Mycobacterium avium subsp. paratuberculosis infection in dairy cattle: development, optimization and evaluation of cell-mediated immune based assays

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Mycobacterium avium subsp. paratuberculosis infection in dairy cattle

Development, optimization and evaluation of cell-mediated immune based assays

PhD thesis by
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2011

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Frederiksborg, April 2011
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Preface

The research presented in this PhD thesis was performed in the Adaptive Immunology and Parasitology Research Group, National Veterinary Institute, Technical University of Denmark, Copenhagen and at the Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Copenhagen from 2007 to 2011.

The thesis consists of a general introduction, materials and methods, results and discussion based on five accompanying manuscripts of which two have been published / available online, two have been accepted for publication and one has been submitted to a peer reviewed journal. Final version of the unpublished paper may change from the current manuscripts presented here. Figure 1 presents how papers and subprojects of this thesis are related.

The thesis is based on the following papers:

Paper I

Paper II

Paper III

Paper IV

Paper V
Mikkelsen, H., Aagaard, C., Nielsen, S.S. and Jungersen, G. Correlation of antigen-specific IFN-γ responses of fresh blood samples from Mycobacterium avium subsp. paratuberculosis infected in cattle with responses of day-old blood samples co-cultured with IL-12 or anti-IL-10 antibodies. Submitted to Veterinary Immunology and Immunopathology.
Fig. 1. Diagram illustrating how manuscripts (blue) and unpublished projects (black) included in this thesis are related.
Abbreviations

CD cluster of differentiation
CMI cell-mediated immunity
DNA deoxyribonucleic acid
ELISA enzyme-linked immunosorbent assay
Fab fragment antigen-binding
FC faecal culture
Foxp3 forkhead box p3
FSC forward scatter
GTP guanosine triphosphate
HPLC high performance liquid chromatography
Hsp heat shock protein
IFN interferon
IgG immunoglobulin G
IL interleukin
MAA Mycobacterium avium subsp. avium
MAP Mycobacterium avium subsp. paratuberculosis
MHC major histocompatibility complex
NK cell natural killer cell
OD optical density
PBMC peripheral blood mononuclear cell
PBS phosphate buffered saline
PCR polymerase chain reaction
PPD purified protein derivate
RIA radioimmunoassay
SEB Staphylococcus enterotoxin B
SSC side scatter
TGF transforming growth factor
T\textsubscript{H}1 T cell T helper cell type 1
T\textsubscript{H}2 T cell T helper cell type 2
T\textsubscript{H}3 T cell T helper cell type 3
T\textsubscript{H}17 T cell T helper cell type 17
TNF tumour necrosis factor
T\textsubscript{reg} cell regulatory T cell
Summary

Paratuberculosis is a chronic infection of the intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Infection of dairy cattle can lead to reduced animal health and welfare, and economic losses through decreased milk yield and premature culling. It is generally believed that cattle are primarily infected as calves, either while still in the uterus or as neonates by ingestion of faecal matter, milk or colostrum from infected cows in the herd. Infection with MAP is characterised by early development of cell mediated immune (CMI) responses with production of the cytokine interferon gamma (IFN-\(\gamma\)), which are then replaced by a serological response with presence of antibodies. The IFN-\(\gamma\) test measure IFN-\(\gamma\) in whole blood samples following culture with MAP antigens and can be used for early identification of MAP infected animals. Most often purified protein derivate of MAP (PPDj) has been used for stimulation of blood samples in the IFN-\(\gamma\) assay, resulting in low specificity as PPDj cross-react with environmental mycobacteria. Furthermore, large scale use of the IFN-\(\gamma\) assay is limited by the time allowed from sampling to start of culture.

The aim of the thesis was to a) investigate the association between early IFN-\(\gamma\) responses and later milk antibody responses in cattle from MAP infected herds, b) review characterised MAP antigens for diagnostic applications, c) evaluate the use of novel recombinant and well defined antigens in the IFN-\(\gamma\) assay for early detection of MAP and d) evaluate the use of novel recombinant antigens in a protocol including day-old blood samples and immunopotentiating cytokines.

For evaluation of MAP exposure of calves in a herd, the IFN-\(\gamma\) test is the only available test. The association between IFN-\(\gamma\) responses to PPDj and later faecal culture status and milk antibody responses was evaluated in a large study including 975 cows from 18 dairy cattle herds. A significant association was observed between early IFN-\(\gamma\) and later faecal culture status with occurrence of antibodies. However, not all IFN-\(\gamma\) positive heifers developed a positive antibody response against MAP later in life as cows and not all MAP antibody positive cows had tested IFN- positive as calves. Knowledge of published MAP antigens for diagnostic application was compiled in a review paper, including 115 different MAP antigens, with focus on antigens for CMI recognition, but also including other antigens organised according to type. No obvious antigen candidate has been described. For evaluation of 14 novel antigens, blood samples were collected from heifers of a MAP non-infected herd and three times from a MAP infected herd, to study the consistency of IFN-\(\gamma\) responses. The same 14 novel antigens were evaluated for use in the day-old sample protocol with addition of recombinant bovine interleukin 12 (IL-12) or anti-bovine interleukin 10 (IL-10) antibodies. The results showed that IFN-\(\gamma\) responses to antigens of samples from the non-infected herd were significantly lower than IFN-\(\gamma\) responses of samples from the infected herd and the IFN-\(\gamma\) responses fluctuated between sample days. IFN-\(\gamma\) results obtained with samples from the non-infected herd were used to calculate cut-offs for all antigens in each protocol to differentiate between test positive and test negative
heifers of the infected herd. Furthermore, heifers of the infected herd, was differentiated into groups of cases and non-cases based on sample day results, to calculate specificity and immunogenicity of antigens. Results of the IFN-γ assay using PPDj did not correlate well with the results using the novel antigens and suggested that PPDj is highly immunogenic but have low specificity. IFN-γ responses of sample day samples showed high correlation with responses to some antigens in day-old samples with addition of IL-12 or anti-IL-10 antibodies to culture, indicating that day old protocols can be used as an alternative to the conventional IFN-γ protocol. Immunogenicity was generally low for day-old samples. Latency antigens were the most promising antigens in all three protocols and could be promising diagnostic antigens as both immunogenicity and specificity was high.

The results in the thesis demonstrate a significant association between early IFN-γ and subsequent faecal culture status with occurrence of antibodies, but only some IFN-γ positive animals will develop a positive antibody response. Furthermore, the IFN-γ test can be optimised by including novel, recombinant and specific MAP antigens and can be used for early detection of MAP infected cattle. Latency proteins might be promising antigens for MAP diagnosis, although a cocktail of antigens might be further investigated for detection of infection. Day-old blood samples can be used as an alternative to the conventional IFN-γ assay, although some reactivity may be lost.

Future studies should focus on evaluation of the most promising antigens in MAP infected herds for detection of exposed calves and the use of a modified IFN-γ protocol with day-old blood samples.
Sammendrag (Danish summary)


Formålet med afhandlingen var at a) undersøge sammenhængen mellem tidlig IFN-γ-svar og senere antistofreaktioner i mælk hos kvæg fra MAP-smittede besætninger, b) give et overblik af karakteriserede MAP-antigener til diagnostiske anvendelser, c) evaluere brugen af nye rekombinante og veldefinerede antigener i IFN-γ-testen til tidlig opdagelse af MAP og d) evaluere anvendelsen af nye rekombinante antigener i en protokol med daggamle blodprøver og immunpotensierende cytokiner.

beregne skillepunkter for alle antigener for hver protokol, for at kunne differentiere test-positive og test-negative kvier i den smittede besætning. Desuden blev kvierne fra den
smittede besætning, opdelt i grupper af ”cases” og ”non-cases”, baseret på resultaterne fra
de friske blodprøver, for at beregne specificitet og immunogenicitet af antigenerne.
Resultaterne af IFN-γ-testen med PPDj korrelerede dårligt med resultaterne med de andre
antigener og antydede, at PPDj er yderst immunogent men har lav specificitet. Korrelationen
imellem IFN-γ-svarene i de friske prøver og IFN-γ-svarene i de daggamle prøver med
tilsætning af IL-12 eller anti-IL-10 antistoffer til kultur var høj for flere antigener og indikerer,
at daggamle protokoller kan bruges som et alternativ til den konventionelle IFN-γ-protokol.
Immunogeniciteten var generelt lav for daggamle prøver. Latens-antigener var de mest
lovende antigener i alle tre protokoller og kunne være lovende diagnostiske antigener, da
både immunogenicitet og specificitet var høj.

Resultaterne i afhandlingen demonstrerer en signifikant sammenhæng mellem tidlig IFN-γ-
svar og efterfølgende fækalkultur-status med forekomst af antistoffer, men kun nogle IFN-γ-
positive dyr udvikler et positiv antistofsvar. Desuden kan IFN-γ testen optimieres ved at
bruge nye, rekombinannte og specifikke MAP-antigener og kan anvendes til tidlig påvisning af
MAP-smittede kvæg. Latens-proteiner kan være lovende antigener til MAP-diagnose, og
brug af en antigen-kombination til påvisning af smitte skal undersøges yderligere. Daggamle
blodprøver kan bruges som et alternativ til den konventionelle IFN-γ test, dog kan noget af
reaktionsevnen forringes.

Fremtidige undersøgelser bør fokusere på evaluering af de mest lovende antigener i MAP-
smittede besætninger til påvisning af udsatte kalve og brug af en modificeret IFN-γ-protokol
med daggamle blodprøver.
1. General introduction

1.1. Mycobacterium avium subsp. paratuberculosis

The first report of paratuberculosis was published in 1895 by Johne and Frothingham describing a peculiar case of tuberculosis in a cow with chronic enteritis and with presence of acid-fast bacilli in sections of the intestinal mucosa (Cocito et al., 1994). In 1906, B. Bang recognised that the disease was an infection distinct from tuberculosis and called it pseudotuberculosis (Clarke, 1997), whereas McFadyean (1906) termed it Johne’s disease. In 1910, O. Bang demonstrated that avian tuberculin could be used for diagnosis of paratuberculosis (Bang, 1910) and in 1912 the etiological agent was cultured, characterised and used for experimental studies by Twort and Ingram (Clarke, 1997).

Paratuberculosis is a chronic, enteric infection of cattle and ruminants that is caused by Mycobacterium avium subsp. paratuberculosis (MAP). MAP are small, Gram-positive, slow-growing and acid-fast bacteria that belong to the mycobacterial species M. avium. This specie also includes M. avium subsp. avium (MAA), M. avium subsp. hominisuis and M. avium subsp. silvaticum (Thorel et al., 1990; Behr, 2008). MAP can be differentiated from MAA and M. avium subsp. silvaticum by its dependence of mycobactin, an iron-chelating agent, for in vivo growth (Thorel et al., 1990) and by the presence of multiple copies of a repetitive DNA sequence, IS900 (Collins and Delisle, 1986). The genome of MAP has now been fully sequenced (Li et al., 2005).

Paratuberculosis is common in the Danish dairy population and the between-herd prevalence of MAP has been estimated to be higher than 50% (Nielsen, 2009). Infection of dairy cattle leads to economic losses because of reduced milk yield, premature culling and reduced slaughter value (Ott et al., 1999). It is believed that cattle are most susceptible to infection as calves (Doyle, 1953) where they can be infected in utero or by ingestion of bacteria through milk, colostrum or faecal matter (Chiodini et al., 1984; Sweeney, 1996). In clinical paratuberculosis, the cell wall of the intestines may be severely thickened because of increased granulomatous inflammation with infiltrations by large numbers of macrophages and lymphocytes. Protein–losing enteropathy takes place and the animal may suffer from chronic or intermittent diarrhea, weight loss, and eventually death (Coussens, 2001).

In Denmark, a voluntary paratuberculosis control programme was established in 2006 and by January 2009 approximately 28% of the dairy herds, including 40% of the cows, participated in the programme (Nielsen, 2009). The programme is a risk-based control programme in which lactating cows are screened for MAP antibodies in milk on four test-dates per year using milk collected through the milk recording scheme. The results of screening are transferred to management lists, where cows are divided into High-Risk and Low-Risk cows. For management of High-Risk cows special recommendations are provided, that focus on preventing within-herd transmission. All expenses are paid by the farmers (Nielsen, 2009). The programme is based on detection of antibodies that are thought to be
produced in later stages of MAP infection and antibodies do not measure if an animal have been exposed to MAP. Therefore, there is a need for a diagnostic tool, which can detect early MAP infection and determine if calves are exposed to MAP.

1.2. Immunological response to MAP infection

Several scenarios for immune responses against MAP have been suggested based on results obtained from in vivo and in vitro studies. However, it is clear that cell mediated immune (CMI) responses mediated by T lymphocytes (T cells) play an important role in immunity to paratuberculosis and other mycobacterial infections. Several T cell subsets have been defined, including the classical T cells that express the αβ-receptor (CD4+ T cells and CD8+ T cells) and the gamma-delta (γδ) T cells that express the γδ T cell receptor on their surface. It is thought, that MAP primarily targets the mucosa-associated lymphoid tissues of the small intestines where the bacteria gain access to the epithelium through M cells of Peyer’s patches (Momotani et al., 1988). In the epithelium, MAP bacteria are primarily phagocytosed by resident macrophages and internalized in phagosomes (Momotani et al., 1988). Macrophages are cells designed to kill invading bacteria and present antigens to other immune effector cells (de Almeida et al., 2008). However, as other pathogenic mycobacteria, MAP can replicate and persist for years within phagosomes. Survival is possible because MAP exploits various mechanisms to escape destruction including prevention of phagosome maturation into an acidic phagolysosome, inhibition of apoptosis and destruction of toxic superoxides secreted by immune cells (Woo and Czuprynski, 2008). Activated macrophages are the main effector cells involved in killing of intracellular MAP and the proinflammatory cytokine interferon gamma (IFN-γ) plays an important role for activation of macrophages. In fact, IFN-γ appears to be the most critical cytokine for controlling mycobacterial infections (Stabel, 2000). Calves have a large proportion of γδ T cells (Vesosky et al., 2003; Kampen et al., 2006) and these cells could be an important early source of IFN-γ, tumour necrosis factor-alpha (TNF-α) and perhaps other cytokines for activation of resting MAP infected macrophages.

Once the MAP infected macrophage is activated, the phagosome containing MAP is fused with lysosomes containing bactericidal agents and MAP is degraded into smaller fragments or peptides. Major histocompatibility complex (MHC) molecules are surface proteins with a peptide-binding groove that can bind a variety of different antigens. MHC class I molecules are present on almost all nucleated cells, whereas MHC class II molecules are only present on specialised antigen presenting cells, such as macrophages. The MHC molecule binds the foreign peptide in an intracellular location and transport it to the cell surface, where it in combination with a ligand can be recognised by T cells (Janeway et al., 2005). The activated macrophage present MAP antigens associated with MHC class I and II on the cell surface to CD8+ and CD4+ T cells, respectively. As a result, CD4+ T helper type 1 cells (Th1) are activated and produce a range of cytokines, including IFN-γ. The Th1 cytokines signal through a
network of receptors and start a cascade of reactions characterising the CMI response (Coussens, 2001). One of the produced cytokines, are interleukin 12 (IL-12) produced by activated macrophages and T\textsubscript{H}1 cells that act to further stimulate IFN-γ production by antigen activated T\textsubscript{H}1 cells (Collins et al., 1999). In fact, two proinflammatory cytokines IL-12 and interleukin 18 (IL-18) play a significant role in the induction of IFN-γ production in T cells, B cells, NK cells and dendritic cells (Tanaka et al., 2005).

As the infection progresses, the CMI response wanes and an antibody-mediated CD4\textsuperscript{+} T helper type 2 (T\textsubscript{H}2) response becomes predominant. Antibody production provides little, if any, protection against MAP and the infection expands without the important T\textsubscript{H}1 cell subset, which is considered to be necessary for protective immunity (Toman et al., 2003). Therefore it appears that IFN-γ producing T\textsubscript{H}1 cells recognizing peptide antigens are the major mediators of specific immunity early in the infection. Hence, specific T cell responses can be exploited for early detection of infection whereas assays of humoral responses may be relevant for detecting the later stages of MAP infection (Stabel, 2000). However, the T\textsubscript{H}1-T\textsubscript{H}2 switch paradigm for MAP immune responses has been questioned as studies with calves (Koets et al., 2001; Waters et al., 2003) and goats (Begg et al., 2011) experimentally infected with MAP have indicated that antibodies and IFN-γ responses are present simultaneously.

Cytokine and chemokine production is critical for development of effective inflammatory and immune responses to mycobacterial infections and these responses must be carefully controlled to limit local tissue damage and prevent systemic inflammatory responses (Weiss and Souza, 2008). For these reasons, both pro-inflammatory and anti-inflammatory cytokines are produced to regulate inflammatory responses and regulatory T (Treg) cells limit the extent of the immune response (de Almeida et al., 2008). Treg cells are characterised by expression of the surface markers CD4 and CD25 and expression of forkhead box P3 (Foxp3) transcription factor and can be further divided into IL-10 producing Tr1 cells and transforming growth factor beta (TGF-β) producing T helper cell type 3 (T\textsubscript{H}3 cells) (Belkaid, 2007). The loss of CMI responses, and hence pro-inflammatory responses, in cattle naturally infected with MAP have been connected with development of Treg cells that effectively limit effector T cell responses (Coussens, 2004; Buza et al., 2004; Weiss et al., 2005b). However, the role of Tregs in MAP infections remains to be further investigated. Another recently indentified T\textsubscript{H}1 cell population with possible importance for paratuberculosis is the T helper cell type 17 (T\textsubscript{H}17 cells). T\textsubscript{H}17 cells are characterised by production of interleukin 17 (IL-17) and might be key inducers of inflammation and tissue damage in human tuberculosis (Torrado and Cooper, 2010) and other infectious and autoimmune diseases.
1.3. Diagnosis
Several types of diagnostic tests for paratuberculosis are available: these are agent detecting tests (culture of faecal or tissue samples), detection of MAP-specific antibodies (serology), detection of MAP-specific CMI responses (CMI based tests) or detection of MAP-specific nucleic acids (polymerase chain reaction, PCR) (Collins, 1996).

The true state of MAP infection can only be established through culture of multiple tissues. However, bacterial growth is slow with test results being available only after months of incubation, and up to 100 tissues may be required to establish the infection status of an animal (Whitlock et al., 1996). Further, shedding of MAP at detectable levels in faeces is irregular (Nielsen and Toft, 2008). Therefore immune-based diagnostic tests are relevant alternatives to faecal culture (FC) and several tests measuring either specific CMI or antibodies have been developed as described in Paper III, which is a review of MAP antigen candidates.

1.3.1. Challenges and limitations of MAP diagnostic tests
1.3.1.1. Sensitivity and specificity issues
Two characteristics that are used to describe the validity of a test are sensitivity and specificity. The sensitivity is the proportion of animals with the condition, which is to be diagnosed, that will be classified as positive and the specificity is the proportion of animals without this condition that will be classified as negative. The true state of infection is described by the reference standard (previously referred to as “gold standard”), which is a test or procedure that perfectly describes the target condition. For paratuberculosis there is no reference standard and in reality, few true reference standards exist or otherwise they require that the animal is dead (Gardner et al., 2011). The sensitivity and specificity for MAP diagnostics tests vary significantly with infection stage (Nielsen and Toft, 2008)

1.3.1.2. Age of animals
Before using any diagnostic tests for detection of MAP the age of the animals should be considered. Because MAP is a chronic infection with a long incubation time and slow development, age will influence the test response. The sensitivity and specificity of diagnostic tests are therefore greatly influenced by the age at testing. For the PPDj IFN-γ test, it has been demonstrated that animals younger than 15 months of age are likely to produce false-positive IFN-γ test results (Jungersen et al., 2002) and animals less than one year have widely fluctuating IFN-γ test results when tested repeatedly (Huda et al., 2004). The observed unspecific IFN-γ production of calves might be produced by natural killer (NK) cells (Boysen and Storset, 2009) or γδ-T cells (Kampen et al., 2006) that are present in high numbers in young animals. The time at which MAP infected cattle seroconvert has been investigated, and showed that cows have the highest probability of testing milk ELISA positive at 2.5 to 4.5 years of age (Nielsen and Ersbøll, 2006). Sensitivity of enzyme-linked immunosorbent assay (ELISA) has been estimated to 0.06 for cows two years of age and 0.5
at five years of age, while specificity was 0.997 at two years of age and 0.93 at five years of age (Nielsen and Toft, 2006). The highest probability of testing FC positive was 2.5 to 5.5 years of age (Nielsen and Ersbøll, 2006). Sensitivity of FC was 0.05 for cows two years of age and 0.21 at five years of age, while specificity was high, ranging 0.964 to 0.984. Hence, sensitivity of ELISA is higher than for FC, but at early age sensitivity is low for both tests.

1.3.1.3. Stage of infection at sampling for diagnosis
Different diagnostic tests can be used at different stages of MAP infection. The stage of MAP infection is related to the age of animals, as cattle are thought to be most susceptible to infection as calves (Doyle, 1953). Figure 2 summarise the various stages of MAP infection, which is related to the age of animals, transmission of MAP bacteria and how the diagnostic tests ELISA, FC and IFN-γ perform at these infection stages.

![Diagram presenting the various stages of infection, transmission of Mycobacterium avium subsp. paratuberculosis bacteria and the utility of diagnostic tests (based on figure in Nielsen and Toft, 2008).](image-url)
1.3.1.4. Blood sampling for IFN-γ assay

The IFN-γ assay is a whole-blood proliferation assay, in which blood is cultured overnight with MAP antigens followed by collection of supernatants (Wood et al., 1989). Bovine tuberculosis and paratuberculosis were the first infections for which this test was used for in veterinary medicine (Wood et al., 1989; Billman-Jacobe et al., 1992; Rothel et al., 1992). Culture with antigens should be initiated within eight to twelve hours of blood sampling to ensure viability of IFN-γ producing T cells (Rothel et al., 1992). Under field conditions this time limitation from collection to culture is a problem that limits the utility of the IFN-γ test. A protocol with day-old blood samples and co-culture with recombinant bovine IL-12 has been developed as an alternative to the conventional IFN-γ protocol using fresh blood samples. In this modified protocol, start of culture with antigens should be initiated within 20 hours of sample collection.

