protein, has been shown as an additional CMI indicator of disease (111). Detection of CMI is speculated to be more accurate than the other methods for detecting early infection (17, 78, 86). However, other infections induce interferon gamma production and cattle need to be in a healthy state when tested, or the specificity is reduced (3, 22, 156). The test is also labor intensive (running the cattle into a head chute twice to inject the PPD and then 48 hours later reading the test and drawing blood) and expensive. Finally, the Johnin PPD can cause serious reaction in personnel in the event of accidental injection.

Recommendations on using these tests for control programs were summarized by Collins et. al. and were accepted by the National Johne's Working Group and JD Committee of the US Animal Health Association during their annual meetings in October 2006 (32). Rather than initially testing individual animals, an accepted strategy is serological analysis of a statistically representative sample of animals within a herd, with follow-up of positive animals with a specific test, culture being the standard. Seedstock producers should take this approach, with continued year to year testing, to maintain a test negative herd status. Another test strategy available to commercial producers is to test the cattle environment and/or bulk milk coming from the establishment. While this strategy will not determine which animals are infected, it is a more cost effective approach as an indicator that MAP is present within the herd and the calves are at risk for JD.

Treatment

Treatment of JD with antibiotics is rarely practiced and used only in cattle showing clinical signs who have genetic value for production (68, 151-153). MAP is susceptible to several kinds of antibiotics, including monensin sodium (71, 72). Reports indicate treatment of MAP with antibiotics reduces pathogen shedding but does not eliminate disease. Due to the inability of detecting subclinical infection, the only alternative is to feed antibiotics in bulk with feed; a dangerous practice that increases antibiotic resistance and decreases antibiotic effectiveness (2, 80, 126, 181).

Available vaccines against JD are tightly regulated and are only administered under special circumstances. If the vaccine is given, producers may lose the ability to distinguish if their herds have been infected with *Mycobacterium bovis* (94, 138) by traditional caudal fold tests. The vaccine efficacy is also limited. Studies have focused on the efficacy as a measure of reduction in cattle that show clinical signs of disease and a reduction in the amount of MAP shed by the animals within a herd (84, 88, 138). The vaccine does not necessarily prevent infection and MAP is still transmitted by fecal shedding. Prevention of disease transmission with-in or between herds is a better measure of control.

Controlling JD: Prevent the Transmission

Biocontainment in the case of JD is the recognition of the infection within a herd, and the practice to contain and eliminate that infection. It is aimed at preventing calves from being exposed to the agent from the adult cow herd. Control measures for eliminating sources of contamination within herd include:

- Culling clinical, shedding cattle
- Eliminating standing water supplies
- Cleaning barns regularly and extensively
- Cleaning feed bunks and feeders regularly
- Avoiding use of loaders and wagons to haul manure that will be used to transport feed
- Avoiding leader-follower grazing practices with older to younger cattle populations
- Preventing cattle from grazing crop land recently fertilized with liquid or dry manure
- Feeding calves pasteurized colostrum if possible
- Avoiding the feeding of pooled sources of milk or colostrum as these sources present a greater risk for MAP exposure
- Segregating calves from cows as soon as possible (within 24 hours) after birth
- Segregating cows showing signs of JD from their calves and the herd

Animals should not be used as replacement stock from a herd deemed infected with MAP. In an infected herd, within herd replacement strategies seem to mask the infection

as older, clinical cattle are potentially replaced with sub-clinical, healthy appearing cattle. A better replacement strategy is to obtain cattle from a test negative herd.

Biosecurity: Preventing Between Herd Transmission

Biosecurity for JD should be aimed at preventing the introduction and purchase of infected cattle. Dairies should avoid sending calves to calf rearing feedlots as there is a greater chance of exposure to MAP (135). Pre-purchase testing is NOT effective at preventing the purchase of infected cattle due to the inaccuracy of the diagnostic tests discussed (56, 96, 128). It is more effective to purchase replacements from test-negative herds even if that animal has not been individually tested. The risk of introducing JD is dependent on the number of animals purchased (Figure 1), but is greatly reduced when purchasing from a herd that has been herd tested and classified negative. The bottom line is that buying an untested animal from a tested negative herd is safer than buying a tested animal from an untested herd (Figure 1).

Pathogenesis

MAP is one of the slowest growing mycobacteria (97). It takes 2 months for MAP colonies to appear on specialized medium (98). These culture plates are kept at constant temperature, oxygen content, and humidity. The incubation period within the host animal is approximately 3 years (174, 177, 179). The duration of each stage of infection depends on age of host at the time of exposure and the dose of microorganisms ingested.

Mycobacterium species that are pathogenic illicit a chronic inflammation characterized by the appearance of granulomas (tubercles) due to the immune response to these intracellular pathogens. The portal of entry for MAP is most often the fecal-oral route. MAP targets the Mucosal Associated Lymphoid Tissues (MALT) of the ileal Peyers patches (103). To attain access to MALT, MAP must traverse the intestinal epithelium and sub-epithelium spaces. MAP induces endocytosis by M-cells with specific adherence to fibronectin binding protein (61, 62, 146, 147). It moves through these M-cells in a non-lytic fashion. Macrophages phagocytose MAP once it traverses the epithelium. Multiplication and death of MAP may occur simultaneously in infected macrophage. The intracellular residence results in consequential cytokine production, recruitment of Th1 cells, and a subsequent cell mediated inflammatory response. Intestinal granuloma formation occurs (141), and this inflammatory process leads to the clinical signs of the disease. Detectable adaptive immunity can occur anywhere from several months to years after infection (121, 158). This variability in immune responses adds to the difficulties of using serological tests to diagnose JD.

After an animal ingests MAP, a strong lymphoproliferative inflammatory response occurs, followed by a long latent period, then a decline in cell mediated immunity (91, 93). The initial lymphoproliferative response shows a marked increase in the expression of IL-2, IL-5, GATA-3, tissue inhibitors of matrix metalloproteinases 1 and 2, and factors promoting apoptosis (141). Tumor necrosis factor alpha (TNF-α) is the dominant cytokine in the pro-inflammatory cascade (141). Chemokines, most notably CCL2 and CXCL10, aid in the recruitment of additional immune inflammatory cells to

the site of infection. These cytokine and chemokines are produced by infected macrophage. The cell signals and cytokines organize the granuloma. A granuloma is a dense focus of infected macrophages, surrounded by foamy macrophages (macrophage undergoing expression of cytokines, chemokines, reactive oxygen species (ROSs), metabolites, and lipids) and non-infected monocytes (141). Natural killer cells T-cells, $CD4^+$, $CD8^+$, and γδ T – cells are also present in the granuloma (141). A fibrous network of collagen, fibronectin, and other extracellular matrix (ECM) components provides a structural framework for containment and adhesion of bacterial and immune cells. The granuloma forms within capillary beds, due to continuous blood and nutritional supply. As the disease progresses the granuloma becomes more densely compacted with the ECM fibrous cuff and the number of blood vessels penetrating the structure diminishes markedly. The density and compaction of the granuloma provides for a dynamic interaction of bacterial and immune cell response. This interaction is characteristic of the containment phase of the disease (141). While the bacteria are contained to the granuloma, they persist, most likely in foamy macrophages, which provide a nutrient rich environment for bacterial replication (40, 141). However, in JD, damage to the intestine integrity is created by the density and compaction of the granuloma. The intestinal wall becomes densely compacted with "scar" tissue, thickening and flattening, losing the ability to uptake liquids and nutrients in a normal fashion. The thickening of areas also causes thinning of other areas, and the intestine in these thinner areas is more prone to perforation and abscess.

The decline in cell mediated immunity seems to occur because of disruption of the gene activation and signaling cascades within the macrophages infected with MAP (93, 141). This can occur with a change in immune status of the host; age, malnutrition, or co-infection with other pathogens. Malnutrition occurs readily in MAP infections due to thickening and hardening of the intestinal epithelium as the inflammatory response persists, as well as when MAP becomes systemic and the animal is dedicating most of its energy to maintaining an immune response. There is a reduction in continued signaling from CD4 T cells associated with the decline in cell mediated immunity. The cytokine profile shifts with an increase in IFN-γ, IL-4, and IL-10 (111, 112, 141). This shift also creates a shift from a cell mediated immune response to humoral immune response and antibody production. The granuloma decays into cellular debris, and MAP can move systemic through the blood stream. The humoral response is unable to contain the infection and most host animals become clinical at this stage of infection.

The effects of macro- or micro-nutrient environments to the manifestations of JD have been recently analyzed (104, 155). These studies determined that altering the nutritional environment by appropriate trace element supplementation and liming may assist in controlling the clinical stage of JD. These findings suggest a role of acidification, excess iron and molybdenum, and deficiencies in copper and selenium in the clinical manifestations of JD. Lower calcium intake by beige mice aided resistance to MAP infection. This is important in the pathogenesis of JD because supplementing cattle with mineral salts and calcium while improving milk production, may hinder the animal's ability to maintain a cell mediated immune response and containment of MAP infection.

Reservoirs of JD include all domestic or wild ruminants, several non-ruminants, birds, and open ecological niche (5, 68, 102). Ruminants, including cattle, sheep, and deer, are primary carriers. MAP has been isolated from fox, stoat, weasel, crow, rook, jackdaw, rat, wood mouse, hare, and badger (16). MAP can seemingly survive in environments such as water sources, dust, and soil (48, 57). Every country has cattle herds affected by MAP, though not every infected herd is isolated (143). It affects an estimated 21–40% of cattle herds in Europe and North America.

Genetics

The sequencing of the MAP genome ushered in a new era of research for finding diagnostic and prognostic indicators, construction of live-attenuated vaccines, and treatment targets for controlling JD (99). The pathogenic strain K-10 (59) was used by Li et. al. as the best candidate of low *in vitro* passages to retain virulence factors and other genes of pathogenic importance. The MAP genome is 4.83 MB in size, encodes 4,344 open reading frames (ORFs), 45 tRNAs, one rRNA operon, and has a 69.3% GC content. Of the ORFs, 60% have known functions, and 25% encode putative proteins of unknown functions. There is a high level of redundancy within the MAP genome due to gene duplication, approximately 50% of genes have duplications. Open reading frame sizes range from 114 bp (a ribosomal subunit encoding gene) to 19155 bp (a peptide synthetase). A total of 52.5% of genes are transcribed with the same polarity as the origin of replication. One hundred and fifty genes are regulatory in function. Insertion elements are abundant throughout the genome (20 different insertion elements with 58 copies) and one unique element (*IS900*) has been used in PCR diagnosis (81, 105). There

are 39 predicted proteins unique to MAP. Important differences with other mycobacterial genomes include: no intact PE-PGRS homologues, 39 PPE genes (68 in *Mycobacterium tuberculosis* (MTB)), and the presence of a truncated salicyl-AMP ligase gene (*mtbA*) which seems to underlie a MAP defect in mycobactin biosynthesis. We pictured the locations of characterized virulence factors, PE and PPE genes, genes for iron utilization, surface polysaccharides, lipopolysaccharides, surface proteins and antigens, heat shock and detoxification proteins, secreted and adaptive proteins, and large sequence polymorphisms (Figure 2), all of which are important diagnostic and prognostic indicators of disease. The sequences of known virulence factors, large sequence polymorphisms, and the PE, PPE proteins map together in clusters. Differences in gene organization may lead to a context-dependent function, as for example, MAP gene homologues in other mycobacterial species may play different roles in pathogenesis. This hypothesis is discussed further in the context of our studies on PE and PPE proteins

PE PPE Proteins

Approximately 1% of the MAP genome encodes members of the PE (Pro-Glu) and PPE (Pro-Pro-Glu) protein families, so denominated by their characteristic motifs at their N-terminal domains. These genes were initially discovered in the MTB genome, which dedicates approximately 10% of its coding capacity to these elements. Cole et al. hypothesized that the PE and PPE families may have immunological importance, being the main source of antigenic variation (29, 159, 165). This quantitative difference in coding capacity seems to be rooted in the evolutionary expansion of these families as microorganisms of the MTB complex diverged from the *M. avium* group (63). MAP

possesses only the ancestral members of these families. Thus, functional analyses of these proteins are significant for the development of vaccines and diagnostics, as well as for the understanding of their roles in JD pathogenesis.

