## *The Diagnosis of Mycobacterium avium Infections Using Serodiagnosis With Novel Lipid Antigens*

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# The Diagnosis of *Mycobacterium avium* Infections Using Serodiagnosis With Novel Lipid Antigens

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## Abstract

*Mycobacterium avium* subspecies *Paratuberculosis* (MAP) is an endemic pathogen in ruminants, present in a high proportion of herds worldwide. Its presence within cattle herds creates an economic burden on both farmers and the wider economy; due to lost milk production and premature culling.

Currently there is a lack of sensitive and rapid detection techniques, as culture can take months to give results and traditional PCR cannot distinguish viable from non-viable cells. Tests utilising defined synthetic mycolic acids and their sugar esters have already shown promise at diagnosing tuberculosis. This will form the basis of work described here, translating those procedures to the detection of MAP using both ELISA and a flow through device.

MAP has also been shown to survive pasteurisation, thus making it into the food chain, and has been proposed as an aetiological agent for the development of Crohn's disease.

- First, a study of strongly positive experimentally infected cattle samples against negative serum from a herd with no history of MAP resulted in a single antigen sensitivity/specificity of 100/100.
- Second, a study of 40 negative and 40 positive, naturally infected cattle samples from Canada resulted in a sensitivity/specificity of 85/75. Combined with the first study and utilising all 5 common antigens for diagnosis resulted in a sensitivity/specificity of 84/93
- Initial testing and translation of flow through procedures from *M. tb* to MAP with a pooled cattle sample resulted in defined red spots, with the control remaining clear.
- MAP specific antigens tested against human Crohn's samples as compared with healthy samples resulted in a single antigen sensitivity/specificity of 91/100

This work has identified promising antigens for further large-scale testing against both MAP in cattle and Crohn's disease. Additionally, the first test of a flow through device shows promise in developing a rapid point of care device for the detection of MAP.

## Contents

## Abbreviations

| Abbreviation | Definition |
|---|---|
| AG | Arabinogalactan |
| AGID | Agar Gel Immunodiffusion Assay |
| AGPT | Agar Gel Immunodiffusion Test |
| ArMM | Arabinose Mono-Mycolate |
| ArTM | Tri-arabinose Di-mycolate |
| ATCC | American Type Culture Collection |
| ATP | Adenosine Triphosphate |
| AUC | Area Under the Curve |
| BVD | Bovine Viral Diarrhea |
| CEMOVIS | Cryo-Electron Microscopy of Vitreous Sections |
| CFP10 | 10 kDa Culture Filtrate Protein |
| CFT | Complement Fixation Test |
| CFU | Colony-Forming Unit |
| CI | Confidence Interval |
| CR | Complement Receptor |
| CRAN | Comprehensive R Archive Network |
| CryoEM | Cryogenic Electron Microscopy |
| DAT | Di-O-acylated Trehaloses |
| d $H_2O$ | Distilled $H_2O$ |
| DIM | Phthiocerol Dimycocerosate |
| DMAG | Dimycolyl Diarabinoglycerol |
| DMT | 6,6P-dimycoloyl Trehalose |
| DNA | Deoxyribonucleic acid |
| DTH | Delayed-Type Hypersensitivity |
| *E. coli* | *Escherichia coli* |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ELISPOT | Enzyme-Linked Immune Absorbent Spot |
| ESAT6 | 6 kDa Early Secretory Antigenic Target |
| GMM | Glucose Monomycolate |
| HPMC | Hydroxypropyl Methylcellulose |

| | |
|---|---|
| HRP | Horseradish Peroxidase |
| HTST | High Temperature Short Time |
| IBD | Inflammatory Bowel Disease |
| IFN-γ | Interferon gamma |
| IgG | Immunoglobulin G |
| IGRA | Interferon Gamma Release Assay |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IS | Insertion Sequence |
| LAM | Lipoarabinomannan |
| LAMP | Loop-Mediated Isothermal Amplification |
| LM | Lipomannan |
| LOD | Limit of Detection |
| LOS | Lipooligosaccharides |
| LTLT | Low Temperature Long Time |
| M | Microfold |
| *M. bovis* | *Mycobacterium bovis* |
| *M. phlei* | *Mycobacterium phlei* |
| *M. tb* | *Mycobacterium tuberculosis* |
| MAC | *Mycobacterium Avium Complex* |
| mAGP | Mycolyl-Arabinogalactan-Peptidoglycan |
| MAP | *Mycobacterium Avium* subspecies *Paratuberculosis* |
| MM | Mycomembrane |
| MMT | 6-monomycoloyl Trehaloses |
| OPD | o-Phenylenediamine |
| PBMC | Peripheral Blood Mononuclear Cell |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase chain reaction |
| PG | Peptidoglycan |
| PGL | Phenolic Glycolipids |
| PIM | Phosphatidylinositol Mannosides |
| PM | Plasma Membrane |
| PPD | Purified Protein Derivative |

| | |
|---|---|
| RNA | Ribonucleic acid |
| ROC | Receiver Operating Characteristic |
| rt | Room Temperature |
| RT-PCR | Real-Time Polymerase chain reaction |
| TAG | Triacylglycerol |
| TDM | Trehalose 6,6′-dimycolate |
| TEM | Transmission Electron Microscopy |
| Th | T Helper cell |
| THF | Tetrahydrofuran |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| TMM | Trehalose Monomycolate |
| TST | Tuberculin Skin Test |
| UK | United Kingdom |
| USA | United States of America |
| WE-TDM | Wax-ester Trehalose 6,6′-dimycolate |
| WE-TMM | Wax-ester Trehalose Monomycolate |
| ZN | Ziehl–Neelsen |

## Declaration

I confirm that no part of the material presented in this thesis has been previously submitted by me or any other person for a degree awarded at this or any other university. In all cases in which it is relevant, material from the work of others has been acknowledged.

## Statement of Copyright

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# 1. Introduction

Paratuberculosis or Johne's disease is a chronic enteric wasting infection caused by *Mycobacterium avium* subspecies paratuberculosis (MAP) [1]. MAP affects, and has been detected in, numerous domestic and wild ruminants worldwide, including cattle, sheep, goats, wild deer and rabbits [2–4]. Of particular note are animals that act as natural reservoirs for the disease such as deer [3,5] and wild rabbits [6] but also food producing animals, especially bovidae; particularly domestic cattle, sheep and goats within this biological family.

MAP is of global interest not only due to animal health and welfare concerns but there are also economic considerations due to reduced milk production. Whilst MAP is more prevalent in dairy herds, it also affects beef cattle because of premature culling, diminished slaughter value and reduced value of calves [7]. Although there is limited prevalence data due to problems associated with diagnosing populations [8], MAP is endemic in the United Kingdom (UK) [9]. The lack of data is partially due to MAP currently being a non-notifiable disease as opposed to *Mycobacterium bovis* (*M. bovis*) in the UK and bovine viral diarrhoea virus (BVD) in Scotland. Secondly, farmers are less aware of endemic diseases, as symptoms can be almost non-existent or vague, leading to acceptance that this is business as normal. The estimated population of UK herds affected with MAP was 34.7% (95% Confidence Interval (CI) 27.6-42.5%) in 2006 [10], with an estimated cost range of £0.327-£10 million per year for cattle [11]. The United States of America (USA) at a similar time period reported a prevalence of 91% within dairy herds [12].

MAP has been suggested as a possible cause or contributing factor to inflammatory bowel disease (Crohn's, ulcerative colitis) in humans [13,14]. This relationship is born of the aetiological similarity, although the connection is not fully understood.

## 1.1. Immunology of MAP Infection

### 1.1.1. Early Infection

The immunological progression of MAP within cattle is not entirely confirmed but, like other mycobacteria, it is an intracellular pathogen, invading and surviving within macrophages. It is thought that initial infection predominantly occurs early in life when calves are most susceptible [15].

The main route for this infection is thought to be through infected grass, either through faeces or other bodily fluids. Horizontal transmission through herds is also possible, with young calves being exposed via milk or colostrum from an infected dam or *in utero* [16,17]. From this point it is plausible that the bacteria enter via oral mucosa in the tonsils, but this has only been demonstrated in high dosage experimental infection models, lower dosage models pointing towards the ileum [18,19]. From ingestion, all areas of the small intestine should be exposed to MAP, however the ileal region appears to provide a unique entry point to establish persistent infections [20,21]. This would also support the theory that calves are at greater risk, as during the first year of life transient Peyer's Patches go into regression giving way for jejunal Peyer's Patches [22,23].

Internalisation of MAP occurs through the epithelial barrier via microfold (M) cells in Peyer's Patches and differentiated epithelial cells [22,24,25]. This mechanism is dependent on the binding to fibronectin and as M cells are enriched with β1 fibronectin, these present a preferential route for the uptake of MAP [26–28]. This mechanism happens quickly [29]; from this point MAP is transported to the submucosa [30] and ingested by macrophages. These intestinal macrophages then transport MAP to the mesenteric lymph nodes [31].

### 1.1.2. Innate Immune Response

The uptake of MAP into sub-epithelial macrophages has been proposed to preferentially occur via the complement receptors (CR1, CR3 and CR4) [32,33]; uptake via this system limits macrophage activation [34]. MAP is phagocytized within macrophages but interferes with maturation, leading to replication of bacilli [31,35]. MAP now replicates within the macrophage, until macrophage death and bursting, releasing the bacteria. This has a snowball effect, as

inflammatory reactions due to the killing of macrophages attract more macrophages, causing a cycle of infection, macrophage death and bursting [1,35]. This process leads to the formation of granulomas that contain macrophages with a high intracellular burden [36].

At this point MAP can shed into faeces and infect the environment, but the mechanism that causes shedding is not fully understood. It has been proposed that low level shedding can occur quite soon after the initial infection (several weeks post infection), then there is a latent infection period were no shedding or low-level shedding occurs, and animals are asymptomatic. Following this at a yet undetermined time, tangible shedding occurs and at later stages where clinical symptoms present continuous shedding can occur; so called 'super shedders' [37].

### 1.1.3. Adaptive Immunity

The humoral response has been shown to be largely delayed compared to the cellular response [38]. The MAP cellular immune response is shown by the production of interferon gamma (IFN-γ) [38,39], which activates macrophages to kill MAP [40].

This generally is not successful, and the disease enters an equilibrium or so called latent phase between MAP and the host immune system. Infrequent shedding of MAP leads to low level stimulation of the humoral response. Shedding later in the infection is simultaneous with a decline in IFN-γ producing cells and an increase in interleukin (IL) 10, IL-4 producing cells, MAP specific antibodies also become apparent [38,40,41]. These antibodies primarily belong to the IgG1 subclass but limited IgG2 responses maybe present in some animals in later disease stages [42]. Following this switch, an analogy to human *Mycobacterium tuberculosis* (*M. tb*) has been drawn, the murine Th1-Th2 paradigm [43]. This suggests that MAP can stimulate both responses, with Th1 predominating in the early infection supressing the humoral Th2 response and limiting MAP replication. In later stages this response changes to a humoral one which limits the cellular response, is less effective against MAP, and leads to higher levels of bacterial shedding [44,45].

There is however conflicting evidence on the cellular and humoral response switch, with some studies suggesting MAP specific antibodies speed

up the disease progress due to the uptake of bacteria by macrophages [46]. Recent studies showed high IL-10 and IFN- γ in the early stages correlated to delayed shedding [47], with other studies indicating cellular and humoral responses are decreased during later disease stages [36]. This conflicts with the Th1-Th2 paradigm and suggests that disease progression is responsible for the shift, rather than competition between these two branches of adaptive immunity [48,49]. This shift from Th1 to Th2 is however associated with progression to clinical disease.

## 1.2. Classification of Infection Stages

### 1.2.1. Exposed

Animals can be exposed to MAP through various direct and indirect means: known infected animals, pasture, shared sheds, *in utero*, milk and colostrum. Exposed animals can easily be identified after infected animals are identified, through farm epidemiological assessment and analysis of surrounding herds. Alternatively boot swabs can be cultured or qPCR used to provide evidence of exposure and detect possibly infected animals within a herd [149].

### 1.2.2. Infected

Definitive infection is defined by histopathology, by culture of MAP, PCR of infected tissues and conclusively by a combination of two or more of the above. Faecal culture can only provide indirect evidence and is not conclusive; MAP bacteria in faeces can be due to pass-through (passive shedding), from ingested environmental MAP and although this provides confirmation of exposure, it is not singularly conclusive of an active infection. Passive shedding occurs for 7-10 days after a single ingestion event [99,150–154]. Multiple detections of MAP bacteria in faeces over an extended period are suggestive of infection. In a study, 80% of cows with >1 faecal sample positives in culture also had a positive culture test result from intestinal tissues, the latter confirming their true infection [155]. ELISA can be applied at this stage but false positive results are possible due to exposure to other environmental mycobacteria [156]; because of this, traffic light systems are normally put in place

with multiple tests taken at defined intervals to increase the confidence in the results.

### 1.2.3. Clinical Disease

Clinical disease is associated with clinical symptoms; weight loss, measured as ≥10% body weight loss over one month [157] or is in relative low body condition compared to the majority of animals in the herd/flock or farm. Objective visual body condition scoring systems are used internationally [158]. Cattle may have other symptoms including diarrhoea, decreased milk production or roughening of the hair coat. Although these are clinical signs of MAP infection they are also generic symptoms of multiple infections, for a definitive MAP diagnosis additional testing would still be required.

### 1.2.4. Sub-clinical Disease

Sub-clinical disease is defined as a diseased animal with no clinical symptoms. In these cases, the disease can only be identified post-mortem or by robust continuous testing via multiple testing methods.

### 1.2.5. Infectious

Infectious is defined as when the animal is shedding viable MAP into the environment, generally via faeces or milk. Viable MAP can be identified by faecal culture which has ≥24 bacilli per gram sensitivity; [159] quantitative PCR can also contribute information on shedding [160], although both of these depend on the sampling method. At this point animals can be classed according to the amount of shedding; light, medium or heavy/super shedders and thus their degree of infectiousness [161,162].

### 1.2.6. Resistant

Animals can possibly be resistant; this is where an animal receives an infectious dose at a time or age they are susceptible but no infection establishes or it remains in a dormant asymptomatic state. A study in 2007 showed that Holstein-Friesian cattle were more resistant to clinical disease and bacterial shedding than goats or sheep [163].

### 1.2.7. Recovered

Recovery from MAP would mean complete elimination of a demonstrable infection [144]. Due to the large latent period in the MAP infection, a confirmed case of recovery could take several years and would normally need tissue examinations when infected and after recovery to be certain. It is possible that recovered animals will show histopathological lesions, but MAP could not be cultured from these sterile granulomas; they would also be of a lower grade than those earlier in the infection.

## 1.3. Transmission to Humans

Transmission of a pathogenic species to humans can be generally by one of three routes. This depends on environmental survivability and the lifecycle of the pathogen, especially as MAP is an obligate pathogen; it can only reproduce within a host. MAP can survive for long periods outside its host environment in harsh conditions, and published work has generally focused around waterborne, food or zoonotic exposure.

### 1.3.1. Waterborne

MAP has numerous animal hosts and extensive shedding from these hosts suggests large quantities of viable infectious bacteria are present in the environment at any given time. Additionally, MAP can survive for extended periods in run off, agricultural slurry and thus in the wider environment, in either a vegetative state or spheroplast [164]. MAP has been detected in freshwaters (32.2% detection rate) receiving domestic and farming runoff in South Wales [165].

Contamination of surface waters (including lakes and rivers [166]) means drinking water contamination is possible and inevitable for animal water sources. In Ohio, USA, MAP DNA has been detected in over 80% of domestic water samples [14]. Research has shown that MAP is resistant to standard water disinfection protocols [167]. It has been reported that 103 CFU of planktonic MAC per 100 mL was present in potable water [168]. Biofilm and sessile communities are extremely hard to eliminate and very resistant to chemical disinfection [169],

biofilms in water piping can pass into domestic water supplies and be aerosolised by taps [170].

This leads to a potential for human exposure [171]. qPCR was used and detected MAP (IS900 gene) in ~90% of water and biofilm samples from 31 cold water taps in Ohio [172], in addition it has been demonstrated that MAC biofilms survive and proliferate in temperatures between 15-45°C and salinities of 0-2% NaCl. There has however not been extensive investigation distinguishing between water contamination, survival of MAP in treated water and human infection. This is most likely due to MAP infection not being notifiable, and no concrete links exist between animal and human infections.

### 1.3.2. Foodborne Transmission

MAP is found in milk and faeces of dairy cattle both clinically and sub clinically infected with MAP [173] and can result in foodborne transmission. It has also been postulated that old dairy cattle used for meat may be an additional source of infection, due to concentrated infection within the tissues [172]. Reviews have demonstrated that MAP can be detected on meat from animals with clinical infection and/or found positive via ELISA, PCR or culture [166]. Reports describe a 1000 CFU/g recovery of MAP from 7 of 15 liver, mesenteric and ileocecal lymph node samples; smaller numbers were isolated from 5 of 15 kidney, superficial and prescapular lymph nodes [174].

Within milk, it has been shown that cattle can shed significant levels of MAP, up to 540 CFU $ml^{-1}$ [175]. Considering milk from a herd will normally be pooled, this contamination has the possibility of affecting a large quantity of a farm's product. Given this, the efficiency of pasteurisation is important in reducing the risk of MAP transmission to humans [176]. Two types of pasteurisation are commonly used, low-temperature long-time (LTLT) and high-temperature short-time (HTST); both have been shown to be insufficient to deactivate MAP when at higher than $1x10^4$ CFU $ml^{-1}$ [177,178]. Following this evidence, processing centres changed the HTST holding time from 15s to 25s; however MAP detected via culture can still be found in pasteurised milk around the globe (1.7% to 6.7%) [179–182]. In 2011 a study of 567 pasteurised milk samples found 11.8% were MAP positive by PCR and additionally 1.8% of samples could be cultured [183]. MAP has also been detected in powdered infant

formula using a phage amplification coupled with PCR; this suggests freeze drying does not remove the bacteria [173]. Most studies have focused on the use of PCR but with varied success rates due to the presence of inhibitory substances within food itself [184]; PCR also only detects DNA and is not a direct indication of viability, for which culture must be used. Culturing of MAP from food samples is difficult as the samples contain a mixed variety of microbial species which could inhibit or outgrow it [185]. Additionally chemical decontamination to eliminate fast growing bacteria causes a 1-2 $\log_{10}$ drop in viable MAP cells [186].

### 1.3.3. Zoonotic

Presently MAP is not considered zoonotic [170] or a notifiable pathogen in animals, however the similarities of the disease to Crohn's means the possibility of human transmission cannot simply be ignored. Studies that have looked into zoonotic transmission show evidence is currently weak but should not be overlooked [13]. They have also shown that direct contact with calves' faeces is the most likely route of transmission; housing environment, colostrum, milk [187] and grazing [164] are all listed as potential risk factors. Of a larger concern is the lengthy asymptomatic period of subclinical infection [167] where the pathogen can be potentially transmitted to humans via contact with excreted faeces and milk [151].

### 1.3.4. Crohn's Disease

Crohn's belongs to the group of chronic inflammatory bowel diseases (IBD) of the intestinal tract with increasing prevalence in high-income countries [190]. It was once thought to be an autoimmune disease, but that mentality has changed to point more towards environmental, genetic and persistent antigenic compounds [191]. It is however not known if these persistent compounds are from pathogenic species or the gut microbiome. Within North America more than 1.4 million people are thought to suffer from Crohn's disease.

Both Crohn's and MAP have common clinical signs including intermittent diarrhoea, weight loss, mucosal ulcerations, granulomas and the

same primary infection site (ileocecal area) [192]. The appearance of the gut wall of Crohn's patients is also similar to animals with MAP [172]. Given these strong similarities and that MAP can make it into the food chain, it has been long proposed as the etiological for Crohn's disease [167]. There is also genetic evidence for zoonotic transmission, coming from whole genome sequence comparisons between MAP isolates from animals and humans with IBD [183], plus strain adaptations between camels and sheep [193]. MAP DNA has been detected with some frequency (46-100%) in biopsies and blood from patients with Crohn's disease [167,194]. It is detected less often in patients with ulcerative colitis and normal individuals. Detectable levels of MAP vary between studies but meta-analysis of numerous studies has shown increased levels of MAP compared with controls [195,196], with an average of 7 times more likely to be detected than either ulcerative colitis or normal controls [195]. Normal controls have still shown MAP DNA in peripheral blood, showing that MAP exposure is common and widespread [194,197]. This evidence does not preclude MAP as the cause for Crohn's disease because approximately one third of the globe's population is infected with tuberculosis but only 5-10% develops a clinical infection [198]. After MAP infected sheep were imported to Iceland in 1938, MAP diagnosis jumped from 0% to 30% within 18 years [199]. This coincided with an increased incidence of IBD over 40 years from 1950 [200].

## 1.4. Current Detection Techniques for MAP infections

### 1.4.1. Microscopy

Microscopy of mycobacteria involves the direct examination of tissues using either Ziehl-Neelsen (ZN) or acid-fast staining techniques. Although this method is inexpensive and simple it cannot be performed in the field. All sample types can be considered for microscopy including tissue, blood, milk and faecal samples.

When either tissue sections (ileocecal valve/lymph nodes) or smears are used, ZN will stain MAP bacilli bright pink with a background counter stain of blue. These bacilli groups can be visualised within macrophages of lesions and are suggestive of MAP infection. Within faecal samples, MAP bacilli clumps can be seen in smears, however concentration of the faecal sample

and decontamination via hexadecylpyridinium chloride, greatly improve the sensitivity of this test [89].

Microscopy is only useful after animals have begun shedding or after slaughter; as such it is unlikely to catch subclinical animals. Furthermore it is dependent on staff experience and saprophytic acid fast bacteria can be easily confused with MAP, compromising specificity [90].

Fluorescent microscopy has also been reported for detecting MAP, using a fluorigenic component which by enzymatic action in live cells can be transformed into carboxy fluorescein. Both macrophage uptake and the defined antigen substrate spheres system have been tested with mycobacteria [91].