1.4. Neopterin

Neopterin is synthesised in vitro by monocyte derived macrophages and dendritic cells in response to stimulation with IFN-γ and is considered an excellent marker of cellular immune activation (Hamerlinck, 1999; Wirleitner et al., 2002). Neopterin belongs to the chemical group known as pterins and was named neopterin when it was first isolated to denote that it might start a new (Greek, neo) epoch in pteridine research. Neopterin is a substance of low molecular mass, derived from guanosine triphosphate (GTP) catabolism by GTP cyclo-hydroxylase I (Hamerlinck, 1999). IFN-γ was until recently considered to be the only cytokine that could induce significant production of neopterin, but in vitro experiments suggested that also interferon-alpha (IFN-α) and interferon-beta (IFN-β) can induce neopterin synthesis of dendritic cells (Wirleitner et al., 2002). Detection of neopterin instead of direct measurement of IFN-γ has various advantages, since IFN-γ is subject to fast degradation and is able to bind to soluble or cell bound receptors, the measured IFN-γ may not represent the actual freely available IFN-γ level. Neopterin is biochemically inert and stable because its half-life in the human body is solely due to renal excretion (Hamerlinck, 1999). Furthermore, neopterin is synthesized one step further down the activation pathway – eliminating the noise from unspecific IFN-γ production by NK- and γδ-cells.

High neopterin levels are observed in different inflammatory diseases and certain malignancies and can be measured in serum and urine (Hamerlinck, 1999). Routine neopterin screening is commonly used in human medicine. As an example, Austrian blood donations have been screened since 1994, and those with elevated levels are excluded for transfusion (Renneberg et al., 2006). The use of neopterin assays in veterinary medicine is limited and has so far only been approached by a few authors. They have measured neopterin in cattle, pigs, llamas, dogs, cats, rabbit and rats (Stang and Koller, 1998; Breinekova et al., 2007). Neopterin is measured by high performance liquid chromatography (HPLC), radioimmunoassay (RIA) and by competitive ELISA.
The IFN-γ test needs to be optimized before it can be used as an early and specific diagnostic tool that can detect MAP exposed calves before they become MAP infectious cows. Optimization might be achieved by inclusion of novel, MAP-specific antigens in the IFN-γ assay as an alternative to PPDj. Furthermore, the use of day-old blood samples in the IFN-γ assay in combination with novel, MAP-specific antigens would improve the utility of the IFN-γ test as blood samples can be received by regular postal service. Furthermore, detection of neopterin may be a more specific indicator of MAP–specific CMI responses compared to detection of IFN-γ.
2. Hypotheses

The work in this thesis is based on the following hypotheses:

a) It is hypothesized that infection with MAP is characterised by early development of CMI responses, which are then replaced by a serological response, which have no effect against MAP infection. Early stage MAP infection of cattle can be detected by measuring CMI responses, such as IFN-γ production of whole blood samples following culture with MAP antigens.

b) Furthermore, it is hypothesized that well-defined MAP antigens can serve as alternatives to PPDj for use in the IFN-γ assay and give early and specific detection of MAP infected cattle. Well-defined antigens can be selected on genome level and based on absence from MAA and M. bovis genomes and experimental knowledge from in vitro and in vivo expression studies with M. tuberculosis or immune recognition of orthologue M. tuberculosis proteins in mice.

c) It is also hypothesized that day-old blood samples can be used in a modified IFN-γ assay protocol as an alternative to fresh blood samples, which is used for the conventional IFN-γ assay protocol. The modified protocol use day-old blood samples and includes co-culture with cytokine IL-12 or anti-IL-10 antibodies, which block IFN-γ reducing effects of the cytokine IL-10.

d) Finally, it is hypothesized that levels of neopterin in cattle serum can be related to the paratuberculosis status of the individual animal. Detection of neopterin could be an alternative to IFN-γ detection as an indicator of CMI in whole blood samples with better specificity than IFN-γ, as neopterin is synthesized one step further down the activation pathway.

These working hypotheses were investigated through studies within four categories:

I) Association between IFN-γ responses and milk antibody of dairy cattle
II) Review of characterised MAP antigens
III) Development, optimization and evaluation of the IFN-γ assay including novel antigens and use of day-old blood samples
IV) Neopterin detection in serum samples from cattle with different MAP status
3. General materials and methods

3.1. Cattle herds
Samples were collected from 22 cattle herds for the studies presented in this thesis. Figure 3 presents an overview of the herds numbered 1 to 22 that were used for studies of paper I, II, IV and V.

Fig. 3. Diagram presenting an overview of the 22 cattle herds included in this thesis and used for studies presented in Papers I, II, IV and V. Furthermore, the number of cattle from each herd is presented. The herds are numbered 1 to 22. The blue boxes show criteria for cattle herd selection.

Herd no. 1-18 included 975 cows from 18 Danish dairy herds and samples were collected during a period of three years through August 2003 to December 2007. A detailed description of the herds and how they were selected is presented in Paper II.

For studies presented in Papers IV and V a non-infected and a MAP infected herd were needed. Two herds were selected: a presumed non-infected herd (herd no. 20), and a herd in which MAP infection had been confirmed by cultivation of MAP (herd no 21). Descriptions and history of the herds are presented in Papers IV and V. Herd no. 19 was selected based on the same criteria as herd no. 20. Herd no. 22 which is included in the studies presented by Paper I, was selected based on a history of clinical paratuberculosis cases.
3.2. IFN-γ assay
The IFN-γ assay is a whole-blood proliferation assay, in which blood is cultured overnight with MAP antigens followed by collection of supernatants (Wood et al., 1989). Supernatants are harvested and IFN-γ levels in supernatants are then quantified by an IFN-γ specific ELISA described in section 3.3. ELISA responses are recorded as optical density (OD) values.

Johnin purified protein derivative (PPDj), which is a crude undefined extract of MAP antigens, is often used for culture in the IFN-γ assay (Jungersen et al., 2002). As PPDj is not commercially available, other closely related mycobacteria PPD’s such as PPDa prepared from MAA and PPDb prepared from M. bovis have often been used as antigens in the test. The IFN-γ response to PPDa relative to the response to PPDb have then been used to examine MAP-specific CMI responses (Jungersen et al., 2002) such as the IDEXX criterion according to which the ratio PPDb/PPDa of less than 0.71 have been interpreted as specific responses (McDonald et al., 1999; Huda et al., 2003).

3.3. IFN-γ specific ELISA
The IFN-γ secretion in supernatants was analysed by an in-house sandwich ELISA, described in detail in Paper II and used in Papers II, IV and V. In this sandwich ELISA the capture antibody is coated onto the microtiter plates (a monoclonal antibody against IFN-γ F(ab)2 fragment), followed by addition of the supernatants with secreted IFN-γ. In the next step, an enzyme-linked capture antibody (biotinylated monoclonal antibody against IFN-γ) is added followed by substrate, which is converted by the enzyme into a measurable form. The level of IFN-γ (pg/ml) was calculated using linear regression on log-transformed readings from two-fold dilution series of a standard with known IFN-γ concentration. The standard dilution series was included on all plates.

3.4. Quality control criteria of IFN-γ assay
Besides MAP-specific antigens, a positive and a negative control are included for culture in the IFN-γ assay. For the studies presented in this thesis the superantigen Staphylococcus enterotoxin B (SEB) was used as a positive control and phosphate buffered saline (PBS) was used as a negative control. Superantigens are produced by many different pathogens and are recognised by T cells without being processed into peptides though MHC pathways. As the name implies superantigens are able to induce a massive production of cytokines by CD4+ T cells, including production of IFN-γ (Janeway et al., 2005). Hence, inclusion of SEB for stimulation of blood is a control to ensure that blood cells are alive and able to produce IFN-γ. IFN-γ responses to positive and negative controls were used for quality control of blood samples to decide whether samples should be excluded or included in the studies in Papers IV and V. Furthermore, the value of IFN-γ produced against the negative control PBS was subtracted the values of IFN-γ produced against antigens of each individual animal because
all antigens were diluted in PBS. Criteria for exclusion or inclusion of samples based on positive and negative controls are presented in the flow diagram in Figure 4. The detailed IFN-γ test protocol is described in Paper IV and the use of day-old samples in this protocol is described in Paper V.

Fig. 4. Quality control criteria for inclusion or exclusion of samples from studies presented in Papers IV and V, based on values of IFN-γ production against positive (SEB) and negative (PBS) controls. Numbers of excluded samples, stratified on the Mycobacterium avium subsp. paratuberculosis (MAP) non-infected and the MAP infected herd presented in Papers IV and V, are listed in the red boxes.
3.5. Antigens for IFN-γ assay

The crude antigen PPDj, which is widely used for culture in the IFN-γ assay, has been prepared from different strains in different laboratories and no standardised protocol has been established. In many cases there is no information regarding the history of the strains used, while other strains have been passaged continuously in the laboratory for more than 70 years (Semret et al., 2006). PPDj might cross-react with environmental mycobacteria such as the closely related (MAA) leading to low specificity of the IFN-γ assay (Magnusson, 1961). Figure 5 illustrates, that PPDs are complex mixtures of a large number of proteins and no clear bands are visible in lanes with PPDj. Identification of well-defined and MAP-specific antigens, which are both highly immunogenic and specific in IFN-γ assay, are therefore needed. PPD, also known as tuberculin, was discovered by the German medical bacteriologist Robert Koch in 1890 and presented as a remedy for tuberculosis which was a widespread and feared disease at the time. However, PPD lacked clinical efficacy but could instead be used for diagnostic purposes in the tuberculin skin test (Gradmann, 2006).

Paper III summarise antigens for CMI assays that have been described to date, and in Paper IV 14 novel recombinant antigens are tested and evaluated. The same 14 antigens are used in Paper V and tested with day-old blood samples.

![Image of PPD derivatives](image.png)

**Fig. 4.** Silver staining of three purified protein derivatives (PPD) from *Mycobacterium avium* subsp. *avium* (PPDa), *M. bovis* (PPDb) and *M. avium* subsp. *paratuberculosis* (PPDj). Lane M: marker of protein size (kDa), the following lanes show 5 µg, 2.50 µg and 1.25 µg PPD of each type.
3.6. Flow cytometry

To investigate the effect of IL-12 potentiation of day-old blood samples from cattle a study using flow cytometry was planned. Potentiation of blood samples with IL-12 may stimulate other cells such as NK cells and γδ T cells to unspecific IFN-γ production besides primed T cells and give false positive IFN-γ reactions. By use of flow cytometry the composition of the cell population in a given sample can be investigated. The various cells types are characterised by their surface receptors and can be visualised by adding receptor-specific antibodies with a fluorescent marker (a fluorochrome). Each fluorochrome emits light when hit by laser light of a particular wavelength. Individual cells of the sample are then passed through one or more laser light beams that cause light to scatter and fluorescent. At the point where the cells passes through the light a detector measure forward scatter (FSC) and several another detectors measures side scatter (SSC). Hence, three types of data are measured, cell size (FSC), cell complexity or granularity (SSC) and fluorescent labelling. Several different cells and fluorochromes can be measured in the same sample.

Intracellular proteins, such as IFN-γ, can be visualised by permeabilising the cells followed by addition of IFN-γ antibody labelled with a fluorochrome. In the present protocol, cells were intracellular stained with IFN-γ combined with various surface staining, giving data of relative IFN-γ production of the different cell types. The IFN-γ test and subsequent ELISA described above were performed in parallel.

Blood samples were collected from cattle experimentally infected with MAP and non-infected cattle at Queens University, Agri-Food and Biosciences Institute, Veterinary Sciences Division, Belfast, UK. Blood samples were collected several times from 12 MAP infected cattle 14 to 20 weeks post infection and from six non-infected control cattle. Both flow cytometry analysis and IFN-γ testing were performed on fresh samples without IL-12 potentiation and on day-old samples with IL-12 potentiation.

The principle protocol for intracellular IFN-γ staining was described by Riber et al. (2011). In the study presented here, whole blood was stimulated with SEB, PBS and PPDj as fresh samples or as day-old samples with IL-12 followed by overnight culture. The next day peripheral blood mononuclear cells (PBMC)s were isolated, adjusted to $1 \times 10^7$ cell/ml and cells were stained with primary monoclonal antibodies against CD2 (clone MCA833F, AbD Serotec, Oxford, UK), CD4 (clone IL-A11, WMRD, Pullman, WA, USA), CD8 (clone ILA-51, WMRD), WC-1 on γδ T cells (clone MCA838S, AbD Serotec) and NKP46(CD335) on NK cells (clone AKS-1, a kind gift from Anne Storset, Norwegian School of Veterinary Science, Oslo, Norway). Secondary antibodies were anti-IgG2a conjugated with Alexa Flour 647 (Invitrogen, Paisley, UK) and streptavidin conjugated with PerCP (BD Biosciences, Broendby, Denmark). Cells were permeabilized and intracellularly stained with IFN-γ antibody (clone MCA1783, AbD Serotec) or with an isotype control antibody (DakoCytomation, Glostrup, Denmark). Fix and Perm solution (BD Sciences) were used for fixation and permeabilization. Flow cytometry analysis was performed on a FACSCanto II with DIVA software (BD Biosciences) at
the National Veterinary Institute, Copenhagen and a FACS Dako MoFlo (DakoCytomation) at the Veterinary Sciences Division, Belfast.

3.7. Neopterin

Neopterin levels in culture supernatants from six cattle in different clinical stages of disease were investigated. A commercial Neopterin ELISA kit was used according to the instructions of the manufacturer (IBL, Hamburg, Germany). The ELISA was a competitive ELISA, in which the unknown amount of neopterin in the samples competed with a fixed amount of enzyme labelled neopterin for the antibody-binding site of rabbit-anti-neopterin. The kit included a neopterin standard solution ranging 1.35-111 nmol/L.
4. General results

4.1. Use of johnin PPD IFN-γ assay in control of bovine paratuberculosis (Paper I)

Different target conditions for diagnostic testing of MAP were evaluated in the second part of Paper I. The diagnostic target condition and the purpose of the diagnostic testing should be considered before applying any diagnostic test for paratuberculosis to give meaningful estimates for sensitivity and specificity. For each of the five previously defined target conditions (Nielsen and Toft, 2008): affected, infectious, infected, non-infected and exposed young stock, the purpose of testing and the use of available diagnostic tests were evaluated. For evaluation of MAP infection in exposed young stock, the IFN-γ assay is the only useful test because FC and antibody ELISA have sensitivities approximating zero for this animal group.

For the conventional IFN-γ test, cultures of whole blood samples with antigen should be initiated within eight hours of blood sampling (Rothel et al., 1992) because IFN-γ responses of blood samples are weakened when time from sampling to culture is extended. In Paper I addition of cytokines IL-12 or IL-2 to cultures of day-old blood were investigated with samples of two MAP infected herds. Addition of IL-12 could be used to rescue IFN-γ responses to PPDj stimulation of day-old blood samples, but addition of IL-2 gave high levels of non-specific IFN-γ levels in cultures with PBS stimulation, which has to be used as a negative control. This modified protocol including IL-12 was applied for analysis of blood samples of 18 MAP infected herds that were pioneers in a paratuberculosis eradication programme. The IFN-γ response to PPDj was significant different in the MAP infected herds compared to two non-infected herds.

4.2. Association between milk antibody and IFN-γ responses in cattle from MAP infected herds (Paper II)

IFN-γ test results obtained prior to first calving were correlated to later results of the milk antibody ELISA test. The ELISA test result was considered positive if the corrected optical density (ODc) was ≥0.3 as used in the Danish paratuberculosis control programme (Nielsen, 2007). Highest predicted ELISA result was obtained for the group of animals that were FC positive and IFN-γ negative, but the difference between IFN-γ positive (range 0.19-0.26 ODc units) and IFN-γ negative (range 0.21-0.30 ODc units) was small. The predicted ELISA test result increased slightly with age at ELISA for all groups of cows.

4.3. Review of MAP antigen candidates with diagnostic potential (Paper III)

This review suggests that the IFN-γ assay and other assays measuring CMI responses are limited by the lack of MAP-specific antigens included in these assays, resulting in poor sensitivity and specificity. In the paper, relevant information of 115 different MAP antigens
were systematically extracted from literature and summarised in 6 tables of CMI antigens, secreted antigens, cell wall and membrane antigens, lipoprotein antigens, heat shock antigens and hypothetical antigens. Of these antigens, 27 different antigens have been evaluated for their use in CMI based diagnostic assays and no obvious diagnostic candidates have been described. CMI antigens included both purified native proteins and recombinant antigens candidates. All purified proteins, were described to recall CMI responses in vitro but all, except AphC and AphD (Olsen et al., 2000), lacked MAP specificity because of orthologues in other mycobacteria species. Of the described recombinant antigen candidates only one, HspX (Bannantine and Stabel, 2000), were described to be specifies specific but unfortunately this antigens could not recall an in vitro CMI response. Hence, several antigens were reported to be immunogenic and could recall CMI responses, but they lacked species specificity compared to mycobacteria such as MAA and M. bovis. A general problem was that all studies, except three (Koets et al., 1999; Olsen et al., 2005; Shin et al., 2005), evaluated the characterised CMI antigens in a low number of animals. Validation of immunogenicity of antigen candidates requires an appropriate sample size otherwise the results cannot be applied to herd conditions with genetically heterogeneous animals demonstrating different stages of MAP infection. To calculate the appropriate sample size of a study with the aim to test a difference between means of two groups, four parameters are needed: significance level, power, a definition of expected difference and standard deviation (Houe et al., 2004).

4.4. Novel antigens for detection of CMI responses to MAP infection in cattle (Paper IV)

To investigate the use of novel antigens in the IFN-γ assay, 14 Novel MAP antigens were selected and produced as recombinants and evaluated repeatedly with blood samples of 30 heifers from a MAP infected herd. Cut-off levels of the 14 novel antigens, Ag85B and PPDj were determined based on IFN-γ results obtained with samples from a MAP non-infected herd. The infected and non-infected herd showed significant different (P<0.05) IFN-γ responses to all the novel antigens investigated.

To evaluate immunogenicity and diagnostic specificity of antigens, a case definition was defined as an animal with ≥ two positive tests for ≥ four antigens resulting in 13 cases and 13 non-cases. IFN-γ levels of cases were significantly (P<0.05) higher than IFN-γ levels of non-cases. IFN-γ test results to PPDj showed low correlation to results obtained with the novel antigens and PPDj gave high IFN-γ responses of samples from animals of both the infected and non-infected herd. However, the difference between IFN-γ responses of the infected and non-infected herd was significant (P=0.0001).

Three latency proteins, LATP-1, LATP-2 and LATP-3 showed highest immunogenicity and diagnostic specificity and the differentiation between cases and non-cases were highly significant for this group. Three of the tested antigens, LATP-2, MAP-1 and MAP-2 had no
homologue sequence in the MAA or M. bovis genomes and correlated highly with the case definition. Consistency of IFN-γ responses to antigens was presented as the number of animals with three and zero positive tests for each antigen. The ESAT-6 family antigen ESAP-4 showed highest consistency, as 18 animals had zero positive tests against this antigen, followed by MAP-1 and MAP-2 with 13 animals with zero positive tests. Consistency of IFN-γ responses to PPDj was low, as nine heifers had zero positive tests and four heifers had three positive tests against PPDj. The gene number for the different antigens is not yet revealed due to IPR issues and the possibility of applying these antigens in a novel diagnostic test.

4.5. Correlation of antigen-specific IFN-γ responses of fresh blood samples from MAP infected cattle with responses of day-old blood samples co-cultured with IL-12 or anti-IL-10 antibodies (Paper V)

To investigate the use of day-old blood samples for the IFN-γ test including novel antigens for stimulation, the same 14 novel antigens, Ag85B and PPDj were applied for stimulation of blood samples in Paper V of the same 30 heifers as used in Paper IV. The work for these two Papers was done in parallel. The correlation coefficient, measured as Spearman’s ρ, between IFN-γ results to antigens of sample day samples and IFN-γ results to antigens of day-old samples added recombinant bovine IL-12 or anti-bovine IL-10 antibodies were examined. The case definition of animals that was defined for analysis of fresh blood samples, for calculation of immunogenicity and specificity of antigens, was also applied for the analysis of day-old samples, but as different animals were excluded in the three test types, the number of animals differed. Valid results for the day-old samples with IL-12 included 10 cases and 13 non-cases, and the day-old samples with anti-IL-10 antibody included 11 cases and 13 non-cases. Generally, there were fewer test positive day-old samples with IL-12 compared to day-old samples with anti-IL-10 antibodies.

IFN-γ responses to antigens of the non-infected herd were considerably higher for day-old samples with IL-12 compared to day-old samples with anti-IL-10, which resulted in high cut-off values for antigens using day-old samples with IL-12. Only two antigens gave statistical significant different IFN-γ responses of samples from the non-infected and infected animals using the day-old protocol with IL-12. Immunogenicity and diagnostic specificity of the 14 novel antigens, Ag85B and PPDj were generally low for day-old samples compared to sample day samples.

Correlation of blood samples cultured on the day of sampling was high (ρ ≥ 0.80) for three antigens, ESAP-2, ESAP-3 and Ag85B compared to day-old blood samples cultured with IL-12 and high (ρ ≥ 0.80) for four antigens, LATP-2, LATP-3, MAP-2 and MAP-3 compared to day-old blood samples cultured with anti-IL-10 antibodies. Correlation of sample day samples and day-old samples stimulated with PPDj were 0.78 for the day-old protocol with IL-12 and 0.75 for the day-old protocol with anti-IL-10.
Immunogenicity of antigens were generally low for both day-old protocols and resulted in low Youden indices, which combines immunogenicity and diagnostic specificity. A Youden index of 0.5 indicates that the diagnostic test has no value. The antigens with the highest Youden indices were the latency antigen LATP-2 (0.80) using IL-12 and latency antigen LATP-1 (0.74) using anti-IL-10 antibodies in the day-old protocol. PPDj gave low Youden indices of 0.20 and 0.29 using day-old protocols, due to very low immunogenicity in both protocols.