Compared with MTB, PE/PPE proteins of MAP have a higher single-base substitution frequency, supporting the hypothesis that they are recognized by the immune system and are subject to positive selection (58, 99). There are no PE-PGRS identified in the K-10 genome, although this subfamily has been identified and associated with PE and PPE proteins in other mycobacteria including *M. bovis* and *M. marinum* (13, 99). PE-PGRS proteins are also absent in *M. avium* and *M. leprae* (29, 99). PE and PPE proteins are also tempting targets for antimicrobial therapy due to their uniqueness and possible unique expression patterns in mycobacterial pathogenesis (99).

PE and PPE proteins are strong humoral and cell mediated immune response antigens (19, 106, 111, 118, 122). In a recent study by Nagata et. al. recombinant MAP41 was shown to induce IL-10 production in calves infected with MAP (111). This is important in the context of their study for two reasons. IL-10 has inhibitory effects on macrophage anti-microbial activity, preventing expression of MHC class II and co-stimulatory molecules needed on the surface of macrophages for Th1 activation. This creates a shift from a cell-mediated immune response to a humoral immune response. As discussed, shifting from a cell-mediated immune response to a humoral immune response in MAP infection is detrimental to the containment phase of the disease. Nagata et. al. used this as a CMI diagnostic indicator, showing that IL-10 production upon stimulation with the

recombinant PPE protein MAP41 was significantly higher in animals infected with MAP than with other mycobacteria, or cattle never exposed to MAP.

Another interesting hypothesis in that this and other PPE proteins may also be responsible for a shift from CMI to the humoral immune response. In the next chapter, we show that animals infected with MAP mount humoral immune response to a different PPE protein, MAP1152, and a protein within the same genetic cluster, MAP1156.

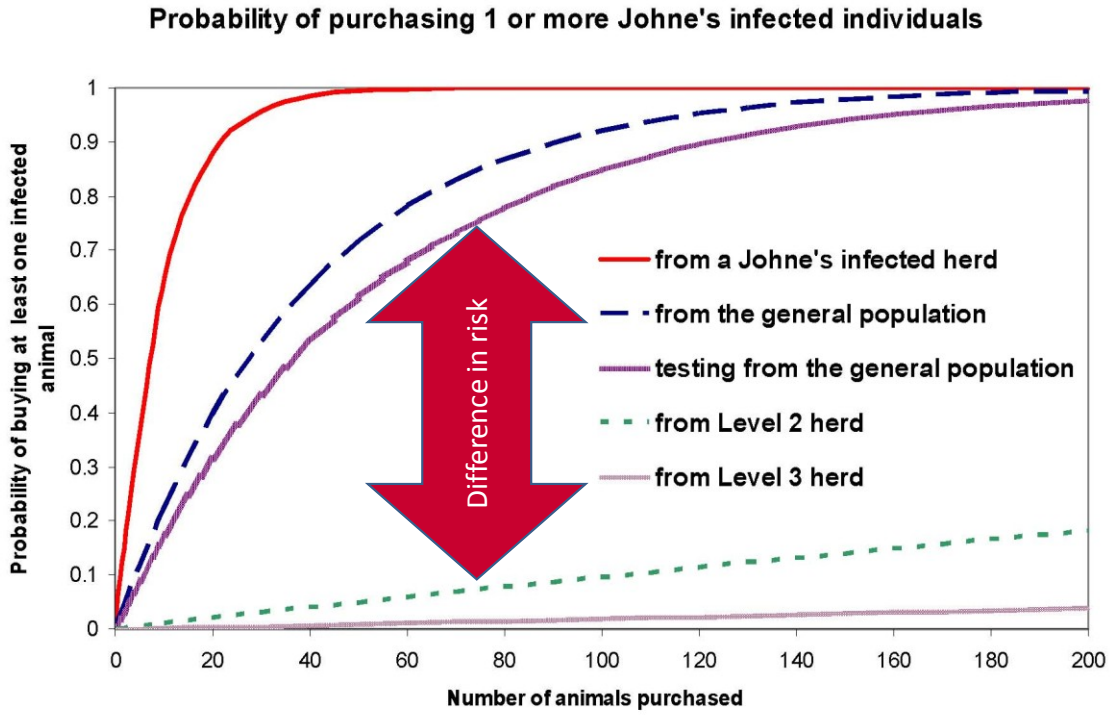

Figure 1. Probability of purchasing one or more MAP infected animals. The probability of purchasing MAP infected animals was modeled under different conditions; from a Johne's infected herd (red line), from a herd of unknown untested status without testing the animal (blue dashed line), from a herd after testing an individual animal (purple line), from a tested herd (green dashed line), from a herd with continued monitoring (gray line).

FIGURE 1

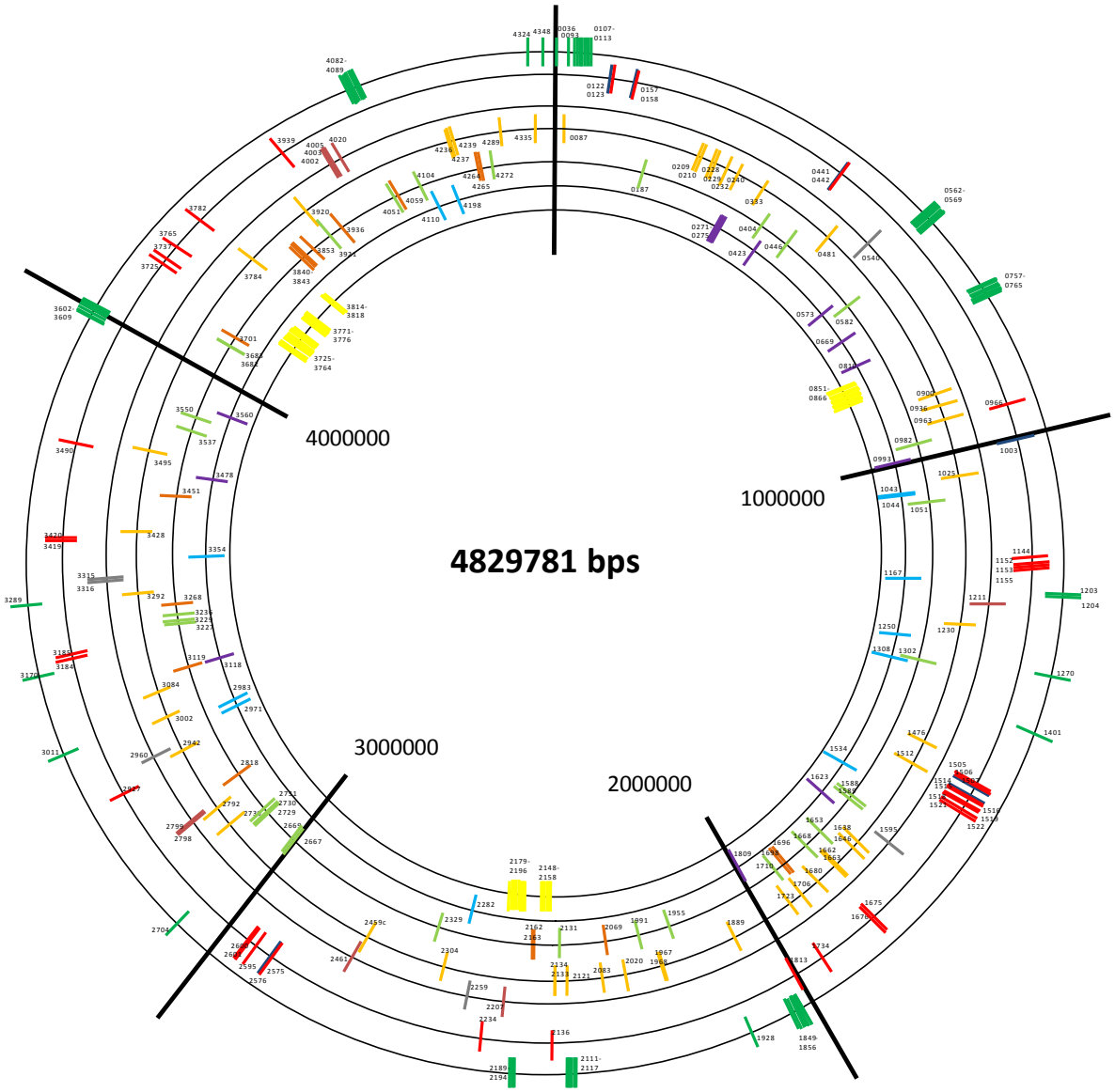

Figure 2. Genomic map indicating locations of key Mycobacterium avium subspecies paratuberculosis ORFs. From the outer most circle genes for characterized virulence factors, PE and PPE genes, genes for iron utilization, surface polysaccharides, lipopolysaccharides, proteins and antigens, Heat shock and detoxification proteins, secreted and adaptive proteins, and large sequence polymorphisms were mapped using NCBI Entrez and Geneious 4.6 (Biomatters Ltd.)

FIGURE 2

CHAPTER 2

IMMUNOGENICITY AND REACTIVITY OF NOVEL *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* PPE MAP1152 AND CONSERVED MAP1156 PROTEINS WITH SERA FROM EXPERIMENTALLY AND NATURALLY INFECTED ANIMALS

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Introduction

Information obtained from the MAP genome sequencing project ushered the development of new tools for diagnosis and disease control (99). In the context of our study, approximately 1% of the MAP genome encodes members of the PE (Pro-Glu) and PPE (Pro-Pro-Glu) protein families, so denominated by their characteristic motifs at their N-terminal domains. These genes were initially discovered in the *Mycobacterium tuberculosis* (MTB) genome, which dedicates approximately 10% of its coding capacity to these elements. Cole *et al.* hypothesized that the PE and PPE families may have immunological importance, being the main source of antigenic variation (29, 159, 165). This quantitative difference in coding capacity seems to be rooted in the evolutionary

[1] AP contributed the ELISA development, methods, and analysis, along with assistance on and confirmation of the gene maps, tables, protein analysis, and overall production of the manuscript.

expansion of these families as microorganisms of the MTB complex diverged from the *M. avium* group (63). MAP possesses only the ancestral members of these families. Thus, functional analyses of these proteins are significant for the development of vaccines and diagnostics, as well as for the understanding of their roles in JD pathogenesis.

This study focused on the MAP1152-MAP1156 gene cluster as the Tn5367 transposon insertion in a colony morphology MAP strain K-10 mutant with an attenuated phenotype in bovine macrophages, was now mapped ca. 0.6 kb upstream from MAP1152. To evaluate the potential role of this gene cluster in MAP immunobiology, we performed further bioinformatic analysis and determined the reactivity of MAP1152 and MAP1156 against sera from experimentally infected mice and rabbits, and experimentally and naturally infected cattle.

Materials & Methods

Cloning, expression, and purification of MAP proteins in *Escherichia coli*. MAP1152 and MAP1156 coding sequences were amplified from MAP K-10 genomic DNA and cloned into the pMAL-c2 translational fusion expression vector using primers 5'- ATCCTCTAGAATGGATTTCGGGTCGTTACCGC-3' and 5'-GCGCAAGCTTCTATTTCGCGTTCGGCG-GAATG-3' for MAP1152 and 5'-ATCCTCTAGAATGAAACGGCTTTCGAGTGTCG-3' and 5'-GCGCAAGCTTCAGCCGGTCTCGCCCGCGGCG-3' for MAP1156. Both primer pairs amplified the full-length coding sequences. The vector and amplification products were digested with XbaI and HindIII. Following overnight ligation at 4°C, the products

were transformed into *E. coli* DH5α and selected on LB agar plates containing 0.10 mg/ml ampicillin. Drug-resistant colonies were screened by and plasmid DNA for sequencing analysis was isolated from positive colonies to confirm each clone. *E. coli* protein lysates from verified clones were prepared as previously described (12, 137). Proteins used in this study were expressed and purified as N-terminal maltose-binding protein fusions (e.g. LacZ, MAP1152, MAP1156 for short thereafter, with the understanding that all recombinant proteins used in experiments are fusion products). These MBP-tagged recombinant proteins were overexpressed by induction of 1.0 L LB broth cultures with 0.3 mM isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Company, St. Louis, MO) for 2.5 h with shaking at 37°C. *E. coli* cells were harvested by centrifugation at 4,000 x g and resuspended in column buffer and the cell suspension was subjected to a freeze-thaw cycle at -20°C and sonicated using conditions described previously (9). The resulting crude extracts were purified by affinity chromatography using an amylose resin (New England Biolabs). Purified protein yields were determined from eluted fractions with a NanoDrop spectrophotometer set at 280 nm. The most concentrated fractions were pooled and dialyzed in Slide-A-Lyzer cassettes (Pierce Biotechnology Inc., Rockford, IL) immersed in 1.5 L phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na2HPO4, pH 7.4) with three exchanges at 4°C. Purified protein aliquots were stored at -20°C. After thawing aliquots, protein yield was re-assessed by a modified Lowry Assay using Bovine Serum Albumin (BSA) as the standard. *E. coli* DH5α harboring the parental plasmid pMAL-c2 was expressed, purified and used as a control. Purified protein from this control strain consists of an MBP fusion (overall MW,

42 kDa) with the LacZ alpha peptide (8 kDa). Each recombinant protein was further evaluated by GelCode Blue (Pierce Biotechnology Inc., Rockford, IL) stained SDS-PAGE gels to assess purity and expected size.