### 1.4.2. Bioluminescence

Oxyluciferin, the excited state oxidation product of the luciferase enzyme catalysed luciferin reaction, gives off a photon of light when returning to its ground state. This bioluminescence reaction found in fireflies requires adenosine triphosphate (ATP) provided by live bacteria and oxygen to complete. Luciferase-encoding mycobacteriophages have been reported for use in the detection of MAP, offering a limit of detection (LOD) of >1000 cells/mL within 24-48 hours [92].

### 1.4.3. Culture

Culture is considered by most to be the ‘gold standard’ for MAP detection [93,94]. It can be undertaken on milk, blood and tissues but the most common by far is faecal culture, due to ease of sample collection from both dairy and beef cattle. Intestinal tissue culture is the most sensitive for individual animals however. Due to the slow growing nature of MAP, liquid media culture is far quicker to reach results at about 7 weeks, compared with solid media which can produce results from 2-4 months or even up to 6-7 months [95]. There is a larger contamination risk with liquid culture incubating for months as can be expected; however it is more sensitive than solid medium culture [94]. All *in vitro* culture of MAP requires the addition of mycobactin J, as it enables iron acquisition. This requirement can be used as a discriminatory test for

confirmation of MAP in cultures [96]. There are also automated systems in use, such as BACTEC MGIT 960 which has an LOD of 10 CFU $ml^{-1}$ and a detection time of 4-7 weeks [97].

Although culture is considered the gold standard, due to intermittent shedding, disease stages and sample decontamination reducing the quantity of viable MAP cells, it unfortunately has imperfect sensitivity [37,98], with different species and disease stages ranging between 16 to 74% [93]. Because of the intermittent shedding [93] regular serial sampling is needed to ensure all shedding animals can be detected.

Radioisotopic culture can also be used for MAP detection and carries with it an LOD of 3 organisms/gram of sample. Time to culture using this method varies between a few days to a few weeks. BACTEC 12B and BACTEC 460 are examples of systems that use this method [99,100]. Due to the use of more hazardous substances and the equipment costs involved, this is not the usually preferred method for detection [95].

### 1.4.4. Polymerase Chain Reaction (PCR)

PCR for the detection of MAP centres around the insertion sequence (IS) 900 which has 1451 base pairs and appears 15-20 times within the MAP genome. Some IS900 like sequences have been found within other environmental mycobacterial genomes [101] but these can be separated through characterization of the amplified segment by sequencing or genotyping via methylation-restriction.

PCR has been reported to have a better sensitivity than culture with an LOD of 10-100 CFU/mL in milk [102,103] and can be used on the same sample types, faeces, milk, blood and tissues. However, PCR is vulnerable to interferences, for example, enzyme inhibitors which make detection of the IS gene probe problematic and can cause false-negative results [104,105]. Isolation of deoxyribonucleic acid (DNA) from the sample is one of the most important parameters for PCR performance especially as samples generally have few organisms; given this laboratories usually have their own optimised method [106,107]. Additionally, no PCR method can distinguish between viable and non-viable MAP cells.

Real-time PCR (RT-PCR) uses fluorescently tagged primers or probes complementary to an intermediate fragment of the target sequence that is amplified. The fluorescence has a direct relationship with the amount of amplifying product and allows near instantaneous quantification of the target with greater sensitivity than culture [108]. Although RT-PCR is very sensitive, IS900 has a varying number of copies within the MAP genome and as such cannot accurately quantify the colony forming unit (CFU) present. However by the use of optimised standard curves it can quantify the starting sample with very high sensitivity [109,110].

More recently, multiplex PCRs have become possible with multiple strains of bacteria analysed within a single reaction tube, rather than successive testing of a single sample. Luminex has a system based on IS900, IS901, IS1245 and dnaJ gene that can detect MAP, *Mycobacterium hominissuis*, *Mycobacterium silvaticum* and more, with an LOD of $10^3$ CFU. Multiplexing however makes optimisation more complex and with speed comes a lowering of the sensitivity due to reagent interference and primer dimer formation [111].

PCR can also be used on *in situ* formalin-fixed, paraffin-embedded tissue sections [112]. This method is especially helpful at confirming MAP DNA in infected tissues as well as detecting spheroplasts. RT-PCR can also be used within this context, is highly specific and is capable of detecting m ribonucleic acid (RNA) expression within infected tissues [113,114]. In situ hybridisation uses a labelled probe to specifically label DNA/RNA on a tissue section; this enables MAP infection to be found within specific tissue sections [115].

Alongside these PCR methods loop-mediated isothermal amplification (LAMP) has also been developed for use on MAP and requires no thermal cycler. Recently this method was shown to provide greater sensitivity than PCR and nested-PCR [116].

### 1.4.5. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a simple, cost effective, technique which requires little specialised equipment and can be carried out on all sample types except tissue. Current ELISAs report high specificity by compromising sensitivity, with

specificities in the range of 97%-99% [117,118]. Due to disease progression, the sensitivity of current ELISA increases over time, with higher antibody loads in later disease stages [119]. To improve the specificity, sensitivity and eliminate cross-reacting antibodies from environmental mycobacteria, *Mycobacterium phlei (M. phlei)* has been used as a pre-absorption step [120].

Although the antigens used within commercial ELISAs are not specified, it is known that at least some use lipoarabinomannan (LAM), protoplasmic antigens (PPA3) and isolates from ATCC19698, VRI316 [93].

Due to ELISA's ease of use, it is generally used on large scale screening of animals with 'traffic light' systems in place, where multiple positive signals are needed over a space of time to indicate MAP infection.

### 1.4.6. Interferon-Gamma Release Assays (IGRAs)

IFN-γ released when peripheral blood mononuclear cells (PBMCs) are stimulated by an antigen, can be measured in various ways: ELISA [121], enzyme-linked immunospot (ELISPOT) [122,123], and qRT-PCR. Measured via ELISA, whole blood is cultured with antigens in a proliferation assay and IFN-γ levels are measured in the supernatant [124,125]. Studies have shown that purified protein derivatives (PPD) used as antigens in calves younger than 15 months give fluctuating IFN-γ levels [125,126]. Issues also arise in IGRAs when non-specific or cross-reactive PPD antigens are used and these can be blamed for the low specificities and sensitivities shown [127,128]. Alongside these issues, the samples also need to be processed quickly as cells need to be alive. IFN-γ is however secreted in large amounts during early infection and is an important tool for detecting subclinical animals.

### 1.4.7. Delayed-type Hypersensitivity (DTH)

DTH is a measure of cell-mediated immunity and is commonly used to test for *M. tb* immunity via the TST skin test. For the DTH skin test, the animal is inoculated with an antigen or antigen cocktail; in the case of MAP it is a PPD of MAP cultures called Johnin [129]. The skin is measured with slide callipers both before and 72 hours after inoculation, and a swelling greater than 2 mm indicates DTH in infected animals. Some animals however produced diffuse

swelling making interpretation difficult [130]. Using Johnin in this fashion has a reported specificity of 88.8%, increasing the cut-off to greater than 4 mm changes this to 93.5%. Although specificity is high, the sensitivity is lacking and minor changes with the PPD antigens batch to batch make its value at the moment questionable [37].

### 1.4.8. Lymphocyte proliferation/transformation assay

This test is a research tool only and measures the ability of peripheral blood mononuclear cells (PBMC) in recognising and responding to MAP antigens post vaccination when pulsed with a protoplasmic antigen. The simulative index value has considerable variation in heavily infected animals; for non-infected animals this value depends on the population and herd history of MAP [131].

### 1.4.9. Immunohistochemistry (IHC)

IHC uses enzyme tagged MAP specific antibodies, which when used with a substrate allows identification of MAP in tissues and spheroplasts [115]. Whilst sensitivity is good in tissues false positives are possible due to environmental mycobacteria and culture has superior sensitivity [132].

### 1.4.10. Flow Cytometry

For flow cytometry, intact MAP bacteria serve as a test antigen and measuring particle. All the MAP surface antigens can be recognised by animal antibodies in this setting. Flow cytometry is quick, taking less than 4 hours to complete. Within experimentally infected animals it has a reported sensitivity of 95% and specificity of 97%, detecting MAP up to 170 days after the infection event [133]. A study has shown IgG1 in flow cytometry to have a 78% sensitivity and 100% specificity; additionally it showed that MAP antibodies could only be detected occasionally in calves less than 1 year of age [134].

### 1.4.11. Complement Fixation Test (CFT)

CFT tests have been used for detecting MAP for some years [135], they are however limited to general screening of a population due to their lower

specificity. Numerous protocols exist but there is no universal pattern sera with standardized complement fixation units for use as a reference. CFT has largely been superseded by agar gel Immune-diffusion (AGID) and ELISA but can still be in demand for countries that import animals.

### 1.4.12. Agar Gel Immune-Diffusion/Precipitation Test (AGID/AGPT)

AGID has been reported to have a higher sensitivity and specificity than ELISA in small ruminants [136,137]; measured against histology this resulted in 99%-100% (95% CI) specificity and 38%-56% (95% CI) sensitivity [138]. AGID can detect antibodies 39 months after shedding [139]. Given its high specificity and low cost by nature it is an important tool for confirmation testing of suspected clinical infection animals.

### 1.4.13. Necropsy

Necropsy or post-mortem examination allows extensive examination of relevant tissues, including the ileum, associated lymph nodes and other intestinal sections. Culture, histopathological, PCR and other methods can be used on these tissues.

Gross pathology of infected animals shows enlargement of the mesenteric and ileocecal lymph nodes, as well as thickening of the intestinal mucosa, but these are not specific to paratuberculosis and they also do not occur in all affected animals[140–142].

Lesions can be scored/categorised with the criteria being widely published[143–147]. All of these methods use multiple categories to describe extent, severity and nature of the granuloma's lesions. Based on biopsies there is a likely progression from mild to severe[144]. Using the Perez et al. system[145] this starts at 1 (mild focal), to 2 (focal), 3a (multifocal) and 3b (multifocal to diffuse, multibacillary) or 3c (multifocal to diffuse, paucibacillary). These lesions are most commonly found within the terminal ileum, ileocecal valve region and nearby lymph nodes. As the disease progresses the lesions extend along the intestines [146,148]. Focal lesions can be difficult to find due to the large

surface area of the intestines; as such thorough examination of intestinal sites is required to confirm whether lesions are present.

### 1.4.14. Commercial Usage

Although a large number of methods are available for the detection of MAP, most are used as research tools, with very few used inside commercial farming. Although culture and necropsy are used, the latter for obvious reasons has limited use within dairy herds. The former is not suitable for large scale timely testing, because results can take a significant amount of time to come to fruition. Faecal PCR and ELISA are available as commercial assays, though both currently have downsides. Faecal PCR cannot distinguish between live or dead cells, is susceptible to passive shedding and sampling issues. ELISA on the other hand is perfect for large scale high throughput testing but currently has sensitivity issues until late stages of infection.

No current detection method is perfect, not even culture, and as such a sensitive test for early disease stages is actively sought after.

## 1.5. Mycolic Acids

The mycolic acids contained within mycobacteria follow a general formula (Fig. 1) and fall into three major classes [81–83]; wax esters also seen here (Fig. 2) feature in MAP whereas the methoxy class does not [84]. The identity of the mycobacterial species can be elicited via the composition of mycolic acids present.

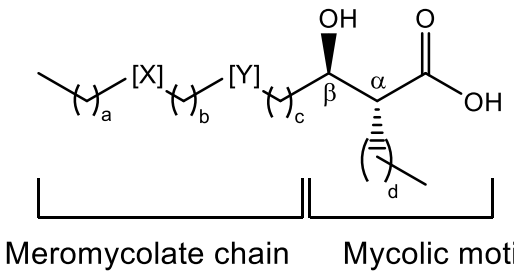


**Figure 1** – Shows the general formula for a mycolic acid. [X] indicates the distal group, whilst [Y] is the proximal group. The methylene chains indicated by a-d can vary in length considerably.

Mycolic acids have been shown to be key antigenic structures within *M. tb*, with trehalose-6,6-dimycolate (TDM) and trehalose 6,6'-monomycolate (TMM) able to stimulate innate and adaptive immune responses [85]. Glucose monomycolate (GMM) has proven to be highly potent as well with T cell receptors being highly specific towards certain conformations [86].

It has also been shown that methoxy and keto classes are more antigenic than the alpha class, with both chain length and the specific stereo chemistry playing an important part [87]. Due to this and the difficulty separating natural mixtures with different stereochemistry, fully synthetic mycolic acids and sugar ester derivatives can be extremely valuable for both diagnosis and vaccine development. Defined synthetic versions have already shown promise for the diagnosis of *M. tb* via immunoassays [88], when compared to natural mixtures.

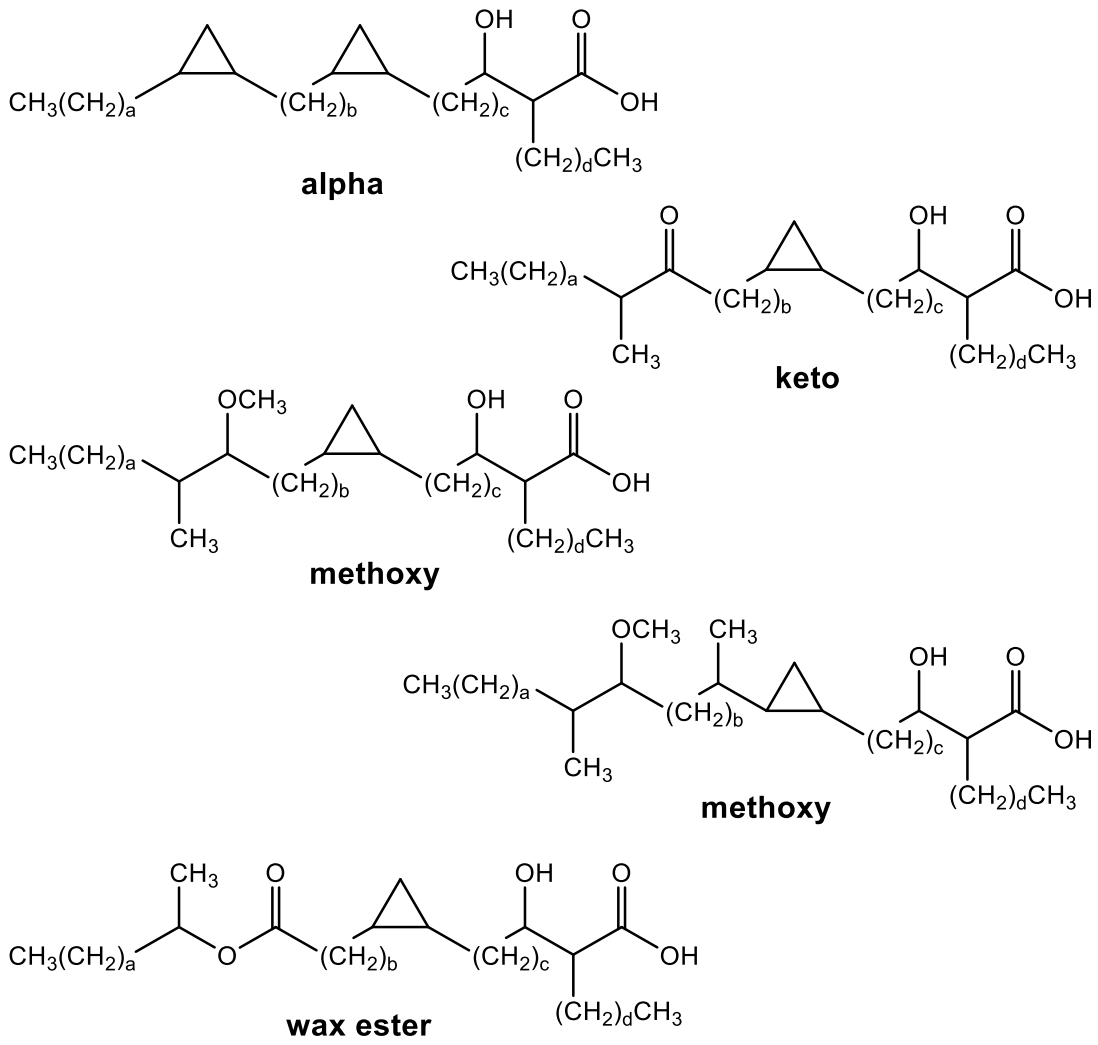


**Figure 2** – The major classes of mycolic acids from *M. tb,* including a wax ester from MAP.

Highly defined compounds have an advantage in eliminating cross reactivity and can be mixed if necessary in specific quantities, whereas natural mixtures can vary batch to batch. Furthermore, given that mycolic acids are unique to mycobacteria it is hoped that they provide an earlier and more specific antibody response than secretory protein antigens.

## 1.6. Cell Envelope

The envelope encompassing mycobacteria is responsible for many of the biological properties that they present, including their shape, mechanical resistance and protection from hostile environments. The uniqueness of the mycobacterium envelope is due to the large proportion of lipids present, constituting 40% by dry weight of the tubercle bacillus [52,53]. The cell wall itself is made up of 60% lipids vs approximately 20% of lipid rich Gram negative bacteria [53]. This characteristic also helps to explain MAP's tendency to grow in clumps and its acid-fastness [54].The key to these lipids is that they include long chain fatty acids, mycolic acids, covalently linked to the cell wall polysaccharide arabinogalactan. These mycolic acids are held responsible for many biological properties the bacteria present [52], which includes their high resistance to most broad spectrum antibiotics with the exception of rifamycins and streptomycin. This resistance is due to the impermeability not only to nutrients and small molecules but also dilute acid, alkali and infected mammalian cells [52]. This makes mycobacteria 100-1000 times less permeable than the most resistant Gram negative bacteria, for example *escherichia coli* (*E. coli*) and *pseudomonas aeruginosa* [55].

Compared to Gram negative bacteria, relatively little is known about the composition and arrangement of the cell envelope. A model for the envelope of mycobacteria was elicited via transmission electron microscopy (TEM)/freeze substitution in the 1990's [56,57] but recently cryo-electron microscopy of vitreous sections (CEMOVIS) has been used to demonstrate this more accurately with fully hydrated unstained cells [58]. This latter method has also directly observed the outer membrane and revealed a symmetrical plasma membrane. Via cryogenic electron microscopy (cryoEM), the capsule,

which is present in pathogenic mycobacteria, not seen with CEMOVIS, has been revealed [59,60]. From these recent models we can divide the envelope into three parts: the outer most layer also called the capsule, the cell wall and the plasma membrane.

### 1.6.1. The Capsule

This outer-most layer; also called the capsule in pathogenic mycobacteria [61], is mainly comprised of polysaccharides [62,63] within slow-growing bacteria, compared to fast-growing which predominately feature proteins [62]. Within slow-growing mycobacteria the capsular and extracellular mainly feature glucans with repeating units of five or six α-(1→4) linked D-glucosyl residues substituted at position 6 with mono- or oligoglucosyl residues [62–65]. Amongst the surface exposed compounds are mannan chains composed of an α–(1→6)-D-mannosyl core, with some units substituted with α-D-mannose at position 2 and D-arabino-D-mannan heteropolysaccharide [66].

Lipids do not feature greatly in the capsule of mycobacteria, most being found on the inner rather than the outer part [67]. Within *M. tb*, the 2 to 3% of surface exposed lipids present are mainly comprised of phenolic glycolipids (PGLs), dimycocerosates of phthiocerols (DIMs), lipooligosaccharides (LOSs), 2,3-diacyl trehaloses (DATs) and phospholipids, with the inner capsule being composed of mainly 6,6′ -dimycoloyl trehaloses (DMTs), triacyl glycerols (TAGs), 6-monomycoloyl trehaloses (MMTs) [67].

Proteins within the capsule also seem to be found to a great degree within the cell wall, which suggests that they could shed and remain confined around the cell within the capsule [52]. From *M. tb* these proteins are a complex mix of polypeptides [63].

### 1.6.2. Cell Wall

The cell wall of mycobacteria comprises an outer membrane bilayer or mycomembrane (MM) as well as arabinogalactan (AG) and peptidoglycan (PG) [52]. These three components form a tripartite cell wall complex called the mycoloyl-arabinogalactan-peptidoglycan (mAGP) complex [52,68]. The MM has an unusual bilayer construction; the inner one is comprised of mycolic acids

esterifying AG which is covalently linked to the PG. The outer layer of the MM is presumably composed of various free lipids, including phospholipids (cardiolipid/phosphatidyl glycerol, phosphatidyl ethanol-amine, and phosphatidyl inositol, plus a few phosphatidylinositol mannosides (PIMs)), trehalose mycolates, glycopeptidolipids, and lipoglycans [69], which are electrostatically bound to the cell lipids [52,70,71]. Despite the MM containing long chain mycolic acids it remains 7-8 nm thick [58,59], which gives clues regarding the exact native conformation of the mycolic acids, because if they were organised as long straight chains in parallel the total thickness would be >40 nm.

The AG is a branched heteropolysaccharide that contains a galactan chain composed of alternating 5- and 6-linked D-galactofuranosyl residues, the substitution of this chain depending on the mycobacterial species [72].

The PG is comprised of repeating units of N-acetylglucosamine and N-acetyl/glycolylmuramic acid cross-linked with a peptide side chain; these side chains are heavily cross linked when compared with *E. coli*.

The cell wall contains porins and membrane machineries [73–75] which are key to secretory proteins and virulence factors including, secretory protein 6-kDa early secretory antigenic target (ESAT6) and 10-kDa culture filtrate protein (CFP10) [76–78]. Finally, the conventional lipid bilayer plasma membrane is separated from the cell wall by a periplasmic space [58].

### 1.6.3. Plasma Membrane

The plasma membrane (PM) follows a similar bilayer construction when compared to other Gram-positive/negative bacteria, and comprises phospholipids and proteins with specific metabolic functions [52]. The outer leaf is thicker, being comprised of lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannosides (PIMs).