4.6. Flow cytometry analysis of day-old samples with IL-12 (unpublished)
This study, with the aim to investigate the effect of IL-12 potentiation of day-old blood samples from cattle experimentally infected with MAP, was only partly conducted. Testing and development of this protocol using blood samples of non-infected cattle at the National Veterinary Institute showed staining of the various cell subsets and of intracellular IFN-γ. In these preliminary studies, the various cell subsets were successfully stained. However, due to technical difficulties and time limitations the study with cattle experimentally infected with MAP did not succeed and no further work is described in this thesis.

4.7. Neopterin detection in vitro as an alternative to IFN-γ assay (unpublished)
A pilot study was conducted for in vitro detection of neopterin as an alternative to IFN-γ as an indicator of CMI. The advantages of detection of neopterin over IFN-γ are that neopterin is more stable and is synthesized one step further down the activation pathway – eliminating the noise from unspecific IFN-γ production by NK- and γδ-cells (Hamerlinck, 1999). Neopterin levels were measured using a commercial Neopterin ELISA kit (Fig. 6A) of stored culture supernatants from six cattle that had been tested by IFN-γ test of supernatants (6B), milk ELISA to measure IFN-γ specific antibodies (Fig. 6C) and by faecal culture to measure shedding of MAP bacteria (Fig. 6D). The samples were collected from cattle in age groups 4 to 55 months of age. Only one study has previously measured neopterin values of cows giving an average value of 2.8 nmol/L (n=18) and of a bull giving a value of 3.44 (Stang and Koller, 1998).

In Figure 6A, a neopterin value of 2.8 nmol/L is indicated by the horizontal dotted line and only cow no. two had a neopterin value above this level, which was 5.3 nmol/L measured at 21 months. At the same time point, a high IFN-γ level (1916 pg/ml) was measured and remained high for the next to sample times, and the cow tested positive at five sample times by milk ELISA and faecal culture positive at the following sampling. Cow number one, two and four tested positive by IFN-γ, milk ELISA and FC and cow no. three tested positive by IFN-γ and FC, but none of these animals produced elevated neopterin levels. Cow five and six did not test positive in any tests, including neopterin. Consequently, neopterin could not be used to discriminate between infected and non-infected animals and no further work was done using the neopterin test.
Fig. 6. Neopterin (nmol/L) values (A) of six cows with different paratuberculosis status measured in supernatants (n= 5-8 samples) at different ages (months). IFN-γ results (B), milk ELISA results (C) and faecal culture results (D) of the same cows. The dotted line in each figure represents the cut-off value to differentiate test positive and test negative samples.
5. General discussion

5.1. Use of johnin PPD IFN-γ assay in control of bovine paratuberculosis (Paper I)

Paper I discusses the issue of establishing the diagnostic target condition and the purpose of testing prior to using and interpreting results of any diagnostic test for paratuberculosis because these parameters may have considerable impact on the utility of the tests. For paratuberculosis, five target conditions were previously defined as: affected, infectious, infected, non-infected and exposed young stock (Nielsen and Toft, 2008). The utility of available diagnostic tests for each of these target conditions are described.

Detection of animals that are affected by paratuberculosis is most often not difficult as both ELISA and FC have high sensitivities and specificities for this condition and in general, sensitivity of ELISA assays for milk or serum increases with progression of disease. Accurate diagnosis of animals in the group that are infectious is more difficult. This group of animals may shed MAP bacteria in high numbers to the environment but shows no clinical signs and no noticeable reduction in milk production. However, diagnosis of the infectious animals can be achieved by frequent FC and used to break transmission of MAP within the herd, but may be costly. The infected animals, which are still not infectious or affected, are even more difficult to diagnose, because FC and ELISA have low sensitivities and the utility of the PPDj IFN-γ assay is modest due to low sensitivity and specificity. The purpose of identifying infected animals should be considered carefully, because some animals may be able to control and potentially eliminate the infection without ever becoming infectious as indicated by results presented in Paper II. The study presented in Paper II included 975 dairy cows, and a large proportion of the heifers tested IFN-γ positive but did not test positive by ELISA or FC as cows. The purpose of identifying infected animals could be identification of animals with low paratuberculosis prevalence prior to trade and possibly culling of animals. However, culling of animals with strong CMI may select for a herd with low CMI capacity. Instead, animals with a positive IFN-γ assay result could be kept under close surveillance and tested repeatedly to follow the IFN-γ status for early identification of the few animals that will become shedders. Paratuberculosis is a slowly developing, chronic infection that may be cleared by immune responses, or the bacteria may hide in tissues without activation of immune responses and there is no available diagnostic test that can certify an animal as non-infected. However, MAP infection status of the exposed young stock may be established by use of the IFN-γ assay with the purpose of evaluation of in-herd transmission and in the future to certify herds as MAP free. To do this, an optimized IFN-γ assay protocol including novel and specific MAP antigens is needed.

Paper I also describe the use of day-old blood samples for the IFN-γ assay including recombinant bovine IL-12 as an alternative to the conventional IFN-γ assay protocol with fresh blood samples and in part fulfil hypothesis c). The results indicated that the IFN-γ assay is reasonably MAP-specific and IFN-γ responses of 717 heifers from 18 MAP infected herds were significant different from 101 heifers from two non-infected herds. Herds infected
with MAP were tested repeatedly by IFN-γ assay and may emphasise that the IFN-γ assay is not suitable for diagnosis of individual animals. Instead, the IFN-γ assay should be considered as a herd test to establish MAP status in a herd to assist herd owners in decisions of management procedures.

5.2. Association between milk antibody and IFN-γ responses in cattle from MAP infected herds (Paper II)
In Paper II the IFN-γ test results obtained prior to first calving were demonstrated to be correlated to later results of the milk antibody ELISA test. However, animals with a negative IFN-γ test on average had a higher OD₃₅ value of the ELISA. Although statistically significant, the IFN-γ test only slightly affected the future antibody status. Thus, only some of the IFN-γ positive heifers developed a positive antibody response against MAP as cows. This may indicate that some animals are able to defeat MAP infections perhaps by CMI responses or it may indicate that CMI responses keep the infection under control and therefore antibodies are not produced. In hypothesis a) it was stated, that infection with MAP is characterised by early development of CMI responses, which are then replaced by a serological response. However, the results of the study presented in Paper II, suggest that hypothesis a) may not be correct and animals that tested positive by IFN-γ in early age, may not necessarily become antibody ELISA positive as cows. Even more surprising, results of Paper II showed that some of the IFN-γ negative calves developed a positive antibody ELISA response as cows. A possible explanation for this could be that a number of animals were tested false-positive in the IFN-γ assay or perhaps the infection had advanced to another stage and no CMI responses could be detected at the time of sample collection, but this is all speculations.

The predicted OD₃₅ value of ELISA positive cows showed the highest increase at the age 2.5-3.5 years and three of the tested groups became ELISA positive in this period. This agrees with previous findings, that the probability of testing ELISA positive were highest from 2.5 to 4.5 years of age (Nielsen and Ersbøll, 2006). Sensitivity and specificity of the ELISA for detection of MAP infected animals vary considerably with age (Nielsen and Toft, 2006). The study was observational and herds were non-randomly selected. However, the 975 cows included in the study were a random selection of animals present in the herds at the time of sampling and may consist of a representative population of these herds. All included herds were MAP infected and therefore specificity of the tests was not evaluated. Another limitation of the study was that relatively few animals were FC positive (5.2%) and as a consequence the distribution of FC positive and FC negative animals were skewed. The overrepresentation of FC negative animals from these MAP infected herds may be due to low sensitivity of FC in animals tested below two years of age or perhaps the tested animals were actually negative. The results of Paper II suggest that MAP infected animals may not
necessarily be characterised by early presence of CMI responses followed by later development of MAP antibodies as stated in hypothesis a). The course of MAP infection may be very complex and the course of infection may vary from animal to animal. However, the IFN-γ assay is the only available test that can be used for young animals before they become infectious to other animals in the herd. Consequently, it might become an important decision support tool for cattle practice advisers in spite of poor specificity of the test. Further refinement of the test is therefore warranted.

5.3. Review of MAP antigen candidates with diagnostic potential (Paper III)

Based partly on hypotheses a) and b) a literature review was conducted in Paper III to investigate if promising diagnostic antigens that were immunogenic and specific for MAP had been described. MAP antigen candidates, with diagnostic potential were evaluated, with special emphasis on antigens for inclusion in CMI based tests. At present, the IFN-γ assay and other assays measuring CMI responses are limited by the lack of MAP-specific antigens included in these assay resulting in poor sensitivity and specificity. For CMI based diagnostic assays, 27 different antigens were evaluated and no obvious diagnostic candidates were described. Based on these findings, hypothesis a) can neither be accepted nor rejected, but hypothesis b) can be accepted as true, because several antigens could induce CMI responses. However, the most promising antigens that were immunogenic and MAP-specific, remains to be further validated before any conclusion regarding their utility in a diagnostic test can be made.

Different types of antigens such as: secreted antigens, cell wall and membrane antigens, lipoprotein antigens, heat shock antigens and hypothetical antigens were included in the review. Secreted antigens are expected to be highly immunogenic or immunodorminant due to their presence in the extracellular environment where they are more likely to encounter sensitized immune cells. This was confirmed in a study comparing secreted antigens from supernatants to antigens of intracellular origin, in which serum from infected cattle reacted more strongly to the secreted antigens (Cho and Collins, 2006). Secreted antigens such as Ag85B, which was used in Paper IV and V, were reported to be immunogenic in several studies, but was not specific for MAP.

Similar to secreted antigens, cell wall and membrane antigens are expected to be highly immunogenic due to their accessibility to components of the immune system. The antigens of this group were reported to induce immune responses, but the majority were not specific for MAP. A comparative study of different mycobacteria species concluded that the major differences between these species were in the gene products constituting the cell wall, especially genes encoding the PE and PPE proteins (Marri et al., 2006). These proteins are unique to mycobacteria, and several PPE proteins have been reported to be unique to MAP compared to other member of the Mycobacterium avium complex (Mackenzie et al., 2009).
Two recombinant PPE proteins, MAP1506 and MAP3420c showed positive immunoblot reaction and could be promising diagnostic antigens if not orthologous genes were found in MAA and *M. avium* subsp. *hominissuis* genomes (Newton et al., 2009). However, other MAP-specific PE or PPE proteins may very well be promising diagnostic antigens if they are immunogenic.

Lipids comprise a large part of the mycobacterial cell wall and similar to cell wall antigens, many of these structures have demonstrated high seroreactivity. Three antigen candidates, L5P (Biet, 2008 266 /id), Para-LP-01 (Eckstein et al., 2006) and PstA (Wu et al., 2009) were reported to be absent in MAA, but their diagnostic values remain to be validated with appropriate sample sizes.

Heat shock protein (Hsp) antigens are highly conserved molecules produced by both eukaryotic and prokaryotic cells. Due to their conserved nature Hsps are likely to be immunogenic but are not ideal antigens for diagnostic purposes as cross reaction towards other bacteria are expected. The antigen HspX was actually reported to be MAP-specific, but did not induce a CMI response (Bannantine and Stabel, 2000).

Many hypothetical antigens have been characterised for MAP, but most of these were only evaluated with sera from few animals. In general, the characterised MAP antigens remain to be validated in appropriate sample sizes in order to validate their utility in a diagnostic test. The genome sequence of MAP (Li et al., 2005) and other closely related mycobacteria (Garnier et al., 2003; Semret et al., 2004; Wu et al., 2006) are now available and with new tools for *in silico* analysis and genomic techniques, specific and immunogenic antigens for paratuberculosis diagnosis should be expected soon.

5.4. Novel antigens for detection of CMI responses to MAP infection in cattle (Paper IV)

The use of novel recombinant antigens in the IFN-γ assay was investigated based on hypothesis b). For the study, 14 novel antigens, the well-known Ag85B and PPDj were used for stimulation of whole blood from a non-infected and a MAP infected cattle herd. As a group, latency proteins gave the most promising results with immunogenicity of 0.85 and diagnostic specificity of 1.00 for the three antigens LATP-1, LATP-2 and LATP-3. Latency proteins are known to be expressed during the latent stage of *M. tuberculosis* and are able to induce IFN-γ responses *in vitro* and therefore these proteins may play a significant role in control of latent *M. tuberculosis* infection (Leyten et al., 2006). The heifers were young (10-21 months of age at first sampling) and may have a latent stage MAP infection, which would support the elevated IFN-γ response to this group of antigens. Three of the tested antigens, LATP-2, MAP-1 and MAP-2 had no homologue sequence in MAA or *M. bovis* and all gave differentiated IFN-γ responses to cases and non-cases and could be useful diagnostic
antigens. However, the antigens MAP-1 and MAP-2 had an immunogenicity of 0.62, which is moderately low.

IFN-γ levels of the non-infected herd were generally low and the presumption that this herd is non-infected may indeed be correct. The true MAP status of individual animals for the infected herd was unknown and there is no reference test for MAP, which prevented calculations of diagnostic specificity and sensitivity. However, the infection pressure in this particular herd was high and it is likely that all heifers had been exposed to MAP. Evaluation of immunogenicity should be evaluated in an exposed population and evaluation of specificity should preferably be done in the same population. Therefore, a case was defined based on the results of multiple antigens as: an animal with ≥two positive tests for ≥ four antigens. Based on this, both immunogenicity and specificity could be validated in a MAP exposed population. Diagnosis of MAP infected animals is often based on diagnostic tests such as FC or milk antibody ELISA, but these tests are not applicable until years after infection with MAP (Nielsen and Toft, 2006). As observed in Paper II, heifers that are IFN-γ positive do not necessarily become milk ELISA positive as cows, and some ELISA positive cows had not tested IFN-γ positive as heifers (Mikkelsen et al., 2009). Because of this, it is not possible to wait some years for a true MAP diagnosis to evaluate immunogenicity and specificity of novel antigens. Alternatively, cases could have been defined based on IFN-γ results against PPDj. If this approach had been used, 17 heifers would have been defined as cases excluding heifer 29, that had positive IFN-γ tests results against 15 of the other antigens and including heifers that had positive IFN-γ tests against one, two or three of the other antigens. Because the case definition was based on IFN-γ test results against the 15 antigens, it might be more likely that animals defined as cases had positive IFN-γ test results against the antigens compared to animal defined as non-cases. If an ideal study of immunogenicity and specificity of novel antigens should be designed the first step would be defining the purpose of testing. If the purpose is to detect infected animals, the study should include experimentally infected animals that are known to be MAP infected. However, if the purpose is to detect exposed animals, the study should include animals of herds, which are known to have MAP infection.

PPDj gave high IFN-γ responses in the non-infected herd, which suggests that PPDj is either highly immunogenic or non-specific, but in both cases PPDj is of limited value for diagnostic purposes because of low specificity. In the infected herd, IFN-γ responses to PPDj did not correlate well with responses to the other antigens. This may be due to low specificity of PPDj, but it may also be due to the case definition, as cases were defined based on IFN-γ responses to multiple antigens except PPDj. Because PPDj was not included in the case definition, cases were less likely to have a positive IFN-γ test result to PPDj compared to the other antigens. On the other hand, PPDj is a mixture of an unknown but massive variety of antigens and are known to be very immunogenic. PPDj probably contains a large variety of epitopes, which can be recognised by the components of the immune system. In contrast,
the 14 novel antigens and Ag85B, on which the case definition was defined, probably contains considerably fewer epitopes compared to PPDj. However, this is merely speculative. In fact, if PPDj had been included in the case definition, just one additional animal would have been defined as a case, and diagnostic specificity of PPD would have been 0.66 instead of 0.62 and immunogenicity would have been 0.93 instead of 0.92. Hence, regardless of PPDj were included in the case definition or not, PPDj would still not correlate well with the case definition or with the other antigens.

Previous studies have shown that the PPDj IFN-γ test can be used specifically for animals older than 15 months, as blood samples from younger animals may produce false-positive IFN-γ responses (Jungersen et al., 2002). This unspecific IFN-γ may be produced by NK cells (Olsen et al., 2005) or γδ-T cells, which are present in high numbers in young cattle (Baldwin et al., 2000). At first sample collection a few animals were younger than 15 months and samples of these animals could be predisposed to false-positive reactions. However, results showed highest IFN-γ responses at the second and third sample collection to the majority of antigens.

Consistency of IFN-γ responses were investigated by collecting blood samples from the same heifers of the MAP infected herd on three days with four and five week interval. The IFN-γ responses fluctuated in samples of the same animals between sample days, which emphasize the need for repetitive sample collection for evaluation of antigen performance in the IFN-γ assay. Furthermore, evaluation and development of diagnostic tests are optimally done using samples from the target population, in this case young cattle exposed to MAP. A group of naturally infected cattle may represent various stages of MAP infection and hence blood samples will produce IFN-γ responses to different antigens due to heterogeneous MHC molecules. Previous studies have demonstrated that MAP express differentiated patterns when environmental conditions change (Gumber et al., 2009; Gumber and Whittington, 2009) and different strains of MAP show different antigen expression patterns. Based on this information, it may be speculated that a cocktail of antigens, as used in the study of Paper IV, is required to correctly diagnose all MAP infected animals of a given population. To detect the animals defined as cases, eight of the novel antigens (ESAP-3, LATP-1, LATP-2, LATP-3, LATP-4, MAP-2, MAP-3 and SECP-1) and Ag85B could be included in a cocktail. A cocktail of antigens should preferably include different antigen groups to detect all MAP infected animals at different stages of disease.

One heifer that had reacted strongly to most of the tested antigens on two repetitive sample days was observed to have a large abscess on her hind leg at the third sample collection day. Hence, samples from this heifer showed elevated IFN-γ responses, which were not due to a MAP antigen specific induction of primed T cells. This animal was excluded from the study population as IFN-γ values of positive and negative controls did not fulfil inclusion criteria. However, this observation emphasize that elevated IFN-γ responses may be due to other infections of the tested animal, but inclusion of positive and negative
controls in the IFN-γ assay should be used to validate samples and to control for unspecific IFN-γ responses.

5.5. Correlation of antigen-specific IFN-γ responses of fresh blood samples from MAP infected cattle with responses of day-old blood samples co-cultured with IL-12 or anti-IL-10 antibodies (Paper V)

Correlations of IFN-γ responses to antigens of blood samples at sample day with responses of day-old blood samples were high for several antigens as presented in Paper V. Hence, the use of these antigens in a day-old IFN-γ protocol could be applied as a promising alternative to the conventional IFN-γ protocol in which culture with PPDj is initiated on the day of blood sampling. Results of both day-old protocols with PPDj stimulation and addition of either recombinant IL-12 (p=0.78) or anti-IL-10 antibodies (p= 0.75) correlated reasonably well with results of sample day. Hence, hypothesis c) might be fulfilled for some of the antigens but to fully investigate this, a larger study including animals from herds of different MAP infection status is needed.

At sample day, three latency proteins, LATP1, LATP-2 and LATP-3 were the most promising, as Youden indices were highest (0.85) for this group. When day-old samples were used in the modified IFN-γ protocol with addition of either IL-12 or anti-IL-10 antibodies, latency proteins seemed promising, but the same antigens did not perform equally well in the two protocols. When correlation, immunogenicity and diagnostic specificity were considered collectively, LATP-2 was the most promising antigen tested with day-old samples with IL-12 and LATP-2 was the most promising antigen tested with day-old samples with anti-IL-10 antibodies.

The day-old protocol with IL-12 addition gave high IFN-γ responses to antigens in samples of both the infected and non-infected herd. Because IFN-γ responses of the non-infected herd were high, cut-off values for antigens were high and this resulted in few test positive animals of the infected herd giving low immunogenicity of antigens. However, there was no indication that IL-12 induced unspecific IFN-γ responses, because IFN-γ responses to the negative control PBS of blood samples from the negative herd were low in both sample day samples and day-old samples with IL-12. One possible explanation could be that IL-12 can amplify even very small responses.

IL-12 is a pleiotropic cytokine that is produced by macrophages and dendritic cells and induce early differentiation of CD4+ T cells and hence can shift immune responses toward CMI responses (Weiss and Souza, 2008; Mendez-Samperio, 2010). On contrary, IL-10 is known to enhance antibody mediated responses and suppress CMI responses partly by inhibiting IL-12 production by macrophages. IL-10 is produced by a range of cells including monocytes, macrophages, different T cell types and B lymphocytes (Moore et al., 2001; Weiss and Souza, 2008). Previous studies showed that neutralisation of IL-10 enhanced
killing capacity of MAP by macrophages (Weiss et al., 2005a), and enhanced IFN-γ production of bovine whole blood stimulated with PPD (Buza et al., 2004). However, another study demonstrated that neutralisation of IL-10 in PPD-stimulated blood samples of MAP infected goats, increased IFN-γ production in unstimulated samples and the IL-10 producing cells were identified to be mainly monocytes and not regulatory T cells as speculated (Lybeck et al., 2009). In the study presented in Paper V, IFN-γ responses to PBS were low in animals of both the infected and non-infected herd. This indicates that IFN-γ is specifically produced in response to antigen stimulation.

From the results, the use of day-old protocols with cytokine addition or inhibition seems promising. The day-old protocol with IL-12 have already been used in studies including a large number of animals (Paper I and II). However, the effect of adding cytokines to blood samples remains to be investigated by flow cytometry. In such a study, the IFN-γ producing cells should be investigated, to document if addition with IL-12 or anti-IL-10 antibodies induces unspecific IFN-γ production.

5.6. Neopterin detection in vitro as an alternative to IFN-γ assay (unpublished)
Detection of neopterin was investigated as an alternative to IFN-γ detection, and correlation between the two parameters had been expected. However, in the pilot study there was no correlation between IFN-γ values and neopterin values of the six cows that were tested. The supernatants used for neopterin detection had been stored for some years at -20°C and if neopterin had been present at some point it may have been degraded at the time of testing. On the other hand, neopterin is reported to be a quite stable molecule in vivo (Hamerlinck, 1999). The commercial neopterin ELISA kit that was used for this study was intended for detection of human neopterin that may differ from bovine neopterin. However, since elevated neopterin was detected for cow no. two, this explanation does not seem likely.