Electrophoresis and immunoblot analysis. Gel electrophoresis was performed using 12% (w/v) polyacrylamide gels. Electrophoretic transfer of proteins onto pure nitrocellulose was carried out using a Bio-Rad Trans Blot Cell (Bio-Rad Laboratories, Richmond, CA, USA) with sodium phosphate buffer (25 mM, pH 7.8) at 0.8 A for 90 min. After transfer, filters were blocked with PBS plus 2% BSA and 0.1% Tween 20 (BSA-PBST). Cattle serum samples (Table 1) were diluted 1:500 in BSA-PBST, and the rabbit and mouse sera were diluted 1:1,000. As necessary, subclinical (stage 2) versus clinical (stage 3) infection was assessed by fecal counts as described previously (175). For detection of MBP, a high titer murine anti-MBP monoclonal antibody was diluted 1:10,000. Each blot was incubated at room temperature for 2 h with gentle rocking. After three washes in PBS plus 0.1 % Tween 20, blots were incubated for 1.5 h in a horse radish peroxidase labeled goat anti-bovine detection antibody that was obtained from Southern Biotechnology diluted 1:10,000 in PBS-BSA. The blots were again washed three times as described above and developed for chemiluminesence using Supersignal detection reagents (Pierce).

Quantitative analysis of band intensity on immunoblots. Band intensity was determined using the Adobe Photoshop CS3 extended application measurement tool. This version has the ability to record pixel gray values using the default measurement scale (1 pixel = 1 pixel). Each spot was measured identically using a defined window area. Values

were exported into a spreadsheet for further analysis. The background statistics were calculated by determining the mean and standard deviations of the bands that had the least signal intensity within each output graph. Each intensity score was normalized to this calculated background intensity.

Enzyme Linked Immunosorbant Assay (ELISA). This test was developed to quantify the reactivity of MAP1152 and MAP1156 proteins with a subset of serum samples from cattle of known exposure to MAP (Table 1). Antigen (0.010 mg per well) was bound to BD Falcon Pro-Bind 96 well plates over night at 4°C. Antigen was diluted in 1.0 M sodium carbonate buffer (pH 9.8) and 0.1 ml of solution was bound in each well. After overnight incubation, the plate was washed 5 times with PBS, and blocked with 0.3 ml BSA-PBST for 2 to 3 h at room temperature. ELISA was performed as per instructions provided by the Idexx HerdChek® ELISA (Idexx Laboratories, One Idexx Drive, Westbrook, ME). Briefly, sera were diluted 1:25 in either BSA-PBST the buffer supplied by Idexx® containing *M. phlei* antigen for clarification. Diluted serum was applied (0.1 ml) to each well and incubated for 30 min at room temperature, plates were washed 5 times with PBS, and secondary antibody conjugate (Horse Radish Peroxidase (HRP) conjugated rabbit anti-bovine IgG) applied (0.1 ml/well) and incubated for 30 min at room temperature. After additional washing steps, 0.1 ml tetramethyl-benzadiene (TMB) was added and the subsequent reaction was stopped with the supplied SDS-stop solution, as appropriate color developed. Absorbance was read at 650 nm with a Molecular Devices Vmax Kinetic Microplate Reader and translated with xChek software from Idexx®. Statistical analysis of ELISA results was performed using SAS 9.2 for

Windows (SAS institute Inc., Cary, NC). To compare average absorbance of all seropositive cattle samples to the seronegative group, mixed model regression (MIXED) analysis was performed factoring the repeated measures for day and serum sample. To evaluate within and between antigen sera combinations, the generalized linear mixed models regression (GLIMMIX) analysis was used. A P value of < 0.05 was considered significant.

Results

Bioinformatic analysis of MAP1152-MAP1156 gene cluster. Nine PE and 37 PPE protein genes are scattered around the MAP chromosome (Fig. 1). The major PPE cluster is at 1.66 Mb in the clockwise direction in the published genome sequence (99) and comprises MAP1514, encoding a PE protein, and six PPE genes. The MAP1152-MAP1156 region of interest for this study is the second major cluster and is located at 1.21 Mb in the clockwise direction. This region encodes the PPE proteins MAP1152 (40.8 kDa), MAP1153 (45.8 kDa), and MAP1155 (32.3 kDa) (Fig. 1, inset). This region also encodes a putative unknown function ORF (MAP1154, 11.8 kDa) and MAP1156 (50.7 kDa), a member of the uncharacterized protein family (UPF0089; http://pfam.sanger.ac.uk/family/PF03007). The MAP genome encodes nine UPF0089 family genes and MAP1156 is the only member that is immediately adjacent to a PPE gene (Fig. 1). This cluster does not encode a PE protein sequence.

It is tempting to speculate that genes in the MAP1152-MAP1156 region are organized in a functional unit since ORFs further upstream and downstream are

transcribed from the complementary strand with short (e.g., < 200 bp) intergenic regions. However, the more stringent predictions indicate that only MAP1152 and MAP1153 are organized in a true operon with a pOp (estimated probability that a pair of genes is in the same operon) value of 0.842 (http://www.microbesonline.org/operons/gnc262316.html (129, 130)). Nonetheless, the genes in this cluster may comprise more than one operon.

The proximal (MAP1152) and distal (MAP1156) ORFs in the cluster were selected for immunological tests of humoral immunity in infected and non-infected animals. MAP1152 encodes a typical PPE protein of 416 amino acids with a predicted molecular mass of 40.8 kDa (Table 2). Protein structure analysis predicts for the best model three trans-membrane helices (http://www.predictprotein.org) (139) consistent with a surface/membrane localization as shown for other PPE proteins (19, 69, 106, 112, 127). MAP1156 is a protein of 464 amino acids with a predicted molecular mass of 50.7 kDa. Protein structural analysis for the best fit model predicts one trans-membrane helix suggesting surface localization (139). Both MAP1152 and MAP1156 have homologous sequences in both MAP and other mycobacterial genomes. Further bioinformatic analysis of the ORFs in the cluster is presented in Table 2.

Seroreactivity of recombinant proteins by immunoblot analysis. Purified recombinant proteins MAP1152 and MAP1156 were subjected to SDS-PAGE and immunoblot analyses. Equal amounts of both proteins were examined by SDS-PAGE to verify that their migration corresponded with molecular size (Fig. 2, SDS-PAGE Panel). Both fusion proteins migrated to the expected positions between the 75-kDa and 100-kDa protein markers, with MAP1156 (90.6 kDa) located slightly higher than MAP1152 (82.8

kDa). Likewise, LacZ (42 kDa) migrated to a position below the 50-kDa marker. These data indicate that MBP method resulted in protein preparations of the necessary purity and yield to carry out further testing. MAP1156 did show multiple bands in contrast to the discrete single bands observed for MAP1152 and LacZ. Immunoblot quality control analysis was also performed using a monoclonal antibody developed to the MBP affinity tag. All three MBP fusion proteins were detected by this antibody (Fig. 2, MBP mAb Panel). The additional bands observed in the MAP1156 preparation likely represent degradation products with the same N-terminus, as the fast-migrating bands reacted also with the MBP monoclonal antibody.

The immunogenicity of the recombinant proteins was tested by immunoblotting using immune mouse (C57BL/6 Black) or rabbit (New Zealand White) serum from animals immunized with a live preparation of MAP, as described previously (11). The mouse serum displayed little to no reactivity to MAP1152, but strong reactivity to MAP1156 with the most intense band migrating between the 50 and 75 kDa markers (Fig. 2, Mouse 160 Panel). The rabbit immune serum showed strong reactivity to both proteins (Fig. 2, Rabbit 273 Panel). Reactivity against the LacZ antigen carrying the MBP-tag was used as a negative control. The mouse serum displayed no MBP-LacZ reactivity (Fig. 2, Mouse Panel, lane 4) and only a weak reaction was observed with the rabbit serum (Fig. 2, Rabbit Panel, lane 4). Thus, the MBP tag yielded no to minimal reactivity in these immunoblots.

Immunoblots were also performed with sera from five cattle naturally infected with MAP (Fig. 3A, Panels 183, 2075, 184, 805, and 45). MAP1156 (Fig. 3A, lane 3, all

Panels) showed higher levels of reactivity compared to MAP1152 (Fig. 3A, lane 2, all Panels). Furthermore, densitometry analysis (Table S1, Supplemental Material) enabled a semi-quantitative comparison and showed that MAP1156 displayed 2- to 5-fold more seroreactivity than MAP1152 among these MAP seropositive samples. Conversely, weak reactivity was observed with sera from cattle experimentally infected with *M. avium* or *M. bovis* (Fig. 3B).

We also followed the reactivity of the proteins against serum samples taken at different time points during natural infection. Samples corresponded to bleeds taken 9-14 months apart, corresponding to disease progression from subclinical to clinical disease. Results obtained with these samples (Fig. 4) indicated that MAP 1152 did not react with any of the serum samples (Fig. 4, Upper Panel, lanes 1-4), while MAP1156 (Fig. 4, Middle Panel, lanes 1-4) and MAP K-10 whole cell extract (Fig. 4, Lower Panel) displayed increasing reactivity with samples from subclinical (Fig. 4, lanes 1 and 2) to clinical stages (Fig. 4, lanes 3 and 4). Both proteins reacted with the anti-MBP monoclonal antibody control (Fig. 4, lane 5), albeit MAP1152 yielded a weaker reaction.

Seroreactivity of recombinant protein with sera from MAP-infected and non-infected cattle by ELISA. As the ELISA technology is readily adaptable to diagnostic applications, we also determined the reactivity of MAP1152 and MAP1156 by this method. In addition, this assay is generally performed allowing the antigen-antibody reaction to take place in the absence of denaturing agents. Thus, both linear and conformational epitopes may contribute to the overall reactivity. Due to limiting amounts

of serum samples, the ELISA was performed using archived serum samples, previously evaluated by the Idexx® test in the Veterinary Diagnostic Laboratory (Table 1).

Each sample was tested in triplicate against LacZ (negative control antigen), and on three different days in duplicate (day 3) or triplicate (days 1 and 2), to evaluate reproducibility and performance, against MAP1152, MAP1156, and a standard MAP antigen control (Idexx® antigen, a proprietary mixture of MAP antigens) included in the Idexx® test kit (Supplemental Material, Table S2). Each antigen showed low reactivity and a small variability as tested against seronegative serum samples from non-infected cattle. As expected, the differences in absorbance means of samples from MAP infected versus non-infected cattle were statistically significant for antigens MAP1152 ($P \leq 0.01$), MAP1156 ($P \leq 0.05$) and Idexx® ($P \leq 0.001$) with more variable results for MAP1156 and the Idexx® antigens (Table S3, Supplemental Material). In contrast, the difference in absorbance means was not significant for the LacZ negative control ($P > 0.05$). However, the reactivity of seropositive samples against LacZ was also more variable as indicated by the error bars (Fig. 5). MAP capture antigens were able to differentiate, in the combined analysis, between the groups of seropositive and seronegative samples. Thus, MAP infection results in a humoral response directed against epitopes present in MAP1152, MAP1156, and the Idexx® antigen. However, absorbance values using MAP1152 and MAP1156 as the capture antigens were lower than those attained with the Idexx® capture antigen. This performance is not surprising because these results were obtained with the standardized Idexx® test protocol.

Comparative analysis of the reactivity of each serum-antigen combination was also carried out (Fig. 6). Reactivity trends were similar for both MAP1152 and MAP1156 as all samples with higher absorbance readings for MAP1152 also yielded higher values for MAP1156. The Idexx® antigen followed a similar trend, except for the positive control as all three MAP antigens yielded similar absorbance. In contrast, three seropositive samples (EDNA, 308 and 2010-07) yielded significantly higher absorbance for the Idexx® antigen than for MAP1152 and MAP1156. No samples were expected to yield above background absorbance readings with LacZ, the negative antigen control carrying the MBP tag. However, the EDNA serum sample displayed approximately 4 times higher absorbance readings than any other sample.