Lipids are also present within this bilayer. Phospholipids are present, mainly phosphatidyl glycerol, PIM, cardiolipid and phosphatidyl ethanolamine. Trehalose monomycolate has also been found here [52].

Numerous proteins are present in this layer (>2000) [69], including those related to the biosynthesis of PIM [79]. Also present is ESAT6 [80], that has been used in detection methods for various mycobacterial infections.

## 1.7. Study Aims and Approaches

The aim of this study is to determine if fully defined synthetic mycolic acids and their sugar esters, can be used for the detection of specific antibody responses to MAP, within cattle serum samples. This will take the form of ELISA based testing with a selected set of serum against a large set of antigens. Continuing from this, a larger set of serum will be tested against a refined set of antigens. Further, the ELISA assay will be translated to a flow through device for initial testing.

In addition, the refined set of antigens will be tested against a small set of Crohn's patient's serum using ELISA.

# 2. General Materials and Methods

## 2.1. Materials

Potassium chloride, sodium chloride, potassium phosphate monobasic, anhydrous sodium phosphate dibasic, o-phenylenediamine (OPD), phosphate-citrate buffer with sodium perborate capsules, (hydroxypropyl)methyl cellulose, tween 20, all molecular biology grade, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Casein according to Hammarsten was purchased from VWR International (Radnor, PA, USA).

Hexane and tetrahydrofuran (THF), both HPLC grade, were purchased from Fisher Scientific (Hampton, NH, USA).

C-bottom, polystyrene, single-break, high binding strip plates (705071) were purchased from Greiner Bio-One (Kremsmünster, Austria).

Breathable plate seals (3345) were from Corning Inc. (New York, USA).

Bovine secondary antibody, affinity purified goat anti-bovine IgG (Fc) horseradish peroxidase conjugated (HRP) (101-035-008-JIR) was purchased from Stratech (Ely, UK).

Human secondary antibody, preabsorbed goat anti-human IgG (Fc) HRP conjugated (ab98624) was purchased from Abcam (Cambridge, UK).

InnovaCoat Gold – 40 nm Protein G nanoparticles (223-1000) was purchased from Expedeon (Cambridge, UK).

Preassembled flow through devices were purchased from mdi Membrane Technologies Inc. (Ambala Cantt, India). Type FT12, size 31mm x 41mm with 8x absorbent pad, type AP080 of size 24.5mm x 36mm, with 1 piece of size 2cm x 2cm of membrane, type CLW-040-SH34, 0.45µm.

## 2.2. Antigens

All synthetic antigens (OTA97 [201], AD123 [202], KB110 [202], ST152 [203], MH175 [202], SMP74 [204], MOD171 [205], JRRR121 [206], JR1080 [207], MOD30 [208], RT237F2 [209], ST151 [203], AD129 [210], MH140 [206], JR1056 [211], SMP75 [204], ST123

[203], ST124 [203], KV059) were prepared by the literature method and provided by Diagnostig Ltd (Gaerwen, UK).

## 2.3. Data Analysis

Microsoft Excel was used for initial data analysis including median values and standard deviations.

R by CRAN was used for statistical analysis. ROC analysis was via the proc package, graphs were plotted with gplots. Optimal cutoffs and sensitivity/specificity (Fig 3) were determined using Youden's index. Random forest and principal coordinate analysis was done using the following packages: gbm, randomForest, ROCR, caret, RColorBrewer.

$$Sensitivity = \frac{number\ of\ true\ positives}{number\ of\ true\ positives + number\ of\ false\ negatives}\ x\ 100$$

$$Specificity = \frac{number\ of\ true\ negatives}{number\ of\ true\ negatives + number\ of\ false\ positives}\ x\ 100$$

**Figure 3** – Equations defining sensitivity and specificity calculations.

A ROC plot shows the sensitivity percentage plotted against the specificity percentage at various threshold values. The AUC calculated from ROC summarises the diagnostic accuracy of a test, greater than 80% is considered very good.

Boxplots display the median, first quartile, third quartile, minimum, and maximum. The median is indicated by the central line, the first quartile by the outer side of the box within the lower data range and the third quartile by the other side of the box within the higher data range. The lines outside the box represent the minimum and maximum values excluding outliers. The individual averaged absorbances for each sample are shown as dots on the boxplots presented here.

Raw data including averaged individual sample results is displayed within the appendix.

# 3. An Investigation of Responses of Strongly Positive Experimentally Infected Samples Compared to Negative 'No History' Samples

## 3.1. Introduction

This study was undertaken to provide initial evidence as to whether specific antibodies were produced against MAP in infected cattle that bind to mycolic acids and their sugar esters.

The ELISA method used closely followed the one used in previous human ELISA work [88] with a few alterations. Firstly, to make it suitable for detecting cattle antibodies an anti-bovine secondary antibody, anti-bovine IgG(Fc) HRP, was used. Although it is presumed that the majority of the response will be from IgG1, using a less specific secondary antibody that recognises all subclasses could help ensure that no potential antigens are discarded due to missed antibody binding. From the human work [88], the Fc specific antibody was found to give better responses and as such will be used here as well. Secondly, the concentration of $H_2O_2$ and OPD was reduced by a factor of five to ensure all values fell within the linear range of the microplate reader and substrate. Although this could diminish the sensitivity/specificity due to reducing the dynamic range, ensuring a linear response is important for the results' validity.

Fifteen antigens (Figs 4-6) were selected for this study based on either their appearance within the MAP bacilli (this includes TMMs, TDMs and wax esters), or on their previous performance in other mycobacterial assays. All three major classes of mycolic acid (alpha, keto, methoxy) were included.

For this series of experiments, a controlled set of serum was selected using that from an experimentally heavily infected cattle herd supplied by CODA-CERVA, and negative serum supplied by the Moredun Institute, from a farm which has no history of MAP. Using this approach of a highly infected set and one which has not had exposure should give the largest separation between positive and negative and will inform the antigen selection for later stages.

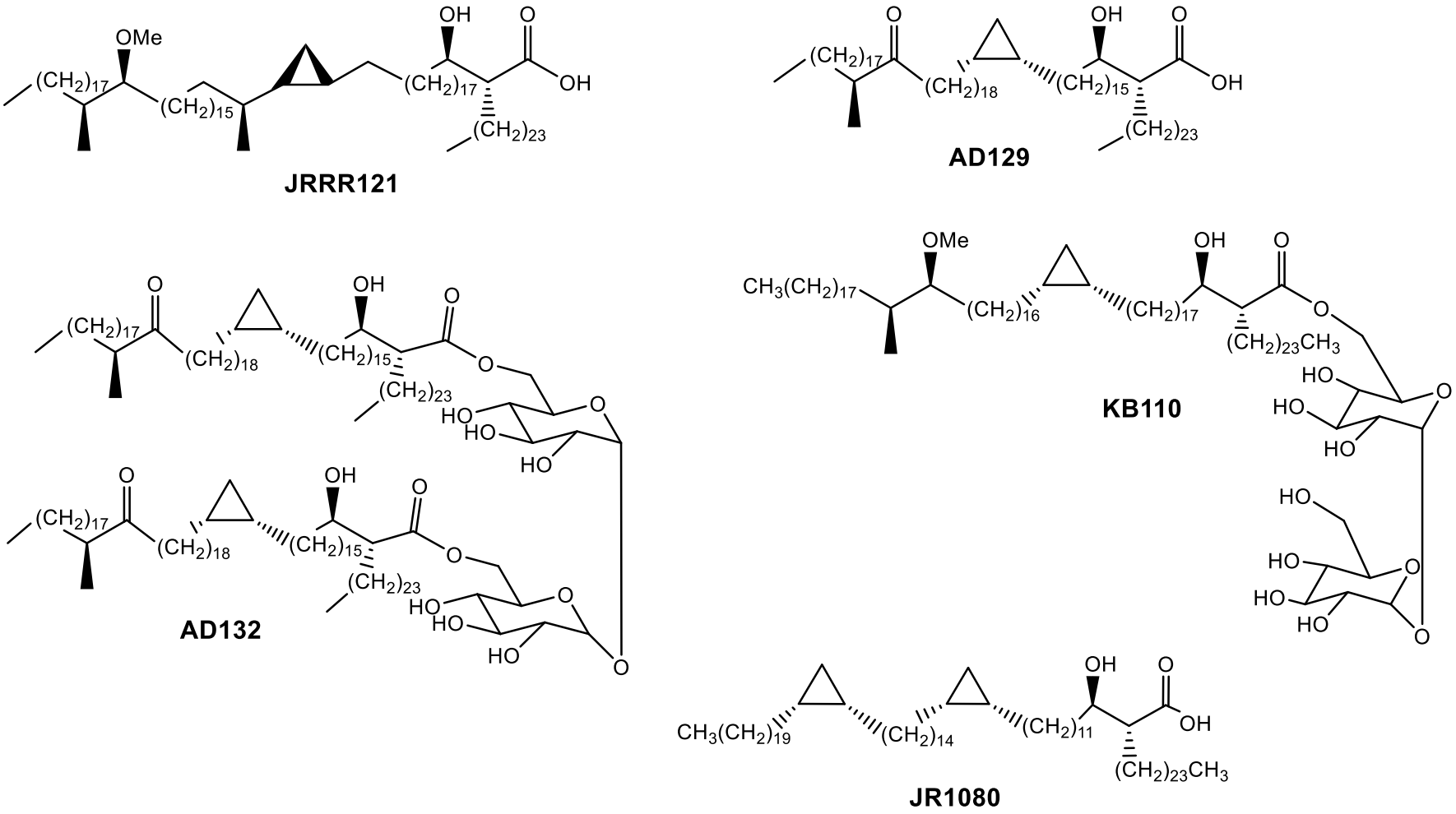


**Figure 4** – JRRR121 is a *trans*-methoxy mycolic acid, AD129 a *cis*-keto mycolic acid, AD132 a *cis*-keto TDM, KB110 a *cis*-methoxy TMM and JR1080 a *cis*-alpha mycolic acid.

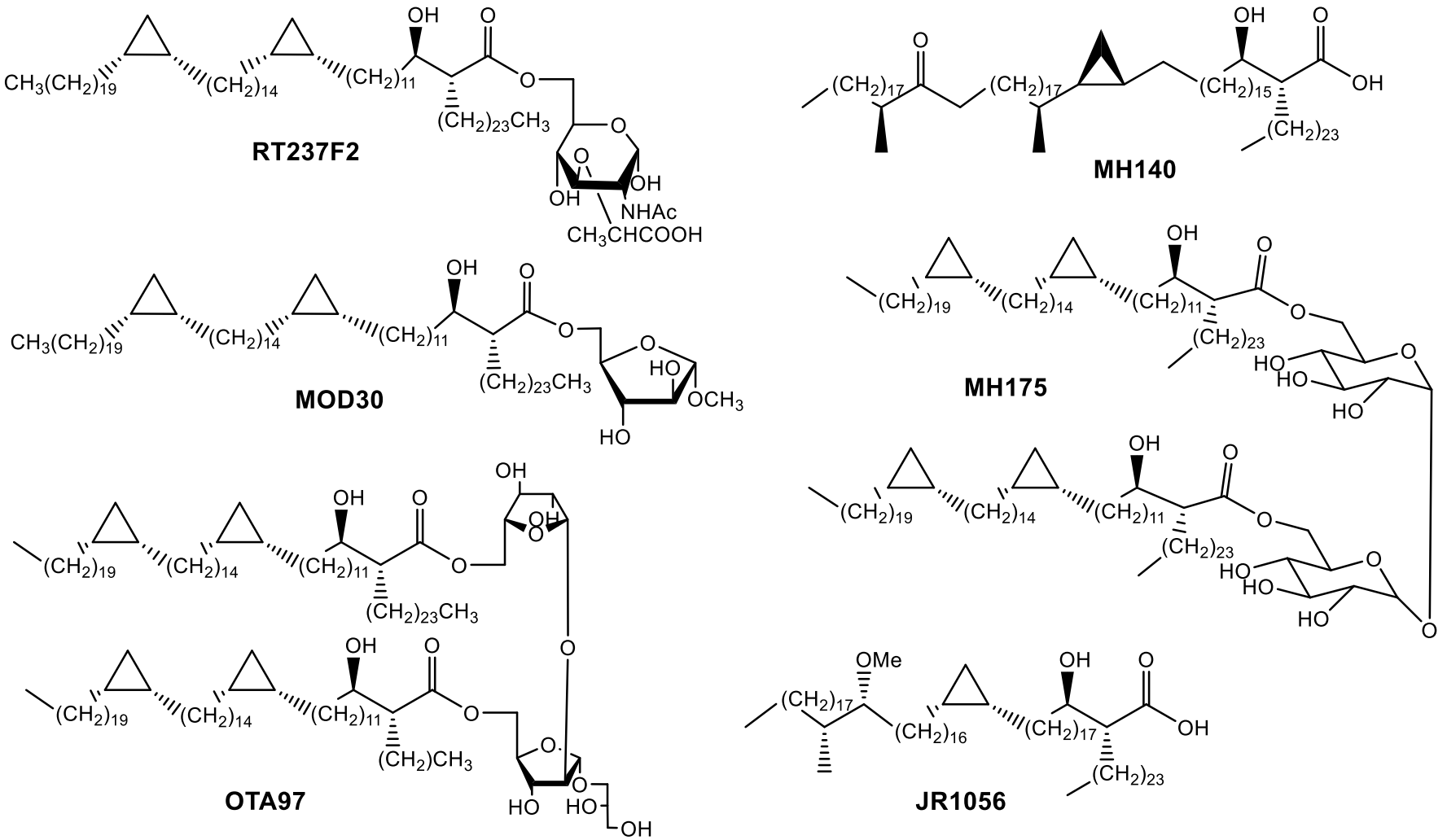


**Figure 5** – RT237 is a *cis*-alpha muramyl, MH140 a *trans*-keto mycolic acid, MOD30 a *cis*-alpha arabinose mono-mycolate (ArMM), MH175 a *cis*-alpha TDM, OTA97 a *cis*-alpha dimycolyl diarabinoglycerol (DMAG) and JR1056 a *cis*-methoxy mycolic acid.

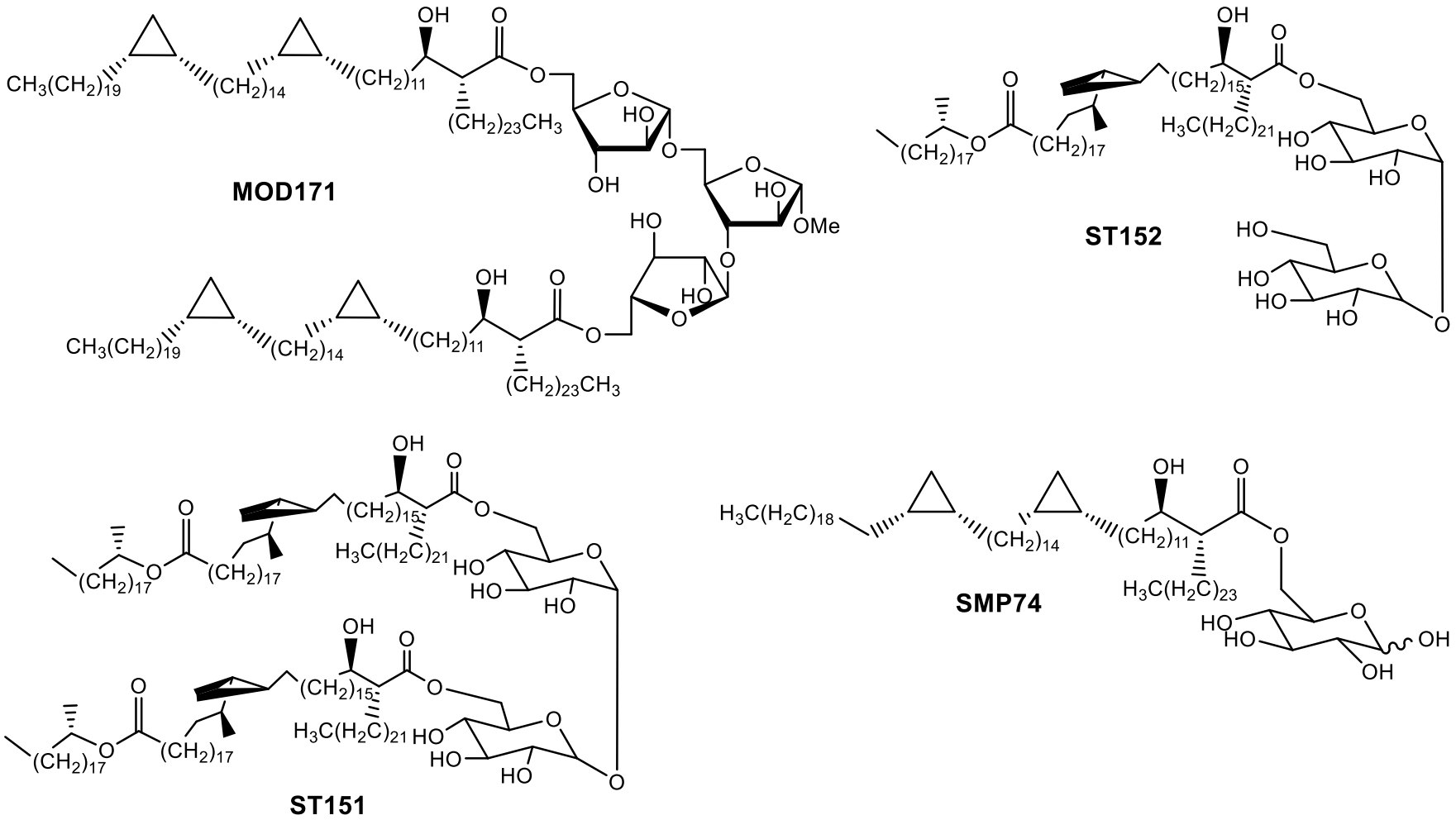


**Figure 6** – MOD171 is a *cis*-alpha tri-arabinose di-mycolate (ArTM), ST152 a wax-ester trehalose monomycolate (WE-TMM), ST151 a wax-ester trehalose 6,6′-dimycolate (WE-TDM) and SMP74 a *cis*-alpha GMM.

## 3.2. Materials

The MAP positive serum samples were supplied by CODA-CERVA (Federal Veterinary and Agrochemical Research Centre, Brussels, Belgium) and were from 35 experimentally infected cattle. All 35 were confirmed positive via culture and Pourquier ELISA. Additionally, all but two were positive against the IDvet paratuberculosis ELISA.

The 29 negative samples were supplied by the Moredun Institute (Penicuik, UK) and were from a remote farm with no history of Johne's disease for many years.

## 3.3. Methods

KCl (0.40 g), $KH_2PO_4$ (0.49 g), anhydrous $Na_2HPO_4$ (2.88 g), NaCl (16.00 g) was dissolved in 1800 mL d $H_2O$, stirred and heated to 50°C. Casein (10 g) was added slowly and the solution was stirred at 50 °C for 2 hours. After reaching room temperature (rt) it was then adjusted to pH 7.4 with NaOH (1 M), made up to 2000 mL and stored in the fridge. This formed the 0.5% casein w/v PBS buffer.

Antigens were diluted to 2.9 µM (Table 1) using THF-hexane (1:50) and 50 µL was centrally pipetted into each well of the ELISA plate, to cells G-H 11-12 only THF:hexane (1:50) solution was added. The plates were allowed to evaporate at rt for 2 hours before being sealed with breathable sealing tape.

The following day, plates were unsealed and 0.5% casein w/v PBS buffer (350 µL) was dispensed using a 96 well plate washer, then incubated at 25°C for 30 minutes.

Serum (5 µL) was diluted to 1:40 with rt 0.5% casein w/v PBS buffer and resuspended by pipette. The casein solution was aspirated off the plates and tapped dry, diluted serum (50 µL) was added to the wells with one duplicate well per sample. Remaining serum was pooled and added (50 µL) to cells A-B and G-H 11-12 for a pseudo positive and negative control respectively, 0.5% casein w/v PBS buffer was added to cells E-F 11-12 for a diluent. The plates were then sealed with a breathable plate seal and incubated at 25°C for 60 minutes.

The plates were then washed three times using 0.5% casein w/v PBS buffer and tapped dry, before adding anti-bovine IgG(Fc) HRP (50 µL) diluted to 2.9 µg/mL using 0.5% casein w/v PBS buffer with a multichannel pipette and then incubated at 25°C for 30 minutes.

Following this the plates were washed three times using 0.5% casein w/v PBS buffer and tapped dry, OPD (5 mg) was dissolved in 25 mL citrate buffer (0.05 M) containing sodium perborate buffer (0.03%) and added (50 µL) to the plates using a multichannel pipette. Plates were incubated at 25°C for 30 minutes.

$H_2SO_4$ (50 µL, 3 M) was added to the wells after the 30 minutes and the results read on a UV-visible ELISA plate reader at 450, 492, 620 nm.

**Table 1** – Molecular weights used for concentration calculations in the first study.

| Antigen | Molecular Weight |
|---|---|
| AD129 | 1238.23 |
| AD132 | 2782.73 |
| JR1056 | 1254.28 |
| JR1080 | 1138.07 |
| JRRR121 | 1296.36 |
| KB110 | 1578.56 |
| MH140 | 1280.31 |
| MH175 | 2582.41 |
| MOD30 | 1284.21 |
| MOD171 | 2668.50 |
| OTA97 | 2287.84 |
| RT237F2 | 1413.33 |
| SMP74 | 1300.21 |
| ST151 | 2842.78 |
| ST152 | 1592.54 |

## 3.4. Results

From this ideal set of serum, 10 out of the 15 antigens gave an AUC over 80, with 8 antigens showing over 90 (Table 2) on ROC analysis (Fig 8). Of these 8, 5 showed a sensitivity/specificity of ≥ 80/80, with MOD171 being 100/100.