5.7. General remarks
As presented in this thesis, both the diagnostic target condition and the purpose of testing should be defined prior to using and interpreting results of any diagnostic test. Furthermore, immune responses against MAP may not necessarily evolve as explained by the classical perception in which early immune responses are characterised by CMI responses that are replaced by later antibody mediated responses (Begg et al., 2011). However, some heifers do have MAP-specific CMI responses, and if the specificity and sensitivity of the IFN-γ test are improved, the test can be used as a herd test for early indication of MAP infection in the herd. However, the current IFN-γ assay cannot be used for diagnosis of individual animals and perhaps IFN-γ responses fluctuate too much to ever achieve this. It might be speculated, that IFN-γ testing should be done within an even more narrow age period than in the studies presented here, where calves were tested at 15-24 months of age, and perhaps this would
increase stability of IFN-γ test results. However, this needs to be further investigated. In addition, the optimal interval for repeated IFN-γ testing needs to be investigated with the aim to obtain a more stable and reliable IFN-γ test result.

MAP infections evolve extremely slowly and due to this wide time perspective, it might be speculated that MAP infection can follow several different paths and develop in different ways because of differences in both hosts responses and MAP strains. To evaluate the use of novel antigens and/or use of the day-old protocols presented here, the perfect test evaluation could be a longitudinal study in which animals were studied over their entire lifetime. In this study, animals would have to be tested regularly by an agent detection test such as FC, a CMI-based test such as the IFN-γ assay and a test to detect antibodies, such as serum antibody ELISA. Finally, the study would end with post-mortem histopathological evaluation of up to 100 tissues per animals, which is required for correct diagnosis (Whitlock et al., 1996). This study would be very expensive, and perhaps it would just confirm that an IFN-γ test positive heifer does not necessarily become a future MAP infected cow. If enough animals were included, it would perhaps give us a better idea of which path the immunological responses follow during MAP infections.

For the future, the utility of the IFN-γ test might be promising if an optimized test protocol can give specific and sensitive IFN-γ results. The test should be used on herd level as an early diagnostic tool to indicate MAP infection of a herd. Early identification of MAP can be used as motivation for herd owners after changes in management, otherwise it take years before positive or negative changes in MAP infection level can be measured by FC or antibody ELISA. Furthermore, the herd owners can keep these animals under close observation before MAP is transmitted to other animals in the herd, MAP contaminate the environment or leads to potential production losses. Finally, perhaps an improved IFN-γ test can be used for future certification of herds as MAP-free which is highly desired by Danish farmers (Nielsen, 2011).

Diagnosis of MAP is complicated, and if eradication or control of MAP is the goal, a vaccination strategy is perhaps a better way to achieve this. The optimal MAP vaccine should not interfere with diagnostic tests but should make it possible to differentiate infected from vaccinated animals (DIVA vaccine). A multi-stage DIVA vaccine against MAP could include a combination of some of the novel antigens presented in this thesis.
6. Conclusion and perspectives

There can be several objectives for testing animals for MAP infection but both the diagnostic target condition and purpose of testing must be defined prior to use and interpretation of any given diagnostic test. If the purpose is to evaluate MAP infection level in a herd and the diagnostic target condition is calves that are exposed to MAP, the IFN-γ assay is the only useful test available. Results of this thesis suggest, that immune responses against MAP may not necessarily evolve as explained by the classical perception in which early CMI responses are replaced by later antibody mediated responses, because not all IFN-γ positive heifers became milk antibody ELISA positive as cows, and not all milk antibody ELISA positive cows had tested IFN-γ positive as heifers. However, many infected heifers did produce MAP-specific CMI responses, and if specificity and sensitivity of the IFN-γ assay are improved, the test can be used as a herd test for early indication of MAP infection in the herd. Such an early diagnosis tool would provide herd owners with an indication of the MAP infection level in young stock and the possibility to keep these animals under close observation before transmission of MAP infection to other animals in the herd, MAP contamination of the environment or potential productions loss.

Current IFN-γ assays use PPDj that give false-positive IFN-γ assay results, but results presented here indicate that novel recombinant MAP antigens can be used as an alternative to PPDj in an optimized IFN-γ assay protocol. The IFN-γ assay including some of these novel antigens have to be further evaluated, but based on presented results, we have some indication of which antigens are promising such as some latency proteins. Selection of animals and herds for evaluation should be considered carefully. If the purpose of testing is to detect MAP infected animals, the IFN-γ assay with novel antigens should be evaluated in cattle experimentally infected with MAP. However, if the purpose of testing is to detect MAP exposed cattle, the IFN-γ assay should be evaluated in MAP infected herds. The results of this thesis also indicate that day-old blood samples can be used as an alternative to the conventional IFN-γ protocol in which blood samples should be cultured with antigens within 10 hours of sample collection, although some reactivity may be lost. The use of day-old blood samples, make it possible to receive blood samples by regular mail and perform large herd screenings including herds that are not close to a laboratory.

Overall, the results emphasise that correct diagnosis of MAP is complicated. The IFN-γ assay may be used as an early tool for evaluation of MAP infection level in young stock and in the future perhaps to certify herds as MAP free. However, if eradication or control of MAP is the goal, a vaccination strategy is perhaps a better way to achieve this and a future MAP vaccine could be based on some of the antigens presented here.


Torrado, E., Cooper, A.M., 2010. IL-17 and Th17 cells in tuberculosis. Cytokine Growth Factor Rev. 21, 455-462.


Accompanying papers

Paper I

Paper II

Paper III

Paper IV

Paper V
Mikkelsen, H., Aagaard, C., Nielsen, S.S. and Jungersen, G. Correlation of antigen-specific IFN-γ responses of fresh blood samples from Mycobacterium avium subsp. paratuberculosis infected in cattle with responses of day-old blood samples co-cultured with IL-12 or anti-IL-10 antibodies. Submitted to Veterinary Immunology and Immunopathology.
Use of the johnin PPD interferon-gamma assay in control of

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Use of the johnin PPD interferon-gamma assay in control of bovine paratuberculosis

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ABSTRACT

Although the interferon-gamma (IFN-γ) assay for measurements of cell-mediated immune (CMI) responses to paratuberculosis PPD (johnin) has been available for close to 20 years, the assay has not yet emerged as the long desired test to identify infected animals at an early time point. Among other issues, this relates to problematic interpretation of the test results and maybe an over-expectation of what can be deducted from this kind of test given the chronic nature and slow development of infection of paratuberculosis. Over a number of years a modified IFN-γ assay with addition of recombinant bovine IL-12 to the PPDj stimulation of blood samples from the heifer group in more than 20 Danish dairy herds which also perform surveillance of MAP antibodies in milk have been performed. The results indicate that IFN-γ assay results are specific for paratuberculosis, but the IFN-γ assay result of an individual animal cannot establish whether the animal is infected or predict the future progression of disease in this animal. The IFN-γ assay should thus be used on a group of animals to test the level of exposure to paratuberculosis bacteria the animals have experienced, and thereby assist in maintaining rational in-herd management procedures and in the establishment of paratuberculosis status of a given herd. Indeed, for any diagnostic test applied in paratuberculosis, both the diagnostic target condition and the purpose of the diagnostic testing must be considered before any meaningful estimates of sensitivity or specificity can be given.

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1. Introduction

Bovine paratuberculosis, caused by chronic infection of the intestine with Mycobacterium avium subsp. paratuberculosis (MAP), continues to be in focus for national or herd-by-herd eradication due to the large economic losses (Ott et al., 1999) associated with the reduced production capacity of infected animals, trade restrictions and, in a few countries, concerns for a possible food born threat to human health. Control programs to manage paratuberculosis in cattle and sheep herds have been launched, and in some cases re-launched, in different countries such as Australia, USA, The Netherlands, Japan and Denmark over the past couple of decades (Benedictus et al., 2000; Nielsen, 2007, 2009). The value of several recent programs still remains to show the effectiveness, since no countries have yet been able to eradicate the infection and there is no international agreement on a formula for success.

A major problem in control and possible eradication of paratuberculosis is the poor ability to identify animals that are infected with MAP bacteria in early stages of the infection. At this infected but not affected disease stage, no clinical symptoms are apparent; and antibodies specific to paratuberculosis, and antibodies specific to paratuberculosis are absent, or at low levels, while MAP bacteria might be shed to the environment (Sweeney et al., 1992; Chiodini, 1996; Toman et al., 2003). Therefore, when a single animal is diagnosed with clinical paratuberculosis the herd of origin is also infected and there are much larger
numbers of unidentified animals which are subclinically infected with paratuberculosis.

It is generally believed that cattle are largely infected with MAP as calves, either in utero or more often as neonates via ingestion of faecal matter, milk or colostrum containing the bacteria (Sweeney, 1996). The early immune response to the infection is dominated by cell-mediated immune responses (CMI) and only if the infection progresses towards a more disseminated and uncontrollable infection will measurable levels of antibodies against MAP occur (Bendixen, 1978; Chiiodini, 1996). Thus, during disease progression from an early subclinical infected stage without shedding of bacteria, through a subclinical infectious stage to the late affected clinical stage, CMI gradually wanes and a strong humoral immune response with antibody formation develops. However, antibodies do not protect the cows against progression of disease and are merely indicative of a poor prognosis for the animal (Toman et al., 2003).

Here we report experiences with cytokine potentiation of the IFN-γ assay cultures to enhance IFN-γ responses of day-old blood samples sent by ordinary postal services before culture, and the application of an IL-12 potentiated IFN-γ assay as a means to evaluate MAP exposure in the pre-calving heifer group in 18 herds infected with paratuberculosis. There are important observations on the predictive value of the IFN-γ assay for individual animals that have implications on the prudent use of the IFN-γ assay in control of paratuberculosis. Taking the biology of MAP and disease characteristics of paratuberculosis into account, we summarise how diagnostic testing for paratuberculosis must be considered in a context of the purpose of testing and which target condition one attempts to diagnose.

2. Cytokine potentiation of IFN-γ assay

Although the IFN-γ assay as an in vitro measure of CMI has been available for 20 years, and bovine tuberculosis and paratuberculosis were the first infections this new test was used for in veterinary medicine (Wood et al., 1991, 1989; Rothel et al., 1990; Billman-Jacobe et al., 1992), the IFN-γ assay using purified protein derivative of MAP (Johnin PPD or PPDj) has not become widely accepted in diagnostic laboratories for paratuberculosis. This lack of acceptance relates to several inherent problems with the IFN-γ assay: (i) laborious handling and high costs per sample; (ii) low specificity of the assay for calves less than 12–15 months of age due to induction of non specific IFN-γ production in NK cells by PPDj antigens (Jungersen et al., 2002; Olsen et al., 2005); (iii) a poor predictive value in terms of identifying which animals that will eventually succumb to the infection as evidenced by development of antibodies or faecal shedding (Huda et al., 2004; Mikkelsen et al., 2009) and (iv) the in vitro production of IFN-γ by cells in the culture is highly sensitive to time from sampling to culture (Rothel et al., 1992; Jungersen et al., 2002).

To address the issue of limited time from sampling to start of culture the effects of addition of recombinant co-stimulatory cytokines to day-old blood samples on the antigen-specific IFN-γ production in response to PPDj have been investigated (Jungersen et al., 2005). Heparin stabilised bovine blood samples (Vacutainer®, Thermofisher, Belgium) were collected from cattle in one Holstein-Friesian and one Jersey herd, both of which were endemic for paratuberculosis. Undiluted whole-blood samples were incubated for 20–22 h in 1 ml cultures at 37°C in 5% CO₂ in the presence of PPDj (10 μg/ml), a negative (PBS) and a positive superantigen control (Staphylococcal enterotoxin B; SEB) (1 μg/ml) as previously described (Jungersen et al., 2002). PPD was prepared by SSI, Copenhagen, Denmark in 1975 from the American laboratory strain “Promise” which we presently have confirmed to be MAP with positive IS900 PCR reaction. For samples cultured the day after collection, unopened tubes were stored at room temperature (20°C). Day-old cultures were potentiated by addition of 10 U/ml recombinant bovine IL-12 (produced at IAH, Compton, UK) and/or 10 U/ml recombinant human IL-2 (Sigma). The levels of secreted IFN-γ in culture supernatants were measured by an in-house mAb sandwich ELISA as previously described (Mikkelsen et al., 2009). Paratuberculosis specific IFN-γ levels were calculated by subtracting IFN-γ levels in PBS cultured samples from IFN-γ levels in PPDj cultured samples. IFN-γ values were log transformed to obtain a normal distribution followed by statistical analyses using GraphPad Prism version 4.00 for Windows (GraphPad Software, USA).

Fig. 1 illustrates the effects of addition of bovine IL-12 and/or human IL-2 to day-old whole blood cultures of adult cows stimulated with PPDj and PBS compared with samples cultured 4 h after sampling without addition of cytokine. A significant decrease in the PPD induced IFN-γ levels in day-old samples were observed compared to levels in fresh cultures (P < 0.0001). Addition of IL-12 enhanced the waning IFN-γ levels of day-old samples and in 7 of 9 animals IFN-γ levels were even higher than in cultures of fresh samples. Addition of IL-2 enhanced some of the waning response, but also induced non-specific IFN-γ production in PBS-stimulated cultures, which invalidates the interpretation of Ag-specific responses. Potentiating with IL-12 and IL-2 in combination induced up to 108 fold increase in PPD induced IFN-γ production, but also induced very high levels of invalidating non-specific IFN-γ production in PBS-stimulated cultures. Titrations of recombinant bovine IL-12 and recombinant mouse IL-2 as culture additives revealed that both cytokines were able to potentiate the paratuberculosis specific IFN-γ response, but murine IL-2 consistently induced higher levels of non-specific IFN-γ in PBS cultures (data not shown). Addition of 10 U/ml bovine IL-12 added together with the stimulating antigen was chosen as the optimal concentration for potentiation of antigen-specific responses of day-old blood samples with low induction of non-specific IFN-γ in PBS cultures (Mikkelsen et al., 2009).

Fig. 2 shows the correlation of IFN-γ results of day-old samples potentiated with bovine IL-12 at 10 U/ml from 31 cows compared with the IFN-γ results of the conventional IFN-γ test on fresh samples and with non potentiated culturing of day-old samples. Statistical analysis (two-tailed paired t-test) documented a significant reduction in paratuberculosis specific IFN-γ responses of non potentiated day-old samples compared with fresh stimulated
3. Screening of heifers by IFN-γ assay

Applying the IL-12 potentiating protocol to day-old heparinised blood samples sent by ordinary mail service to the laboratory allowed us to investigate if IFN-γ response to PPD could be used as an early indicator of later antibody results of milk samples in cattle with different faecal culture status (Mikkelsen et al., 2009). Over a period of 3
years, a group of 975 cows from 18 Danish MAP infected dairy herds was studied. Prior to first calving, cell-mediated immune responses to MAP were measured in blood samples at the age of 15–24 months by the use of IL-12 potentiated IFN-γ assay protocol. Following calving, milk samples were collected and analysed for MAP specific antibodies by ELISA and faecal samples were cultured. The relationship between the variables IFN-γ and faecal culture and the outcome of ELISA (corrected optical density, ODc) was assessed using generalised additive models and revealed that a small, but significant ($P<0.0001$), correlation exists between IFN-γ test results obtained prior to first calving and milk antibody ELISA ODc value after calving. Most surprisingly, the observed correlation was towards a negative IFN-γ test predicting the higher OD value and, although significant, the IFN-γ test result only slightly affected the future antibody status. Given the relatively short observation period of 3 years it was not surprising that many of the heifers that tested IFN-γ positive prior to calving never became ELISA positive as cows in the study period. However, of the heifers that did develop a positive milk ELISA response, a small majority were IFN-γ test-negative as heifers. As expected, there was a strong and significant correlation between FAECAL CULTURE positive and high ELISA ODc.

After the completion of the study above, the Danish paratuberculosis eradication programme has changed the antibody test to a more specific commercial ELISA (Nielsen, 2009). After this change, ELISA results from another 352 heifers, of which 11 cows have produced one or more ELISA positive results of milk samples, corroborates the previous analysis that IFN-γ test results are not predictive of later antibody formation. Only one cow has produced consistently high ELISA test results, and this animal was IFN-γ test negative when analysed as a heifer. These results are also consistent with an earlier prospective study employing standard procedures for IFN-γ assay of fresh blood samples and antibody analyses of both serum and milk antibodies of 371 animals performed over a 2-year period (Huda et al., 2004). Although the IFN-γ assay proved to have a higher sensitivity than ELISA or faecal culture in young animals below 3 years of age, IFN-γ assay results of animals 1–2 years of age were not predictive of later infection status. Of the eight cows that eventually were faecal culture positive and had been included in the study at a young age, only four had a positive IFN-γ test result before first calving.

In Fig. 3(left) are shown all 2008 IL-12 potentiated PPDj-specific IFN-γ results from analysis of 717 heifers from 17 MAP-exposed herds engaged in an active eradication programme of paratuberculosis. Fig. 3(right) show IL-12 potentiated IFN-γ results of 101 heifers and cows from closed herds without historical, serological or clinical evidence of paratuberculosis (non-exposed herds). The difference in level of IFN-γ response between these exposed and non-exposed animals is highly significant ($P<0.0001$) and a tentative cut-point based on mean value + 2SD of the non-exposed animals can be set at 1135 pg/ml. There are no other available diagnostic tests which can measure this difference in exposure to MAP between the two groups of animals. Therefore, although it may not be possible to use the early IFN-γ test result of an individual animal in a MAP-contaminated environment to assign it into infected or non-infected subgroups, the IFN-γ assay may thus have a very important application as a means to evaluate the level of MAP exposure in a group of young animals.

Fig. 4 shows examples of repeated testing of the heifer group in three of the MAP-exposed herds. In herd 9 only small changes are observed and there are continuously several animals with IFN-γ production above the tentative cut-point, indicating that the group of heifers have been exposed to MAP throughout the study period. In herd 14, the IFN-γ assay results of the first samplings are high and indicative of a highly MAP-contaminated calf-rearing environment. However, at the last sampling only few animals are high and it may appear that control mechanisms to reduce MAP transmission to calves have been improved and are more effective in this herd. In herd 18, the level of MAP exposure to calves appears to have been low at the time of entry into the programme and has, with some
Fig. 4. Repeated IFN-γ testing of the heifer group in infected herds indicates changes in level of exposure to paratuberculosis. A subsample of up to 45 heifers between 15 and 24 months of age was analysed at the indicated time points by the IL-12 potentiated IFN-γ assay on day-old whole-blood samples. Graph shows three examples of the development in IFN-γ responses after the initiation of a paratuberculosis management programme accompanied by serological milk ELISA of adult cows.

Some of the animals which are exposed or infected at young ages are unable to control the infection, and thus become shedders or show evidence of clinical paratuberculosis. However, since relatively few animals will experience this progressive paratuberculosis, sensitive and specific identification of antibodies in serum or milk at the earliest time point is highly warranted before the immunological control over the infection is lost and the animal is becoming infectious and will contaminate its environment.

In the ideal setting, positive identification of MAP by faecal or tissue culture should only be necessary to use for positive identification of MAP as the cause of disease in affected animals when herds are identified with paratuberculosis for the first time. Unfortunately these expectations are not met with currently available tests. As described above, the IFN-γ assay cannot be used to ascribe a status as infected or non-infected for single animals and current antibody tests may be specific for MAP infection, but the sensitivity is very low at the early transition from infected to infectious (Nielsen and Toft, 2008; Nielsen, 2008). The low sensitivity does not necessarily relate to poor tests for detection of antibodies, but may be a reflection of an inherent characteristic with paratuberculosis, that antibodies only appear at very low levels in early stages of infection, and although there is a continued search for new and more sensitive antigens, we are currently faced with making the best of the tests we have.

Recently, Nielsen and Toft (2008) have reviewed accuracies of IFN-γ, ELISA and faecal culture techniques for different animal species and different target conditions. Using the target conditions as defined by Nielsen and Toft (2008), Table 1 summarises different characteristics of currently available tests in the different contexts where they can be applied. The most important message is that the diagnostic target condition and the purpose of testing have significant impact on the utility of the different tests. Making the right diagnosis in animals AFFECTED by paratuberculosis is often not so difficult and both faecal culture and ELISA have high sensitivities and specificities for this condition. It is more difficult to identify animals which are shedding the bacteria and thus are INFECTIOUS to the herd and may contaminate their environment without any clinical signs or apparent reduced production parameters. However, since these animals are often shedding bacteria in extremely high numbers, it is important to make a diagnosis of these animals as early as possible and remove the animal from the herd to break the transmission of paratuberculosis within a herd. The sensitivity of the ELISA assays for milk or serum increases rapidly with progression of disease (Nielsen and Toft, 2008). Hence, it becomes a matter of test frequency to identify the animals at the earliest time point after the infection has progressed to a stage where antibodies have emerged. It is our experience that animals which are antibody positive and IFN-γ negative are more prone to develop clinical paratuberculosis compared to animals which are both antibody and IFN-γ positive.

The INFECTED animals which are not yet infectious or affected, are much more difficult to identify as sensitivity of both faecal culture and antibody ELISA is very low.
Table 1
Diagnostic testing for paratuberculosis with varying target conditions.