Both statistical analysis using GLIMMIX and calculation of sample to positive (S/P) ratios for the Idexx® antigen yielded the same classification as the original test (Tables S2 and S4, Supplemental Material). As cutoff values for S/P ratios are dependent on the particular assay procedure, capture antigen, and selection of an appropriate positive control serum, GLIMMIX provides the more accurate analysis for the recombinant antigens. Absorbance means for each antigen–serum combination were compared to each other and samples were classified as positive or negative based on the P value obtained for the comparison against the negative control serum for the test antigen. This analysis indicated that the non-specific MBP-LacZ antigen control identified all samples, except EDNA, as seronegative; in contrast, antigens MAP1152 and MAP1156 misclassified only the seropositive sample 2010-07 as seronegative (Table S4,

Supplemental Material). Thus overall, all MAP antigens yielded a classification of serum samples consistent with the original test.

Discussion

Development of effective strategies for the control of JD remains as one of the most challenging issues in animal health (33, 46). Advances in MAP genomics and molecular genetics provide an integrated rational approach to solve this problem (9, 12, 99). In this context, the search for new diagnostic tests is most effective if combined with novel approaches to vaccination and knowledge of what the test indicates regarding the underlying mechanisms of disease pathogenesis. Targeting the MAP1152-MAP1156 gene cluster was based on the identification of an attenuating mutation possibly related to the expression of this cluster, and a potential role of the encoded proteins as B- or T-cell antigens. An attenuated mutant may serve as a candidate live-attenuated vaccine strain and the protein as a subunit vaccine or diagnostic indicator; all relevant aspects are combined into an integrated approach.

In this study, we demonstrate that both MAP1152 and MAP1156 encode reactive B-cell epitopes, a result predicted from the surface localization implied by *in-silico* analysis (Table 2). This finding is also consistent with prior experimental evidence on other PE/PPE proteins. For example, the MTB protein Rv2430c (PE25) was identified as a strong B-cell antigen (26) and the seroreactivity of MAP Rv1818c was demonstrated in cattle (113). In contrast, antigens MAP1518 (Map41, ortholog of Rv1808 [PPE32]) and

MAP3184 (Map39, ortholog of Rv3135 [PPE50]), the *M. avium* strain 104 MaPE protein, and the cell-wall associated MTB protein Rv3873 (PPE68) serve as T-cell antigens (112, 118, 122).

MAP1152 and related PPE proteins MAP1153, and MAP1155 are members of the ancestral PPE sublineage IV (63). The genes encoding these proteins belong to an ancestral cluster that underwent duplication events from ancestral ESAT-6 clusters but without a concomitant duplication of the ESAT-6 genes and thus, these paralogs are no longer associated with the ESAT-6 genes. PE/PPE genes are usually organized in operons encompassing at least several PE and/or PPE members (136, 166). Biochemical evidence indicates that PE and PPE function in pair-wise combinations of interacting proteins exposed to the cell surface (42, 136, 161) with the larger size PPE proteins providing a pocket for the PE partner (161). However, MAP1152 is organized in a cluster devoid of coding sequences for PE proteins. Because the probability of PPE proteins participating in pair-wise associations decreases for unlinked PE and PPE genes (136), the function of MAP1152 may or may not require a PE partner. Instead, MAP1152 may play additional roles in the mechanisms of pathogenesis, as shown for other PE/PPE proteins. For example, MTB transposon mutants of the PE/PPE genes Rv1807 (PPE31), Rv3872(PPE35), and Rv3873 (PPE68) are attenuated in mice (145). Likewise, a transposon mutant in the *M. avium* PPE gene homologous to Rv1787 (PPE25) displayed impaired growth in macrophages, reduced virulence in mice, and failed to prevent phagosome acidification (82, 100). Thus, a group of PE and PPE proteins may be necessary for intracellular survival.

MAP1156 is also a B-cell antigen, stronger than MAP1152 in Western blot reactivity, a property also consistent with its inferred surface localization. MAP1156 belongs to the uncharacterized protein family UPF0089. MTB encodes 15 members of this family that include proteins with triacylglycerol synthase (TGS) activity (38). The *M. tuberculosis* ORF Rv1425 is the closest homologue to MAP1156 (Table 2), but this ORF has been shown not to possess significant TGS activity, at least under the conditions tested. One possible role consistent with a strong humoral reaction is that this ORF was hitchhiked by MAP into a co-expression unit that modulates the immune response. Microorganisms of the MTB complex possess a large number of PE-PGRS proteins that are associated with immunoregulatory roles (13, 41). However, as indicated above, the MAP evolutionary lineage separated prior to the further expansion of the ESAT-6 Region V that gave rise to the PE-PGRS and PPE-MPTR sublineage V PE/PPE proteins (63). Thus, the absence of PE-PGRS proteins may require the recruitment of other proteins, for example MAP1156, to play this immuno-modulatory role. Future experiments will be directed to identify T-cell epitopes in both MAP1152 and MAP1156. These tests are indispensable to determine the major role of these antigens in immunopathogenesis. For example, it is possible that the PPE protein MAP1152, the weaker B-cell antigen in Western immunoblotting analyses, may elicit a predominant T-cell response early in infection, with MAP1156 exerting a counter-modulating B-cell response, most favorable for MAP to maintain a chronic infection. This hypothesis is consistent with the increasing MAP1156 seroreactivity observed with serum samples withdrawn as JD progressed from subclinical to clinical stage (Fig. 4). However, a more detailed study of

linear and conformational epitopes in these proteins, especially for MAP1152, should be undertaken, as conformational epitopes may not be reactive in Western immunoblots.

The B-cell reactivity of both MAP1152 and MAP1156 in Western blots and ELISA with serum samples from MAP-infected animals is likely associated with private rather than crossreactive or shared epitopes from related MAP proteins. In this context, the closest paralog to MAP1152 is MAP1518 with 47% identity. Likewise, the closest paralog to MAP1156 is MAP1969c with 38% identity (Table S5, Supplemental Material). The low degree of reactivity of MAP1152 in the immunoblots against sera from *M. bovis*-infected animals may be explained in a similar manner as the closest homologue (Mb1837) shares only 50% identity. In contrast, the low reactivity of MAP1156 against the *M. bovis* serum, and MAP1152 and MAP1156 against *M. avium hominissuis* serum, may be associated with low expression levels in these microorganisms. Otherwise, the corresponding proteins are highly homologous with 86 to 99% identity (Table S5, Supplemental Material) and thus, likely to be highly crossreactive as most epitopes are shared. Moreover, the MAP1152-MAP11156 cluster organization is conserved between MAP and *M. avium* 104 (a sequenced isolate of *M. avium* subsp *hominissuis*), both genomes possessing highly homologous genes within this region. However, sequence divergence with *M. avium* 104 occurs upstream from MAP1152 that could affect gene regulation. Nonetheless, to substantiate these findings for the development of diagnostic tests, it would be necessary to test a larger group of cattle infected with *M. bovis*, *M. avium* subspecies, and other environmental species such as *Mycobacterium kansasii*.

Interestingly, MAP1152, MAP1156 and the Idexx® antigen yielded similar absorbance values against the standardized Idexx® positive control bovine serum included in the Idexx® kit that was from a single naturally infected Holstein cow (Table 1). However, the other three seropositive clinical samples reacted significantly stronger with the Idexx® antigen (Fig. 6). As indicated above, these results may be due to methodological aspects, as the overall assay optimization was based on the Idexx® protocol. Alternatively, or by compounding effects, the increased reactivity in these samples against the Idexx® antigen may in part reflect the presence of crossreactive antibodies against the various MAP proteins present in the Idexx® antigen.

In summary, MAP infected cattle mount a humoral response to both MAP1152 and MAP1156. Further research is needed to determine the value of these recombinant proteins as diagnostic capture antigens, subunit vaccines, markers of disease progression, or their suitability for the development of DIVA (diagnosis of infected from vaccinated animal) tests coupled with the development of live attenuated deletion mutant marker vaccines (27, 170).

Table 1. Cattle serum samples used in the study.

Serum Sample	Original classification (Idexx test)	Type of infection	Source
Cow # 183	+	Natural	NADC[1]
Cow # 2075	+	Natural	NADC[1]
Cow # 184	+	Natural	NADC[1]
Cow # 805	+	Natural	NADC[1]
Cow # 45	+	Natural	NADC[1]
Cow # 193	-	Experimental with *M. avium hominissius*	NADC[1]
Cow # 2291	-	Experimental with *M. bovis* strain 95-1315	NADC[1]
Positive Control[2]	+	Experimental with MAP	Idexx Laboratories

EDNA	+	Natural	NADC[1]
Cow #308	+	Natural	NADC[1]
2010-07	+	Natural	Nebraska Dairy[3]
Negative Control[4]	-	None	Idexx Laboratories
J53-90	-	None	Nebraska Beef Herd[3]
Cow #559	-	None	NADC[1]
3438-08	-	None	Nebraska Dairy[3]

[1]NADC, National Animal Disease Center, Ames, Ia.

[2]Positive control serum provided with the Idexx kit.

[3]Veterinary Diagnostic Center, Lincoln, NE

[4]Negative control serum provided with the Idexx kit.

Table 2. Characterization of MAP1152-MAP1156 ORFs

MAP ORF	Size [1]	MTB H37R$_V$ homologue/ E-value [1]	Domains and Motifs [1,2]	Comments
MAP1152	416, 40.7	Rv1808 (PPE32)[3]/ 8.0 e^{-78}	PPE, GxxSVPxxW	Three membrane helices (MH)[4]
MAP1153	454, 45.7	Rv1809 (PPE33)[3]/ 1.0 e^{-84}	PPE, GxxSVPxxW	Coding sequence starts 7 bp downstream from MAP 1152. Three MH[4].
MAP1154	117, 11.7	Rv1810/ 8.0 e^{-19}	DUF732 super family	Hypothetical protein. No MH[4]
MAP1155	320, 32.2	Rv1807(PPE31)/ 4.0 e^{-24}	PPE, GxxSVPxxW	Attenuating mutation in *M. tuberculosis* homologue (49). Two MH[4]
MAP1156	464, 50.6	Rv1425/ 0.0	UPF0089	*M. tuberculosis* homologue encodes enzyme with low TGS activity (16). Possibly one MH[4]

[1] Data entries based on the National Center for Biotechnology Information (NCBI) output. Size given in no. of amino acids, mol wt (kDa). E-values (blastp suite) are formatted as described in the Blast help manual (http://www.ncbi.nlm.nih.gov/blast/blast_help.shtml).

[2] Data entries based on the Pfam database (http://pfam.sanger.ac.uk/family).

[3] Most homologous *M. tuberculosis* are not necessarily orthologs (22).

[4] Based on the PredictProtein server (http://www.predictprotein.org) for protein analysis (PHDhtm output).