Of the 5 pure mycolic acid types tested the *trans*-methoxy type (JRRR121) gave the best results with an AUC of 94 and sensitivity/specificity of 79/100. The *trans*-keto (MH140) acid gave an AUC >80 but showed low absorbances in general contributing to little variation between median values. Both *cis* conformations of the methoxy (JR1056) and keto (AD129) acid gave low AUCs (<80), with AD129 in particular giving barely any difference in median values between positive and negative serum. Again, both of these *cis* acids showed generally low absorbances. JR1080 the alpha acid, followed similarly to JRRR121 with an AUC >90 but with a high confidence interval and sensitivity/specificity of 90/100. All acids with the exception of AD129 displayed very good grouping of negative results.

Both of the TMMs tested KB110 and ST152 showed below average results, but interestingly KB110 displayed a slightly negative skew (Fig 7) with a median positive result of 1.86 and median negative of 2.14.

Between the TDMs tested (AD132, MH175, ST151), the *cis*-keto AD132 and wax ester ST151 produced very similar results with both having AUCs >90, sensitivity/specificities of 90/80, 90/83 respectively and median values over 1.00 apart. MH175 in comparison gave a large overlap of positive and negative samples resulting in a low confidence interval and sensitivity/specificity of 90/63.

The two arabinose based compounds (MOD30, MOD171) gave rather different absorbance ranges with MOD171, giving a perfectly clear distinction with an AUC of 100, sensitivity/specificity of 100/100 and confidence interval of 100-100. MOD30 gave good grouping of negatives but with a median positive absorbance of 0.33 and AUC of 93.

Of the three remaining compounds SMP74 and RT237F2 both displayed an AUC >90 and sensitivity/specificity >80/80, with OTA97 falling only slightly behind in both aspects. RT237F2 showed similar results to

MOD171, almost perfect but with very low response values. Whilst SMP74 and OTA97 displayed greater difference between positive and negative medians.

The results from all 15 antigens were combined using a Random Forest classifier and plotted via principal co-ordinate analysis (Fig 9). Different combinations of the results with each antigen are represented by the two axes. From this, two groups are clearly separated, with the no history negatives on the left and the experimentally infected positives on the right. If a line was drawn y=-x only one negative would end up on the positive side. The contribution or weighting of individual antigens within the random forest gave MOD171 as the most significant followed by RT237F2, JR1080, JRRR121, SMP74, MOD30. This pattern closely follows the AUC values.

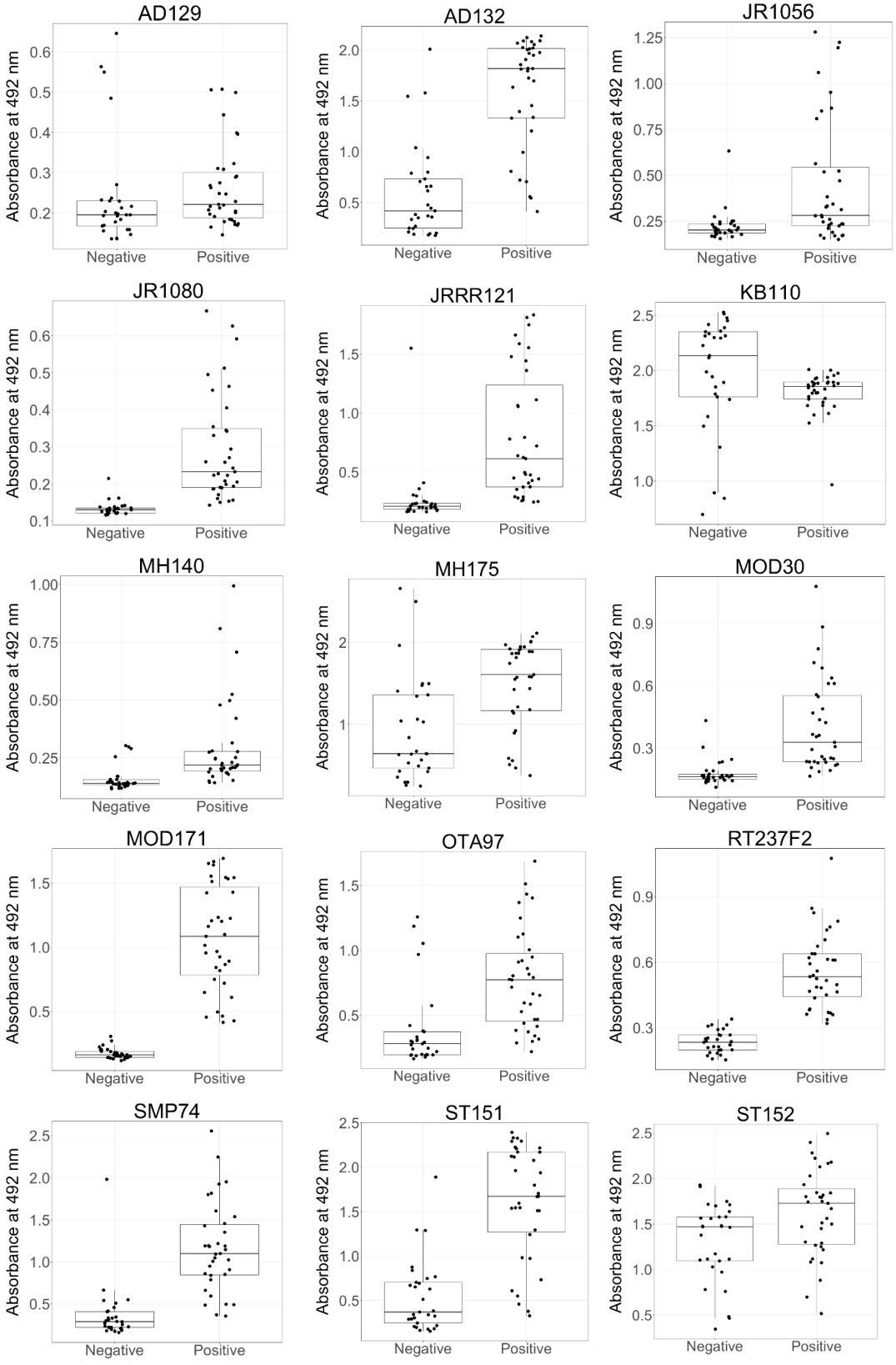


**Figure 7** – Shows ELISA responses and boxplots of culture positive and negative sample sets, for all 15 antigens in the first study of experimentally infected positives and no history negatives.

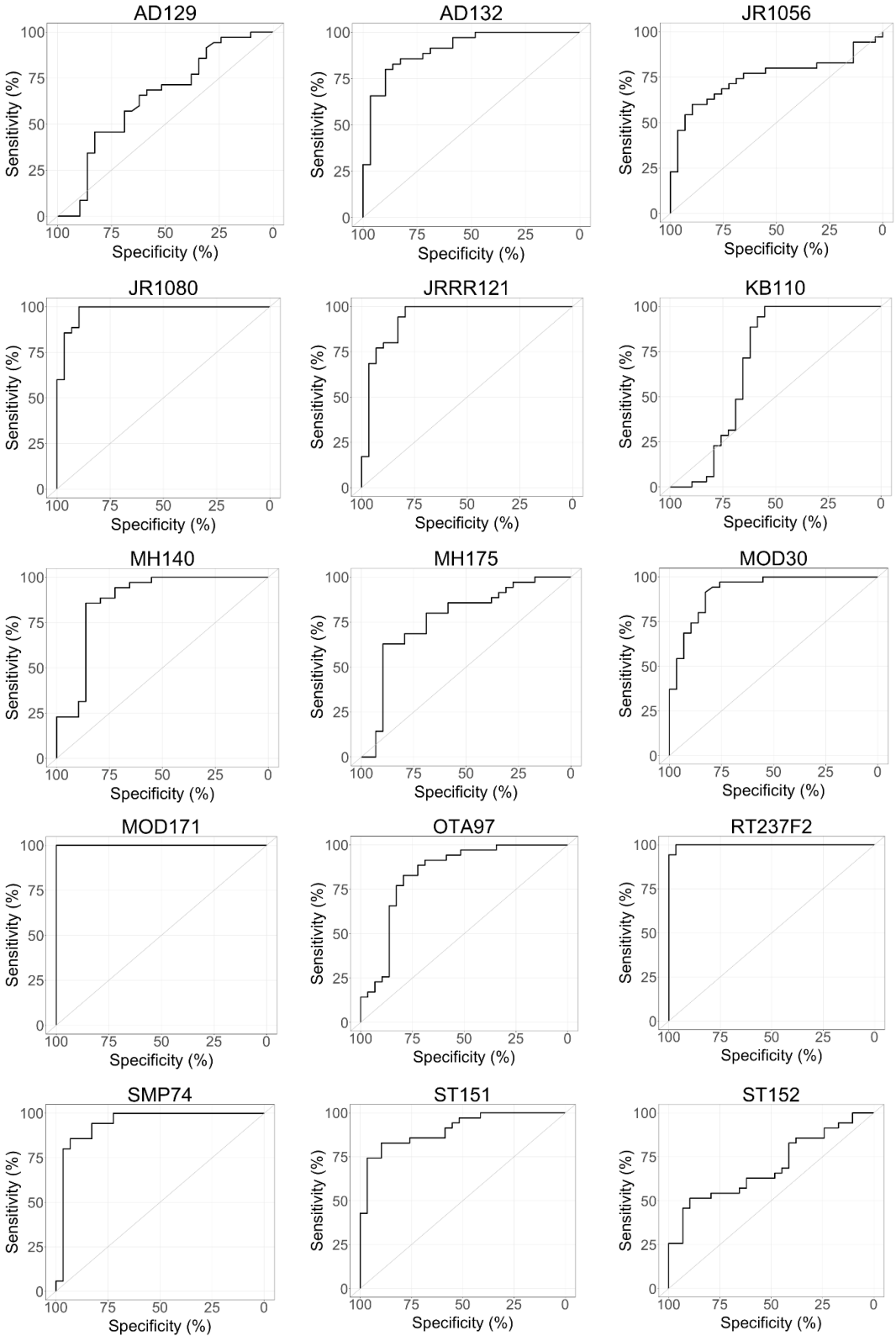


**Figure 8** – Shows ROC diagrams with sensitivity % plotted against specificity % at various thresholds, for all 15 antigens in the first study of experimentally infected positives and no history negatives.

**Table 2** – ELISA results for the 15 antigens in the first study.

| Antigen | Antigen Type | Median, Positives | Median, Negatives | AUC (%) | 95% CI (%) | Threshold | Sensitivity/ Specificity | 95% CI (%) |
|---|---|---|---|---|---|---|---|---|
| AD129 | *cis*-keto acid | 0.22 | 0.20 | 64.43 | 50-79 | 0.24 | 46/83 | 29-63, 69-97 |
| AD132 | *cis*-keto TDM | 1.82 | 0.42 | 90.84 | 84-98 | 1.12 | 80/90 | 66-91, 79-100 |
| JR1056 | *cis*-methoxy acid | 0.28 | 0.20 | 74.88 | 62-87 | 0.26 | 60/90 | 43-77, 76-100 |
| JR1080 | alpha acid | 0.23 | 0.13 | 97.73 | 95-100 | 0.14 | 100/90 | 100-100, 79-100 |
| JRRR121 | *trans*-methoxy acid | 0.61 | 0.21 | 93.69 | 87-100 | 0.25 | 100/79 | 100-100, 66-93 |
| KB110 | *cis*-methoxy TMM | 1.86 | 2.14 | 68.87 | 53-84 | 2.06 | 100/55 | 100-100, 38-72 |
| MH140 | *trans*-keto acid | 0.22 | 0.14 | 87.19 | 77-97 | 0.17 | 86/86 | 74-97, 72-97 |
| MH175 | alpha TDM | 1.61 | 0.64 | 76.85 | 64-89 | 1.52 | 63/90 | 46-80, 76-100 |
| MOD30 | alpha-ArMM | 0.33 | 0.16 | 92.56 | 86-99 | 0.20 | 91/83 | 80-100, 69-97 |
| MOD171 | alpha-ArTM | 1.09 | 0.16 | 100.00 | 100-100 | 0.36 | 100/100 | 100-100, 100-100 |
| OTA97 | alpha-DMAG | 0.77 | 0.28 | 83.65 | 73-95 | 0.38 | 83/79 | 69-94, 62-93 |
| RT237F2 | alpha muramyl | 0.53 | 0.23 | 99.80 | 99-100 | 0.32 | 100/97 | 100-100, 90-100 |
| SMP74 | alpha GMM | 1.10 | 0.29 | 93.99 | 87-100 | 0.57 | 86/93 | 74-97, 83-100 |
| ST151 | WE-TDM | 1.67 | 0.37 | 90.64 | 84-98 | 0.93 | 83/90 | 69-94, 79-100 |
| ST152 | WE-TMM | 1.73 | 1.47 | 69.26 | 56-82 | 1.72 | 51/90 | 34-69, 76-100 |

Footnotes: AUC – Area under the curve, CI – confidence interval, commercial ELISA sensitivity/ specificity: 94/100

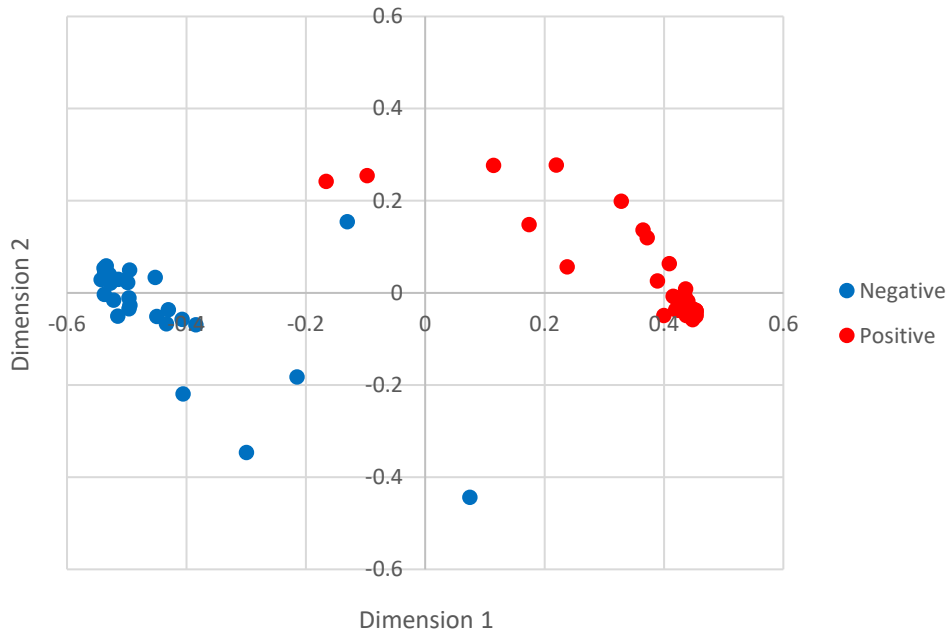


**Figure 9** – Proximity of individual cases using Random Forest classifier, reduced to 2-dimensions using principal co-ordinate analysis, for the 15 antigens in the first study of experimentally infected positives and no history negatives.

## 3.5. Discussion

This study was aimed at determining if mycolic acids and their sugar esters were plausible antigens for diagnosis of MAP. The sera selected were designed to give the greatest distinction possible, with an experimentally heavily infected set and a negative set from a farm with no history of MAP within the herd. The antigens chosen gave a wide range of both acid types and sugars attached, some which are present within MAP and others which have shown promise in previous assays for different mycobacteria.

Of the 15 antigens tested it is clear some show promise for the detection of MAP with 10 out of the 15 showing around or over 80/80 sensitivity/specificity, in addition these 10 antigens had an AUC over 80. MOD171 gave a clear distinction between positive and negative, however this is unlikely to be the case within naturally infected samples; for that purpose, a way of combining the antigens results is needed.

Given the wide variety of mycolic acid classes tested there is surprisingly little difference between them. The alpha acid, *trans*-methoxy and *trans*-keto (JR1080, JRRR121 and MH140) all gave a sensitivity/specificity

over 80/80. However, the *cis*-keto and *cis*-methoxy (AD129, JR1056) produced appreciably lower results, this is most likely due to the prominence of *trans* acids with MAP. This result can also be seen from the *cis*-methoxy TMM (KB110), which gives a slight negative skew but also displays weak differentiation between positive and negative. Interestingly, the difference in absorbance between the acid (JR1056) and the TMM (KB110) is very large (>1.5, Table 2) indicating that a sugar group might help greatly in binding antibodies.

Considering that wax esters are more unique to MAP and *Mycobacterium avium* (*M. avium*) than other mycobacteria, it is unfortunate that they did not produce better results. Ideally these should be more specific to MAP than a TDM.

A Random Forest Classifier was used to combine the results from all antigens. It generates a large number of decision trees that operate as an ensemble. This model works best with large data sets and as such will become more accurate with more data, it also protects against bad data in case there are interferences with one antigen. From this data a 2D graph can be formed via principal co-ordinate analysis, which allows you to visualise the combined result of all results across the antigens. It is clear from this that these antigens with this sample set give good separation, with only a few samples being moderately borderline.

The negative sample that shows up at -0.13, 0.15 (Fig 9) remains divisive even when the results are narrowed down to the top 10 antigens and run through the Random Forest Classifier. This could be due to cross reactivity with other infections or a number of factors. Given that immunoassays are an indirect detection method having just one sample outside the correct grouping is good at this stage.

# 4. A Study of Naturally Infected Positive and Negative Cattle Samples From Canada

## 4.1. Introduction

For this second study a larger set of 40 naturally infected cattle samples were compared to 40 negatives, tested against a reduced set of antigens. The sera from positive animals all gave positive faecal PCR assays, with moderate cycle numbers of 21 to 37. Only some (14) were positive by commercial ELISA assays. The negatives were negative in both assays.

Using a naturally infected set, all of which are sourced from the same geographically region, will allow us to gauge the real-world sensitivity/specificity of the assay. In comparison to the experimentally infected it is expected that the hormonal immune response will be lower, due to the comparative difference in infection dosage.

A few changes were made to the antigen selection for this set of serum. Both wax esters (ST151, ST152) were switched for ST123 and ST124 (Fig 10), the only difference between these sets is the carbon chain length, moving from 17, 17, 15, 21 to 17, 15, 17,21. This change should have no effect on the response to the antigen. In addition both KV059; a keto-TDM and SMP75; a keto-GMM, were added to this set (Fig 10).

A slight change was also made to the buffer, which involved making a stock solution and introducing a biocide Proclin 300, to ensure longer term storage and enabling a larger throughput of ELISA plates. Proclin 300 works via interrupting the Krebs cycle.

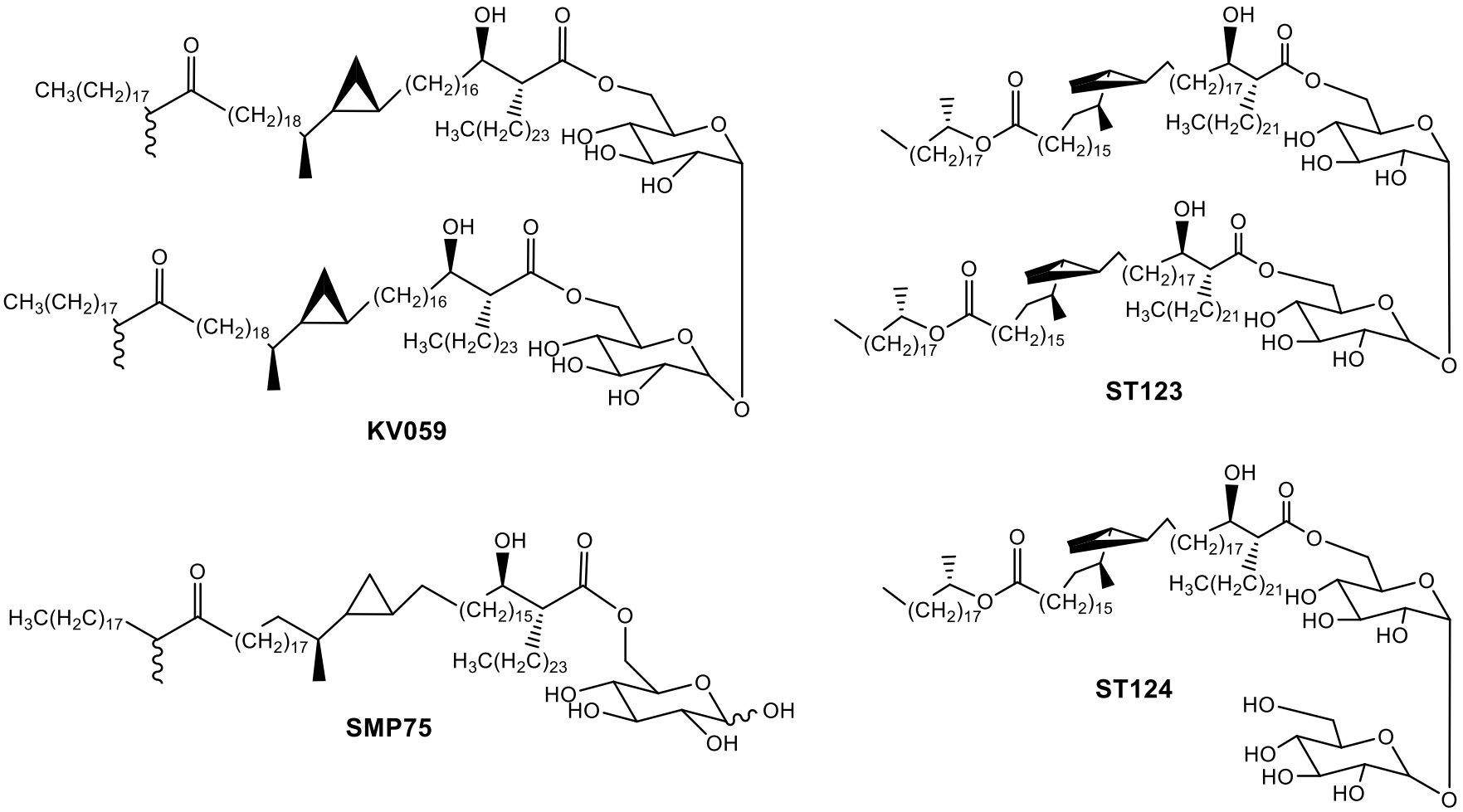


**Figure 10** – KV059 is a *trans*-keto TDM, ST123 a WE-TDM, SMP75 a *trans*-keto GMM and ST124 a WE-TMM.

## 4.2. Materials

Serum samples were supplied by the University of Prince Edward Island, Canada. 40 positive samples were confirmed by faecal culture, of which only 14 were serum ELISA positive. 40 negative samples were confirmed negative by faecal culture and serum ELISA.