<table>
<thead>
<tr>
<th>Diagnostic target condition</th>
<th>Disease condition</th>
<th>Purpose of testing</th>
<th>Faecal culture</th>
<th>Antibody ELISA</th>
<th>PPDj IFN-γ assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) AFFECTED</td>
<td>Diarrhoea, weight loss, high shedding</td>
<td>New diagnosis of herd, Verify clinical diagnosis</td>
<td>High Sp High Se</td>
<td>High Sp High Se</td>
<td>Negative results carry a poor prognosis</td>
</tr>
<tr>
<td>(2) INFECTIOUS, not (1)</td>
<td>No illness, reduced production, increased shedding</td>
<td>Stop transmission of MAP, avoid production loss</td>
<td>High Sp Medium Se</td>
<td>High Sp Medium Se</td>
<td>Negative results carry a poor prognosis</td>
</tr>
<tr>
<td>(3) INFECTED, not (2)</td>
<td>No illness, normal production, low or absent shedding, MAP resting in tissues</td>
<td>Culling prior to shedding or disease, Trade</td>
<td>Sp affected by passive transmission Low Se</td>
<td>High Sp Low Se</td>
<td>Low Sp Medium Se</td>
</tr>
<tr>
<td>(4) NON-INFECTED</td>
<td>No MAP in tissues</td>
<td>Certify individual animal freedom of infection</td>
<td>Biology of paratuberculosis makes it impossible to link negative results of any current diagnostic test with freedom of infection</td>
<td>Se close to 0</td>
<td>Se close to 0</td>
</tr>
<tr>
<td>(5) EXPOSED YOUNG STOCK</td>
<td>Unknown. MAP may have been eliminated or may be progressing</td>
<td>Evaluate in-herd transmission, certify herd MAP-free</td>
<td></td>
<td>Only useful test</td>
<td></td>
</tr>
</tbody>
</table>

and both the sensitivity and specificity of the PPDj IFN-γ assay for individual animals is mediocre. However, since many of these animals will be able to control and probably eliminate the infection without ever shedding any bacteria (Sweeney et al., 1992; Chiodini, 1996; Toman et al., 2003), the purpose of testing and identifying these animals must be considered carefully. Positive identification of animals with a relatively early infection may be very relevant for trade and possibly culling in herds with very low prevalence of paratuberculosis. In herds with higher prevalences, culling of all animals with a CMI reaction towards MAP is, however, undesirable as the CMI response is important for the immunological control of MAP and it might be speculated, that such a culling strategy will breed for a herd with low CMI capacity, which will be detrimental for the in herd resistance to paratuberculosis.

Importantly, as paratuberculosis is a slow chronic infection where MAP may be cleared by effective immune responses or hiding in tissues in a resting state without generation of immune responses (Woo and Czuprynski, 2008), there are no tests for paratuberculosis for which a negative result can positively identify an individual as NON-INFECTED and certify it to be free of infection. Therefore, it will be of very high value to be able to evaluate in-herd transmission and level of MAP EXPOSURE TO YOUNG STOCK in order to improve management efforts to break transmission and at some point certify freedom of infection in a herd. The only current test with the potential to provide herd owners with an early identification of MAP exposed animals in the herd is the IFN-γ assay.

Conflict of interest statement
None of the authors for this manuscript have any financial or personal relationships with other people or organizations with interests in commercial tests for paratuberculosis or other areas with potential interests in the topics covered in the manuscript.

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References


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Association between milk antibody and interferon-gamma responses in cattle from *Mycobacterium avium* subsp. *paratuberculosis* infected herds

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ABSTRACT

Paratuberculosis is a chronic infection of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is possible to detect infection with paratuberculosis at different stages of disease by means of various diagnostic test strategies. The objective of the present study was to evaluate if early cell-mediated immunity could predict the antibody results of milk samples in cattle with different faecal culture (FC) status. A group of 975 cows from 18 Danish MAP infected dairy herds was studied during a 3-year period. Cell-mediated immunity was measured in blood samples from heifers by use of an IL-12 potentiated IFN-γ protocol. Following calving, milk samples were collected and analysed for MAP specific antibodies by ELISA and faecal samples were cultured. The relationship between the variables IFN-γ and FC and the outcome of ELISA was assessed using generalised additive models. The results of the study showed that a significant association exists between early IFN-γ and later FC status with occurrence of antibodies. In addition, the early IFN-γ and FC status affect the antibody ELISA result at different stages post calving. We observed that only some IFN-γ positive animals developed a positive antibody response against MAP, which indicate that cell-mediated immune responses can control or eradicate MAP in many animals.

1. Introduction

Paratuberculosis is a chronic, granulomatous enteric infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in ruminants. Infection of dairy cattle leads to economic losses through premature culling and reduced milk yield (Ott et al., 1999). It is believed that cattle are largely infected as calves, either in utero or more often as neonates via ingestion of faecal matter, milk or colostrum containing the bacteria (Chiodini et al., 1984; Sweeney, 1996). Cell-mediated immunity (CMI) is critical to host defence against intracellular MAP and is initially elicted at an early stage in infection where antibodies are still not detected (Chiodini, 1996; Toman et al., 2003). During disease progression from the subclinical to the clinical stage, CMI gradually wanes and a strong humoral immune response with antibody formation develops. However, antibodies do not protect the cows against progression of disease (Stabel, 2000).

An important tool for management and control of MAP is diagnostic testing to detect infectious cows that pose a potential risk to calves in the herd. Diagnosis of MAP infected animals is usually not possible before 2 years of age by detection of specific serum/milk antibodies or by faecal culture (FC) (Nielsen and Toft, 2006). However, measurement of the CMI by the IFN-γ test poses an alternative, which would enable detection of infected and infectious animals at an early stage of disease.

The IFN-γ test can be used specifically for animals older than 15 months, while analyses of IFN-γ responses in...
younger animals are prone to non-specific false positive reactions (Jungersen et al., 2002). This is possibly due to activation of NK cells (Olsen et al., 2005).

A diagnostic test that can identify animals before they become infectious is desirable. To accomplish this, a highly specific diagnostic test, or refinement of the existing IFN-γ test, should be further developed. Given the current understanding of the pathogenesis of paratuberculosis, a positive correlation between early CMI, followed by a serological response, bacteriological shedding and later clinical disease is expected. Therefore, our objectives were to: (a) describe an IFN-γ method using IL-12 for early detection of MAP infected animals; and (b) to study early IFN-γ to predict the antibody ELISA results of milk samples in cattle with different FC status.

2. Materials and methods

2.1. Herds and animals

The present study included 975 cows from 18 Danish dairy herds during a period of 3 years through August 2003 to December 2007. The herds consisted of a non-random sample, where practising veterinarians had suggested their inclusion in a study of intervention against paratuberculosis. During the study period, two of the included herds ceased production. A brief description of the herds is given in Table 1. Information on dates of birth, dates of calving and breed was obtained from the Danish Cattle Database.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow (years)</td>
<td>ECM (kg/cow year)</td>
<td>Cow (years)</td>
</tr>
<tr>
<td>1</td>
<td>209.4</td>
<td>10,478</td>
</tr>
<tr>
<td>2</td>
<td>203.6</td>
<td>8,364</td>
</tr>
<tr>
<td>3</td>
<td>132.2</td>
<td>10,838</td>
</tr>
<tr>
<td>4</td>
<td>104.8</td>
<td>8,510</td>
</tr>
<tr>
<td>5</td>
<td>136.2</td>
<td>8,887</td>
</tr>
<tr>
<td>6</td>
<td>129.6</td>
<td>9,519</td>
</tr>
<tr>
<td>7</td>
<td>67.2</td>
<td>7,644</td>
</tr>
<tr>
<td>8</td>
<td>130.2</td>
<td>11,115</td>
</tr>
<tr>
<td>9</td>
<td>110.1</td>
<td>11,392</td>
</tr>
<tr>
<td>10</td>
<td>117.6</td>
<td>11,302</td>
</tr>
<tr>
<td>11</td>
<td>117.9</td>
<td>9,012</td>
</tr>
<tr>
<td>12</td>
<td>95.5</td>
<td>8,408</td>
</tr>
<tr>
<td>13</td>
<td>120.8</td>
<td>9,719</td>
</tr>
<tr>
<td>14</td>
<td>122.9</td>
<td>9,076</td>
</tr>
<tr>
<td>15</td>
<td>71.5</td>
<td>8,717</td>
</tr>
<tr>
<td>16</td>
<td>157.6</td>
<td>8,757</td>
</tr>
<tr>
<td>17</td>
<td>128.1</td>
<td>9,040</td>
</tr>
<tr>
<td>18</td>
<td>84.0</td>
<td>8,982</td>
</tr>
</tbody>
</table>

Table 1 Description of the 18 herds included in the study: herd size and production level for two periods.

kg ECM was estimated from the milk yield control scheme carried out in the herds 11 times per year.

* In the period October 1, 2003 to September 30, 2004.
* In the period October 1, 2006 to September 30, 2007.
* Ceased production April 2005.

2.2. Sampling

The study was conducted as an observational longitudinal study with a sampling frame as illustrated in Fig. 1. One heparinised whole-blood sample was collected by the vaccutainer system from each animal in the period 15–24 months of age and samples were subject to IFN-γ testing. After first calving, annual faecal samples for detection of MAP were collected by milk quality advisors from rectum, using one glove per cow. This resulted in minimum one faecal sample per cow. Milk samples for detection of antibodies were collected 4 times per year in each herd via the Danish milk recording scheme as done in the Danish paratuberculosis control programme (Nielsen, 2007). A total of 5545 milk ELISA results from 975 cows in 18 dairy herds was included in the study. Of these, 144 cows had less than 4 tests, 136 had 4 tests, 185 had 5 tests, 172 had 6 tests and 338 had 7 or more ELISA tests (minimum 1, median 5 and maximum 12 samples per cow). The number of ELISA samples per cow varied due to the observational design of the study; new cows entered at first calving, and older cows left when they were sold, culled or died. Distribution of milk samples in different age groups is given in Table 2.

2.3. Diagnostic testing

2.3.1. ELISA analysis for milk antibody

Milk samples were tested for the presence of MAP antibodies using an ELISA performed as described elsewhere (Nielsen, 2002). The ELISA is based on a commercially available antigen (Allied Monitor, Fayette, Missouri, USA) designated ‘M. paratuberculosis Strain 18’ and the ELISA detects both IgG1 and IgG2.

2.3.2. Analysis for cell-mediated immunity

One of the major impediments for using the IFN-γ test is that the time span from collection of the blood sample to the start of culture influences the viability of the cells. To obtain valuable results it is therefore recommended to start culture within 8–12 h of sampling (Robbe-Austerman et al., 2006). Studies performed prior to the study evaluated various procedures, which increased the time period available (Jungersen et al., 2005). These studies resulted in a new protocol in which whole blood collected in heparinised vaccutainer tubes, was shipped by postal freight service and received at the laboratory the following day. At arrival, the whole blood was cultured with antigens with addition of co-stimulatory cytokine IL-12. Adding IL-12 to samples within 20 h of collection rescues a weakened IFN-γ response (Jungersen et al., 2005). This IL-12
potentialized IFN-γ test was used for CMI analysis in the present study.

To each well in a 24-well culture plate (Greiner Bio-one, Heidelberg, Germany), 1.5 ml blood was cultured with 10 μg/ml Johnine purified protein derivative (PPDj) antigens (National Veterinary Institute, Copenhagen, Denmark), 1 μg/ml superantigen staphylococcal enterotoxin B (SEB) and PBS (nil antigen) with addition of 10 U/ml recombinant IL-12 (a kind gift from Chris Howard and Jayne Hope, The Institute of Animal Health, Compton, UK). Following stimulation for 18 h at 37°C/5% CO2, the culture plates were centrifuged and approximately 0.8 ml supernatant was harvested and stored in 96-well storage plates (Greiner Bio-One, Heidelberg, Germany) at −20°C until further analysis.

The antigen-specific IFN-γ secretion in supernatants was determined by use of an in-house ELISA. MaxiSorpTM microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl/well anti-bovine IFN-γ F(ab)2 monoclonal antibody (clone 6.19) (National Veterinary Institute, Copenhagen, Denmark) at a concentration of 0.3 μg/ml. Subsequently, the wells were blocked for 1 h with 200 μl/well blocking buffer (PBS, 0.05% Tween 20, 0.1% Casein) and thereafter washed 5 times with washing buffer (PBS, 0.05% Tween 20). To each well 50 μl blocking buffer was added first, followed by 50 μl supernatant or a two-fold standard dilution series. The reference standard was prepared from calf plasma stimulated overnight with 0.2 μg/ml SEB resulting in a known IFN-γ concentration of 5,140 pg/ml. The plates then incubated for 1 h at room temperature with gentle agitation followed by 5 washings. Biotinylated secondary anti-bovine IFN-γ monoclonal antibody (clone 6.22) (National Veterinary Institute, Copenhagen, Denmark) was diluted in blocking buffer and 100 μl/well was added in a final concentration of 1.76 μg/ml followed by 1 h incubation at room temperature and 5 washings. Next, the wells incubated with 100 μl/well ZyMAX horseradish peroxidase-conjugated streptavidin (ZYMED® Laboratories, Invitrogen, Carlsbad, CA, USA) diluted 1:10,000 in blocking buffer, for 1 h at room temperature. Finally, the wells were washed 5 times and o-phenylenediamine dihydrochloride substrate (Sigma, St. Louis, MO, USA) was added and incubated in the dark for 20 min. The enzyme reaction was stopped by adding 100 μl/well 0.5 M H2SO4 and the optical density (OD) was read at 490 nm with 650 nm reference subtraction. The level of IFN-γ (pg/ml) was calculated using linear regression on log–log transformed readings from the two-fold dilution series of the reference standard with known IFN-γ concentration.

2.3.3. Bacteriological culture of faecal samples

The faecal specimens were decontaminated and cultivated as described previously (Nielsen et al., 2004). Briefly, samples were decontaminated in 4% NaOH/15 min, 5% oxalic acid + 0.1% malachite green/15 min, neomycin sulphate + amphotericin B (50 μg/ml)/overnight. Drops of decontaminated faecal samples were applied to four tubes containing Herrold’s egg yolk medium (HEYM) (Difco/Becton Dickinson, Sparks, MD, USA). Following 8 and 12 weeks of incubation the cultures were inspected using an illuminated magnifier. All positive isolates were confirmed for the presence of IS900 insertion sequence by PCR.

2.4. Interpretations of diagnostic procedures

The ELISA test result was considered positive if ODcorrected (ODC) was ≥0.3 as used in the Danish paratuberculosis control programme (Nielsen, 2007). The IFN-γ test was considered positive if IFN-γ levels in PPDj stimulated samples ≥1 ng/ml. Furthermore, a sample needed a SEB-stimulated IFN-γ level >1 ng/ml and a PBS-stimulated IFN-γ level <1 ng/ml to be considered valid. Estimates of sensitivity and specificity for the IL-12 potentiated IFN-γ gamma test are not currently available and as such no fixed cut-points have been determined for the exact interpretation. The values for cut-points in this study have been decided based on our best guess. This guess has been based on levels of IFN-gamma in non-stimulated samples as well as the levels in PPDj stimulated samples in herds with different levels of MAP exposure. Animals were considered FC-negative if all samples were negative at culture.

Table 2

Distribution of 5545 milk samples tested using ELISA from 975 Danish dairy cows included in the study. Observations are cross-tabulated by age of the cows from which the milk samples were obtained. Number given was no. of samples in the group. Values in parentheses represent median predicted optical density (ODc) value of cows in the group.

<table>
<thead>
<tr>
<th>FC IFN-γ</th>
<th>ELISAa</th>
<th>Age group</th>
<th>2–3 years</th>
<th>3–4 years</th>
<th>&gt;4 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Negative</td>
<td>Negative</td>
<td>47 (0.00)</td>
<td>942 (0.00)</td>
<td>758 (0.00)</td>
<td>291 (0.00)</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>53 (0.02)</td>
<td>311 (0.01)</td>
<td>185 (0.20)</td>
<td>110 (0.30)</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>23 (0.02)</td>
<td>1087 (0.00)</td>
<td>837 (0.00)</td>
<td>219 (0.00)</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>2 (0.19)</td>
<td>206 (0.03)</td>
<td>149 (0.22)</td>
<td>37 (0.40)</td>
</tr>
<tr>
<td>Positive Negative</td>
<td>Negative</td>
<td>4 (0.06)</td>
<td>21 (0.00)</td>
<td>16 (0.00)</td>
<td>3 (0.10)</td>
</tr>
<tr>
<td>Positive Negative</td>
<td>Positive</td>
<td>0</td>
<td>37 (0.12)</td>
<td>29 (0.33)</td>
<td>13 (0.40)</td>
</tr>
<tr>
<td>Positive Positive</td>
<td>Negative</td>
<td>1 (0.05)</td>
<td>52 (0.01)</td>
<td>29 (0.00)</td>
<td>3 (0.00)</td>
</tr>
<tr>
<td>Positive Positive</td>
<td>Positive</td>
<td>4 (1.45)</td>
<td>47 (0.11)</td>
<td>25 (0.60)</td>
<td>4 (0.80)</td>
</tr>
</tbody>
</table>

FC = faecal culture test; IFN-γ = interferon-gamma test.

a ELISA-status was determined from analysis of all available ELISA results of each animal.
2.5. Statistical analysis

Descriptive statistics were performed by grouping the 975 animals into eight groups based on results from FC, IFN-γ and final ELISA status of the cow. The ELISA-profile for each cow within each group was plotted as a function of age at testing to check for unlikely values (data not shown), and the median corrected optical density OD_C value was calculated in each group for descriptive purposes.

The generalised additive model (GAM) procedure in SAS v. 9.1 (SAS Institute, Cary, NC, USA) were applied to explore the functional relationship between the outcome, OD_C, and the explanatory variables FC and IFN-γ. GAM is an extension of generalised linear models, in which variables are assumed to have a linear relationship with the outcome and are included additively. Generalised linear models use a so-called link function and are used to do regression modelling for non-normal data. In GAM, the usual linear function of a covariate is replaced with a smoothing function (Hastie and Tibshirani, 1991). Hence, in GAMs the variables are not assumed to have a linear relationship with the outcome. In the present analysis, we made predictions of the antibody-response as a function of age by semiparametric regression using GAM including the effect of FC and IFN-γ.

The model used was as follows
\[
\text{OD}_C = \beta_0 + I_i + \text{FC}_j + S(\text{Age})
\] (1)
where OD_C was the corrected OD-value from the ELISA; \(\beta_0\) was the baseline value of the OD_C; \(I_i\) was the fixed effect of IFN-γ (where \(i\) could be either positive or negative); \(\text{FC}_j\) was the fixed effect of FC (where \(j\) could be either positive or negative); \(S(\text{Age})\) was the smoothing function of age in years, estimated using 4 degrees of freedom.

Finally, the graphs showing predicted OD-values for different combinations of IFN-γ and FC results were made. Because many cows did not become antibody positive, the above model was repeated while the final ELISA-status (E) was also included as a covariate. Thereby, the average antibody profile of the different groups can better be appreciated.

3. Results

The distribution of samples within FC, IFN-γ and E-groups is shown in Table 2, along with median OD_C-values of cows in each group. Parameter estimates for the model predicting the antibody-response as a function of age, with the effect of FC and IFN-γ as covariates, are shown in Table 3. Predicted OD_C was significantly affected by both FC (\(p < 0.0001\)) and IFN-γ (\(p < 0.0001\)). The parameter FC- was negatively associated with predicted OD_C, whereas IFN-γ- was positively associated, although to a minor degree, with predicted OD_C. The predicted OD_C as a function of age at ELISA test for the four different groups of cows combining FC and IFN-γ test are shown in Fig. 2. FC+ cows had highest OD_C-values. Highest predicted OD_C values were obtained for group FC+ IFN-γ+. The difference in OD_C between IFN-γ- and IFN-γ+ was small (~0.02 OD_C-units). The predicted OD_C increased slightly with age at ELISA for all four groups of animals.

Fig. 3 illustrates predictions of OD_C on cow level also including the final ELISA status. The highest average OD_C values were predicted in the group including FC+ IFN-γ+ E+ followed by the group FC+ IFN-γ- E+. These two groups resulted in a higher predicted OD_C response than any of the other groups presented in Fig. 3.

![Fig. 2. Predicted corrected optical density (OD_C) value in Mycobacterium avium subsp. paratuberculosis milk antibody ELISA of cows, at ages from 2 to 5 years, based on previous interferon gamma (IFN-γ) and faecal culture (FC) results. Predictions are shown for four groups of cows: IFN-γ− FC+, IFN-γ− FC−, IFN-γ+ FC− and IFN-γ+ FC− based on data from 18 Mycobacterium avium subsp. paratuberculosis infected dairy cattle herds.](image)
however, although statistically significant, the IFN-gamma response to a non-MAP PPD, will not solely reflect paratuberculosis-induced activity and specificity becomes complicated. The IFN-gamma test for detecting infected animals varies considerably with age.

4. Discussion

We demonstrate that IFN-gamma test results obtained prior to first calving and FC status are correlated to antibody ELISA OD_c value at different ages after calving (Fig. 2). Surprisingly, the observed correlation was towards a negative IFN-gamma test predicting the higher OD value, however, although statistically significant, the IFN-gamma test result only slightly affected the future antibody status. Thus, many of the cows that tested IFN-gamma positive as calves never became ELISA positive and some IFN-gamma test-negative heifers developed an ELISA response. MAP infections are by some considered incurable, but no evidence exists to support this. Cell-mediated immune responses may be able to eradicate MAP from some animals. Thereby, antibodies would never be expected to occur. Although the current data cannot prove this hypothesis, antibody profiles in Fig. 3 suggest that it may be a likely explanation. However, it is also likely that the animals with positive IFN-gamma and negative ELISA are merely controlling the infection, which will later erupt. It has previously been demonstrated that most animals shedding MAP will become ELISA-positive (Nielsen and Ersbøll, 2006). Therefore, both options are possible.

One of the problems with diagnosis of MAP is the lack of a reference test, whereby evaluation of diagnostic sensitivity and specificity becomes complicated. The IFN-gamma test is known to have a low specificity and hence a better correlation between IFN-gamma, ELISA and FC may not be expected until this test has been further optimised. Comparison to a non-MAP mycobacterial antigen in the IFN-gamma test is often recommended, but in relation to MAP it is not straightforward to interpret (Jungersen et al., 2002). The IFN-gamma response to a non-MAP PPD, will not solely reflect the non-specific mycobacterial background production, but will also reflect paratuberculosis-induced responses, because of high cross-reactivity. Denmark has never become ELISA positive and some IFN-gamma positive calves have become ELISA-positive (Nielsen and Ersbøll, 2006). Thereby, MAP infections are by some considered incurable, but no evidence exists to support this. Cell-mediated immune responses may be able to eradicate MAP from some animals. Thereby, antibodies would never be expected to occur. Although the current data cannot prove this hypothesis, antibody profiles in Fig. 3 suggest that it may be a likely explanation. However, it is also likely that the animals with positive IFN-gamma and negative ELISA are merely controlling the infection, which will later erupt. It has previously been demonstrated that most animals shedding MAP will become ELISA-positive (Nielsen and Ersbøll, 2006). Therefore, both options are possible.