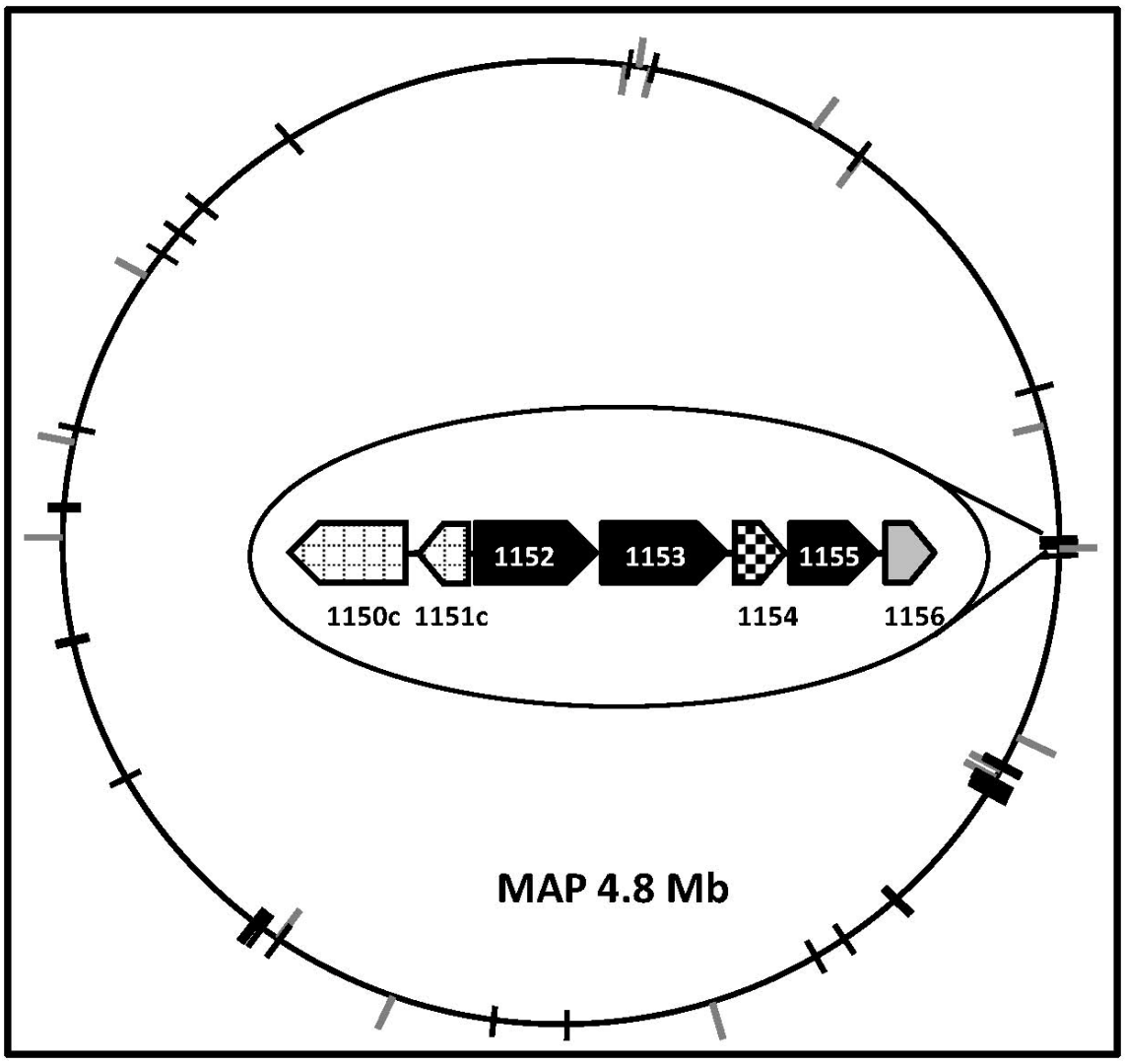

Figure 1. Genomic map of PE, PPE, and UPF encoding genes in MAP K-10. Genes encoding for PE (inside bars), PPE (crossing bars), and UPF (outside bars) protein family members are shown. No PGRS protein encoding sequences were found. Inset: MAP1152-MAP1156 genomic region indicating genes encoding PPE proteins MAP1152, MAP1153 and MAP1155 (black boxes), UPF protein MAP1156 (grey box), hypothetical protein MAP1154 (dark patterned box), and MAP1150c and MAP1151c (light patterned boxes). Arrowed boxes were used to indicate the direction of transcription.

FIGURE 1

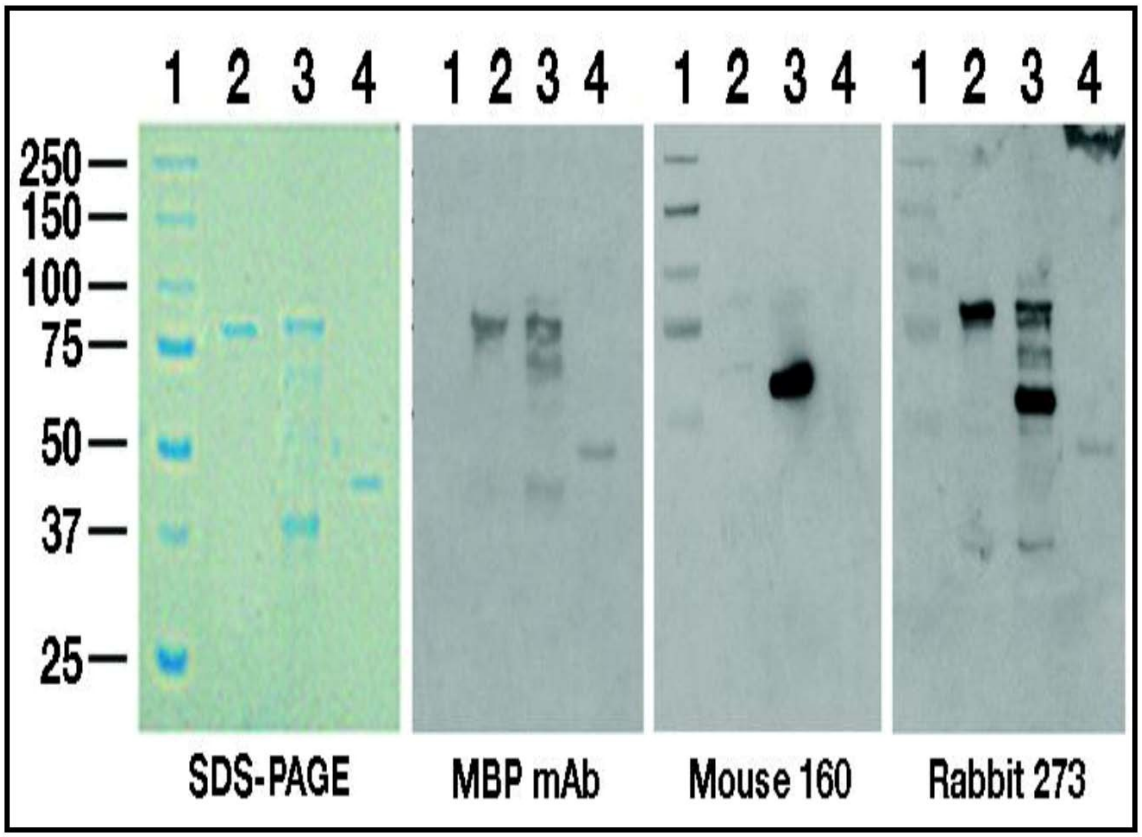

Figure 2. SDS-PAGE and immunoblot analysis of recombinant *M. avium* subsp *paratuberculosis* MAP1152 and MAP1156 proteins. Shown is a 12% SDS-PAGE gel, stained with GelCode Blue, along with three corresponding immunoblots containing purified recombinant fusion proteins. Antibody or serum samples used to probe the immunoblot are indicated beneath: MBP mAb, monoclonal antibody against the maltose binding protein; Mouse 160, serum derived from a mouse experimentally infected with MAP K-10; Rabbit 273, serum derived from a rabbit experimentally infected with MAP K-10. Size standards, reported in kDa, are indicated to the left. Assignments for the gel and blots were: lane 1, protein size standards; lane 2, MAP1152; lane 3, MAP1156; and lane 4, LacZ.

FIGURE 2

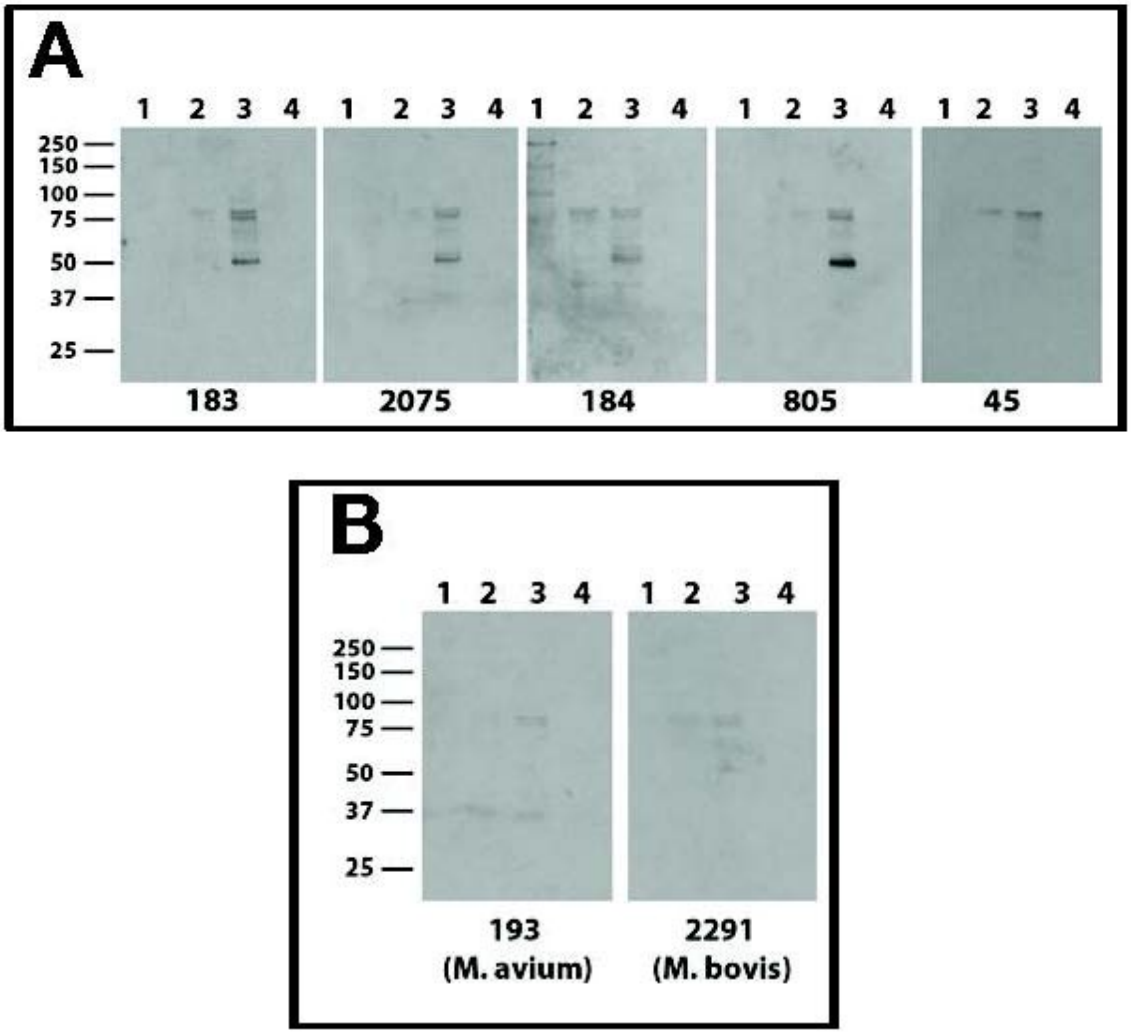

Figure 3. Western blot analysis of antibody responses to MAP1152 and MAP1156 in naturally infected cattle. Immunoblots containing MAP1152 and MAP1156 were probed with sera from (A) five cows (183, 2075, 84, 805 and 45) naturally infected with Johne's disease, and (B) two additional cows experimentally infected with *M. avium* or *M. bovis*. Size standards, reported in kDa, are indicated to the left. Assignments for the blots were: lane 1, protein size standards; lane 2, MAP1152; lane 3, MAP1156; and lane 4, LacZ.

FIGURE 3

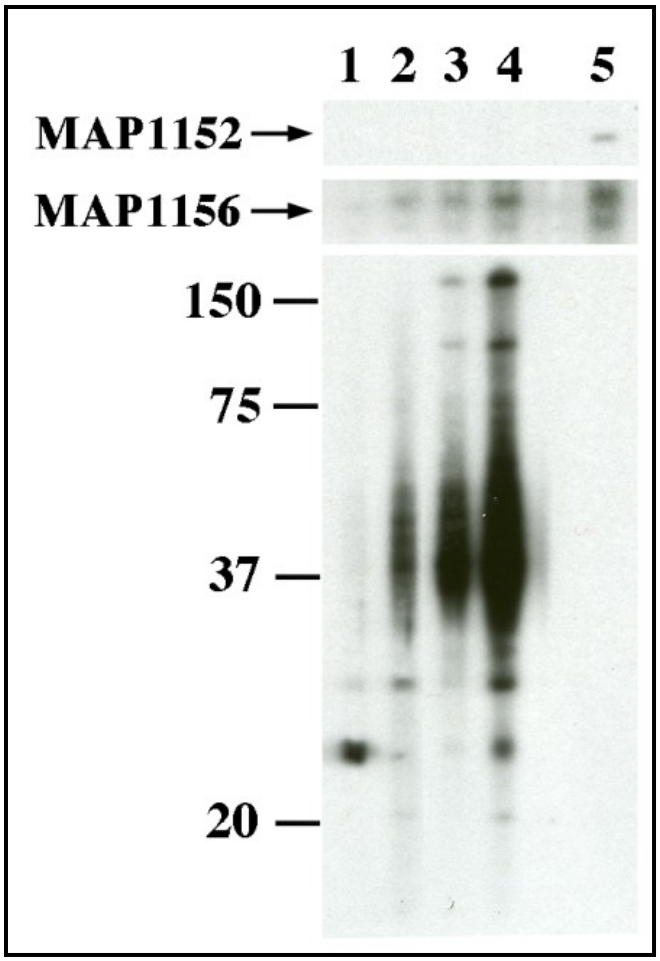

Figure 4. Western blot analysis of antibody responses to MAP1152 and MAP1156 during the course of JD. Immunoblots of MAP1152 (upper panel), MAP1156 (middle panel), and K-10 whole cell extract (lower panel) were probed with serum samples withdrawn from a naturally infected cow (cow#47) at various times during the course of infection: first bleed (time zero), subclinical infection (lane 1); 12 months, borderline clinical/subclinical infection (lane 2); 26 month, clinical infection (lane 3); 35 months, clinical infection (lane 4); and anti-MBP monoclonal antibody control (lane 5). Size standards (kDa) are indicated to the left.