## 4.3. Methods

KCl (1.01 g), $KH_2PO_4$ (1.23 g), anhydrous $Na_2HPO_4$ (7.10 g), NaCl (40.03 g) was dissolved in 1800 mL d $H_2O$, stirred and heated to 50°C. After which NaOH (1 M, 12.5 mL) was added. Casein (25 g) was added and the solution was stirred at 50 °C for 2 hours. After removing from the heat and reaching rt, Proclin 300 (2.5 mL) was added and the solution made up to 2000 mL, then stored in the fridge. This formed the 2.5x 0.5% casein w/v PBS buffer.

Antigens were diluted to 2.9 µM (Tables 3) using THF-hexane (1:50) and 50 µL was centrally pipetted into each well of the ELISA plate, to cells G-H 11-12 only THF:hexane (1:50) solution was added. The plates were allowed to evaporate at rt for 2 hours before being sealed with breathable sealing tape.

The following day, plates were unsealed and 1x 0.5% casein w/v PBS buffer (350 µL) was dispensed using a 96 well plate washer, then incubated at 25°C for 30 minutes.

Serum (5 µL) was diluted to 1:40 with rt 0.5% casein w/v PBS buffer and resuspended by pipette. The casein solution was aspirated off the plates and tapped dry, diluted serum (50 µL) was added to the wells with one duplicate well per sample. Remaining serum was pooled and added (50 µL) to cells A-B and G-H 11-12 for a pseudo positive and negative control respectively, 1x 0.5% casein w/v PBS buffer was added to cells E-F 11-12 for a diluent. The plates were then sealed with a breathable plate seal and incubated at 25°C for 60 minutes.

The plates were then washed three times using 0.5% casein w/v PBS buffer and tapped dry, before adding anti-bovine IgG(Fc) HRP (50 µL) diluted to 2.9 µg/mL using 1x 0.5% casein w/v PBS buffer with a multichannel pipette and then incubated at 25°C for 30 minutes.

Following this the plates were washed three times using 1x 0.5% casein w/v PBS buffer and tapped dry, OPD (5 mg) was dissolved in 25 mL citrate buffer (0.05 M) containing sodium perborate buffer (0.03%) and added (50 µL) to the plates using a multichannel pipette. Plates were incubated at 25°C for 30 minutes.

$H_2SO_4$ (50 µL, 3 M) was added to the wells after the 30 minutes and the results read on a UV-visible ELISA plate reader at 450, 492, 620 nm.

**Table 3** – Molecular weights used in concentration calculations for the second study.

| Antigen | Molecular Weight |
|---|---|
| JRRR121 | 1296.36 |
| KV059 | 2866.89 |
| MOD171 | 2668.50 |
| OTA97 | 2287.84 |
| RT237F2 | 1412.33 |
| SMP74 | 1300.21 |
| SMP75 | 1442.45 |
| ST123 | 2842.78 |
| ST124 | 1592.54 |

## 4.4. Results

From this set of serum and antigens, the results were rather different to the experimentally infected set (Table 4). JRRR121, MOD171 and SMP74 all gave good a grouping of the negative samples (Fig 11), however there was overlap with the positive samples even though the median positive value was outside the IQR of the negative samples.

The keto-TDM (KV059), vaguely grouped the negatives around 0.5 but with the positive spread over a large range only resulted in a sensitivity/specificity of 73/73, when using culture as the reference.

OTA97 showed poor results this time with an AUC of 53, with no distinction between positive and negative medians, both 0.65 and 0.64 respectively.

ST124 displayed similar results but on the other end of the spectrum; positive and negative medians being 2.18 and 2.11, with an AUC of 59. However, its TDM counterpart (ST123) gave appreciable separation with an AUC of 78 and sensitivity/specificity of 65/80.

SMP75 a keto-GMM gave the lowest AUC (51), CI (38-64%) and specificity (30), signifying a large overlap of negative and positive results.

Finally, RT237F2 followed a similar pattern to KV059, however its range of positive results was much decreased resulting in low sensitivity/specificity (65/68).

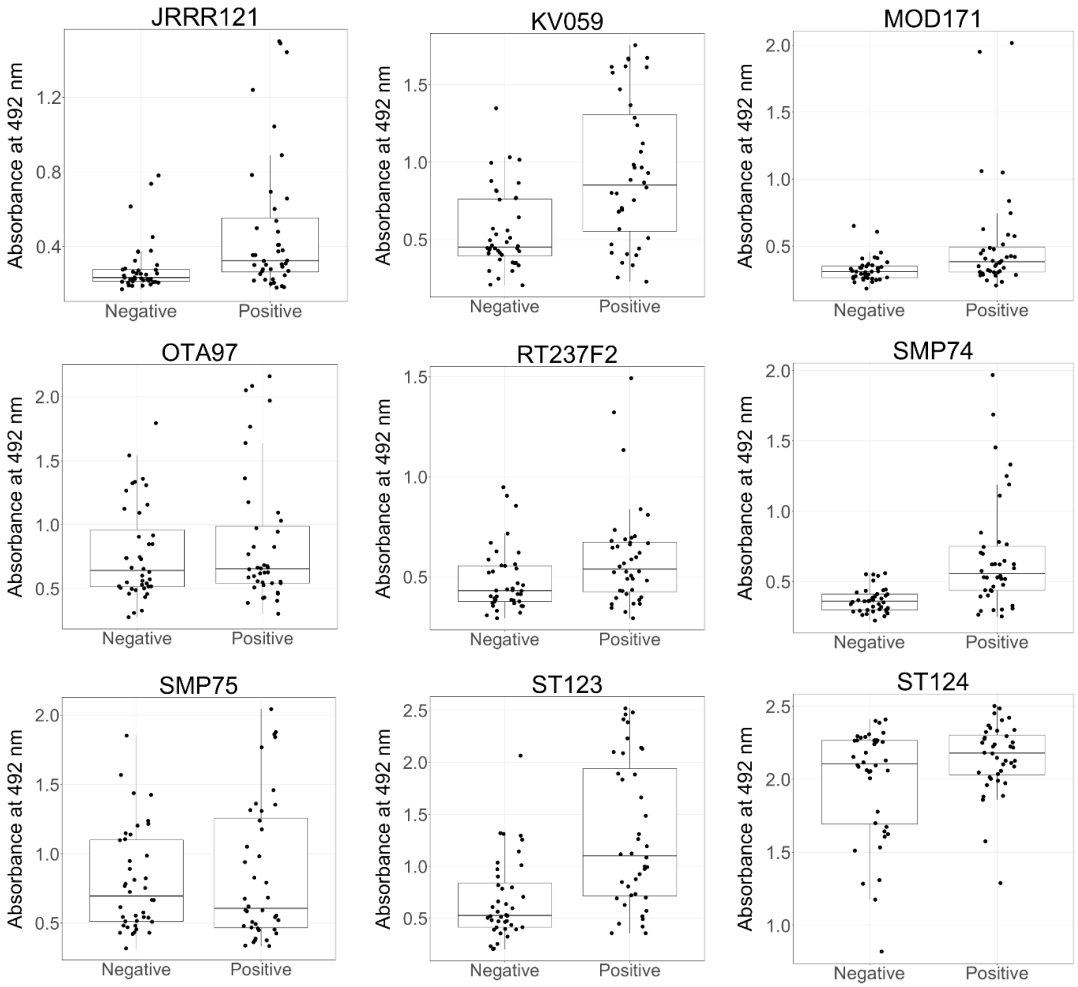


**Figure 11** - Shows ELISA responses and boxplots of culture positive and negative sample sets, for all 9 antigens in the second study of naturally infected positive samples and negative samples from Canada.

From the principal co-ordinate analysis (Fig 13) there is a clustering of positive results on the left-hand side and a broader clustering of negatives on

the right. There is an overlap of results between x=-0.2 and x=0.2 and 6 positive results appear well within the negative cluster.

The random forest analysis gave the most significance to SMP74 by a large margin followed by ST123, KV059, JRRR121 and MOD171, again closely following the AUC values.

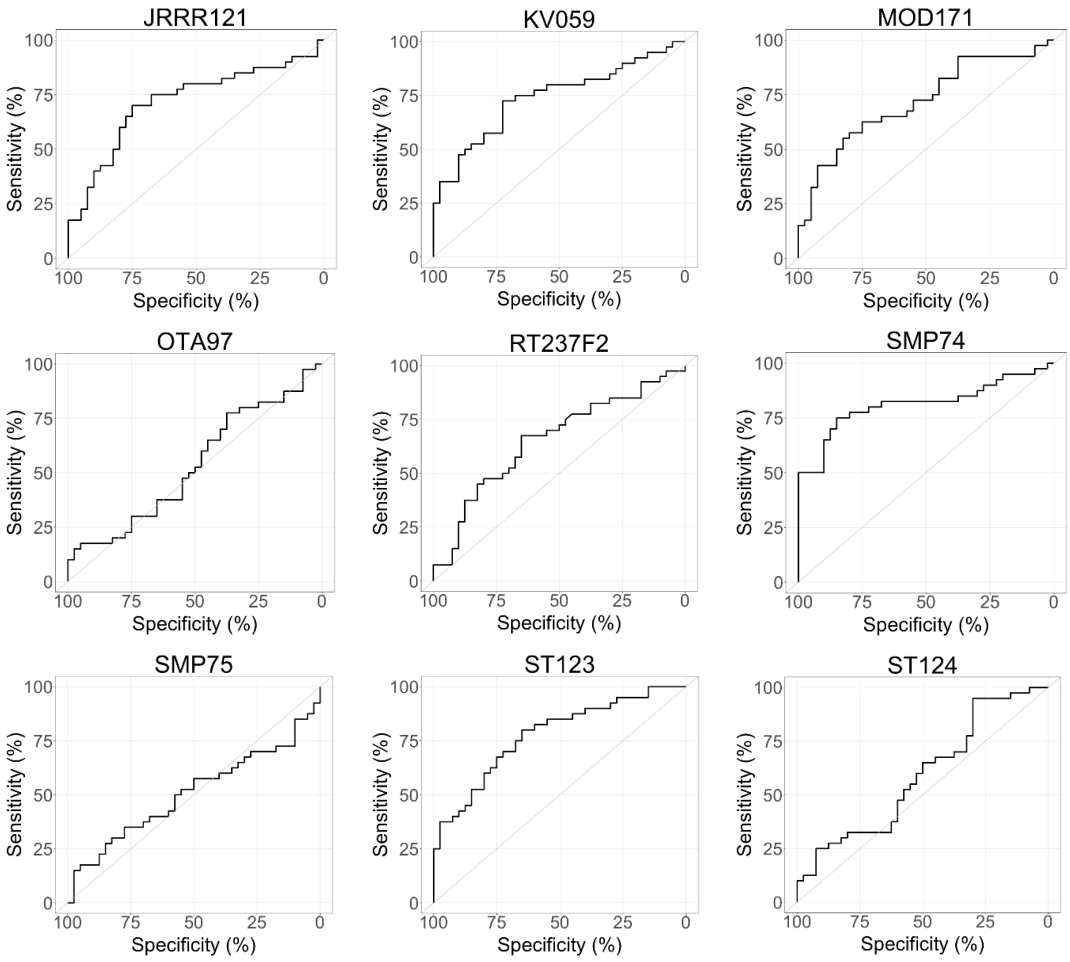


**Figure 12** - Shows ROC diagrams with sensitivity % plotted against specificity % at various thresholds, for all 9 antigens in the second study of naturally infected positive samples and negative samples from Canada.

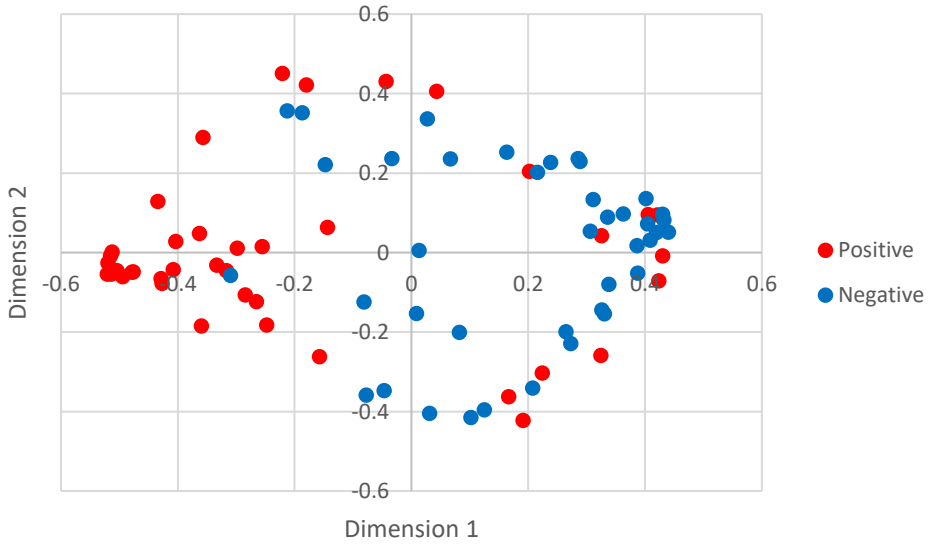


**Figure 13** - Proximity of individual cases using Random Forest classifier, reduced to 2-dimensions using principal co-ordinate analysis. For the 9 antigens in the study of 40 naturally infected positive samples and 40 negative samples from Canada

**Table 4** - ELISA results for the 9 antigens in the second study.

| Antigen | Antigen Type | Median, Positives | Median, Negatives | AUC (%) | 95% CI (%) | Threshold | Sensitivity/ Specificity | 95% CI (%) |
|---|---|---|---|---|---|---|---|---|
| JRRR121 | *trans*-methoxy acid | 0.32 | 0.23 | 71.88 | 60-84 | 0.28 | 75/70 | 55-83, 60-88 |
| KV059 | keto-TDM | 0.85 | 0.45 | 74.00 | 63-85 | 0.66 | 73/73 | 58-85, 58-85 |
| MOD171 | alpha-ArMM | 0.38 | 0.31 | 71.81 | 60-83 | 0.36 | 80/58 | 43-73, 68-93 |
| OTA97 | alpha-DMAG | 0.65 | 0.64 | 53.44 | 41-66 | 0.54 | 38/78 | 65-90, 23-53 |
| RT237F2 | alpha-muramyl | 0.54 | 0.43 | 65.41 | 53-78 | 0.47 | 65/68 | 53-83, 50-80 |
| SMP74 | alpha-GMM | 0.56 | 0.36 | 81.31 | 71-91 | 0.44 | 85/75 | 60-88, 73-95 |
| SMP75 | keto-GMM | 0.61 | 0.70 | 50.75 | 38-64 | 0.48 | 83/30 | 18-45, 70-93 |
| ST123 | WE-TDM | 1.10 | 0.53 | 77.62 | 67-88 | 0.68 | 65/80 | 68-93, 50-80 |
| ST124 | WE-TMM | 2.18 | 2.11 | 59.06 | 46-72 | 1.82 | 30/95 | 88-100, 18-45 |

Footnotes: AUC – Area under the curve, CI – confidence interval, commercial ELISA sensitivity/ specificity: 38/100

Combining the results from the first study of experimentally infected samples (Section 3) with this latest set of naturally infected samples gave the following results (Table 5). Interestingly the thresholds for optimal separation of positive and negative barely changed. The random forest (Fig 14) antigen significance for the combined set of serum were as follows, MOD171, SMP74, RT237F2 JRRR121, OTA97.

When using the combined set of data to train the random forest and applying this matrix to predict the diagnosis of these samples; effectivity combining all antigens for an optimal diagnosis, this resulted in a sensitivity/specificity of 84/93.

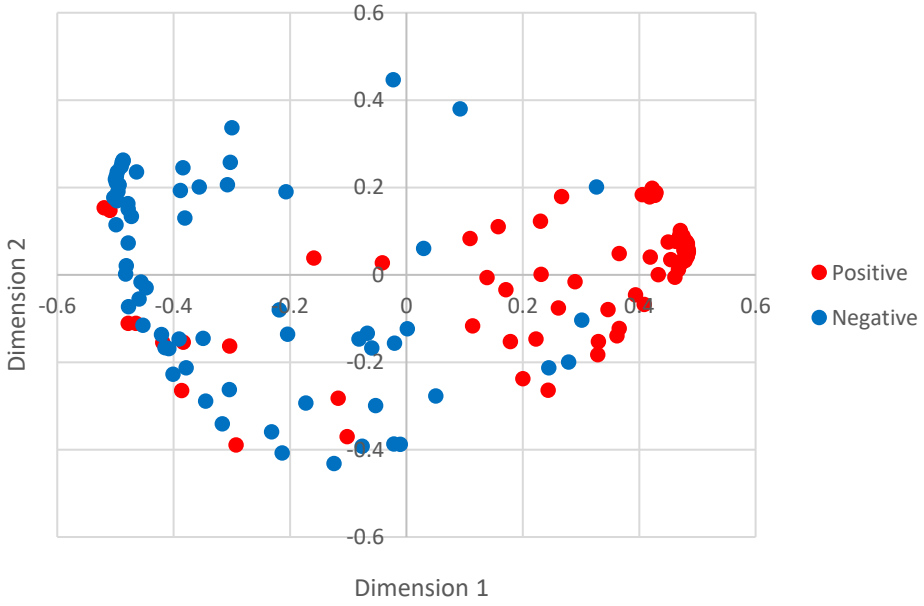


**Figure 14** - Proximity of individual cases using Random Forest classifier, reduced to 2-dimensions using principal co-ordinate analysis. For the combined results from the study experimentally infected and no history samples (Section 3), and the second study of 40 naturally infected positives and 40 negatives from Canada.

**Table 5** - ELISA results for a combination of strong positives and negatives from the first study (Section 3) and 40 naturally infected faecal PCR positives and 40 negatives from Canada.

| Antigen | Antigen Type | Median, Positives | Median, Negatives | AUC (%) | 95% CI (%) | Threshold | Sensitivity/ Specificity | 95% CI (%) |
|---|---|---|---|---|---|---|---|---|
| JRRR121 | *trans*-methoxy acid | 0.41 | 0.23 | 83.01 | 76-90 | 0.28 | 78/79 | 69-88, 68-87 |
| MOD171 | alpha-ArMM | 0.58 | 0.26 | 90.69 | 86-95 | 0.37 | 90/76 | 67-85, 83-96 |
| OTA97 | alpha-DMAG | 0.67 | 0.52 | 66.20 | 57-75 | 0.54 | 57/72 | 61-81, 45-68 |
| RT237F2 | alpha-muramyl | 0.53 | 0.35 | 79.66 | 72-87 | 0.46 | 78/69 | 59-80, 68-87 |
| SMP74 | alpha-GMM | 0.77 | 0.33 | 88.37 | 83-94 | 0.46 | 86/81 | 72-89, 77-93 |

Footnotes: AUC – Area under the curve, CI – confidence interval, commercial ELISA sensitivity/ specificity: 67/100

## 4.5. Discussion

Following on from the experimentally infected serum, this study of naturally infected serum set was aimed at evaluating a more refined set of antigens against realistic samples.

It is clear and expected that naturally infected samples pose a greater challenge when it comes to diagnosis, with the best antigen (SMP74) showing a sensitivity/specificity of 85/75 and AUC of 81. This contrasts with the first experimentally infected set were MOD171 gave 100/100 and SMP74 gave 86/93. However, this is a markedly improved result compared to the commercial serum ELISA results provided with the serum, which only diagnosed 14 of the culture positive samples. This shows that even though the results are worse when compared with the experimentally infected set, within the currently available commercial tests, defined mycolic acids have an advantage.

When using Random Forest Classifier which improves the more data points it has, the combined data set leads to a more refined final analysis. Once the matrix has been trained it can be used for prediction. Using it to predict the results of the naturally infected resulted in a final sensitivity/specificity of 84/93 with 5 antigens. This is in comparison to the commercial ELISA results supplied with the serum samples, which result in a sensitivity/specificity of 35/100. A sensitivity/specificity of 94/93 is acceptable for a diagnostic test, as the high specificity ensures cattle are not incorrectly culled losing to economic loses. A sensitivity of 84% is good enough to ensure most positives are caught and periodic testing through the year has the potential to pick up the rest. In comparison smear microscopy in *M. tb* generally has a sensitivity of 50-60% and specificity of >99% [212]. It must also be noted that the sensitivity/specificity figures are calculated based on a single positive faecal PCR; for some samples, this might be caused by pass-through shedding or detection of dead mycobacteria.

Combining the data from the experimentally infected set and this naturally infected set has various benefits. The negatives from the first set have been confirmed to have no prior exposure to MAP for several years, thus offering a very clean baseline. The positives are confirmed positive for MAP

with no other infections present. Using these highly controlled samples alongside the naturally infected, helps to guide the learning algorithm when weaker or more borderline samples are used.

There are numerous uncontrollable issues when it comes to testing naturally infected samples. Firstly and most importantly, there is no perfect diagnostic method available for MAP in live animals, both culture and PCR being prone to sampling issues and false positives from passive shedding, or false negatives from intermittent shedding. Secondly, with an indirect method as immunoassays are, they could potentially be picking up false positives due to exposure within the herd or from the environment. On the other hand this could be a benefit, as detecting exposure is also a valuable tool next to single animal diagnosis.