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paratuberculosis is common. For one, the sensitivity of faecal culture is on average low in animals tested below 2 years of age (0.05) but increases (0.21) at 5 years of age (Nielsen and Toft, 2006). In this study, a large proportion of the samples tested originated from cows in the age group 2–3 years (48.7%). Second, the animals could be truly negative; however such a low prevalence is unlikely since the within-herd prevalence in Danish herds has been suggested to be 33% (Nielsen et al., 2007).

Several studies have evaluated diagnostic tests detecting CMI in response to paratuberculosis. These studies have been performed on experimentally infected cattle (Lepper et al., 1989; Collins and Zhao, 1995; McDonald et al., 1999) at various infection levels and in naturally infected herds (Huda et al., 2003, 2004; Nielsen and Toft, 2006). Experimental infection studies contribute with new knowledge of infection mechanisms, but it can be argued that the optimal study objects for evaluation of diagnostic tests are the target population in the natural environment. In the present study, we followed the same animals from calves to becoming adult cows.

For this study we used an IFN-γ test protocol in which we co-stimulated day-old blood samples with IL-12, in order to rescue IFN-γ production by Th1 cells in response to culture with MAP antigens. By using this protocol, we circumvent the major problems and logistics of stimulating blood samples in the laboratory within 8 h of sampling at the farms. This is a major advantage in large studies and makes it possible to collect samples from farms outside the near vicinity of the laboratory. However, we face another problem since co-stimulation with IL-12 may induce non-specific false positive reactions. False positive IFN-γ production may be due to NK cells producing IFN-γ in response to IL-12 (Jungersen et al., 2005). NK cells are part of the innate immune system and are observed at highest numbers in calves in which they constitute 5–10% of cells in peripheral blood (Kulberg et al., 2004). The impact of NK cells producing IFN-γ without antigen-specific stimulation has not yet been described for this protocol.

Control for paratuberculosis in a herd involves breaking transmission routes by changing management practices. Most important is strict management of calves (Groenenendaal et al., 2002). Calves are especially vulnerable and needs to be protected against exposure of faeces and milk from infectious cows. Breaking transmission routes will help controlling paratuberculosis over time and result in a lower prevalence. However, for fast eradication, an early diagnostic tool is necessary to identify infected calves, which may have been infected irrespective of the measures taken (Benedictus et al., 2008) before they become shredders of MAP and a risk to other animals. Such identification can be achieved by detection of the early CMI response in calves using IFN-γ testing. The IFN-γ test is in theory applicable at an early age and may be an important decision support tool for advisors in cattle practice. However, previous studies reported that the CMI responses measured by the IFN-γ test fluctuate widely in young cattle, especially in calves younger than 6 months (Jungersen et al., 2002; Huda et al., 2003, 2004).

Further evaluation and optimisation of the IFN-γ test using new and more specific antigens is necessary for diagnosis of paratuberculosis in young animals. In the present IFN-γ test protocol we stimulated blood samples with PPDj which is a crude protein product that results in large variation in IFN-γ response among animals. Evaluation of a cocktail of MAP specific recombinant antigens, as well as the significance of NK cells producing IFN-γ in this in vitro assay remains to be explored.

5. Conclusion

In the present study we investigated the association between IFN-γ, FC and milk antibody ELISA in paratuberculosis infected dairy herds. There was significant association between early IFN-γ and subsequent FC status with occurrence of antibodies. However, the results indicate that only some IFN-γ animals will develop an antibody ELISA response, which suggest that cell-mediated immune responses control or eradicated MAP in many animals.

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References


Paper III

Review of *Mycobacterium avium* subsp. *paratuberculosis* antigen candidates with diagnostic potential

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Review

Review of *Mycobacterium avium* subsp. *paratuberculosis* antigen candidates with diagnostic potential

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**ABSTRACT**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a slow growing bacterium that can infect ruminants and remain latent for years without development of any clinical signs or disease. Diagnosis is often based on detection of MAP antibodies in milk or serum samples or culture of bacteria from faeces; however, these diagnostic tools are often not applicable until years after infection. Detection of MAP specific cell-mediated immune (CMI) responses can serve as an alternative and be implemented in a diagnostic tool. CMI responses can be measured at an early stage of infection, prior to development of antibodies and shedding of detectable amounts of MAP. At present, available diagnostic assays are limited by the lack of MAP specific antigens included in these assays resulting in poor specificity. The objective of this review is to provide a systematic overview of diagnostic MAP antigen candidates described to date with special emphasis on antigen candidates tested for CMI responses. Relevant information on 115 different MAP antigens was systematically extracted from literature and summarized in 6 tables of CMI antigens, secreted antigens, cell wall and membrane antigens, lipoprotein antigens, heat shock antigens and hypothetical antigens. Strategies for evaluation of novel antigen candidates are discussed critically. Relatively few of the described antigens were evaluated for their use in CMI based diagnostic assays and so far, no obvious candidate has been identified for this application. Most of the novel diagnostic candidates were evaluated in few animals and it is recommended that an appropriate sample size is included for evaluation of antigen candidates in future studies.
1. Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis in ruminants. MAP is part of the large and diverse *Mycobacterium* family that is defined by their acid-fast properties, mycolic acid-containing cell walls, and high genomic C+G content (61–71%). The family currently counts more than 130 established and validated species and subspecies (Turenne et al., 2007). Besides ruminants, MAP can infect a large range of host species including birds, rabbits, foxes and badgers (Beard et al., 2001). Infection of dairy cattle leads to economic losses because of reduced milk yield, premature culling and reduced slaughter value (Ott et al., 1999). Cattle are most susceptible to infection as calves where they are infected while still in the uterus or by ingestion of bacteria through milk, colostrum or faecal matter (Chiodini et al., 1984; Sweeney, 1996). Development of a cell-mediated immune (CMI) response is essential to the host in defence against intracellular MAP and is elicited at an early stage of infection before antibodies are detected. As the disease progress from subclinical to clinical, the CMI response wanes and is replaced by a strong humoral immune response characterized by antibody formation (Chiodini, 1996; Stabel, 2000). Antibodies do not protect the animal against the intracellular pathogen and in the final stages of infection suppression of the immune system and a state of anergy may occur (Waters et al., 1999). Diagnosis of MAP infected animals is frequently based on detection of antibodies in milk or serum, or by cultivation of bacteria from faeces, but these diagnostic methods are usually not applicable until years after infection at an advanced stage of disease (Nielsen and Toft, 2008). Contrary, CMI based diagnostics are applicable at an early stage of infection, prior to antibody formation and bacterial shedding in faeces. Hence, by using various diagnostic test strategies it is possible to detect infection with MAP at different stages of disease. The main limitation of available diagnostic tools is the lack of MAP specificity of included antigens resulting in poor specificity.

The aim of the current review was to provide an overview of MAP antigen candidates that has been used for diagnostic application to date. The emphasis will be on diagnostics measuring CMI, although humoral based approaches and different types of antigen will also be discussed.

2. Literature review

A review of MAP antigen candidates was carried out by searching the databases Web of Science and Medline through PubMed. The search terms were paratuberculosis; Johne’s or Johnes combined with antigen and generated 1169 hits including duplicate records. The number of publications was reduced to 96 by exclusion of duplicate records and exclusion of studies, where the abstract indicated that the study did not test antigens for MAP diagnosis or did not characterize MAP antigens. From available publications the following information was extracted and tabulated:

(a) Antigen name, locus and gene name, if available; (b) size in kDa; (c) characteristic; (d) whether the antigen candidate was purified from MAP or recombinant; (e) immunogenicity of antigen candidate and type of assay, i.e. *in vitro* CMI, skin test, enzyme linked immunosorbent assay (ELISA); (f) animal species and (g) species specificity. These publications describe 115 different antigen candidates originating from MAP, antigen candidates from mycobacteria other than MAP used for MAP diagnosis, antigen candidates of different specificity and antigen discovery and characterization by various approaches.

3. Immune-based test platforms for MAP diagnostics

The true state of infection can often only be established through culture of multiple tissues. However, bacterial growth is slow with test results being available only after months of incubation, and up to 100 tissues may be required to establish the infection status of an animal (Whitlock et al., 1996). Further, shedding of MAP at detectable levels in faeces is irregular (Nielsen and Toft, 2008). Therefore immune-based diagnostic tests are relevant alternatives to faecal culture and several tests measuring either specific CMI or antibodies have been developed.

3.1. Host immune response to MAP

MAP infected macrophages present antigens associated with major histocompatibility complex classes I and II on the cell surface to CD8+ and CD4+ T cells. As a result CD4+ T helper type 1 cells (T_{H1}) are activated and produces a range of cytokines, including interferon-γ (IFN-γ). The T_{H1} cytokine signals through a network of receptors and starts a cascade of reactions characterizing the CMI response (Coussens, 2001). As the infection progress, the CMI response wanes and an antibody-mediated CD4+ T helper type 2 (T_{H2}) response becomes predominant. Antibody production provides little, if any, protection against MAP and the infection expands without the important T_{H1} cell subset, which is considered to be necessary for protective immunity (Toman et al., 2003). Therefore it appears that IFN-γ producing T_{H1} cells recognizing peptide antigens are
the major mediators of specific immunity early in the infection. Hence, specific T cell responses can be exploited for early detection of infection whereas assays of humoral responses may be relevant for detecting the later stages of MAP infection (Stabel, 2000).

3.2. CMI based diagnostics

The intradermal skin test measures the delayed-type hyper-sensitivity response 72 h after intradermal injection of purified protein derivative (PPD) (Kalisi et al., 2003; Antognoli et al., 2007). PPDs are crude undefined extracts of mycobacterium antigens of different origin such as: MAP (PPDj or Johnin), M. avium subsp. avium (MAA) (PPDa) or M. bovis (PPDb) (Gilot and Cocito, 1993; Semret et al., 2006). For MAP diagnostics the situation is complicated by the lack of standardization of the PPD used. For many years the alleged reference strain 18 was used for preparation of antigens including PPDj, although it was later shown to be a laboratory adapted strain of M. avium that is not a representation of field strains of MAP isolated from cattle (Chiodini, 1993). Lack of standardized PPDs is of concern, because the genomic variability is considerably high in the organisms used for preparation of different PPDs (Semret et al., 2006). A standardized production of PPDs, including a detailed production protocol and the use of the same strains, would enable comparison of results from various laboratories.

Development of a positive skin test against PPDj is considered an indication of MAP infection. However, the current skin test protocol based upon PPDs not only cross-reacts with environmental mycobacteria but also with currently used MAP and M. bovis vaccines (Köhler et al., 2001). Another drawback of the skin test is that each individual animal needs to be restrained twice within a period of 72 h, first for the application of the test, and later for the reading of the results. As a consequence, the skin test has to some extent been replaced by an in vitro CMI assay measuring secreted IFN-γ in response to an antigen (Wood et al., 1989).

The IFN-γ assay requires only one intervention for blood collection. Whole blood samples are cultured with MAP antigens in a proliferation assay, and released IFN-γ is measured in the supernatant by ELISA (Wood et al., 1989). Similar to the skin test, the current IFN-γ MAP assay is based on PPD as antigen for stimulation and will therefore inherently have the same specificity problems. Furthermore, large scale use of the IFN-γ assay for routine diagnostics is limited by the short timeframe of less than 24 h allowed from blood sampling at the farm to stimulation with antigens in the laboratory (Jungersen et al., 2005). Finally, fluctuating IFN-γ responses to PPDs in calves younger than 15 months puts another limitation of the usefulness of the IFN-γ assay in its current form (Jungersen et al., 2002; Huda et al., 2003). We have previously investigated if the IFN-γ response to PPDj could be used as an early indicator of later antibody results in milk samples of cattle with different faecal culture status (Mikkelsen et al., 2009). Samples were collected during a period of three years from 975 cows from 18 Danish MAP infected dairy herds. The study showed that a small but significant negative association exists between IFN-γ test results obtained prior to first calving and milk antibody ELISA results at different ages after calving. In addition, we observed that only some of the IFN-γ positive animals developed a positive antibody response against MAP, which may indicate that CMI responses can control or eradicate MAP in many animals.

3.3. Serology based diagnostics

Antibodies to MAP in serum can be detected by different methods such as: complement fixation (Morris and Stevens, 1977), agar gel immuno-diffusion (AGID) (Sherman et al., 1990) and ELISA (Reichel et al., 1999). AGID can reach a specificity of 100% but it is less sensitive than ELISA which is capable of detecting small amounts of antibodies and therefore show the highest sensitivity of the serological tests for MAP (Harris and Barletta, 2001). Specificity of ELISA tests has been improved by removing cross-reacting antibodies by absorbing sera with Mycobacterium phlei. Using this method, studies have reported ELISA specificity of 98.8% (Cox et al., 1991) and 99.7% (Reichel et al., 1999). However, this absorption step compromises assay sensitivity. Sensitivity of serological tests is highest for animals with clinical symptoms as antibodies develop in the later stages of infection (Sweeney et al., 1995; Nielsen and Toft, 2008). Consequently, the main limitation of these antibody based assays is that they cannot be used for diagnosis of animals at an early stage of infection. The major advantages of antibody based diagnostic assays are that it is relatively easy to implement into a format that is useful for large scale screening, and can be used in the field without any expensive equipment or highly trained personnel.

4. Mycobacterium avium subsp. paratuberculosis antigen candidates

4.1. The ideal diagnostic antigen

To improve on CMI and serology based diagnostic assays it will be important to identify well-defined antigens contributing to high test sensitivity and specificity. In general, there is a lack of well-defined and standardized antigens for use in diagnostic assays even though a number of antigens have been described in the literature. The ideal characteristics of a new diagnostic MAP antigen are that it is antigenic, unique to MAP, recognized by MAP infected animals in the early subclinical stages of infection and that it remains detectable throughout the course of the disease. Thus far, no such antigen has been discovered for MAP infections. In practice, it is likely that a diagnostic test for MAP will require a cocktail of antigens due to the range of heterogeneous MHC molecules of out-bred animals (Kathaperumal et al., 2009) and due to differentiated antigen expression patterns of MAP bacteria (Radosевич et al., 2007). The same antigens will most likely not be useful for both CMI and serology assays.

4.2. Antigen candidates for CMI diagnostics

Specificity and sensitivity of CMI based assays are poor primarily because of the applied antigens. Most studies...
have been conducted using PPDs (Köhler et al., 2001). However, a number of studies have described novel well-defined MAP antigens for measuring CMI responses. Table 1 summarizes these antigen candidates based on publications from 1989 through 2009. The antigen candidates include secreted antigens, cell wall and membrane antigens, lipoproteins, heat shock proteins and hypothetical proteins. The earliest studies used native proteins purified from MAP: Avi-3 (Abe et al., 1989), A36-complex (Gilot et al., 1992), P30 (Burrells et al., 1995), alkyl hydroperoxid reductase C (AhpC) and alkyl hydroperoxid reductase D (AhpD) (Olsen et al., 2000a), MPP14 (Olsen and Storset, 2001; Olsen et al., 2005) and MPB70 (Olsen et al., 2005). All of these antigens were described to recall CMI responses in in vitro assays but they all lacked MAP specificity because of orthologues in other mycobacteria species. Two exceptions that were specific to MAP were antigens AphC and AphD, as demonstrated by immunoblot reaction of monospecific rabbit antiserum against the two antigens with 9 MAP strain lysates but not with lysates from 20 other mycobacterial species, except M. gordonae that produced weak cross-reactive bands (Olsen et al., 2000a). The diagnostic potential of AphC and AphD were evaluated in four goats experimentally infected with MAP. T cells from all four goats produced IFN-γ to in vitro stimulation with either antigen, whereas a non-infected control animal did not respond. ApHC and ApHD are the most promising CMI antigens among the natively purified MAP antigens as they appear to be species specific with a high sensitivity.

In recent years recombinant antigen candidates have replaced purified antigen candidates in the development of diagnostic assays. The advantages of recombinant antigens over purified antigens are higher product yield, high homology of antigen preparation and faster production. Several recombinant antigen candidates have been evaluated for detection of CMI against MAP. In some studies the species specificity was validated by comparison of the recombinant antigen sequence with available sequences of other mycobacteria (Koets et al., 1999; Nagata et al., 2005; Rosseels et al., 2006). Only the 67 kDa heat shock protein (HspX) was described to be species specific (Bannantine and Stabel, 2000). The remaining recombinant antigen candidates were either highly homologous to proteins in other mycobacteria species or the homology was not reported.

Unfortunately, the HspX protein did not produce a CMI recall response when tested in cell culture from 10 infected cows (Bannantine and Stabel, 2000). Apart from HspX and heat shock protein 65 (Hsp65) (Koets et al., 1999; Nagabhushanam et al., 2001), which induced only a low CMI response in both mice (Nagabhushanam et al., 2001) and cattle (Koets et al., 1999) all the described recombinant antigen candidates in Table 1 were reported to produce a CMI response.

To study recombinant heat shock proteins 65 (Hsp65) and 70 (Hsp70) blood samples were collected from 179 cows and distributed to groups of: cows naturally infected and vaccinated against MAP (n = 30), naturally infected and not vaccinated (n = 79), clinical cases (n = 11) and non-infected animals (n = 59) all from dairy farms (Koets et al., 1999). Each group was further divided into groups of faecal culture positive or negative cows. From blood, peripheral blood mononuclear cells were purified to evaluate proliferative responses following stimulation with Hsp65 or Hsp70 compared to PPDj. Proliferative responses to Hsp65 were generally low in all groups of cows whereas responses to Hsp70 varied between groups. Highest proliferative responses were observed for the group of faecal culture positive cows that were not vaccinated (88% positive), followed by faecal culture negative cows that were vaccinated (93.3% positive), 54 faecal negative cows that were non-vaccinated (74.1% positive) and 11 clinical cases of faecal culture positive cows (72.2% positive). In the control group, 30.5% produced a positive response to Hsp70 and 16.9% produced a positive response to Hsp65, which indicate low specificity of both antigens.

Another large study including 54 young clinical healthy cattle was conducted by Olsen et al. (2005) evaluating IFN-γ responses to recombinant ESAT-6, PPDb, purified antigen MPP14 and purified antigen MPB70. The study investigated the role of unspecific IFN-γ production by natural killer (NK) cells in young animals and concluded that NK cells were responsible for a large proportion of the IFN-γ producing cells in peripheral blood mononuclear cells from uninfected calves. A third study including a decent number of animals was the investigation of recombinant Ag85A, Ag85B and Ag85C. This study included 18 healthy control cows, 16 low shedders and 4 medium shedders and resulted in highest responses by cows from the group of medium shedders and lowest responses in the healthy control cows (Shin et al., 2005). The remaining studies in Table 1 included a much lower number of animals. Validation of immunogenicity of antigen candidates requires an appropriate sample size otherwise the results cannot be applied to herd conditions with genetically heterogeneous animals demonstrating different stages of MAP infection. Further, some of the listed antigens in Table 1 remain to be validated in bovine species, as they have only been validated in other species such as mice, rabbits or guinea pigs.

### 4.3. Types of characterized MAP antigen candidates

The majority of characterized or described MAP antigen candidates were not tested as CMI diagnostic antigens but either tested for immunogenicity in humoral assays or no records of immune reactivity were published. These antigen candidates are grouped and listed as: secreted antigens (Table 2), cell wall and membrane antigens (Table 3), lipoprotein antigens (Table 4), heat shock antigens (Table 5) and hypothetical antigens (Table 6). Due to the limited number of animals included in most studies it is impossible to validate the influence of variance of host immune response between animals, changes in MAP protein expression profiles during infection progress and influence of disease stage.