Figure 4

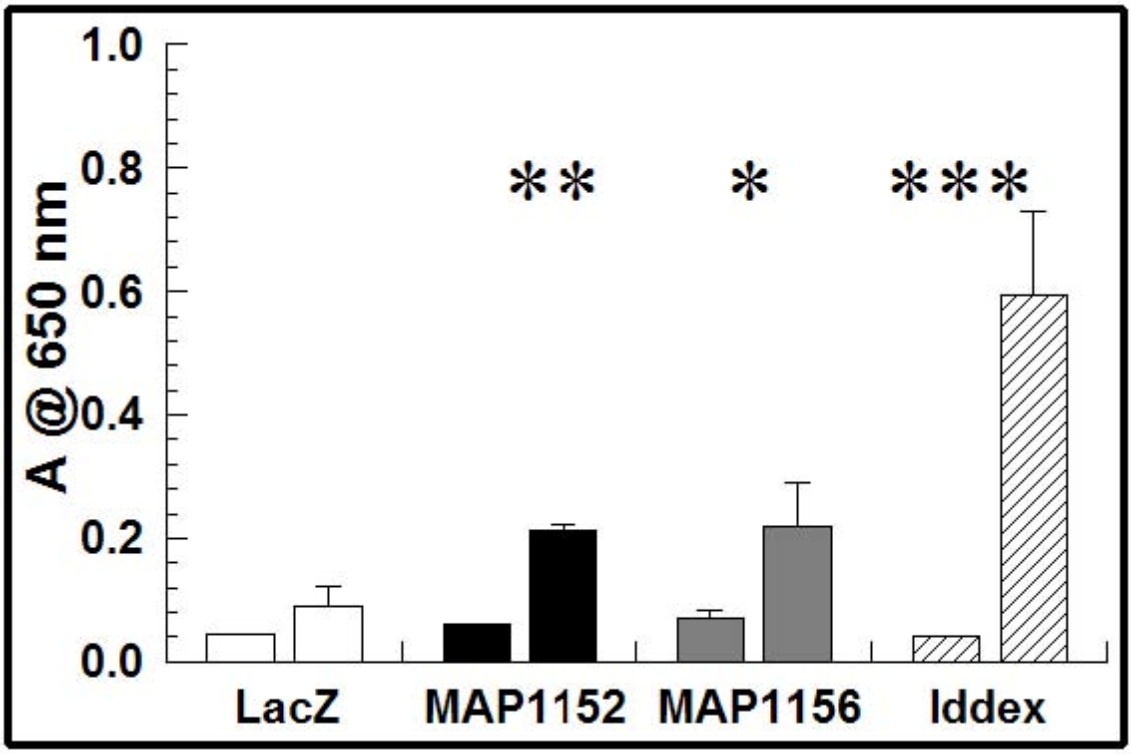

Figure 5. Seroreactivity of MAP1152 and MAP1156 recombinant proteins to infected and non-infected cattle. Antigen (0.010 mg) reactivity was evaluated by ELISA against sera from cattle naturally and experimentally infected with MAP and sera from culture-negative cattle. Each serum was diluted 1:25 in Idexx dilution buffer. Each column represents absorbance means (triplicate wells per plate and sample assayed on three different days for four serum samples) per antigen ± standard errors of the mean from non-infected (left column for each antigen) or infected cattle (right column), as evidenced from the original classification of the corresponding animals. Significance levels are indicated: *, $P \leq 0.05$, **, $P \leq 0.01$, and *** $P \leq 0.001$.

FIGURE 5

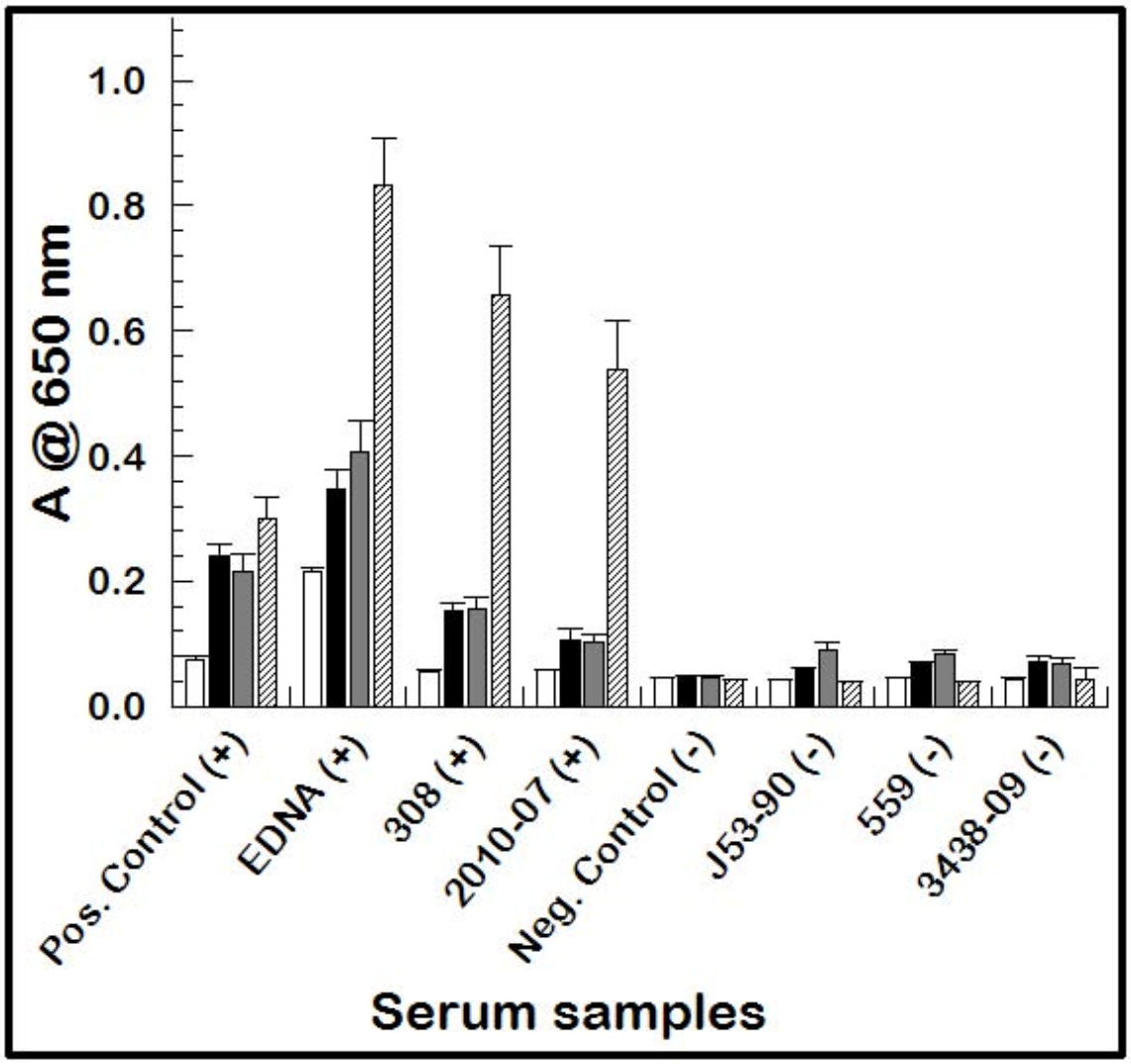

Figure 6: Individual response of serum samples from infected and non-infected cattle to recombinant antigens. Antigen (0.010 mg) reactivity was evaluated by ELISA against sera from cattle naturally and experimentally infected with MAP and sera from culture-negative cattle. Each serum was diluted 1:25 in Idexx dilution buffer. Each column represents absorbance means (triplicate wells per plate and sample assayed on three different days for each serum sample) per antigen ± standard errors of the mean from non-infected (-) or infected (+) cattle, as evidenced from the original classification of serum samples. Reaction to each antigen is indicated by open (LacZ), black (MAP1152), grey (MAP1156) and striped (Idexx antigen) columns.

FIGURE 6

SUPPLEMENTAL TABLES OF STATISTICAL ANALYSIS

Table S1. Density data Western blots

Label/Sample	Gray Value (Mean)	Gray Value (Median)	Integrated Density	Mean Intensity
MAP1152-ascites	140.010256	148	57724	4.989744
MAP1156-ascites	57.051282	44	22250	87.948718
MBP/LacZ-ascites	144.561538	144	56379	0.438462
MAP1152-rabbit	88.089744	82	34355	56.910256
MAP1156-rabbit	67.064103	52	26155	77.935897
MBP/LacZ-rabbit	134.953846	137	52632	10.046154
MAP1152-mAb	115.85641	114	45184	29.14359
MAP1156-mAb	119.8	118	46722	25.2
MBP/LacZ-mAb	134.787179	138	52567	10.212821
MAP1152-cow 183	145.889706	146	19841	18.110294
MAP1156-cow 183	124.080882	129	16875	39.919118
MBP/LacZ-cow 183	153.330882	153	20853	10.669118
MAP1152-cow 2075	145.25	146	19754	18.75
MAP1156-cow 2075	129.235294	134	17576	34.764706
MBP/LacZ-cow 2075	151.014706	151	20538	12.985294
MAP1152-cow 184	138.654412	137	18857	25.345588
MAP1156-cow 184	129.551471	130	17619	34.448529
MBP/LacZ-cow 184	163.727941	164	22267	0.272059
MAP1152-cow 805	148.257353	148	20163	15.742647
MAP1156-cow 805	99.573529	101	13542	64.426471
MBP/LacZ-cow 805	142	142	19312	22
MAP1152-cow 45	155.301471	119	16497	8.698529
MAP1156-cow 45	121.301471	155	21121	42.698529
MBP/LacZ-cow 45	159.338235	152	20582	4.661765
MAP1152-cow 193	155.419118	156	21137	8.580882
MAP1156-cow 193	155.926471	156	21206	8.073529
MBP/LacZ-cow 193	144.191176	144	19610	19.808824
MAP1152-cow 2291	142.698529	143	19407	21.301471
MAP1156-cow 2291	154.316176	154	20987	9.683824
MBP/LacZ-cow 2291	153.275	154	24524	10.725

Table S2. ELISA absorbance data with descriptive statistics								
LacZ Antigen								
	Serum Samples/Absorbance @ 650 nm							
	Control +	EDNA	308	2010-07	Control -	J53-90	559	3438-08
Day 1	0.085	0.207	0.055	0.058	0.049	0.04	0.047	0.047
	0.07	.	0.061	0.057	0.045	0.043	0.044	0.04
	0.069	0.222	0.053	0.055	0.042	0.043	0.043	0.039
Avg:	0.075	0.215	0.056	0.057	0.045	0.042	0.045	0.042
S.D.:	0.0090	0.0106	0.0042	0.0015	0.0035	0.0017	0.0021	0.0044
S.E.M.:	0.0052	0.0061	0.0024	0.0009	0.0020	0.0010	0.0012	0.0025
C.V.:	12.0	4.9	7.4	2.7	7.7	4.1	4.7	10.4
S/P Ratio:		0.57	0.04	0.04		-0.01	0.00	-0.01

Table S2 (Continued)								
MAP1152 Antigen								
	Serum Samples/Absorbance @ 650 nm							
	Control +	EDNA	308	2010-07	Control -	J53-90	559	3438-08
Day 1	0.285	0.426	0.184	0.163	0.044	0.067	0.065	0.049
	0.274	0.45	0.179	0.168	0.045	0.069	0.07	0.049
	0.268	0.439	0.169	0.157	0.044	.	0.072	0.052
Day 1 Avg:	0.276	0.438	0.177	0.163	0.044	0.068	0.069	0.050
S.D.:	0.0086	0.0120	0.0076	0.0055	0.0006	0.0014	0.0036	0.0017
S.E.M.:	0.0050	0.0069	0.0044	0.0032	0.0003	0.0008	0.0021	0.0010
Day 2	0.136	0.287	0.116	0.056	0.044	0.049	0.054	0.061
	0.189	0.238	0.102	0.061	0.046	0.056	0.059	0.063