## 4.6. Future Work

The work described here on detecting MAP in cattle has set the stage for further work both in optimisations and deeper analysis.

The experimentally infected set showed different results than the naturally infected set; although this can be explained by infectious dose, it could also be due to different strains of MAP being present. Further analysis could look at the different strains of MAP and whether this has an effect on antigen selection for diagnosis. This would also come into greater focus when looking worldwide as different strains will predominate in different countries. In addition to different strains, the antibody response to protein and lipid antigens at different time points could also be examined. It is plausible due to their different properties, that protein and lipid components of mycobacteria develop antibody responses at different times during infection.

As for optimisations within the existing protocol, there are many possibilities, as every step within the ELISA can be optimised for reagent concentration, volume and incubation time. The largest gains in sensitivity however are likely to be by switching from OPD as the chromophore to 3,3',5,5'-Tetramethylbenzidine (TMB). TMB is much more sensitive than OPD, both however display a similar LOD. Due to this sensitivity TMB does have a lower dynamic range, which implies that the assay could need per antigen

optimisations to ensure the absorbances fall within the correct range. TMB can be formulated as a one-step solution, replacing the entire OPD, $H_2O_2$ mixture, greatly simplifying the procedure and also reduces possible mistakes. From a commercial perspective TMB is also more suitable due to its environmental credentials. OPD is toxic, a health hazard and environmental hazard whereas TMB is not known to be hazardous.

An avenue to try and minimise the false positives is possible via detergents. Tween, Igepal and Triton are common detergents used within assays to strip away weakly bound antibodies leaving only the strongly bound specific antibodies behind. The issue with this method is that this type of detergent has also been said to strip away mycolic acids from the surface of ELISA plates, as such a gentler method might be more appropriate. This could take the form of a more strongly buffered wash buffer, by increasing the salt concentration non-specific binding or weak interactions can be discouraged.

Another method which is commonly seen within commercial MAP ELISAs is to preabsorb the serum with *M. phlei*. *M. phlei* is a fast-growing mycobacterium and the preabsorbing works by absorbing any non-specifically binding or competing antibodies allowing for only MAP specific antibodies to bind to the antigens on the surface. However, due to the fact that currently commercial ELISAs use protein antigens, this optimisation is unlikely to yield the same results as it currently does. It does indicate the possibility that fast-growing or environmental mycobacteria could be used for a first step in the assay, to remove some unwanted interactions.

# 5. Evaluation of a Flow Through Test With Pooled Strongly Positive MAP Cattle Serum

## 5.1. Introduction

Point of care diagnostics are the ultimate end point in disease tests. Compared to lab-based methods, they allow end users to carry out the tests themselves resulting in quicker turn-around time. Additionally, they require lower sample volumes in the final devices, as serum can be filtered directly from capillary blood on site; alternatively, other less invasive sample types can be used. In point of care settings, immunoassays have an edge due to ease of use and simplicity compared to lab-on-chip varieties.

This small experiment was undertaken to see if existing protocols for detection of *M. tb* could be translated to MAP [213]. The existing protocol is based on patented [213] flow through methodology, which is similar to the more commonly used lateral flow type (for example a pregnancy test) but instead the sample flows through multiple vertical layers (Fig 15).

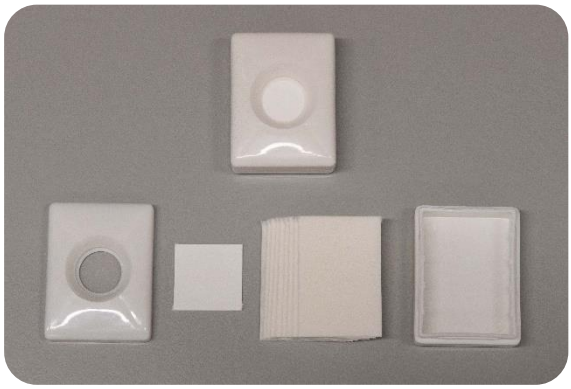

**Figure 15** – An assembled flow through chip above with the constituent parts below, left to right shows the plastic top housing and reagent well, then the nitrocellulose membrane, the absorbent pads and the plastic bottom housing.

Within a flow through device the antigen is applied to the top of the membrane in an appropriate membrane-compatible solvent. The procedure then follows similar steps to an ELISA, but the chromophore and secondary antibody are combined into one step; in this case protein G coated gold nanoparticles. The unbound nanoparticles pass through the membrane and

absorbed below. Protein G was used in this situation as it can bind strongly to the Fc region of IgG and will potentially enable multi-species compatibility for future test development.

A gel is also used within this methodology, having the effect of slowing down the flow rate to allow the antibodies extra binding time, leading to an increase in test sensitivity. It also helps to compensate for any flow difference between the differently polarised antigen spots.

For this test a pooled positive control was used, to help mitigate any individual serum effects that could be present and also allow for easier optimisation across multiple antigens in the future. It will be tested with ST124 and MOD171; although ST124 is not the best antigen from previous tests it has been shown to give high absorbances when compared to MOD171. In addition to the two antigens a negative control spot will be used; this will be the solvent used to apply the antigens to ensure there is no interference with the membrane. These spots will be in a triangular pattern with the control at the top, ST124 on the lower left and MOD171 on the lower right.

## 5.2. Materials

Pooled positive sample was made from the experimentally infected CODA-CERVA (Federal Veterinary and Agrochemical Research Centre, Brussels, Belgium) cattle serum confirmed positive via culture and serum ELISA.

## 5.3. Methods

10 OD 40 nm protein G coated gold nano particles were prepared according to the manufacturer's instructions.

Membranes were prepared by pipetting antigen (1 µl, 1 mg/ml) in THF:hexane (1:50) onto the membrane without touching the surface, in addition THF:hexane (1 µl, 1:50) was pipetted in the same fashion as a control spot. These were then allowed to dry for 2 hours before use.

A stock solution of HPMC was prepared by heating d $H_2O$ (150 mL) to 92°C, then HPMC powder (5 g) was added. Once the powder was well

dispersed, additional $H_2O$ (350 mL) was added and the heat removed. The solution was then stirred continuously overnight.

NaCl (4 g), KCl (0.10 g), $KH_2PO_4$ (0.12 g), $Na_2HPO_4$ (0.72 g), tween 20 (0.55 g) were added to d $H_2O$ and warmed to 50°C. Once the temperature was reached, casein (2.5 g) was added and the mixture stirred for 90 minutes. After this time the solution was allowed to cool to rt and the pH adjusted to 7.4 using NaOH (1 M). Following this, 50 mL of the HPMC was added and the solution made up to 500 mL.

Serum (10 µL) was diluted to 1:50 with rt 0.1% HPMC w/v 0.5% casein w/v 0.1% tween 20 v/v PBS buffer and mixed by resuspension. 10 OD antibody coated nanoparticles were diluted to 1 OD with 0.1% w/v HPMC 0.5% casein w/v 0.1% tween 20 v/v PBS buffer. Diluted serum (500 µL) was added to the membrane and allowed to pass through. Afterwards d $H_2O$ (1 mL) was added and allowed to flow through. Following this, coated nanoparticles (1 mL, 1 OD) were pipetted onto the membrane and allowed to flow through. Finally, the membrane was washed by adding d $H_2O$ (1 mL) and allowing it to pass through the membrane.

The membranes were photographed for qualitative results.

## 5.4. Results

The result of this first test showed a positive result with both antigen spots showing a result and the control spot staying white (Fig 16).

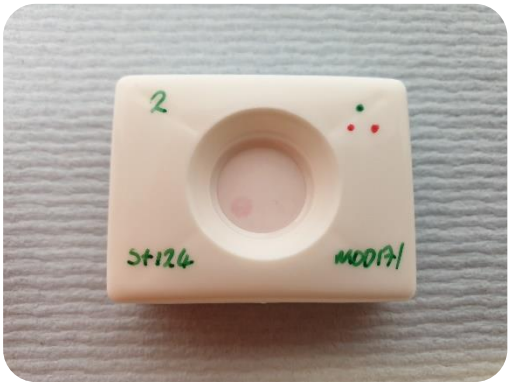


**Figure 16** – The flow through chip after the final wash. ST124 shows a spot on the lower left, MOD171 shows an extremely faint spot on the lower right. The top control spot shows no response.

ST124 showed the stronger result with a clear red dot visible on the lower left. MOD171 however only left a very faint dot in the lower right. Both of these responses are consistent with the median absorbances seen with ELISA.

The top control dot which was purely solvent gave no response that is visible, indicating that the solvent used did not alter the membrane and allow antibodies to bind directly.

Before the final wash, after the nanoparticle solution had passed through the membrane, both spots appeared more visible than pictured here (Fig 16).

## 5.5. Discussion

This simple experiment was undertaken to examine if existing procedures for the detection of *M. tb* could be adapted for the detection of MAP at point of care. Both antigens used here showed a positive result, if a very weak one, and the control showed no response.

ST124 which gave a median result of 2.18 with the naturally infected serum set, gave a clear response. Whilst MOD171 gave an absorbance of 0.38 with the same set. This explains the very weak spot produced by MOD171 on the membrane. If the antigens were applied by machine both spots would be a lot stronger, as a smaller volume of solvent can be used; the resultant spots would be small with a denser concentration of antigen, resulting in a stronger final colour.

The protein G coated nanoparticles appear to work well within this test and should pave the way for a wider reaching test both within Bovidae and other natural reservoirs for MAP. One point to keep in mind however is that protein G binding strength will change per species, with sheep antibodies only having a medium binding strength when compared with cattle antibodies.

The other option compared with flow through would be a lateral flow test, this method could work out better for dissimilar antigens. As the liquids flow through the membrane they are likely to take the path of least resistance, which means that a large proportion could be flowing around the spots not through them. In addition, there also hydrophobicity implications due to the

antigens being lipids. Finally, competitive binding could be coming into play with both antigens attempting to bind the same antibodies, hopefully the antigens are specific enough that they bind different antibodies but this is difficult to determine. This is where a lateral flow could be beneficial, as the entire flow must move through a line of antigen absorbed onto the membrane, thus eliminating multiple issues.

Clearly a single device does not give many data points to analyse and a larger test would be needed. However this first device gives a good starting point for initial optimisations to take place, more precisely to ensure the results are more visible.

## 5.6. Future Work

From this preliminary experiment it is clear that optimisations must take place. Firstly, to increase the strength of the antigen spots and secondly, an application of wax to direct flow. This can be done by both using an automated system to apply the antigen thus requiring less volume of solvent to spot the same amount of antigen. The effect of this would be that the solvent would not absorb so widely through the membrane reducing spot size. In addition, applying a wax on top of the membrane with holes where the antigen is applied to the membrane, would direct the flow of the liquids only through the antigen coated areas creating less wasted liquid and increasing the amount of bound gold.

Following these steps, the serum dilution can be optimised to ensure all spots are visible, this is the most critical step as the gold is applied in excess.

After optimisations have taken place a larger test would need to be undertaken with individual serum and the results compared to the absorbances seen within ELISA. If setup correctly, with the same lighting conditions and positioning, it would also be possible to compare the intensity of the spots between each device for semi quantitative results.

# 6. Crohn's Samples Tested Against MAP Antigens

## 6.1. Introduction

This study aims to look at immune reactions to MAP specific antigens within a set of human Crohn's patient's serum.

The antigens used strongly reflect ones that provided positive results within the previous cattle work, including MOD171, RT237F2 and SMP74. Three of the antigens included (AD132, MH175 and KB110) have also been tested in published work [88] for their effectiveness at diagnosing *M. tb*; this will serve as valuable comparative data for evaluating the test effectiveness.

Within this serum set are seven positive Crohn's samples and eleven negatives. Although these numbers are low it should allow an initial indication whether abnormal responses are present.

The method used here more closely resembled the one used in previous human *M. tb* work [88]. Using anti-human IgG(Fc) HRP and the 5 fold decreased $H_2O_2$ and OPD used in the first experimentally infected cattle study.

## 6.2. Materials

7 Crohn's positive samples (comprised of serum and plasma) were supplied by the Liverpool Bio-Innovation Hub Biobank (Liverpool, UK).

Negative samples were supplied by the Italian Ministry of Health, all 11 were confirmed healthy donors.

All samples supplied and experiments had ethical approval.

## 6.3. Methods

KCl (0.40 g), $KH_2PO_4$ (0.49 g), anhydrous $Na_2HPO_4$ (2.88 g), NaCl (16.00 g) was dissolved in 1800 mL d $H_2O$, stirred and heated to 50°C. Casein (10 g) was added slowly and the solution was stirred at 50 °C for 2 hours. After reaching rt it was then adjusted to pH 7.4 with NaOH (1 M), made up to 2000 mL and stored in the fridge. This formed the 0.5% casein w/v PBS buffer.

Antigens were diluted to 2.9 µM (Table 6) using THF-hexane (1:50) and 50 µL was centrally pipetted into each well of the ELISA plate, to cells G-H 11-12 only THF:hexane (1:50) solution was added. The plates were allowed to evaporate at rt for 2 hours before being sealed with breathable sealing tape.

**Table 6 -** Molecular weights used in concentration calculations for the Crohn's study

| Antigen | Molecular Weight |
|---|---|
| AD132 | 2782.73 |
| JR1080 | 1138.07 |
| JRRR121 | 1296.36 |
| KB110 | 1578.56 |
| MH175 | 2582.41 |
| MOD171 | 2668.50 |
| OTA97 | 2287.84 |
| RT237F2 | 1413.33 |
| SMP74 | 1300.21 |

The following day plates were unsealed and 0.5% casein w/v PBS buffer (350 µL) was dispensed using a 96 well plate washer, then incubated at 25°C for 30 minutes.

Serum (5 µL) was diluted to 1:40 with rt 0.5% casein w/v PBS buffer and resuspended by pipette. The casein solution was aspirated off the plates and tapped dry, diluted serum (50 µL) was added to the wells with one duplicate well per sample. Remaining serum was pooled and added (50 µL) to cells A-B and G-H 11-12 for a pseudo positive and negative control respectively, 0.5% casein w/v PBS buffer was added to cells E-F 11-12 for a diluent. The plates were then sealed with a breathable plate seal and incubated at 25°C for 60 minutes.

The plates were then washed three times using 0.5% casein w/v PBS buffer and tapped dry, before adding anti-human IgG(Fc) HRP (50 µL) diluted to 2.9 µg/mL using 0.5% casein w/v PBS buffer with a multichannel pipette and then incubated at 25°C for 30 minutes.

Following this the plates were washed three times using 0.5% casein w/v PBS buffer and tapped dry, OPD (5 mg) was dissolved in 25 mL citrate

buffer (0.05 M) containing sodium perborate buffer (0.03%) and added (50 µL) to the plates using a multichannel pipette. Plates were incubated at 25°C for 30 minutes.

$H_2SO_4$ (50 µL, 3 M) was added to the wells after the 30 minutes and the results read on a UV-visible ELISA plate reader at 450, 492, 620 nm.

## 6.4. Results

As with the cattle sample sets MOD171 is one of the better antigens, with an AUC of 87 and sensitivity/specificity of 82/86. Sensitivity and specificity is not used in the traditional case here, as we are looking for abnormal immune responses in Crohn's patients and not a mycobacterial disease. RT237F2 was better with an AUC of 94, sensitivity/specificity of 91/100 and showed tight grouping (Fig 17) considering the difference between median positive and negative values was 0.03 (Table 7).

Both JR1080 and MH175 had AUCs over 70 but they also had very wide CIs, with JR1080 having little difference between positive and negative and with sensitivity/specificities of 73/86 and 82/71 respectively. Interestingly JR1080 achieved this with a negative skew; showing a lower median value on the positives than the negatives.

In contrast the *trans*-methoxy acid showed little difference between positive and negative with a large overlap of positive and negative values and AUC of 40.

Three antigens (AD132, KB110, SMP74) gave identical sensitivity/specificity values, this is likely due to the low number of samples tested. They also produced very similar CI values with very wide ranges (40-98%, 29-98% and 37-96% respectively) and AUC values in the mid 60's.

OTA97 the alpha DMAG, came second to last in this set barely giving a separation with an AUC of 55 and sensitivity/specificity of 100/43; resulting in a large number of false positives.

Within this set some individual sera gave very large responses compared with other results, this can be seen within all antigens except JRRR121 and OTA97. The serum in each of these cases was different with the exception of AD132 and KB110 where the same sample was responsible.

When combining the results of these antigens using the Random forest classifier a separation can be gained with one positive amongst the negatives and two negatives on the positive side (Fig 19). As has held true of the other sample sets the weighting followed the AUC values, with RT23F2 contributing the most then MOD171, JR1080, MH175, OTA97, KB110, SMP74, AD132, JRRR121.

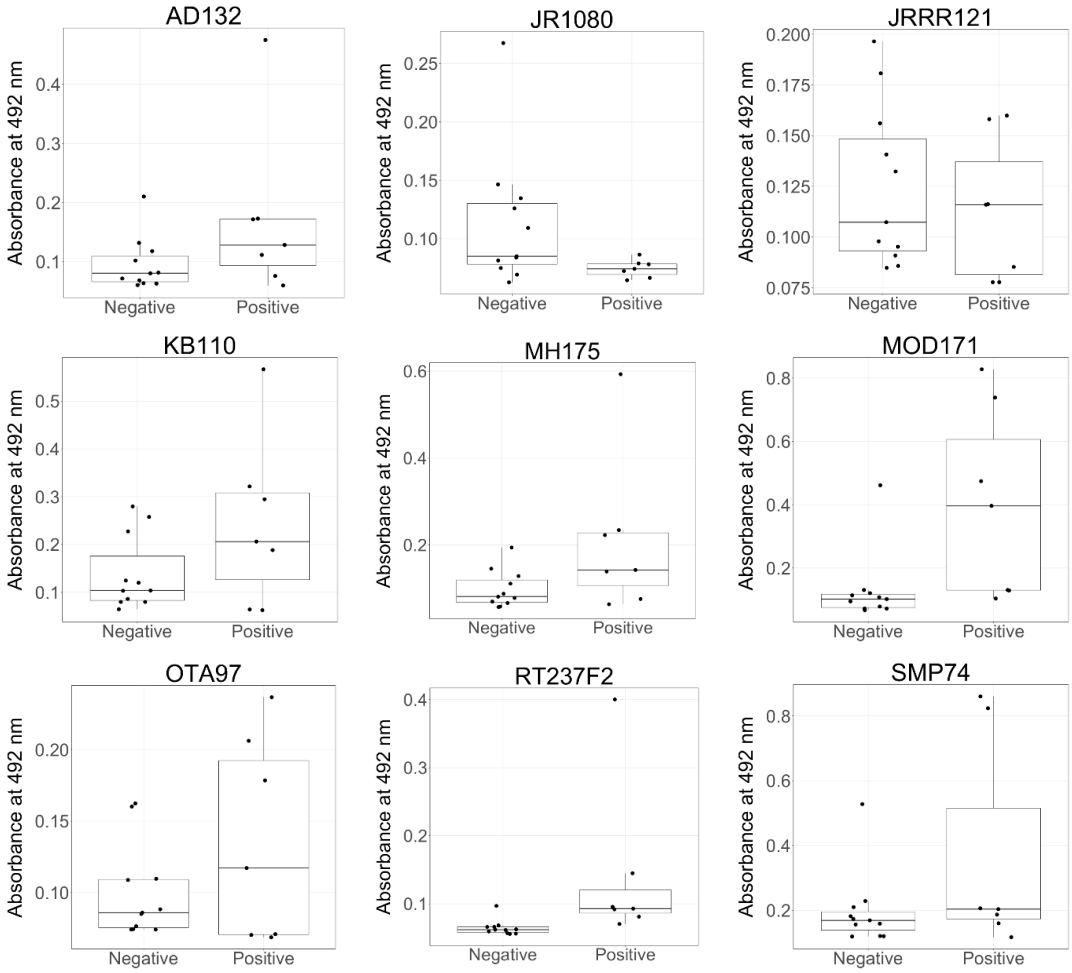


**Figure 17** - Shows ELISA responses and boxplots of Crohn's samples and healthy sample sets for all 9 antigens in the Crohn's study.

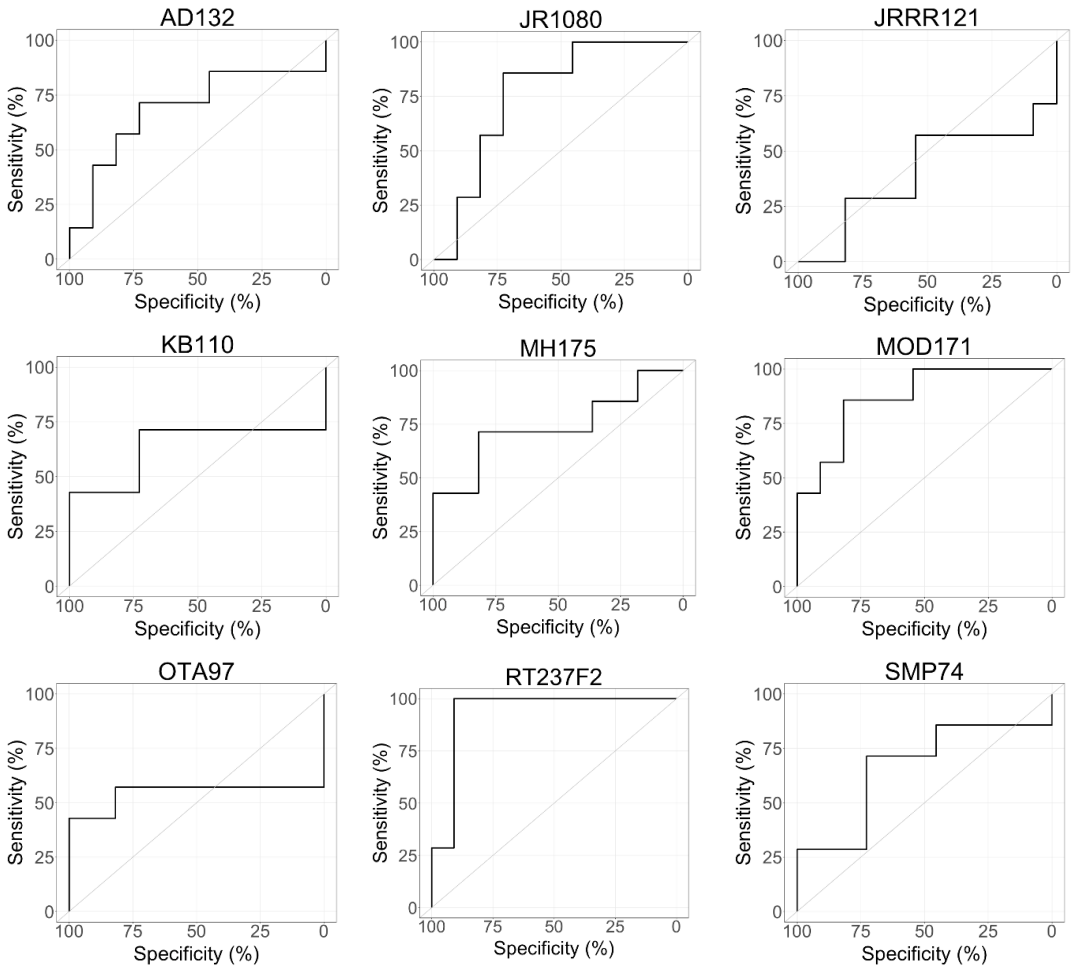


**Figure 18** - Shows ROC diagrams with sensitivity % plotted against specificity % at various thresholds, for all 9 antigens in the Crohn's study.