#### 4.3.1. Secreted antigen candidates

Secreted antigens (Table 2) are expected to be highly immunogenic or immunodominant due to their presence in the extracellular environment where they are more
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Locus</th>
<th>Gene</th>
<th>Size (kDa)</th>
<th>Characteristic</th>
<th>Antigen</th>
<th>Test assay</th>
<th>MAP specific</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted MPP14</td>
<td>–</td>
<td>–</td>
<td>14</td>
<td>Protein from MAA complex</td>
<td>Purified</td>
<td>IFN-γ test, resp. of 14 calves during two months, pos: 5/7 pos and 0/7 neg</td>
<td>Specific for MAA</td>
<td>Olsen et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>MPP14</td>
<td>–</td>
<td>14</td>
<td>Protein of MAIS complex</td>
<td>Purified</td>
<td>IFN-γ test, pos: 6/8 MAP inf, 1/7 control 23 weeks p.i.</td>
<td>No</td>
<td>Olsen and Storset (2001)</td>
</tr>
<tr>
<td>MPT-TP</td>
<td>–</td>
<td>–</td>
<td>16.7</td>
<td>Hypothetical thiol peroxidase</td>
<td>Recombinant</td>
<td>IFN-γ response of pooled mice spleen cells from 24 to 60 mice</td>
<td>Not reported</td>
<td>Mullerad et al. (2003)</td>
</tr>
<tr>
<td>SOD</td>
<td>–</td>
<td>–</td>
<td>28</td>
<td>Superoxide dismutase</td>
<td>Recombinant</td>
<td>IFN-γ signf. higher of 20 FC pos vs. 18 FC neg cows</td>
<td>81–93% homologous to M. TB and M. leprae, M. habana</td>
<td>Mullerad et al. (2002a)</td>
</tr>
<tr>
<td>P30</td>
<td>–</td>
<td>–</td>
<td>30</td>
<td>Protein</td>
<td>Purified</td>
<td>IFN-γ test, pos: 2/2 MAP inf., 0/2 uninf. sheep</td>
<td>Homologous to other mycobacteria</td>
<td>Burrells et al. (1995)</td>
</tr>
<tr>
<td>Ag85A</td>
<td>MAP0216</td>
<td>fhpA</td>
<td>30–32</td>
<td>Protein, T-cell epitope</td>
<td>Recombinant</td>
<td>IFN-γ test, pos: 5 inf. mice (Ag85A) and 5 inf. calves (Ag85 complex)</td>
<td>Identical sequence in M. bovis</td>
<td>Rosseels et al. (2006)</td>
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<tr>
<td>Ag85A</td>
<td>MAP0216</td>
<td>fhpA</td>
<td>30–32</td>
<td>Protein, T-cell epitope</td>
<td>Recombinant</td>
<td>IFN-γ signf. higher of 20 FC pos vs. 18 FC neg cows</td>
<td>Identical sequence in M. bovis</td>
<td>Shin et al. (2005)</td>
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<tr>
<td>Ag85B</td>
<td>MAP1609c</td>
<td>fhpB</td>
<td>30–32</td>
<td>Protein, T-cell epitope</td>
<td>Recombinant</td>
<td>IFN-γ test, pos: 5 inf. mice (Ag85B) and 5 inf. calves (Ag85 complex)</td>
<td>Identical sequence in M. bovis</td>
<td>Rosseels et al. (2006)</td>
</tr>
<tr>
<td>Ag85B</td>
<td>MAP1609c</td>
<td>fhpB</td>
<td>30–32</td>
<td>Protein, T-cell epitope</td>
<td>Recombinant</td>
<td>IFN-γ signf. higher of 20 FC pos vs. 18 FC neg cows</td>
<td>Identical sequence in M. bovis</td>
<td>Shin et al. (2005)</td>
</tr>
<tr>
<td>Ag85C</td>
<td>MAP3531c</td>
<td>fhpC2</td>
<td>30–32</td>
<td>Protein, T-cell epitope</td>
<td>Recombinant</td>
<td>IFN-γ test, pos: 5 inf. mice (Ag85C, low resp.) and 5 inf. calves (Ag85 complex)</td>
<td>Identical sequence in M. bovis</td>
<td>Rosseels et al. (2006)</td>
</tr>
<tr>
<td>Ag85C</td>
<td>MAP3531c</td>
<td>fhpC2</td>
<td>30–32</td>
<td>Protein, T-cell epitope</td>
<td>Recombinant</td>
<td>IFN-γ signf. higher of 20 FC pos vs. 18 FC neg cows</td>
<td>Identical sequence in M. bovis</td>
<td>Shin et al. (2005)</td>
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<tr>
<td>ESAT-6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Early secretory antigenic target 6 of M. TB complex</td>
<td>Recombinant</td>
<td>IFN-γ test, resp. of 14 calves during two months, pos: 7/7 pos and 1/7 neg</td>
<td>No</td>
<td>Olsen et al. (2005)</td>
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<tr>
<td>MPB70</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Protein from M. TB complex</td>
<td>Purified</td>
<td>IFN-γ test, resp. of 14 calves during two months, pos: 0/7 pos and 0/7 neg</td>
<td>No</td>
<td>Olsen et al. (2005)</td>
</tr>
<tr>
<td>SOD</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Superoxide dismutase</td>
<td>Recombinant</td>
<td>IFN-γ signf. higher of SOD imm. mice vs. control mice. Pooled samples of 24–60 mice</td>
<td>No</td>
<td>Shin et al. (2005)</td>
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<tr>
<td>Cell wall and membrane</td>
<td>Map39</td>
<td>Map3184</td>
<td>–</td>
<td>39</td>
<td>Possible T-cell Ag, homologous to PPE protein family</td>
<td>Recombinant</td>
<td>IFN-γ test, pos*: 5/5 pos and 0/5 neg</td>
<td>Homologous to MAA</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>Map41</td>
<td>Map1518</td>
<td>–</td>
<td>41</td>
<td>Possible T-cell Ag, homologous to PPE protein family</td>
<td>Recombinant</td>
<td>IFN-γ test, pos*: 5/5 pos and 0/5 neg</td>
<td>Homologous to MAA</td>
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<td>Antigen</td>
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<td>Gene</td>
<td>Size (kDa)</td>
<td>Characteristic</td>
<td>Antigen</td>
<td>Test assay</td>
<td>MAP specific</td>
<td>Reference</td>
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<td>A36-complex</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>TMA family, part of PPD</td>
<td>Purified</td>
<td>Skin test: reaction to A36 by sensitized rabbits</td>
<td>94% homology to MAA</td>
<td>Gilot et al. (1992)</td>
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<tr>
<td>Lipoproteins</td>
<td>MAP0261c</td>
<td>MAP0261c</td>
<td>19</td>
<td>Homologue protein of M TB is immunodominant in mice and humans</td>
<td>Recombinant</td>
<td>INF-γ test: low resp, but signf. difference between 21 naturally inf and 9 neg control cows</td>
<td>Homologues in M. bovis, MAA and M. intracellulare</td>
<td>Huntley et al. (2005)</td>
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<tr>
<td></td>
<td>MAP1138c</td>
<td>lprG</td>
<td>22</td>
<td>Exported lipoprotein (LppX/LprAFG family)</td>
<td>Recombinant</td>
<td>Proliferation assay, no signf. difference between MAP FC pos (n = 12) and FC neg (n = 14) cows. Serum antibody levels: signf. difference between groups</td>
<td>67% homology with M. bovis</td>
<td>Santema et al. (2009)</td>
</tr>
<tr>
<td>P22</td>
<td>MAP1138c</td>
<td>lprG</td>
<td>22</td>
<td>Exported lipoprotein (LppX/LprAFG family)</td>
<td>Recombinant</td>
<td>INF-γ test, pos: 8/9 MAP vaccinated and 0/5 control sheep</td>
<td>75% homology with M. leprae and M. TB</td>
<td>Dupont et al. (2005)</td>
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<tr>
<td>Heat shock proteins</td>
<td>Hsp65</td>
<td>–</td>
<td>65</td>
<td>From M. bovis BCG</td>
<td>Recombinant</td>
<td>Positive PBMC proliferation: 48% of FC pos, 20.4% of FC neg, 16.9% of control (FC neg), and 60% of MAP vaccinated cows</td>
<td>Highly conserved</td>
<td>Koets et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Hsp65</td>
<td>–</td>
<td>65</td>
<td>From MAA</td>
<td>Recombinant</td>
<td>ELISPOT, INF-γ pos: 0/5 MAP infected mice</td>
<td>Highly homologous to other mycobacteria</td>
<td>Nagabhushanam et al. (2001)</td>
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<td></td>
<td>HspX/MBP</td>
<td>hspX</td>
<td>67</td>
<td>May be involved in adhesion or heat shock. Protein is not secreted.</td>
<td>Purified/recombinant</td>
<td>INF-γ test, pos: 0/10 inf. and 0/3 control cattle. Serum antibody positive: 4/24 inf. and 0/3 control cows</td>
<td>Specific for MAP</td>
<td>Bannantine and Stabel (2000)</td>
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<tr>
<td></td>
<td>Hsp70</td>
<td>–</td>
<td>70</td>
<td>From M. bovis BCG</td>
<td>Recombinant</td>
<td>Positive PBMC proliferation: 88% of FC pos, 74.1% of FC neg, 30.5% of control (FC neg), and 93.3% of MAP vaccinated cows</td>
<td>Highly conserved</td>
<td>Koets et al. (1999)</td>
</tr>
<tr>
<td>Hypothetical proteins</td>
<td>Map10</td>
<td>–</td>
<td>10</td>
<td>Possible T-cell Ag. Part of fusion protein of β-galactosidase</td>
<td>Recombinant</td>
<td>INF-γ test, pos²: 4/5 pos and 0/5 neg</td>
<td>Not reported</td>
<td>Nagata et al. (2005)</td>
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<tr>
<td></td>
<td>AhpD</td>
<td>MAP1588c</td>
<td>ahpD</td>
<td>19</td>
<td>Alkyl hydroperoxid reductase D</td>
<td>Purified</td>
<td>INF-γ test, pos: 3/3 inf and 0/1 control goats. Serum antibody pos: 0/4 inf and 0/1 control goats ELISA pos: 7/56 pos, 0/10 neg cattle</td>
<td>Specific compared to 20 different mycobacteria strains</td>
</tr>
<tr>
<td>Ag85B</td>
<td>MAP1609c</td>
<td>fbpB</td>
<td>34.9</td>
<td>Mycolyl transferase, involved in cell wall assembly</td>
<td>Recombinant</td>
<td>IFN-γ response of pooled mice spleen cells from 24-60 mice</td>
<td>No</td>
<td>Mullerad et al. (2002b)</td>
</tr>
<tr>
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<td>P35</td>
<td>–</td>
<td>–</td>
<td>35</td>
<td>Associated with invasion of bovine intestinal epithelial cells</td>
<td>Recombinant</td>
<td>IFN-γ test, pos: 3/3 pos and 0/3 neg</td>
<td>Not reported</td>
<td>Basagoudanavar et al. (2006)</td>
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<tr>
<td>35 kDa</td>
<td>–</td>
<td>–</td>
<td>35</td>
<td>Associated with invasion of bovine intestinal epithelial cells</td>
<td>Recombinant</td>
<td>Proliferation assay, IFN-γ signif. higher in 20 FC pos vs. 18 FC neg cows</td>
<td>Not reported</td>
<td>Shin et al. (2005)</td>
</tr>
<tr>
<td>AhpC</td>
<td>MAP1589c</td>
<td>ahpC</td>
<td>45</td>
<td>Alkyl hydroperoxid reductase C</td>
<td>Purified</td>
<td>IFN-γ test, pos: 3/3 inf. and 0/1 control goats Serum antibody pos: 4/4 inf. and 0/1 control goats ELISA pos: 13/56 pos, 0/10 neg cattle</td>
<td>Specific compared to 20 different mycobacteria strains</td>
<td>Olsen et al. (2000a)</td>
</tr>
<tr>
<td>Apa</td>
<td>MAP1569</td>
<td>modD</td>
<td>50–60</td>
<td>Alanine and proline rich Ag or fibronectin attachment protein</td>
<td>Recombinant</td>
<td>IFN-γ test, pos: 6/45 FC pos and 0/45 FC neg. cows. Not significant.</td>
<td>98% homologous to MAA</td>
<td>Gioffre et al. (2009)</td>
</tr>
<tr>
<td>74F</td>
<td>MAP3527 and MAP1519</td>
<td>pepA-</td>
<td>74</td>
<td>Polypeptide of MAP3527 (a peptidase) and MAP1519 (a hypothetical protein)</td>
<td>Recombinant</td>
<td>Proliferation of pooled spleen cells of 24 mice. IFN-γ signif. higher of MAP74F immunised vs. control mice</td>
<td>Homologous to M. TB</td>
<td>Chen et al. (2008)</td>
</tr>
<tr>
<td>MAP1718c</td>
<td>MAP1718c</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Purified/ recombinant</td>
<td>Proliferation assay, no signif. difference between MAP FC pos (n = 12) and FC neg (n = 14) cows</td>
<td>99% homologous to MAA</td>
<td>Santema et al. (2009)</td>
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<tr>
<td>MAP3515c</td>
<td>MAP3515c</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Purified/ recombinant</td>
<td>Proliferation assay, no signif. difference between MAP FC pos (n = 12) and FC neg (n = 14) cows</td>
<td>No</td>
<td>Santema et al. (2009)</td>
</tr>
</tbody>
</table>

**Abbreviations:** Ag: antigen; BCG: Bacillus Calmette-Guérin; ELISPOT: enzyme linked immunosorbent spot; FC: faecal culture; IFN-γ: interferon gamma; imm.: immunised; inf.: infected; MAA: Mycobacterium avium subsp. avium; MAIS: M. avium-intracellulare-scrofulaceum; MAP: Mycobacterium avium subsp. paratuberculosis; M. TB: Mycobacterium tuberculosis; neg: negative; TMA: thermostable macromolecular antigen; PBMC: peripheral blood mononuclear cells; p.i.: post infection; pos: positive; PPD: purified protein derivate; PPE: proline-proline-glutamate; resp.: response; signif.: significant.

* Number of positive and negative animals extracted from available data.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Locus</th>
<th>Gene</th>
<th>Size (kDa)</th>
<th>Characteristic</th>
<th>Antigen</th>
<th>Test assay</th>
<th>MAP specific</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP2609</td>
<td>MAP2609</td>
<td>–</td>
<td>9</td>
<td>Conserved hypothetical protein</td>
<td>Recombinant</td>
<td>Immunoblot pos: 11/11 pos</td>
<td>Homologous to M. TB and M. bovis</td>
<td>Willemsen et al. (2006)</td>
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<tr>
<td>MAP0593c</td>
<td>MAP0593c</td>
<td>–</td>
<td>14.7</td>
<td>Hypothetical protein. Degradation of proteins, peptides, and glycopeptides</td>
<td>Recombinant</td>
<td>ELISA pos: 24/41 pos and 5/41 neg</td>
<td>Not reported</td>
<td>Gumber et al. (2009)</td>
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<tr>
<td>14 kDa</td>
<td>–</td>
<td></td>
<td>14</td>
<td>Putative signal peptide</td>
<td>Purified</td>
<td>ELISA pos: 13/30 pos; 1/10 neg</td>
<td>Homologus in all species of MAC</td>
<td>Olsen et al. (2000b, 2001)</td>
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<tr>
<td>MAP2411</td>
<td>MAP2411</td>
<td>–</td>
<td>15.5</td>
<td>Hypothetical protein. Biosynthesis of cofactors, prosthetic groups, and carriers: pyridoxine</td>
<td>Recombinant</td>
<td>ELISA pos</td>
<td>Not reported</td>
<td>Olsen et al. (2000b, 2001)</td>
</tr>
<tr>
<td>MAP2168c</td>
<td>MAP2168c</td>
<td>–</td>
<td>15.95</td>
<td>Hypothetical protein</td>
<td>Purified/recombinant</td>
<td>Immunoblot and ELISA pos</td>
<td>Not reported</td>
<td>Cho et al. (2006, 2007)</td>
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<tr>
<td>GreA</td>
<td>MAP1027c</td>
<td>greA</td>
<td>17.8</td>
<td>Transcription elongation factor GreA</td>
<td>Recombinant</td>
<td>ELISA pos: 10/41 pos and 2/41 neg</td>
<td>Not reported</td>
<td>Gumber et al. (2009)</td>
</tr>
<tr>
<td>Ppa</td>
<td>MAP0435c</td>
<td>ppa</td>
<td>18.5</td>
<td>Inorganic pyrophosphatase involved in metabolism</td>
<td>Recombinant</td>
<td>ELISA pos: 14/41 pos and 2/41 neg</td>
<td>Not reported</td>
<td>Gumber et al. (2009)</td>
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<tr>
<td>ClpP</td>
<td>MAP2281c</td>
<td>clpP</td>
<td>21.6</td>
<td>ATP-dependent Clp protease proteolytic subunit. Degradation of proteins, peptides, and glycopeptides</td>
<td>Recombinant</td>
<td>ELISA pos: 19/41 pos and 2/41 neg</td>
<td>Not reported</td>
<td>Gumber et al. (2009)</td>
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<tr>
<td>SOD</td>
<td>MAP0187c</td>
<td>sodA</td>
<td>23</td>
<td>Superoxide dismutase</td>
<td>Purified</td>
<td>Not tested</td>
<td>Not reported</td>
<td>Liu et al. (2001)</td>
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<td>MAP3199</td>
<td>MAP3199</td>
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<td>26</td>
<td>Hypothetical protein. Microtubule-associated</td>
<td>Purified/recombinant</td>
<td>ELISA pos: 0/7 pos and 0/3 neg</td>
<td>Absent in M. bovis</td>
<td>Leroy et al. (2007)</td>
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<td>Antigen-A</td>
<td>–</td>
<td>–</td>
<td>31</td>
<td>Extracted from culture filtrate and sonicate</td>
<td>Purified</td>
<td>ELISA pos: 18/22 pos at sp = 90, AGID pos 4/22 pos</td>
<td>Similar to BCG85 complex Ags of M. bovis</td>
<td>Sugden et al. (1991)</td>
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<tr>
<td>PepA</td>
<td>MAP3527</td>
<td>pepA</td>
<td>31.8</td>
<td>Serine protease</td>
<td>Purified/recombinant</td>
<td>Immunoblot and ELISA pos</td>
<td>Not reported</td>
<td>Cho et al. (2006, 2007)</td>
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<tr>
<td>32 kDa</td>
<td>–</td>
<td>–</td>
<td>32</td>
<td>Fibronectin binding properties</td>
<td>Purified</td>
<td>Not tested</td>
<td>Not reported</td>
<td>ElZaatari et al. (1994)</td>
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<td>ModD</td>
<td>MAP1569</td>
<td>modD</td>
<td>32.3</td>
<td>Associated with MHC II and fibroinectin attachment</td>
<td>Recombinant</td>
<td>Immunoblot and ELISA pos</td>
<td>Homologous to M. TB and M. bovis</td>
<td>Cho et al. (2006, 2007)</td>
</tr>
<tr>
<td>MAP1272c</td>
<td>MAP1272c</td>
<td>–</td>
<td>33.3</td>
<td>Putative invasin, NlpC/P60 superfamily</td>
<td>Recombinant</td>
<td>Immunoblot, ELISA pos: 7/7 pos, 0/2 neg</td>
<td>Homologous to MMP-I of MAA and M. leprae</td>
<td>Li et al. (2007)</td>
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<tr>
<td>Map0210c</td>
<td>Map0210c</td>
<td>pirG</td>
<td>34</td>
<td>–</td>
<td>Recombinant</td>
<td>Immunoblot pos in 10/11</td>
<td>Homologous to M. TB and M. bovis</td>
<td>Willemsen et al. (2006)</td>
</tr>
<tr>
<td>Ag85C</td>
<td>MAP3531c</td>
<td>βlpC2</td>
<td>34.1</td>
<td>Putative mycolyl transferase</td>
<td>Purified/recombinant</td>
<td>Immunoblot and ELISA pos</td>
<td>Highly conserved</td>
<td>Cho et al. (2006, 2007)</td>
</tr>
<tr>
<td>MAP4308c</td>
<td>MAP4308c</td>
<td>36</td>
<td>Possible L-lactate-2-monooxygenase</td>
<td>Purified/recombinant</td>
<td>ELISA pos(^a): 4/7 pos and 0/3 neg</td>
<td>Absent in M. bovis</td>
<td>Leroy et al. (2007)</td>
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<td>44.3 kDa</td>
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<td>44.3</td>
<td>Soluble protein</td>
<td>Purified</td>
<td>Not tested</td>
<td>Not reported</td>
<td>Mutharia et al. (1997)</td>
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<td>Antigen-D</td>
<td>–</td>
<td>400</td>
<td>Peroxidase, extracted from culture filtrate + sonicate</td>
<td>Purified ELISA pos: 22/22 pos at sp = 90, AGID pos: 18/22 pos</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Sugden et al. (1991)</td>
<td></td>
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<tr>
<td>Antigen-D</td>
<td>–</td>
<td>400</td>
<td>Peroxidase, extracted from culture filtrate + sonicate</td>
<td>Purified ELISA pos: 22/22 pos at sp = 90, AGID pos: 18/22 pos</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Sugden et al. (1991)</td>
<td></td>
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<tr>
<td>Ag85A</td>
<td>MAP0216</td>
<td>fbpA</td>
<td>– T-cell epitope</td>
<td>Purified</td>
<td>Not tested</td>
<td>Not reported</td>
<td>Homologous to MAA</td>
<td>Brooks et al. (1991)</td>
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<tr>
<td>Ag85B</td>
<td>MAP1609c</td>
<td>fbpB</td>
<td>– T-cell epitope</td>
<td>Purified</td>
<td>Not tested</td>
<td>Not reported</td>
<td>Homologous to MAA</td>
<td>Dheenadhayalan et al. (2002)</td>
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<tr>
<td>Ag85C</td>
<td>MAP3531c</td>
<td>fbpC2</td>
<td>– T-cell epitope</td>
<td>Purified</td>
<td>Not tested</td>
<td>Not reported</td>
<td>Homologous to MAA</td>
<td>Dheenadhayalan et al. (2002)</td>
</tr>
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<td>Ag85C</td>
<td>MAP3531c</td>
<td>fbpC2</td>
<td>– T-cell epitope</td>
<td>Purified</td>
<td>Not tested</td>
<td>Not reported</td>
<td>Homologous to MAA</td>
<td>Dheenadhayalan et al. (2002)</td>
</tr>
<tr>
<td>MAP0586c</td>
<td>MAP0586c</td>
<td>–</td>
<td>Hypothetical protein. Possible transglycosylase SLT domain</td>
<td>Purified/recombinant</td>
<td>ELISA pos(^a): 0/7 pos and 0/3 neg</td>
<td>Absent in M. bovis</td>
<td>Leroy et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>MAP2677c</td>
<td>MAP2677c</td>
<td>–</td>
<td>Hypothetical protein, glyoxylase</td>
<td>Purified/recombinant</td>
<td>ELISA pos(^a): 4/7 pos and 0/3 neg</td>
<td>Absent in M. bovis</td>
<td>Leroy et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>–</td>
<td>–</td>
<td>Superoxide dismutase, induce B-cell response</td>
<td>Purified/recombinant</td>
<td>ELISA pos(^a): 49/60 pos and 2/22 pos</td>
<td>Not reported</td>
<td>Shin et al. (2004)</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** As in Table 1; MAC: *Mycobacterium avium* complex; MHC II: major histocompatibility complex class II.

\(^a\) Number of positive and negative animals extracted from available data.