	0.192	0.262	0.126	0.076	0.057	0.067	0.068	0.064
Day 2 Avg:	0.172	0.262	0.115	0.064	0.049	0.057	0.060	0.063
S.D.:	0.0315	0.0245	0.0121	0.0104	0.0070	0.0091	0.0071	0.0015
S.E.M.:	0.0182	0.0141	0.0070	0.0060	0.0040	0.0052	0.0041	0.0009
Day 3	0.279	0.349	0.186	0.088	0.043	0.05	0.077	0.112
	0.291	0.335	0.161	0.082	0.043	0.049	0.077	0.108
Day 2 Avg:	0.285	0.342	0.1735	0.085	0.043	0.0495	0.077	0.11
S.D.:	0.0085	0.0099	0.0177	0.0042	0.0000	0.0007	0.0000	0.0028
S.E.M.:	0.0060	0.0070	0.0125	0.0030	0.0000	0.0005	0.0000	0.0020
Between Day Avg:	0.244	0.348	0.155	0.104	0.045	0.058	0.069	0.074
S.D.:	0.0625	0.0881	0.0351	0.0518	0.0032	0.0093	0.0083	0.0316
S.E.M.:	0.0361	0.0509	0.0203	0.0299	0.0018	0.0054	0.0048	0.0183
Overall Avg:	0.239	0.348	0.153	0.106	0.046	0.058	0.068	0.070
S.D.:	0.0583	0.0829	0.0333	0.0478	0.0047	0.0092	0.0082	0.0256
S.E.M.:	0.0206	0.0293	0.0118	0.0169	0.0016	0.0033	0.0029	0.0090
Between Day C.V.:	25.6	25.3	22.6	50.0	7.1	15.5	12.1	42.6
Overall C.V.:	24.4	23.8	21.8	45.0	10.2	15.9	12.1	36.7
S/P:		1.46	0.64	0.44		0.11	0.17	0.19

Table S2 (Continued)

MAP1156 Antigen

	Serum Samples/Absorbance @ 650 nm							
	Control +	EDNA	308	2010-07	Control -	J53-90	559	3438-08
Day 1	0.269	0.559	0.215	0.134	0.046	0.117	0.094	0.054

	0.253	0.559	0.203	0.133	0.05	0.113	0.095	0.051
	0.302	0.545	0.198	0.151	0.049	0.125	0.091	0.056
Day 1 Avg:	0.275	0.554	0.205	0.139	0.048	0.118	0.093	0.054
S.D.:	0.0250	0.0081	0.0087	0.0101	0.0021	0.0061	0.0021	0.0025
S.E.M.:	0.0144	0.0047	0.0050	0.0058	0.0012	0.0035	0.0012	0.0015
Day 2	0.098	0.274	0.095	0.056	0.046	0.051	0.059	0.054
	0.127	0.277	0.099	0.059	0.049	0.055	0.059	0.06
	0.139	0.215	0.104	0.076	0.046	0.074	0.058	0.08
Day 2 Avg:	0.121	0.255	0.099	0.064	0.047	0.060	0.059	0.065
S.D.:	0.0211	0.0350	0.0045	0.0108	0.0017	0.0123	0.0006	0.0136
S.E.M.:	0.0122	0.0202	0.0026	0.0062	0.0010	0.0071	0.0003	0.0079
Day 3	0.262	0.457	0.161	0.102	0.044	0.099	0.095	0.102
	0.265	0.353	0.173	0.098	0.042	0.093	0.109	0.098
Day 3 Avg:	0.264	0.405	0.167	0.100	0.043	0.096	0.102	0.100
S.D.:	0.0021	0.0735	0.0085	0.0028	0.0014	0.0042	0.0099	0.0028
S.E.M.:	0.0015	0.0520	0.0060	0.0020	0.0010	0.0030	0.0070	0.0020
Between Day Avg:	0.220	0.405	0.157	0.101	0.046	0.091	0.085	0.073
S.D.:	0.0855	0.1495	0.0537	0.0378	0.0028	0.0294	0.0229	0.0242
S.E.M.:	0.0494	0.0863	0.0310	0.0218	0.0016	0.0170	0.0132	0.0140
Overall Avg:	0.214	0.405	0.156	0.101	0.047	0.091	0.083	0.069
S.D.:	0.0791	0.1425	0.0499	0.0359	0.0027	0.0282	0.0204	0.0210
S.E.M.:	0.0280	0.0504	0.0176	0.0127	0.0010	0.0100	0.0072	0.0074
Between Day C.V.:	37.0	22.5	26.2	43.7	5.8	32.1	26.8	32.9
Overall C.V.:	36.9	35.2	32.0	35.5	5.9	31.0	24.8	30.2
S/P:		1.89	0.73	0.47		0.26	0.21	0.14

Table S2 (Continued)								
Idexx Antigen								
	Serum Samples/Absorbance @ 650 nm							
	Control +	EDNA	308	2010-07	Control -	J53-90	559	3438-08
Day 1	0.369	0.717	0.884	0.339	0.043	0.039	0.042	0.048
	0.35	0.96	0.815	0.342	0.042	0.041	0.04	0.053
	0.347	1.151	0.9	.	0.044	0.041	0.041	0.043
Day 1 Avg:	0.355	0.943	0.866	0.341	0.043	0.040	0.041	0.048
S.D.:	0.0119	0.2175	0.0452	0.0021	0.0010	0.0012	0.0010	0.0050
S.E.M.:	0.0069	0.1256	0.0261	0.0012	0.0006	0.0007	0.0006	0.0029
Day 2	0.149	0.663	0.396	0.452	0.041	0.038	0.038	0.036
	0.166	0.606	0.401	0.497	0.038	0.037	0.038	0.039
	.	0.592	0.393	0.447	.	0.037	0.039	0.043
Day 2 Avg:	0.158	0.620	0.397	0.465	0.040	0.037	0.038	0.039
S.D.:	0.0120	0.0376	0.0040	0.0275	0.0021	0.0006	0.0006	0.0035
S.E.M.:	0.0069	0.0217	0.0023	0.0159	0.0012	0.0003	0.0003	0.0020
Day 3	0.352	0.992	0.772	0.911	0.047	0.036	0.041	0.042
	0.367	0.984	0.696	0.786	0.049	0.041	0.042	0.042
Day 3 Avg:	0.360	0.988	0.734	0.849	0.048	0.039	0.042	0.042
S.D.:	0.0106	0.0057	0.0537	0.0884	0.0014	0.0035	0.0007	0.0000
S.E.M.:	0.0075	0.0040	0.0380	0.0625	0.0010	0.0025	0.0005	0.0000
Between Day Avg:	0.288	0.852	0.678	0.572	0.043	0.038	0.040	0.044
S.D.:	0.1132	0.2019	0.2483	0.2997	0.0038	0.0020	0.0015	0.0047
S.E.M.:	0.0653	0.1166	0.1434	0.1730	0.0022	0.0012	0.0009	0.0027
OVERALL Avg:	0.300	0.833	0.657	0.539	0.043	0.039	0.040	0.044
S.D.:	0.0978	0.2129	0.2248	0.2221	0.0041	0.0019	0.0015	0.0047
S.E.M.:	0.0346	0.0753	0.0795	0.0785	0.0014	0.0007	0.0005	0.0017
Between Day C.V.:	39.3	23.7	36.6	52.4	8.7	5.4	3.8	10.7

Overall C.V.:	32.6	25.6	34.2	41.2	9.4	4.8	3.8	10.7
S/P:		2.8	2.2	1.8		-0.02	-0.01	0.00

Table S3. Mixed model regression analysis of mean absorbance differences between sero-positive and sero-negative samples

Antigen	Sero-Positive Average	Sero-Positive S.D.	Sero-Positive S.E.M.	Sero-Negative Average	Sero-Negative S.D.	Sero-Negative S.E.M.
MBP-LacZ	0.101	0.0765	0.0382	0.044	0.0810	0.0405
IDEXXa	0.582	0.2237	0.1118	0.042	0.3388	0.1694
MAP1152	0.212	0.1064	0.0532	0.060	0.1309	0.0654
MAP1156	0.219	0.1322	0.0661	0.072	0.1583	0.0791

Table S3 (Continued) Statistical Parameters[1]

Antigen	LSMeans[2] Difference	LSMeans[2] S.E.M.	*P* value
MBP-LacZ	0.057	0.0382	0.1564
IDEXXa	0.541	0.1139	< 0.0001
MAP1152	0.151	0.0535	0.0069
MAP1156	0.146	0.0668	0.0323

1. Data generated using SAS subroutine:

```
DATA DATA1;
SET WORK.ELISA;
PROC MIXED;
CLASS SEROPOS ANTIGEN DAY SERA
MODEL ABS = SEROPOS/SOLUTION;
RANDOM DAY SERA;
RUN;
```

2. Least Squared Mean Difference between sero-positive and sero-negative samples

Table S4. Generalized Linear Mixed Models Regression Analysis (GLIMMIX): Serum-antigen pair seroreactivity

Sera*Antigen Pairs[1]		Overall Absorbance Avg.	Overall S.D.	Overall S.E.M.	LS Means Difference[2]	*P* value[3]
Control-	**LacZ**	0.045	0.0035	0.0020	.	.
	Idexx	0.043	0.0041	0.0014	-0.00191	0.9747
	MAP1152	0.046	0.0047	0.0016	0.00042	0.9944
	MAP1156	0.047	0.0027	0.0010	0.00117	0.9842
Control+	LacZ	0.075	0.0090	0.0052	0.02933	0.6797
	Idexx	0.300	0.0978	0.0346	0.25467	< 0.0001
	MAP1152	0.239	0.0583	0.0206	0.19392	0.0012
	MAP1156	0.214	0.0791	0.0280	0.16904	0.0045
EDNA	LacZ	0.215	0.0106	0.0061	0.16917	0.0343
	Idexx	0.833	0.2129	0.0753	0.78779	< 0.0001
	MAP1152	0.348	0.0829	0.0293	0.30292	< 0.0001
	MAP1156	0.405	0.1425	0.0504	0.35954	< 0.0001
308	LacZ	0.056	0.0042	0.0024	0.01100	0.8769
	Idexx	0.657	0.2248	0.0795	0.61179	< 0.0001
	MAP1152	0.153	0.0333	0.0118	0.10754	0.0691
	MAP1156	0.156	0.0499	0.0176	0.11067	0.0615
2010-07	LacZ	0.057	0.0015	0.0009	0.01133	0.8732
	Idexx	0.539	0.2221	0.0785	0.49381	< 0.0001

		MAP1152	0.106	0.0478	0.0169	0.06104	0.3007	
		MAP1156	0.101	0.0359	0.0127	0.05579	0.3440	
	J53-90	LacZ	0.039	0.0019	0.0007	-0.00333	0.9626	
		Idexx	0.039	0.0021	0.0007	-0.00208	0.9719	
		MAP1152	0.058	0.0092	0.0033	0.02442	0.6776	
		MAP1156	0.091	0.0282	0.0100	-0.00330	0.9110	
	559	LacZ	0.045	0.0021	0.0012	-0.00333	0.9626	
		Idexx	0.040	0.0015	0.0005	-0.00658	0.9111	
		MAP1152	0.068	0.0082	0.0029	0.01281	0.8313	
		MAP1156	0.083	0.0204	0.0072	0.04554	0.4403	
	3438-08	LacZ	0.042	0.0044	0.0025	-0.00067	0.9925	
		Idexx	0.042	0.0044	0.0025	-0.00521	0.9301	
		MAP1152	0.070	0.0256	0.0090	0.02242	0.7041	
		MAP1156	0.069	0.0210	0.0074	0.03717	0.5281	

Table S4 (Continued)

	Sera*Antigen Pairs[1]		**Overall Absorbance Avg.**	**Overall S.D.**	**Overall S.E.M.**	**LS Means Difference[2]**	***P* value[3]**	
	Control-	**Idexx**	0.043	0.0041	0.0014	.	.	
		LacZ	0.045	0.0035	0.0020	0.00191	0.9747	
		MAP1152	0.046	0.0047	0.0016	0.00232	0.9589	
		MAP1156	0.047	0.0027	0.0010	0.00307	0.9456	