**Table 7** - ELISA results for the 9 antigens in the Crohn's study.

| Antigen | Antigen Type | Median, Crohn's | Median, Healthy | AUC (%) | 95% CI (%) | Threshold | Sensitivity/ Specificity | 95% CI (%) |
|---|---|---|---|---|---|---|---|---|
| AD132 | *cis*-keto TDM | 0.13 | 0.08 | 68.83 | 40-98 | 0.11 | 73/71 | 43-100, 45-100 |
| JR1080 | alpha acid | 0.07 | 0.09 | 76.62 | 53-100 | 0.08 | 73/86 | 57-100, 45-100 |
| JRRR121 | *trans*-methoxy acid | 0.12 | 0.11 | 40.26 | 9-71 | 0.11 | 55/57 | 14-86, 27-82 |
| KB110 | *cis*-methoxy TMM | 0.21 | 0.10 | 63.64 | 29-98 | 0.16 | 73/71 | 43-100, 45-100 |
| MH175 | alpha TDM | 0.14 | 0.08 | 74.03 | 47-100 | 0.13 | 82/71 | 43-100, 55-100 |
| MOD171 | alpha-ArMM | 0.40 | 0.10 | 87.01 | 70-100 | 0.13 | 82/86 | 57-100, 55-100 |
| OTA97 | alpha-DMAG | 0.12 | 0.09 | 54.55 | 16-93 | 0.17 | 100/43 | 14-86, 100-100 |
| RT237F2 | alpha-muramyl | 0.09 | 0.06 | 93.51 | 80-100 | 0.07 | 91/100 | 100-100, 73-100 |
| SMP74 | alpha-GMM | 0.20 | 0.17 | 66.23 | 37-96 | 0.18 | 73/71 | 43-100, 45-73 |

Footnotes: AUC – Area under the curve, CI – confidence interval

Sensitivity/Specificity: At distinguishing abnormal responses in Crohn's patients vs healthy controls

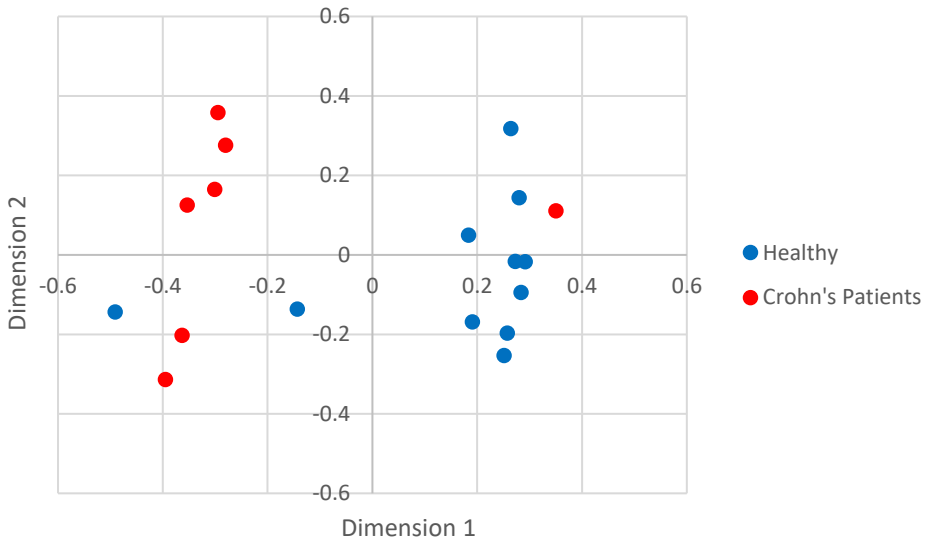


**Figure 19** - Proximity of individual cases using Random Forest classifier, reduced to 2-dimensions using principal co-ordinate analysis. For the 9 antigens in the Crohn's study.

## 6.5. Discussion

This experiment was designed to determine if Crohn's patients had specific antibody responses to MAP antigens. The antigens used here reflected the ones used with the cattle testing plus a couple that have proven effective at detecting *M. tb*.

Both MOD171 and RT237F2 gave good results with a sensitivity/specificity of 82/86 and 91/100 respectively. Although this gives a strong indication that there is an abnormal immune response within Crohn's patients to MAP antigens, the small sample size prevents larger conclusions being drawn. It does however indicate that a larger sample set should be run to gauge how significant this evidence is.

The question then goes in the direction that the cattle study did and that is exposure vs infection. Given that MAP can pass into human food via products such as milk (or milk itself), it is reasonable that both the Crohn's patients and the healthy samples have had exposure to MAP at some point within their life. This rules out simple exposure as a cause for the positive results, though very recent exposure via unpasteurised milk/milk products could explain the two 'false positives'; only very detailed records would be able to confirm this.

Whether or not MAP is a causative agent for Crohn's has been discussed many times, the results shown here suggest that there is a link. But there are many possibilities beyond MAP acting as a causative agent; mycobacteria are widely present in the environment and a large proportion are opportunistic when it comes to infection. For example this can happen through breaks in the skin for weakened immune systems, therefore it is possible that, due to the progression of Crohn's disease, MAP simply follows this trend. Both MAP acting as a causative agent or as an opportunistic secondary infection would produce an abnormal response via an immune assay, and answers to this which would give MAP a role either in disease diagnosis or as a disease progression marker.

## 6.6. Future Work

Given the small set of samples tested the next step would be to test against a larger set of samples, but with a more refined set of antigens.

Ideally this set of serum would have highly detailed patient and dietary information which would help greatly in discovering the differences between sample results.

# 7. Conclusions

A set of 64 cattle samples comprised of 35 experimentally infected with MAP and 29 negative from a farm with no history of MAP, whose diagnosis was confirmed via culture. This gave a sensitivity/specificity of >80/80 on 8 individual antigens out of the 15 tested, with 3 antigens going above 90/90.

A second naturally infected set of samples containing 80 samples of which 40 were positive and 40 negative, when run against a refined set of 9 antigens gave worse individual antigen results with the best 3 antigens giving a sensitivity/specificity of 85/75, 65/80 and 73/73 respectively. However, when combining the results with those of the first experimentally infected set and using all 5 common antigens for a combined diagnosis, a sensitivity/specificity of 84/93 resulted.

These two studies allowed the most effective antigens to be identified according to their sensitivity/specificity. Surprisingly these were not the wax ester compounds that are more unique to MAP; additionally they contained alpha type mycolic acids which according to literature are less immunogenic than oxygenated acid types.

A flow through point of care test for *M. tb* was successfully translated and applied to MAP in cattle serum. The pooled positive tested showed a clear response, whilst the control spot was unaffected and stayed blank. Although this was a numerically small test it shows promise towards a point of care test for MAP, which given the prevalence of MAP in cattle herds has the potential to bring down testing costs and simplify the procedure.

Given the similarities between MAP infection progression and Crohn's disease, the MAP ELISA was also tested against positive Crohn's serum. This resulted in two antigens giving very good results, RT237F2 and MOD171, with a 'sensitivity/specificity' of 91/100 and 82/86 respectively for distinguishing Crohn's from non-Crohn's serum. These two antigens also showed promise within the cattle ELISA, indicating that there could be a common link between MAP in cattle and Crohn's in humans.

## 7.1. Importance and Impact

Currently, Johne's disease is not a notifiable disease in cattle and as such the diagnostics within the area are underdeveloped. However, if the link to Crohn's disease became more concrete, both diagnostics and therapeutics for MAP and Crohn's would become important.

The work shown here demonstrates that cell wall components of MAP can be used for the detection of MAP immune responses within cattle. It also shows that, responses to the same antigens exist with Crohn's patients to a higher degree than a healthy population. This adds evidence to a controversial debate regarding MAP as a causative agent for Crohn's disease in humans.

# 8. Appendix

## 8.1. Raw Data For The A Study of Naturally Infected Positive and Negative Cattle Samples From Canada

| Serum ID | Diagnosis | OTA97 | AD132 | KB110 | ST152 | MH175 | SMP74 | MOD171 | JRR121 | JR1080 | MOD30 | RT237F2 | ST151 | AD129 | MH140 | JR1056 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Averaged Absorbances | | | | | | | | | | | | | | |
| MRI 135 | Negative | 0.38 | 0.66 | 2.36 | 1.57 | 1.06 | 0.27 | 0.24 | 0.17 | 0.14 | 0.43 | 0.18 | 0.63 | 0.27 | 0.15 | 0.18 |
| MRI 136 | Negative | 0.31 | 0.94 | 2.51 | 1.64 | 1.41 | 0.54 | 0.31 | 0.21 | 0.16 | 0.25 | 0.24 | 0.88 | 0.23 | 0.25 | 0.16 |
| MRI 137 | Negative | 0.97 | 0.48 | 1.85 | 1.12 | 0.64 | 0.31 | 0.22 | 0.24 | 0.16 | 0.19 | 0.34 | 0.32 | 0.20 | 0.14 | 0.19 |
| MRI 138 | Negative | 0.28 | 0.27 | 1.58 | 1.10 | 0.64 | 0.33 | 0.16 | 0.20 | 0.13 | 0.15 | 0.29 | 0.29 | 0.20 | 0.12 | 0.19 |
| MRI 140 | Negative | 0.19 | 0.44 | 2.33 | 1.58 | 1.50 | 0.21 | 0.15 | 0.22 | 0.13 | 0.14 | 0.18 | 0.77 | 0.23 | 0.12 | 0.21 |
| MRI 141 | Negative | 0.30 | 1.58 | 2.48 | 1.91 | 2.50 | 0.66 | 0.16 | 0.36 | 0.13 | 0.17 | 0.24 | 1.29 | 0.65 | 0.17 | 0.25 |
| MRI 142 | Negative | 0.30 | 0.18 | 1.50 | 0.78 | 0.29 | 0.22 | 0.17 | 0.20 | 0.13 | 0.18 | 0.21 | 0.18 | 0.56 | 0.14 | 0.19 |
| MRI 143 | Negative | 0.58 | 0.21 | 2.35 | 1.71 | 0.36 | 0.51 | 0.20 | 0.21 | 0.13 | 0.23 | 0.22 | 0.20 | 0.55 | 0.12 | 0.23 |
| MRI 144 | Negative | 0.34 | 0.18 | 0.70 | 0.48 | 0.25 | 0.26 | 0.15 | 0.16 | 0.12 | 0.15 | 0.22 | 0.15 | 0.49 | 0.13 | 0.20 |
| MRI 145 | Negative | 0.27 | 0.62 | 1.79 | 0.97 | 0.84 | 0.42 | 0.15 | 0.41 | 0.14 | 0.17 | 0.25 | 0.51 | 0.22 | 0.17 | 0.32 |
| MRI 146 | Negative | 0.19 | 0.25 | 1.74 | 1.03 | 0.43 | 0.21 | 0.14 | 0.19 | 0.13 | 0.16 | 0.20 | 0.25 | 0.20 | 0.14 | 0.22 |
| MRI 147 | Negative | 0.38 | 0.33 | 2.14 | 1.48 | 0.57 | 0.30 | 0.20 | 0.25 | 0.14 | 0.17 | 0.31 | 0.37 | 0.19 | 0.12 | 0.25 |
| MRI 148 | Negative | 0.31 | 0.79 | 2.29 | 1.47 | 1.50 | 0.34 | 0.19 | 0.31 | 0.14 | 0.17 | 0.30 | 0.69 | 0.20 | 0.13 | 0.23 |
| MRI 149 | Negative | 0.17 | 1.04 | 2.45 | 1.72 | 1.34 | 0.16 | 0.12 | 0.17 | 0.12 | 0.11 | 0.15 | 0.84 | 0.24 | 0.13 | 0.17 |
| MRI 150 | Negative | 1.26 | 0.80 | 2.12 | 1.46 | 1.48 | 0.29 | 0.14 | 0.18 | 0.12 | 0.14 | 0.21 | 0.71 | 0.18 | 0.13 | 0.19 |
| MRI 152 | Negative | 0.42 | 2.01 | 2.53 | 1.93 | 2.66 | 1.98 | 0.27 | 1.55 | 0.21 | 0.31 | 0.31 | 1.89 | 0.21 | 0.30 | 0.63 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MRI 153 | Negative | 0.22 | 0.42 | 2.23 | 1.56 | 0.64 | 0.19 | 0.18 | 0.20 | 0.13 | 0.16 | 0.20 | 0.38 | 0.20 | 0.13 | 0.21 |
| MRI 155 | Negative | 0.20 | 0.25 | 2.39 | 1.75 | 0.47 | 0.18 | 0.14 | 0.20 | 0.13 | 0.16 | 0.31 | 0.21 | 0.23 | 0.14 | 0.27 |
| MRI 156 | Negative | 0.20 | 0.19 | 0.89 | 0.46 | 0.26 | 0.41 | 0.13 | 0.18 | 0.12 | 0.14 | 0.27 | 0.18 | 0.16 | 0.14 | 0.20 |
| MRI 158 | Negative | 0.18 | 1.55 | 2.42 | 1.70 | 1.96 | 0.23 | 0.16 | 0.16 | 0.12 | 0.24 | 0.17 | 1.29 | 0.16 | 0.14 | 0.17 |
| MRI 397 | Negative | 0.25 | 0.73 | 2.31 | 1.48 | 1.03 | 0.55 | 0.19 | 0.20 | 0.13 | 0.17 | 0.20 | 0.75 | 0.17 | 0.14 | 0.18 |
| MRI 398 | Negative | 0.20 | 0.71 | 1.94 | 1.17 | 1.04 | 0.33 | 0.16 | 0.23 | 0.12 | 0.15 | 0.19 | 0.65 | 0.19 | 0.14 | 0.20 |
| MRI 399 | Negative | 0.20 | 0.20 | 1.99 | 1.47 | 0.30 | 0.18 | 0.14 | 0.23 | 0.12 | 0.14 | 0.25 | 0.16 | 0.17 | 0.16 | 0.20 |
| MRI 400 | Negative | 0.24 | 0.36 | 1.31 | 0.76 | 0.49 | 0.46 | 0.18 | 0.23 | 0.13 | 0.16 | 0.27 | 0.34 | 0.15 | 0.15 | 0.17 |
| MRI 402 | Negative | 0.20 | 0.37 | 1.89 | 1.09 | 0.67 | 0.20 | 0.16 | 0.23 | 0.12 | 0.16 | 0.16 | 0.29 | 0.14 | 0.30 | 0.18 |
| MRI 403 | Negative | 1.05 | 0.19 | 0.84 | 0.34 | 0.53 | 0.29 | 0.17 | 0.30 | 0.12 | 0.16 | 0.21 | 0.22 | 0.18 | 0.29 | 0.25 |
| MRI 404 | Negative | 1.19 | 0.38 | 1.76 | 1.11 | 0.63 | 0.22 | 0.13 | 0.19 | 0.13 | 0.14 | 0.25 | 0.34 | 0.16 | 0.14 | 0.24 |
| MRI 405 | Negative | 0.19 | 0.35 | 2.31 | 1.58 | 0.43 | 0.24 | 0.14 | 0.18 | 0.13 | 0.19 | 0.23 | 0.31 | 0.14 | 0.14 | 0.20 |
| MRI 406 | Negative | 0.29 | 0.66 | 2.30 | 1.38 | 1.36 | 0.22 | 0.20 | 0.23 | 0.13 | 0.17 | 0.27 | 0.67 | 0.14 | 0.16 | 0.23 |
| MAP 3 | Positive | 0.95 | 2.07 | 1.88 | 2.50 | 2.11 | 1.80 | 1.20 | 0.43 | 0.23 | 0.49 | 0.62 | 2.29 | 0.21 | 0.18 | 0.26 |
| MAP 4 | Positive | 1.69 | 0.81 | 1.80 | 1.51 | 0.90 | 0.85 | 1.67 | 1.66 | 0.59 | 0.31 | 0.37 | 0.46 | 0.51 | 0.81 | 1.28 |
| MAP 6 | Positive | 0.79 | 2.01 | 1.86 | 1.75 | 1.91 | 1.10 | 1.02 | 0.44 | 0.19 | 0.26 | 0.59 | 2.12 | 0.17 | 0.15 | 0.33 |
| MAP 7 | Positive | 0.91 | 2.09 | 1.90 | 2.03 | 1.74 | 1.02 | 1.23 | 0.72 | 0.35 | 0.64 | 1.08 | 1.94 | 0.31 | 0.28 | 0.34 |
| MAP 8 | Positive | 1.10 | 1.73 | 1.71 | 1.30 | 1.58 | 1.05 | 1.54 | 1.83 | 0.51 | 0.61 | 0.83 | 1.55 | 0.40 | 0.52 | 1.06 |
| MAP 14 | Positive | 0.67 | 1.34 | 1.68 | 1.25 | 1.16 | 0.91 | 0.87 | 0.38 | 0.22 | 0.37 | 0.56 | 0.97 | 0.20 | 0.18 | 0.23 |
| MAP 18 | Positive | 0.44 | 1.64 | 1.68 | 1.45 | 1.43 | 1.19 | 0.82 | 0.37 | 0.19 | 0.36 | 0.49 | 1.67 | 0.22 | 0.19 | 0.23 |
| MAP 28 | Positive | 0.59 | 1.82 | 1.86 | 1.84 | 1.55 | 0.59 | 0.93 | 0.48 | 0.21 | 0.36 | 0.64 | 1.80 | 0.27 | 0.20 | 0.28 |
| MAP 9 | Positive | 1.43 | 1.98 | 1.96 | 1.93 | 1.97 | 0.86 | 1.65 | 1.48 | 0.41 | 0.56 | 0.70 | 2.12 | 0.32 | 0.42 | 0.81 |
| MAP 10 | Positive | 0.66 | 1.91 | 1.86 | 1.67 | 1.87 | 0.79 | 0.89 | 0.48 | 0.23 | 0.44 | 0.64 | 1.96 | 0.20 | 0.19 | 0.24 |
| MAP 11 | Positive | 0.92 | 1.81 | 1.98 | 1.80 | 1.86 | 1.09 | 1.23 | 0.62 | 0.33 | 0.71 | 0.61 | 1.60 | 0.26 | 0.24 | 0.31 |
| MAP 12 | Positive | 0.60 | 1.40 | 1.74 | 1.08 | 1.21 | 0.95 | 1.42 | 1.06 | 0.27 | 0.24 | 0.61 | 1.24 | 0.25 | 0.27 | 0.52 |
| MAP 16 | Positive | 0.72 | 2.02 | 1.82 | 2.17 | 1.92 | 2.56 | 1.09 | 0.50 | 0.26 | 0.55 | 0.53 | 2.39 | 0.22 | 0.21 | 0.25 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MAP 17 | Positive | 1.25 | 2.01 | 1.88 | 2.28 | 2.01 | 1.22 | 1.53 | 1.75 | 0.63 | 0.47 | 0.48 | 2.33 | 0.51 | 0.99 | 1.22 |
| MAP 34 | Positive | 0.30 | 1.33 | 1.61 | 0.88 | 1.18 | 1.35 | 0.61 | 0.26 | 0.15 | 0.19 | 0.36 | 1.29 | 0.29 | 0.14 | 0.17 |
| MAP 35 | Positive | 0.77 | 2.05 | 1.80 | 2.40 | 2.07 | 1.95 | 1.21 | 1.11 | 0.26 | 0.61 | 0.75 | 2.08 | 0.29 | 0.28 | 0.47 |
| MAP 20 | Positive | 1.40 | 2.02 | 1.89 | 2.18 | 2.00 | 2.25 | 1.69 | 1.81 | 0.50 | 1.08 | 0.47 | 2.17 | 0.40 | 0.71 | 1.19 |
| MAP 24 | Positive | 0.37 | 1.70 | 1.68 | 1.22 | 1.58 | 1.54 | 0.75 | 0.28 | 0.19 | 0.23 | 0.52 | 1.54 | 0.17 | 0.17 | 0.17 |
| MAP 29 | Positive | 0.78 | 0.99 | 1.83 | 1.29 | 0.92 | 0.66 | 0.84 | 0.61 | 0.21 | 0.42 | 0.53 | 0.73 | 0.21 | 0.21 | 0.28 |
| MAP 30 | Positive | 0.53 | 1.95 | 1.88 | 1.80 | 1.87 | 1.19 | 0.72 | 0.45 | 0.17 | 0.33 | 0.61 | 2.21 | 0.20 | 0.25 | 0.22 |
| MAP 31 | Positive | 0.42 | 2.09 | 2.00 | 2.13 | 1.94 | 1.18 | 1.10 | 0.78 | 0.20 | 0.25 | 0.50 | 2.22 | 0.17 | 0.22 | 0.27 |
| MAP 33 | Positive | 0.22 | 0.41 | 0.97 | 0.52 | 0.38 | 0.36 | 0.46 | 0.25 | 0.16 | 0.17 | 0.32 | 0.33 | 0.15 | 0.20 | 0.15 |
| MAP 41 | Positive | 0.82 | 1.86 | 1.84 | 1.47 | 1.57 | 1.43 | 0.65 | 0.35 | 0.19 | 0.29 | 0.44 | 1.67 | 0.17 | 0.21 | 0.19 |
| MAP 32 | Positive | 0.29 | 0.55 | 1.76 | 0.70 | 0.47 | 0.37 | 0.46 | 0.29 | 0.14 | 0.21 | 0.34 | 0.38 | 0.18 | 0.23 | 0.16 |
| MAP 5 | Positive | 0.34 | 1.79 | 1.90 | 1.74 | 1.81 | 1.19 | 0.96 | 0.79 | 0.19 | 0.24 | 0.39 | 1.51 | 0.18 | 0.20 | 0.33 |
| MAP 13 | Positive | 1.13 | 2.12 | 1.79 | 1.82 | 1.89 | 1.82 | 1.51 | 1.06 | 0.46 | 0.88 | 0.85 | 2.29 | 0.22 | 0.22 | 0.52 |
| MAP 19 | Positive | 0.86 | 1.82 | 1.75 | 1.12 | 1.61 | 1.01 | 1.43 | 1.55 | 0.45 | 0.22 | 0.36 | 1.54 | 0.44 | 0.50 | 0.95 |
| MAP 23 | Positive | 0.39 | 0.72 | 1.94 | 1.50 | 0.51 | 0.50 | 0.43 | 0.26 | 0.16 | 0.23 | 0.38 | 0.55 | 0.23 | 0.15 | 0.17 |
| MAP 39 | Positive | 0.47 | 1.97 | 1.93 | 1.85 | 1.89 | 1.61 | 0.50 | 0.41 | 0.22 | 0.25 | 0.37 | 2.17 | 0.19 | 0.19 | 0.24 |
| MAP 46 | Positive | 0.81 | 2.14 | 2.01 | 1.73 | 1.92 | 1.46 | 1.16 | 0.64 | 0.34 | 0.69 | 0.79 | 2.22 | 0.28 | 0.25 | 0.38 |
| MAP 47 | Positive | 0.47 | 0.56 | 1.60 | 1.27 | 0.56 | 0.49 | 0.97 | 0.25 | 0.24 | 0.25 | 0.54 | 0.98 | 0.18 | 0.14 | 0.21 |
| MAP 56 | Positive | 1.51 | 2.08 | 1.89 | 2.22 | 1.94 | 1.92 | 1.64 | 1.44 | 0.67 | 0.78 | 0.67 | 2.33 | 0.50 | 0.48 | 0.87 |
| MAP 26 | Positive | 0.32 | 1.20 | 1.87 | 1.79 | 1.14 | 0.84 | 0.42 | 0.29 | 0.15 | 0.19 | 0.46 | 1.71 | 0.18 | 0.21 | 0.17 |
| MAP 25 | Positive | 1.01 | 1.45 | 1.93 | 1.56 | 1.44 | 1.15 | 1.54 | 1.59 | 0.29 | 0.23 | 0.45 | 1.51 | 0.25 | 0.23 | 0.56 |
| MAP 58 | Positive | 1.37 | 0.70 | 1.53 | 1.07 | 0.59 | 0.49 | 1.55 | 1.36 | 0.34 | 0.22 | 0.76 | 0.61 | 0.31 | 0.31 | 0.85 |

## 8.2. Raw Data For The Naturally Infected Positive and Negative Cattle Samples From Canada

| Serum ID | Faecal PCR (CT) | Serum ELISA (S/P) | SMP74 | RT237F2 | SMP75 | MOD171 | ST123 | ST124 | KV059 | JRRR121 | OTA97 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Averaged Absorbances | | | | | | | | |
| PEI 1 | 34.6 | 0.048 | 0.65 | 0.52 | 0.33 | 0.30 | 1.83 | 2.04 | 1.24 | 0.60 | 0.63 |
| PEI 7 | 0.0 | 0.034 | 0.36 | 0.46 | 1.85 | 0.31 | 0.57 | 2.06 | 0.48 | 0.26 | 1.36 |
| PEI 11 | 0.0 | 0.029 | 0.55 | 0.29 | 1.10 | 0.65 | 0.52 | 2.31 | 0.30 | 0.38 | 1.54 |
| PEI 12 | 0.0 | 0.019 | 0.56 | 0.85 | 0.55 | 0.34 | 0.47 | 2.30 | 0.43 | 0.74 | 1.12 |
| PEI 13 | 0.0 | 0.060 | 0.29 | 0.35 | 0.54 | 0.41 | 1.31 | 2.40 | 1.02 | 0.22 | 1.32 |
| PEI 14 | 0.0 | 0.069 | 0.41 | 0.95 | 0.82 | 0.45 | 0.52 | 1.78 | 0.53 | 0.78 | 0.75 |
| PEI 30 | 0.0 | 0.063 | 0.32 | 0.44 | 0.43 | 0.42 | 0.90 | 2.01 | 0.76 | 0.28 | 1.09 |
| PEI 34 | 0.0 | 0.048 | 0.44 | 0.47 | 0.72 | 0.31 | 0.41 | 1.29 | 0.35 | 0.19 | 1.16 |
| PEI 37 | 0.0 | 0.063 | 0.42 | 0.72 | 0.81 | 0.33 | 1.01 | 1.64 | 0.77 | 0.28 | 0.63 |
| PEI 39 | 0.0 | 0.054 | 0.34 | 0.38 | 0.78 | 0.26 | 0.79 | 2.39 | 0.46 | 0.17 | 0.65 |
| PEI 41 | 0.0 | 0.171 | 0.41 | 0.39 | 1.57 | 0.33 | 0.80 | 2.09 | 0.57 | 0.37 | 1.31 |
| PEI 42 | 0.0 | 0.032 | 0.29 | 0.53 | 1.20 | 0.37 | 1.32 | 2.15 | 1.03 | 0.27 | 0.74 |
| PEI 52 | 0.0 | 0.162 | 0.37 | 0.56 | 1.44 | 0.41 | 2.06 | 2.05 | 1.35 | 0.24 | 0.91 |
| PEI 58 | 0.0 | 0.126 | 0.39 | 0.44 | 0.75 | 0.26 | 1.04 | 2.26 | 0.56 | 0.20 | 0.52 |
| PEI 85 | 0.0 | 0.060 | 0.54 | 0.43 | 1.14 | 0.27 | 1.14 | 2.27 | 0.82 | 0.32 | 0.51 |
| PEI 94 | 0.0 | 0.134 | 0.55 | 0.90 | 1.24 | 0.38 | 0.60 | 2.18 | 0.44 | 0.25 | 0.92 |
| PEI 95 | 0.0 | 0.025 | 0.29 | 0.39 | 0.89 | 0.25 | 0.61 | 1.61 | 0.87 | 0.19 | 0.43 |
| PEI 99 | 0.0 | 0.088 | 0.36 | 0.63 | 0.67 | 0.25 | 1.26 | 2.27 | 0.88 | 0.22 | 0.33 |
| PEI 101 | 0.0 | 0.102 | 0.36 | 0.62 | 0.43 | 0.25 | 1.30 | 2.29 | 1.00 | 0.23 | 0.50 |
| PEI 126 | 0.0 | 0.113 | 0.51 | 0.54 | 0.32 | 0.27 | 0.23 | 1.70 | 0.42 | 0.27 | 0.73 |
| PEI 128 | 0.0 | 0.060 | 0.37 | 0.43 | 0.58 | 0.26 | 0.25 | 1.18 | 0.25 | 0.45 | 0.52 |
| PEI 129 | 0.0 | 0.017 | 0.40 | 0.31 | 0.52 | 0.18 | 0.41 | 1.31 | 0.33 | 0.30 | 0.28 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| PEI 154 | 0.0 | 0.034 | 0.29 | 0.38 | 0.47 | 0.28 | 0.39 | 1.67 | 0.35 | 0.23 | 0.85 |
| PEI 156 | 0.0 | 0.028 | 0.26 | 0.41 | 0.48 | 0.34 | 0.39 | 1.53 | 0.35 | 0.26 | 0.54 |
| PEI 160 | 0.0 | 0.077 | 0.44 | 0.40 | 1.10 | 0.31 | 0.52 | 2.27 | 0.51 | 0.37 | 0.57 |
| PEI 161 | 0.0 | 0.076 | 0.38 | 0.52 | 1.43 | 0.35 | 0.47 | 2.10 | 0.41 | 0.22 | 0.60 |
| PEI 164 | 0.0 | 0.097 | 0.36 | 0.56 | 0.42 | 0.31 | 0.21 | 2.09 | 0.21 | 0.21 | 0.52 |
| PEI 171 | 0.0 | 0.131 | 0.35 | 0.40 | 0.45 | 0.29 | 0.20 | 2.28 | 0.21 | 0.23 | 0.55 |
| PEI 181 | 0.0 | 0.082 | 0.31 | 0.38 | 1.15 | 0.27 | 0.33 | 2.41 | 0.30 | 0.21 | 0.56 |
| PEI 182 | 0.0 | 0.046 | 0.44 | 0.43 | 1.22 | 0.35 | 0.48 | 1.51 | 0.45 | 0.25 | 0.66 |
| PEI 184 | 0.0 | 0.122 | 0.38 | 0.37 | 0.99 | 0.34 | 0.44 | 2.06 | 0.44 | 0.61 | 0.85 |
| PEI 186 | 0.0 | 0.177 | 0.25 | 0.32 | 0.54 | 0.31 | 0.51 | 2.26 | 0.40 | 0.22 | 0.53 |
| PEI 187 | 0.0 | 0.082 | 0.32 | 0.37 | 0.48 | 0.27 | 0.47 | 2.13 | 0.37 | 0.19 | 0.50 |
| PEI 189 | 0.0 | 0.069 | 0.28 | 0.35 | 0.61 | 0.36 | 0.71 | 2.06 | 0.77 | 0.21 | 0.74 |
| PEI 192 | 0.0 | 0.077 | 0.39 | 0.67 | 0.77 | 0.34 | 0.82 | 2.26 | 0.64 | 0.26 | 1.27 |
| PEI 197 | 0.0 | 0.139 | 0.30 | 0.59 | 0.43 | 0.26 | 0.53 | 2.24 | 0.43 | 0.22 | 0.46 |
| PEI 211 | 0.0 | 0.040 | 0.30 | 0.38 | 0.95 | 0.29 | 0.97 | 2.27 | 0.81 | 0.21 | 1.79 |
| PEI 212 | 0.0 | 0.065 | 0.27 | 0.35 | 0.54 | 0.23 | 0.40 | 1.62 | 0.43 | 0.21 | 0.31 |
| PEI 213 | 0.0 | 0.029 | 0.34 | 0.55 | 0.51 | 0.29 | 0.66 | 2.32 | 0.46 | 0.22 | 0.46 |
| PEI 216 | 0.0 | 0.230 | 0.22 | 0.33 | 0.51 | 0.23 | 0.64 | 2.12 | 0.50 | 0.20 | 1.33 |
| PEI 218 | 0.0 | 0.022 | 0.30 | 0.41 | 0.67 | 0.61 | 0.36 | 0.82 | 0.44 | 0.23 | 0.49 |
| PEI 217 | 20.7 | 2.329 | 0.78 | 0.36 | 0.94 | 0.57 | 2.23 | 2.22 | 1.67 | 1.44 | 0.83 |
| PEI 48 | 22.5 | 2.774 | 1.69 | 0.62 | 1.86 | 1.95 | 2.09 | 2.01 | 1.67 | 1.50 | 2.05 |
| PEI 144 | 23.1 | 2.653 | 1.19 | 0.40 | 1.77 | 0.31 | 2.13 | 2.18 | 1.66 | 0.78 | 1.64 |
| PEI 64 | 23.9 | 2.280 | 1.45 | 0.69 | 0.83 | 0.63 | 2.39 | 2.34 | 1.47 | 0.69 | 1.03 |
| PEI 46 | 24.3 | 1.724 | 1.25 | 0.37 | 1.24 | 1.05 | 1.89 | 2.22 | 1.37 | 0.54 | 1.36 |
| PEI 114 | 24.3 | 2.178 | 0.47 | 0.60 | 0.47 | 1.06 | 1.19 | 2.32 | 0.70 | 0.48 | 1.97 |
| PEI 103 | 25.5 | 2.318 | 1.97 | 0.73 | 2.04 | 2.02 | 1.66 | 1.97 | 0.96 | 1.49 | 2.16 |
| PEI 163 | 26.3 | 2.264 | 0.85 | 0.48 | 1.18 | 0.42 | 2.46 | 2.28 | 1.75 | 1.24 | 0.65 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| PEI 123 | 26.7 | 2.820 | 0.29 | 0.41 | 0.49 | 0.24 | 1.88 | 2.45 | 1.07 | 0.22 | 1.09 |
| PEI 9 | 27.3 | 2.152 | 0.63 | 0.68 | 1.36 | 0.51 | 2.14 | 2.50 | 1.61 | 0.35 | 1.18 |
| PEI 122 | 29.6 | 1.877 | 1.11 | 0.55 | 1.84 | 0.58 | 2.52 | 2.15 | 1.58 | 1.04 | 0.97 |
| PEI 73 | 30.1 | 1.611 | 0.75 | 0.59 | 0.59 | 0.41 | 2.41 | 2.25 | 1.62 | 0.50 | 0.54 |
| PEI 173 | 31.8 | 0.557 | 0.30 | 0.34 | 0.79 | 0.23 | 0.85 | 2.37 | 0.76 | 0.18 | 0.30 |
| PEI 134 | 33.2 | 0.110 | 0.25 | 0.29 | 0.42 | 0.21 | 0.63 | 1.89 | 0.57 | 0.21 | 0.46 |
| PEI 69 | 33.7 | 0.069 | 1.33 | 0.69 | 1.31 | 0.49 | 0.70 | 1.99 | 0.51 | 0.89 | 0.68 |
| PEI 208 | 33.8 | 1.492 | 0.30 | 0.39 | 0.45 | 0.29 | 0.69 | 2.18 | 0.47 | 0.19 | 0.54 |
| PEI 82 | 34.7 | 0.060 | 0.44 | 0.81 | 0.36 | 0.30 | 2.48 | 2.13 | 1.61 | 0.27 | 0.83 |
| PEI 200 | 34.9 | 1.151 | 0.39 | 0.43 | 0.54 | 0.28 | 0.73 | 1.96 | 0.41 | 0.28 | 0.40 |
| PEI 4 | 35.1 | 0.191 | 0.40 | 0.36 | 0.58 | 0.30 | 1.12 | 2.33 | 0.98 | 0.25 | 0.53 |
| PEI 2 | 35.5 | 0.052 | 0.58 | 1.13 | 0.39 | 0.31 | 0.99 | 2.22 | 0.89 | 0.38 | 0.39 |
| PEI 74 | 35.8 | 0.015 | 0.44 | 0.43 | 1.35 | 0.31 | 0.52 | 2.35 | 0.42 | 0.28 | 1.77 |
| PEI 35 | 35.9 | 0.030 | 0.48 | 0.53 | 0.52 | 0.48 | 0.36 | 1.29 | 0.34 | 0.29 | 0.63 |
| PEI 83 | 35.9 | 0.048 | 0.53 | 0.51 | 0.51 | 0.32 | 1.26 | 2.25 | 0.93 | 0.30 | 0.43 |
| PEI 38 | 36.1 | 0.022 | 0.77 | 0.67 | 1.31 | 0.36 | 0.93 | 2.30 | 0.80 | 0.31 | 0.67 |
| PEI 96 | 36.2 | 0.036 | 0.33 | 0.53 | 0.45 | 0.35 | 1.00 | 2.40 | 0.68 | 0.22 | 0.77 |
| PEI 139 | 36.2 | 0.820 | 0.62 | 0.38 | 0.68 | 0.34 | 1.09 | 2.05 | 0.84 | 0.35 | 0.47 |
| PEI 152 | 36.3 | 0.024 | 0.62 | 0.65 | 0.55 | 0.84 | 0.81 | 2.11 | 0.80 | 0.66 | 0.59 |
| PEI 97 | 36.4 | 0.036 | 0.70 | 0.70 | 0.62 | 0.75 | 0.49 | 1.58 | 0.40 | 0.41 | 0.68 |
| PEI 224 | 36.4 | 0.135 | 0.27 | 0.49 | 0.34 | 0.28 | 0.42 | 2.06 | 0.26 | 0.18 | 0.51 |
| PEI 18 | 36.7 | 0.047 | 0.43 | 0.84 | 0.48 | 0.47 | 1.31 | 2.24 | 0.97 | 0.30 | 0.95 |
| PEI 54 | 36.7 | 0.012 | 0.60 | 0.57 | 0.59 | 0.39 | 0.57 | 1.86 | 0.44 | 0.41 | 0.56 |
| PEI 70 | 36.7 | 0.021 | 0.54 | 1.32 | 1.05 | 0.43 | 1.49 | 2.13 | 1.12 | 0.30 | 0.66 |
| PEI 91 | 36.7 | 0.113 | 0.62 | 1.49 | 1.88 | 0.44 | 2.10 | 2.42 | 1.29 | 0.22 | 0.60 |
| PEI 196 | 37.1 | 0.013 | 0.52 | 0.32 | 1.46 | 0.38 | 0.88 | 2.49 | 0.69 | 0.32 | 2.08 |
| PEI 150 | 37.2 | 0.101 | 0.53 | 0.49 | 0.46 | 0.38 | 0.72 | 2.10 | 0.70 | 0.27 | 0.66 |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| PEI 157 | 37.2 | 0.037 | 0.53 | 0.43 | 0.37 | 0.42 | 0.45 | 1.88 | 0.35 | 0.32 | 0.43 |
| PEI 205 | 37.2 | 0.007 | 0.31 | 0.66 | 0.98 | 0.28 | 0.36 | 2.00 | 0.23 | 0.20 | 0.55 |
| PEI 8 | 37.3 | 0.062 | 0.71 | 0.65 | 0.68 | 0.35 | 0.98 | 2.28 | 0.87 | 0.25 | 0.62 |
| PEI 31 | 37.3 | 0.257 | 0.52 | 0.67 | 0.38 | 0.39 | 1.12 | 2.09 | 0.68 | 0.37 | 0.55 |

## 8.3. Raw Data For The Crohn's Samples Tested Against MAP Antigens

| Sample ID | Diagnosis | SMP74 | MOD171 | JRRR121 | RT237F2 | JR1080 | MH175 | AD132 | KB110 | OTA97 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Averaged Absorbances | | | | | | | | |
| CR1 | Crohn's | 0.21 | 0.13 | 0.16 | 0.09 | 0.08 | 0.22 | 0.17 | 0.29 | 0.07 |
| CR2 | Crohn's | 0.12 | 0.10 | 0.08 | 0.07 | 0.06 | 0.23 | 0.17 | 0.32 | 0.07 |
| CR3 | Crohn's | 0.82 | 0.74 | 0.08 | 0.10 | 0.07 | 0.06 | 0.06 | 0.06 | 0.07 |
| CR4 | Crohn's | 0.19 | 0.47 | 0.12 | 0.08 | 0.07 | 0.14 | 0.11 | 0.19 | 0.21 |
| CR5 | Crohn's | 0.16 | 0.40 | 0.12 | 0.40 | 0.07 | 0.14 | 0.13 | 0.21 | 0.24 |
| CR6 | Crohn's | 0.20 | 0.13 | 0.16 | 0.09 | 0.08 | 0.59 | 0.47 | 0.57 | 0.18 |
| CR7 | Crohn's | 0.86 | 0.83 | 0.09 | 0.14 | 0.09 | 0.07 | 0.08 | 0.06 | 0.12 |
| CR8 | Healthy | 0.17 | 0.09 | 0.09 | 0.06 | 0.11 | 0.08 | 0.08 | 0.08 | 0.08 |
| CR9 | Healthy | 0.16 | 0.07 | 0.16 | 0.06 | 0.13 | 0.09 | 0.08 | 0.28 | 0.11 |
| CR10 | Healthy | 0.53 | 0.11 | 0.20 | 0.07 | 0.27 | 0.19 | 0.21 | 0.26 | 0.16 |
| CR11 | Healthy | 0.16 | 0.13 | 0.11 | 0.07 | 0.09 | 0.07 | 0.07 | 0.10 | 0.09 |
| CR12 | Healthy | 0.17 | 0.46 | 0.09 | 0.10 | 0.08 | 0.06 | 0.06 | 0.06 | 0.07 |
| CR13 | Healthy | 0.23 | 0.11 | 0.14 | 0.06 | 0.08 | 0.07 | 0.06 | 0.08 | 0.09 |
| CR14 | Healthy | 0.12 | 0.07 | 0.18 | 0.06 | 0.07 | 0.06 | 0.06 | 0.23 | 0.16 |
| CR15 | Healthy | 0.18 | 0.10 | 0.13 | 0.06 | 0.13 | 0.15 | 0.13 | 0.12 | 0.11 |
| CR16 | Healthy | 0.21 | 0.12 | 0.10 | 0.07 | 0.15 | 0.13 | 0.12 | 0.12 | 0.09 |
| CR17 | Healthy | 0.12 | 0.07 | 0.08 | 0.06 | 0.07 | 0.08 | 0.07 | 0.09 | 0.07 |
| CR18 | Healthy | 0.12 | 0.08 | 0.10 | 0.06 | 0.06 | 0.11 | 0.10 | 0.10 | 0.07 |

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