	Control +	Idexx	0.300	0.0978	0.0346	0.25657	< 0.0001	
		LacZ	0.075	0.0090	0.0052	0.03124	0.6029	
		MAP1152	0.239	0.0583	0.0206	0.19582	< 0.0001	
		MAP1156	0.214	0.0791	0.0280	0.17095	0.0002	
	EDNA	Idexx	0.833	0.2129	0.0753	0.78970	< 0.0001	
		LacZ	0.215	0.0106	0.0061	0.17107	0.0150	
		MAP1152	0.348	0.0829	0.0293	0.30482	< 0.0001	
		MAP1156	0.405	0.1425	0.0504	0.36145	< 0.0001	
	308	Idexx	0.657	0.2248	0.0795	0.61370	< 0.0001	
		LacZ	0.056	0.0042	0.0024	0.01291	0.8298	
		MAP1152	0.153	0.0333	0.0118	0.10945	0.0159	
		MAP1156	0.156	0.0499	0.0176	0.11257	0.0132	
	2010-07	Idexx	0.539	0.2221	0.0785	0.49571	< 0.0001	
		LacZ	0.057	0.0015	0.0009	0.01324	0.8255	
		MAP1152	0.106	0.0478	0.0169	0.06295	0.1632	
		MAP1156	0.101	0.0359	0.0127	0.05770	0.2010	
	J53-90	Idexx	0.039	0.0019	0.0007	-0.00018	0.9968	
		LacZ	0.042	0.0017	0.0010	-0.00143	0.9810	
		MAP1152	0.058	0.0092	0.0033	0.02632	0.5589	
		MAP1156	0.091	0.0282	0.0100	0.02595	0.5646	
	559	Idexx	0.040	0.0015	0.0005	-0.00468	0.9172	

		LacZ	0.045	0.0021	0.0012	-0.00143	0.9810	
		MAP1152	0.068	0.0082	0.0029	0.01471	0.7517	
		MAP1156	0.083	0.0204	0.0072	0.04745	0.2927	
	3438-08	Idexx	0.044	0.0047	0.0017	-0.00330	0.9415	
		LacZ	0.042	0.0044	0.0025	0.00191	0.9835	
		MAP1152	0.070	0.0256	0.0090	0.02432	0.5892	
		MAP1156	0.069	0.0210	0.0074	0.03907	0.3859	

Table S4 (Continued)

	Sera*Antigen Pairs[1]		**Overall Absorbance Avg.**	**Overall S.D.**	**Overall S.E.M.**	**LS Means Difference[2]**	***P* value[3]**	
	Control-	**MAP1152**	0.046	0.0047	0.0016	.	.	
		Idexx	0.043	0.0041	0.0014	-0.00232	0.9589	
		LacZ	0.045	0.0035	0.0020	-0.00042	0.9944	
		MAP1156	0.047	0.0027	0.0010	0.00075	0.9862	
	Control +	MAP1152	0.239	0.0583	0.0206	0.19350	< 0.0001	
		Idexx	0.300	0.0978	0.0346	0.25425	< 0.0001	
		LacZ	0.075	0.0090	0.0052	0.02892	0.6235	
		MAP1156	0.214	0.0791	0.0280	0.16863	< 0.0001	
	EDNA	MAP1152	0.348	0.0829	0.0293	0.30250	< 0.0001	
		Idexx	0.833	0.2129	0.0753	0.78738	< 0.0001	
		LacZ	0.215	0.0106	0.0061	0.16875	0.0149	

		MAP1156	0.405	0.1425	0.0504	0.35913	< 0.0001	
	308	MAP1152	0.153	0.0333	0.0118	0.10713	0.0146	
		Idexx	0.657	0.2248	0.0795	0.61138	< 0.0001	
		LacZ	0.056	0.0042	0.0024	0.01058	0.8574	
		MAP1156	0.156	0.0499	0.0176	0.11025	0.0120	
	2010-07	MAP1152	0.106	0.0478	0.0169	0.06063	0.1644	
		Idexx	0.539	0.2221	0.0785	0.49339	< 0.0001	
		LacZ	0.057	0.0015	0.0009	0.01092	0.8529	
		MAP1156	0.101	0.0359	0.0127	0.05538	0.2040	
	J53-90	MAP1152	0.058	0.0092	0.0033	0.02400	0.5812	
		Idexx	0.039	0.0019	0.0007	-0.00250	0.9542	
		LacZ	0.042	0.0017	0.0010	-0.00375	0.9492	
		MAP1156	0.091	0.0282	0.0100	0.02363	0.5871	
	559	MAP1152	0.068	0.0082	0.0029	0.01239	0.7831	
		Idexx	0.040	0.0015	0.0005	-0.00700	0.8721	
		LacZ	0.045	0.0021	0.0012	-0.00375	0.9492	
		MAP1156	0.083	0.0204	0.0072	0.04513	0.3002	
	3438-08	MAP1152	0.070	0.0256	0.0090	0.02200	0.6131	
		Idexx	0.042	0.0044	0.0025	-0.00563	0.8971	
		LacZ	0.042	0.0044	0.0025	-0.00108	0.9853	
		MAP1156	0.069	0.0210	0.0074	0.03675	0.3986	

Table S4 (Continued)

Sera*Antigen Pairs[1]		Overall Absorbance Avg.	Overall S.D.	Overall S.E.M.	LS Means Difference[2]	*P* value[3]
Control-	**MAP1156**	0.047	0.0027	0.0010	.	.
	Idexx	0.043	0.0041	0.0014	-0.00307	0.9456
	LacZ	0.045	0.0035	0.0020	-0.00117	0.9842
	MAP1152	0.046	0.0047	0.0016	-0.00075	0.9862
Control +	MAP1156	0.214	0.0791	0.0280	0.16788	0.0002
	Idexx	0.300	0.0978	0.0346	0.25350	< 0.0001
	LacZ	0.075	0.009	0.0052	0.02817	0.6325
	MAP1152	0.239	0.0583	0.0206	0.19275	< 0.0001
EDNA	MAP1156	0.405	0.1425	0.0504	0.35838	< 0.0001
	Idexx	0.833	0.2129	0.0753	0.78663	< 0.0001
	LacZ	0.215	0.0106	0.0061	0.16800	0.0154
	MAP1152	0.348	0.0829	0.0293	0.30175	< 0.0001
308	MAP1156	0.156	0.0499	0.0176	0.10950	0.0126
	Idexx	0.657	0.2248	0.0795	0.61063	< 0.0001
	LacZ	0.056	0.0042	0.0024	0.00983	0.8674
	MAP1152	0.153	0.0333	0.0118	0.10638	0.0153
2010-07	MAP1156	0.101	0.0359	0.0127	0.05463	0.2101
	Idexx	0.539	0.2221	0.0785	0.49264	< 0.0001

		LacZ	0.057	0.0015	0.0009	0.01017	0.8629	
		MAP1152	0.106	0.0478	0.0169	0.05988	0.1697	
	J53-90	MAP1156	0.091	0.0282	0.0100	0.02288	0.5991	
		Idexx	0.039	0.0019	0.0007	-0.00325	0.9404	
		LacZ	0.042	0.0017	0.0010	-0.00450	0.9391	
		MAP1152	0.058	0.0092	0.0033	0.02325	0.5931	
	559	MAP1156	0.083	0.0204	0.0072	0.04438	0.3083	
		Idexx	0.04	0.0015	0.0005	-0.00775	0.8586	
		LacZ	0.045	0.0021	0.0012	-0.00450	0.9391	
		MAP1152	0.068	0.0082	0.0029	0.01164	0.7959	
	3438-08	MAP1156	0.069	0.021	0.0074	0.03600	0.4083	
		Idexx	0.042	0.0044	0.0025	-0.00638	0.8835	
		LacZ	0.042	0.0044	0.0025	-0.00183	0.9752	
		MAP1152	0.07	0.0256	0.0090	0.02125	0.6252	

1. Absorbance values for each serum-antigen pair were contrasted against the upper pair (bold)
Random effect of one to three repeated measures per day across 3 days: LacZ: n =3; 1 day, triplicate; MAP1152, MAP1156 and Idexx: n=8; 2 days, triplicate; 1 day, duplicate

2. Significance is determined as Restricted Estimated Maximum Likelihood (REML) ratio of Least Squared Means (LSMeans)

3. Data generated using SAS subroutine:

```
DATA DATA1;
SET WORK.ELISA;
PROC GLIMMIX;
CLASS CLASS ANTIGEN DAY SERA;
MODEL ABS = SERA*ANTIGEN/SOLUTION;
RANDOM DAY;
RUN;
```

Table S5. Percent identity and similarity of MAP1152 and MAP1156 with closest *M. avium* 104, *M. bovis* and MAP orthologs and paralogs.

MAP ORF	Ortholog/ Paralogs	Species	E-value[1]	% Identity[1]	% Similarity[1]
MAP1152	MAP1152	MAP	0	100	100
	MAV_3356 [2]	*M. avium 104*	3.0 e^{-173}	98	98
	Mb1837 (PPE32)	*M. bovis* AF2122/97	8.0 e^{-78}	50	64
	Rv1808 (PPE32)	MTB	8.0 e^{-78}	50	64
	MAP1518	MAP	6.0 e^{-46}	47	58
MAP1156	MAP1156	MAP	0	100	100
	MAV_3352	*M. avium 104*	0	99	99
	Mb1460	*M. bovis AF2122/97*	0	86	91
	Rv1425	MTB	0	85	91
	MAP1969c	MAP	5.0 e^{-78}	38	55

1. Output from NCBI Blast (http://blast.ncbi.nlm.nih.gov)

2. True orthologous sequence.

APPENDIX I

Permissions

November 4, 2010

David Smith
126 VBS
Fair St. & East Campus Loop
Lincoln, NE 68506

Dear Dr. Smith,

This letter is to request permission to use all or in-part slide 43 “Probability of purchasing 1 or more Johne’s infected individuals” of lecture “Applying population dynamics to the diagnosis and control of Johne’s disease”, a lecture for VBMS 996 “Population Approaches to Medicine”, Spring of 2006.

Sincerely,

Avery Paulson

November 4th, 2010

Raul Barletta
233 VBS
Fair St. & East Campus Loop
Lincoln, NE 68506

Dear Dr. Barletta,

This letter is to request permission to use all or in-part manuscript CVI00297-10 "Immuogenicity and reactivity of novel Mycobacterium avium subspecies paratuberculosis PPE MAP1152 and conserved MAP1156 proteins with sera from experimentally and naturally infected animals".

Sincerely,

Avery Paulson

November 24, 2010

ASM Journals Department
1752 N Street, N.W.
Washington, D.C. 20036-2904

Dear Diane Smith,

I respectfully request permission to reproduce the final accepted copy and all supplements, manuscript CVI00297-10 entitled “Immunogenicity and Reactivity of Novel *Mycobacterium avium* subsp. *paratuberculosis* PPE MAP1 l52 and Conserved MAPI156 Proteins with Sera from Experimentally and Naturally Infected Animals”, CVI accepted and published on-line ahead of print 17 November 2010, as Chapter 2 of my Master of Science Thesis.

The cover page of Chapter 2 of my thesis will include the citation "Copyright @ American Society for Microbiology, Clinical and Vaccine Immunology, CVI Accepted, published online ahead of print on 17 November 2010, doi:10.1128/CVI.00297-10". All authors have granted permission (attached e-mails) and my thesis will be held in confidentiality until ASM permission is granted. I passed my Thesis Defense, November 19th, 2010. My thesis will be deposited with the Dean of Graduate Studies and the University of Nebraska – Lincoln Libraries, November 30th, 2010. Graduation of Graduate Students for Fall 2010 at the University of Nebraska – Lincoln is December 18th.

Sincerely,

Avery Paulson, B.S., M.S.
Master of Science Candidate
School of Veterinary and Biomedical Sciences
University of Nebraska – Lincoln
Fair and East Campus Loop
Lincoln, NE 68583 – 0905

APPENDIX II

Acronyms and Definitions

Acronyms

MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MTB	*Mycobacterium tuberculosis*
JD	Johne's disease
ELISA	Enzyme Linked Immunosorbent Assay
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
PPE	Proline-Proline-Glutamine
MBP	Maltose Binding Protein
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
DNA	Deoxyribose Nucleic Acid
PPD	Purified Protein Derivative
MALT	Mucosal Associated Lymphoid Tissue
JDIP	Johne's Disease Integrated Program
ECM	Extracellular Matrix

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