\(^b\) Based on available data on number of animals, specificity and sensitivity.
Table 3
Characterized cell wall and membrane antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Locus</th>
<th>Gene</th>
<th>Size (kDa)</th>
<th>Characteristic</th>
<th>Antigen</th>
<th>Test assay</th>
<th>MAP specific</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP1087</td>
<td>MAP1087</td>
<td>–</td>
<td>15.4</td>
<td>Putative surface protein. Peptide/nickel transport system permease</td>
<td>Recombinant</td>
<td>Immunoblot pos: 2/2 MAP infected cattle, 0/2 prior to infection</td>
<td>Homologous to MAA and <em>M. smegmatis</em></td>
<td>Bannantine et al. (2008a)</td>
</tr>
<tr>
<td>MAP1087</td>
<td>MAP1087</td>
<td>–</td>
<td>15.4</td>
<td>Putative surface protein. Peptide/nickel transport system permease</td>
<td>Recombinant</td>
<td>Immunoblot: high spot intensity in 6/6 infected cattle</td>
<td>Cross-reaction with <em>M. bovis</em> infected sera</td>
<td>Bannantine et al. (2008b)</td>
</tr>
<tr>
<td>34 kDa-C</td>
<td>–</td>
<td>–</td>
<td>18</td>
<td>Cell wall Ag, carboxyl termini of 34 kDa protein, putative heparin binding hemagglutinin protein</td>
<td>Purified/recombinant</td>
<td>ELISA pos: 18/18 pos, 0/50 neg</td>
<td>Not reported</td>
<td>Malamo et al. (2006)</td>
</tr>
<tr>
<td>MAP3968</td>
<td>MAP3968</td>
<td>–</td>
<td>30</td>
<td>Hypothetical protein, putative heparin binding hemagglutinin protein</td>
<td>Recombinant</td>
<td>ELISA pos: 9/23 pos, 4/46 neg</td>
<td>92% homology to MAA</td>
<td>Sechi et al. (2006)</td>
</tr>
<tr>
<td>a362 P34</td>
<td>–</td>
<td>–</td>
<td>34</td>
<td>B-cell epitope (part of A36, TMA complex)</td>
<td>Recombinant</td>
<td>ELISA pos: 25/25 pos, 0/7 neg</td>
<td>Homologous to <em>M. leprae</em>, but two species specific epitopes</td>
<td>Dekesel et al. (1993)</td>
</tr>
<tr>
<td>P34</td>
<td>–</td>
<td>–</td>
<td>34</td>
<td>B-cell epitope (part of A36, TMA complex)</td>
<td>Purified</td>
<td>Not tested</td>
<td>Specific based on EMBL data bank</td>
<td>Gilot et al. (1993)</td>
</tr>
<tr>
<td>P34-p5</td>
<td>–</td>
<td>–</td>
<td>34</td>
<td>B-cell epitope peptide A</td>
<td>Recombinant</td>
<td>ELISA pos: 6/10 pos, 0/10 neg</td>
<td>Cross-reaction with <em>M. bovis</em> infected sera (0/6)</td>
<td>Ostrowski et al. (2003)</td>
</tr>
<tr>
<td>P34-p8</td>
<td>–</td>
<td>–</td>
<td>34</td>
<td>B-cell epitope peptide B</td>
<td>Recombinant</td>
<td>ELISA pos: 8/10 pos, 0/10 neg</td>
<td>Cross-reaction with <em>M. bovis</em> infected sera (0/6)</td>
<td>Ostrowski et al. (2003)</td>
</tr>
<tr>
<td>34 kDa</td>
<td>–</td>
<td>–</td>
<td>34</td>
<td>B-cell epitope (part of A36, TMA complex)</td>
<td>Purified</td>
<td>Immunoblot: high spot intensity in 3/3 infected cattle</td>
<td>Low cross-reaction with MAA, <em>M. bovis</em> and <em>M. phlei</em></td>
<td>Dekesel et al. (1992)</td>
</tr>
<tr>
<td>MAP2121c</td>
<td>MAP2121c</td>
<td>–</td>
<td>35</td>
<td>Major membrane protein 1, putative role in invasion of epithelial cells</td>
<td>Recombinant</td>
<td>Immunoblot: high spot intensity in 3/3 infected cattle</td>
<td>Homologous to <em>M. leprae</em></td>
<td>Bannantine et al. (2008d)</td>
</tr>
<tr>
<td>P35</td>
<td>–</td>
<td>–</td>
<td>35</td>
<td>–</td>
<td>Recombinant</td>
<td>Immunoblot reaction with anti-MAP rabbit serum and skin reaction in guinea pigs</td>
<td>Cross-reaction with MAA in skin test</td>
<td>Basagoudanavar et al. (2004)</td>
</tr>
<tr>
<td>MMP</td>
<td>–</td>
<td>–</td>
<td>35</td>
<td>T-cell Ag (homologous to major membrane protein, MMP)</td>
<td>–</td>
<td>Not tested</td>
<td>Homologous to <em>M. leprae</em> and MAA</td>
<td>Banasure et al. (2001)</td>
</tr>
<tr>
<td>MMP</td>
<td>–</td>
<td>–</td>
<td>35</td>
<td>Major membrane protein. Role in invasion of epithelial cells.</td>
<td>Recombinant</td>
<td>Not tested</td>
<td>Homologous to <em>M. leprae</em> and MAA</td>
<td>Bannantine et al. (2003a)</td>
</tr>
<tr>
<td>TMA</td>
<td>–</td>
<td>–</td>
<td>100</td>
<td>Cytoplasmic protein of TMA complex</td>
<td>Purified</td>
<td>Not tested, comparison of TMA from MAP, MAA and <em>M. bovis</em></td>
<td>Homologues in MAA and <em>M. bovis</em></td>
<td>Bruneteau et al. (1992)</td>
</tr>
<tr>
<td>a362</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Ag containing B-cell epitope. Polypeptide of carboxyl termini of 34 kDa protein</td>
<td>Recombinant</td>
<td>ELISA pos; 84/208 pos, 9/175 neg, 2/38 <em>M. bovis</em> pos</td>
<td>Specific compared to MAA, <em>M. bovis</em> and <em>M. phlei</em>. Low cross-reaction with <em>M. bovis</em></td>
<td>Vannuffel et al. (1994)</td>
</tr>
<tr>
<td>MAP1204</td>
<td>MAP1204</td>
<td>–</td>
<td>–</td>
<td>Hypothetical protein, putative invasin</td>
<td>Recombinant</td>
<td>Immunoblot: high spot intensity in 6/6 infected cattle</td>
<td>Cross-reaction with <em>M. bovis</em> cow sera</td>
<td>Bannantine et al. (2008b)</td>
</tr>
<tr>
<td>MAP1506</td>
<td>MAP1506</td>
<td>–</td>
<td>–</td>
<td>Hypothetical protein, PPE protein family</td>
<td>Purified peptide</td>
<td>Not tested</td>
<td>Homologous in <em>M. avium</em> subsp. <em>hominissuis</em> and MAA</td>
<td>Newton et al. (2009)</td>
</tr>
<tr>
<td>Antigen</td>
<td>Locus</td>
<td>Gene</td>
<td>Size (kDa)</td>
<td>Characteristic</td>
<td>Test assay</td>
<td>MAP specific</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>---------</td>
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<td>---------------</td>
<td>------------</td>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Lpp34</td>
<td>-</td>
<td>-</td>
<td>20.8</td>
<td>Putative lipoprotein</td>
<td>Recombinant</td>
<td>Immunoblot: high spot intensity in 5/5 pos cattle</td>
<td>Homologous to MAA and E. coli, but absent in M. TB complex</td>
<td>Gioffre et al. (2006)</td>
</tr>
<tr>
<td>L5P</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Lipopentapeptide, a non-ribosomal synthase</td>
<td>Purified</td>
<td>Immunoblot pos:a: 4/4 FC pos, 0/4 FC neg; 3/4 tissue pos, 1/3 tissue neg. ELISA pos: 1 pos in all 3 ELISA/4 FC pos.</td>
<td>Not present in MAA or M. smegmatis.</td>
<td>Biet et al. (2008)</td>
</tr>
<tr>
<td>LAM</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Lipoarabinomannan</td>
<td>Purified</td>
<td>AGID pos: 1/1 pos</td>
<td>Not reported</td>
<td>Sugden et al. (1987)</td>
</tr>
<tr>
<td>Para-LP-01</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Cell wall lipopeptide, likely major component of outer part of cell envelope</td>
<td>Purified</td>
<td>ELISA pos:a: 5/6 pos, 0/3 neg</td>
<td>Not present in MAA</td>
<td>Eckstein et al. (2006)</td>
</tr>
<tr>
<td>Polar GPL-1</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Glycopeptidolipid, belongs to polar mycoside C glycopeptidolipid family</td>
<td>Purified</td>
<td>ELISA pos: 1(high)/9 pos, 0(?) neg</td>
<td>Not reported</td>
<td>Camphausen et al. (1985)</td>
</tr>
<tr>
<td>PstA</td>
<td>-</td>
<td>pstA</td>
<td></td>
<td>Carboxyl termini of 34 kDa protein</td>
<td>Recombinant</td>
<td>Production of monoclonal antibodies</td>
<td>Common epitopes of MAP, MAA and M. intracellulare</td>
<td>Malamo et al. (2007)</td>
</tr>
</tbody>
</table>

**Table 4**
Characterized lipoprotein antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*.

*Abbreviations: As in Table 1.*

*Number of positive and negative animals extracted from available data.*
likely to encounter sensitized immune cells. This was confirmed in a study comparing secreted antigens from supernatants and antigens of intracellular origin (Cho and Collins, 2006). Serum from infected cattle was used for immunoblot analysis which showed that infected sera reacted more strongly to the secreted antigens compared to the antigens of intracellular origin. The majority of the antigen candidates were evaluated with relatively few positive and negative control sera. However, five recombinant antigens, MAP2411, ClpP (MAP2281c), Ppa (MAP0593c) and GreA (MAP1027c), were tested for immunogenicity with sera from 41 sheep with a known MAP infection and 41 non-infected control sheep (Gumber et al., 2009b). Two of these antigens, MAP0593c and ClpP reacted against 58.5% and 46.3% test positive sera and 12.1% and 4.9% of the negative control sera. MAP2411 could not distinguish between MAP positive or negative serum samples. The specificity of these antigen candidates towards MAP was not reported and hence it is difficult to evaluate their usefulness. An earlier study tested recombinant antigens of Ag85A, Ag85B, Ag85C and SOD in an ELISA with sera from 60 MAP shedding cows and 22 non-shedding cows (Shin et al., 2004). Antigens of the Ag85 complex and SOD showed high reactivity against sera from the MAP shedding cows and little reactivity against sera from the non-shedding cows. Conserved proteins such as antigens of the Ag85 complex (Dheenadhayalan et al., 2002) are found in all mycobacteria species. At the protein level the three Ag85 components of MAP share 99% sequence identity with MAA (Rosseels et al., 2006). For vaccine design the use of conserved proteins may provide cross protection towards other infections as a positive side-effect. However, for diagnostic purposes this cross reactivity is undesirable as high specificity and sensitivity is essential. The specificity for MAP was not reported for a large part of the antigen candidates listed in Table 2.

4.3.2. Cell wall and membrane antigens

Antigen candidates originating from cell wall or membrane are listed in Table 3. Similar to secreted antigens, cell wall and membrane antigens are expected to be highly immunogenic due to their accessibility to components of the immune system. Several of the reported studies have characterized and tested a 34 kDa membrane protein antigen candidate (p34) (Gilot et al., 1992; Ostrowski et al., 2003) or parts of the protein (Dekesel et al., 1993; Vannuffel et al., 1994; Malamo et al., 2006) that belongs to the major antigen complex A36. The p34 protein was reported to elicit a predominant humoral immune response (Dekesel et al., 1992) and to be MAP specific (Gilot et al., 1993). In general, the characterized antigen candidates were evaluated with sera of few animals. However, the polypeptide a36.2, which represent the carboxyl termini of p34, was evaluated by ELISA with sera from 208 MAP positive and 175 MAP negative cattle (Vannuffel et al., 1994). The peptide reacted with 40.4% of the positive samples and 5% of the negative samples and hence showed low sensitivity but high specificity. The majority of the listed antigen candidates were reported to cross react with other mycobacteria species (Table 3).
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Locus</th>
<th>Gene</th>
<th>Size (kDa)</th>
<th>Characteristic</th>
<th>Antigen</th>
<th>Test assay</th>
<th>MAP specific</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP3155c</td>
<td>MAP3155c</td>
<td>–</td>
<td>9</td>
<td>–</td>
<td>Recombinant</td>
<td>Immunoblot: high spot intensity in 5/5 pos cattle, high in pos rabbit and low in pos mouse</td>
<td>Not reported</td>
<td>Bannantine et al. (2008d)</td>
</tr>
<tr>
<td>Ag6</td>
<td>Overlap MAP0864 and MAP0865</td>
<td>–</td>
<td>16</td>
<td>T-cell epitope, putative protein</td>
<td>Recombinant</td>
<td>ELISA pos²; 8/18 pos, 1/48 neg</td>
<td>Specific compared to MAA. Low cross-reaction with M. bovis (1/48)</td>
<td>Leroy et al. (2009)</td>
</tr>
<tr>
<td>MAP3857</td>
<td>MAP3857</td>
<td>umpA</td>
<td>18.7</td>
<td>UmpA (orotate phosphoribosyltransferase), biosynthesis</td>
<td>Recombinant</td>
<td>ELISA pos² in 0/6 pos, Dot blot pos, Western blot neg</td>
<td>Not reported</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>21K</td>
<td>MAP2685</td>
<td>–</td>
<td>21</td>
<td>Unknown function</td>
<td>Recombinant</td>
<td>Not tested</td>
<td>Specific compared to MAA. Low cross-reaction with M. bovis (1/48)</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>Ag5</td>
<td>–</td>
<td>–</td>
<td>22.5</td>
<td>T-cell epitope, putative protein</td>
<td>Recombinant</td>
<td>ELISA pos²; 5/18 pos, 1/48 neg</td>
<td>Specific compared to MAA. Low cross-reaction with M. bovis (1/48)</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP1564c</td>
<td>MAP1564c</td>
<td>–</td>
<td>23.1</td>
<td>Short chain dehydrogenase</td>
<td>Recombinant</td>
<td>ELISA pos²; 6/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP3627</td>
<td>MAP3627</td>
<td>–</td>
<td>23.1</td>
<td>Hypothetical protein involved in metabolism</td>
<td>Recombinant</td>
<td>ELISA pos²; 6/6 pos, Dot blot neg, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP268c</td>
<td>MAP268c</td>
<td>–</td>
<td>23.8</td>
<td>Hypothetical conserved protein</td>
<td>Recombinant</td>
<td>ELISA pos²; 6/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP3491</td>
<td>MAP3491</td>
<td>–</td>
<td>25.2</td>
<td>Hypothetical hydrolase</td>
<td>Recombinant</td>
<td>ELISA pos²; 6/6 pos, Dot blot pos, Western blot neg</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP1204</td>
<td>MAP1204</td>
<td>–</td>
<td>25.4</td>
<td>Putative exported p60 protein, virulence factor</td>
<td>Recombinant</td>
<td>Immunoblot pos: 2/2 infected cattle, 0/2 prior infection</td>
<td>Present in MAA and M. smegmatis</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP1297</td>
<td>MAP1297</td>
<td>hisA</td>
<td>25.4</td>
<td>HisA (phosphoribosyl isomerise)</td>
<td>Recombinant</td>
<td>ELISA pos²; 6/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP2878c</td>
<td>MAP2878c</td>
<td>dapB</td>
<td>25.6</td>
<td>DapB (dihydriodipicolinate reductase), amino acid biosynthesis</td>
<td>Recombinant</td>
<td>ELISA pos²; 5/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP0865</td>
<td>MAP0865</td>
<td>–</td>
<td>30.9</td>
<td>Hypothetical protein</td>
<td>Recombinant</td>
<td>ELISA²: 3/3 sheep anti-MAP sera recognised antigen</td>
<td>Unique to MAP</td>
<td>Bannantine et al. (2008c)</td>
</tr>
<tr>
<td>MAP1365</td>
<td>MAP1365</td>
<td>ardF</td>
<td>33.6</td>
<td>ArgF (ornithine carbamoyltransferase)</td>
<td>Recombinant</td>
<td>ELISA pos²; 5/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
</tr>
<tr>
<td>MAP0334</td>
<td>MAP0334</td>
<td>–</td>
<td>34.5</td>
<td>Cytoplasmic protein</td>
<td>Recombinant</td>
<td>ELISA pos²; 6/6 pos, Dot blot pos, Western blot pos</td>
<td>Cross-reaction with tested mycobacteria</td>
<td>Hughes et al. (2008)</td>
</tr>
</tbody>
</table>

*Table 6* Characterized hypothetical proteins, antigen candidates of unknown function or other antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*. 

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<table>
<thead>
<tr>
<th>Antigen</th>
<th>Locus</th>
<th>Gene</th>
<th>Size (kDa)</th>
<th>Characteristic</th>
<th>Antigen</th>
<th>Test assay</th>
<th>MAP specific</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P35</td>
<td>–</td>
<td>–</td>
<td>35</td>
<td>–</td>
<td>Recombinant Immunoblot pos: 31/36 pos sheep/goats/cows, 0/3 M. bovis infected, 0/3 inoculated cows, 0/15 healthy controls</td>
<td>Cross-reaction with anti-M. bovis and anti-MAA serum</td>
<td>ElZaatari et al. (1997)</td>
<td></td>
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<tr>
<td>35 kDa</td>
<td>–</td>
<td>–</td>
<td>35</td>
<td>–</td>
<td>Recombinant ELISA pos: 56/60 pos, 3/22 neg</td>
<td>Not reported</td>
<td>Shin et al. (2004)</td>
<td></td>
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<tr>
<td>MAP1012c</td>
<td>MAP1012c</td>
<td>–</td>
<td>37.5</td>
<td>–</td>
<td>Recombinant ELISA pos: 6/9 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
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</tr>
<tr>
<td>Ag7</td>
<td>MAP1637c</td>
<td>–</td>
<td>40</td>
<td>T-cell epitope. Biosynthesis of cofactors, prosthetic groups, and carriers</td>
<td>Recombinant ELISA pos: 12/18 pos, 1/48 neg</td>
<td>Specific compared to MAA. Low cross-reaction with M. bovis (1/48)</td>
<td>Leroy et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>MAP3932c</td>
<td>MAP3932c</td>
<td>moaA3</td>
<td>41.4</td>
<td>MoaA3 (molybdopterin biosynthesis protein MoeA). Biosynthesis of cofactors, prosthetic groups and carriers</td>
<td>Recombinant ELISA pos: 6/9 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>MAP3175c</td>
<td>MAP3175c</td>
<td>prfB</td>
<td>41.5</td>
<td>Peptide chain release factor 2, protein synthesis</td>
<td>Recombinant ELISA pos: 0/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>42 kDa</td>
<td>–</td>
<td>–</td>
<td>42</td>
<td>Cytoplasmic protein</td>
<td>Purified Not tested</td>
<td></td>
<td>White et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>MAP4147</td>
<td>MAP4147</td>
<td>–</td>
<td>42.2</td>
<td>Hypothetical protein involved in energy metabolism, electron transport</td>
<td>Recombinant ELISA pos: 0/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>MAP3457</td>
<td>MAP3457</td>
<td>metC</td>
<td>47.6</td>
<td>MetC(o-acetylhomoserine aminocarboxypropyltransferase) involved in metabolism</td>
<td>Recombinant ELISA pos: 0/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>MAP1293</td>
<td>MAP1293</td>
<td>hisD</td>
<td>49.4</td>
<td>HisD (histidinol dehydrogenase)</td>
<td>Recombinant ELISA pos: 0/6 pos, Dot blot pos, Western blot pos</td>
<td>Higher expression in MAP &gt; MAA</td>
<td>Hughes et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>MAP0860</td>
<td>MAP0860</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Recombinant Immunoblot pos: 0/1 pos mouse, 0/2 pos rabbit and 0/2 pos cattle</td>
<td>Unique to MAP</td>
<td>Paustian et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>MAP0860c</td>
<td>MAP0860c</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Recombinant Immunoblot: high spot intensity in 3/5 pos cattle, high in pos rabbit and low in pos mouse</td>
<td>Unique to MAP</td>
<td>Bannantine et al. (2008d)</td>
<td></td>
</tr>
<tr>
<td>MAP0862</td>
<td>MAP0862</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Recombinant Immunoblot pos: 1/1 pos mouse, 2/2 pos rabbit and 2/2 pos cattle</td>
<td>Unique to MAP</td>
<td>Paustian et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>MAP0862</td>
<td>MAP0862</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Recombinant ELISA pos: 9/11 pos, ELISA pos: 1/3 sheep anti-MAA sera recognised antigen</td>
<td></td>
<td>Bannantine et al. (2008c)</td>
<td></td>
</tr>
<tr>
<td>MAP1643</td>
<td>MAP1643</td>
<td>aceAB</td>
<td>–</td>
<td>Putative isocitrate lyase</td>
<td>Recombinant Immunoblot: high spot intensity in 6/6 infected cattle</td>
<td>Not reported</td>
<td>Bannantine et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>MAP1730c</td>
<td>MAP1730c</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Recombinant Immunoblot pos: 1/1 pos mouse, 1/2 pos rabbit and 0/2 pos cattle</td>
<td>Unique to MAP</td>
<td>Paustian et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td>Type</td>
<td>Immunoblot:</td>
<td>Specificity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------</td>
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<tr>
<td>MAP2182c</td>
<td>Hypothetical conserved protein</td>
<td>Recombinant</td>
<td>high spot intensity in 4/5 pos cattle, high in pos mouse and rabbit</td>
<td>Highly conserved protein</td>
<td>Bannantine et al. (2008d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP2963c</td>
<td>Hypothetical conserved protein containing heme binding domain</td>
<td>Recombinant</td>
<td>Immunoblot pos: 1/1 pos mouse, 2/2 pos rabbit and 2/2 pos cattle</td>
<td>Unique to MAP</td>
<td>Paustian et al. (2004)</td>
<td></td>
<td></td>
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<tr>
<td>MAP3732c</td>
<td></td>
<td>Recombinant</td>
<td>Immunoblot pos: 1/1 pos mouse, 2/2 pos rabbit and 2/2 pos cattle</td>
<td>Unique to MAP</td>
<td>Paustian et al. (2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP-antigen</td>
<td>Protoplasmic antigen</td>
<td>Purified</td>
<td>ELISA pos: 50/60 pos, 5/44 neg cattle</td>
<td>Cross-reaction with several bacteria</td>
<td>Abbas et al. (1983)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP-gene56</td>
<td>Protein of unknown function produced by MAP</td>
<td>Recombinant</td>
<td>Immunoblot pos: 5/6 pos, 0/4 neg cattle</td>
<td>Specific compared to 9 mycobacteria species</td>
<td>Bannantine et al. (2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP-gene135</td>
<td>Protein of unknown function produced by MAP</td>
<td>Recombinant</td>
<td>Immunoblot pos: 4/6 pos: 1/4 neg cattle</td>
<td>Specific compared to 9 mycobacteria species</td>
<td>Bannantine et al. (2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP-gene218</td>
<td>Protein of unknown function produced by MAP</td>
<td>Recombinant</td>
<td>Immunoblot pos: 5/6 pos, 0/4 neg cattle</td>
<td>Specific compared to 9 mycobacteria species</td>
<td>Bannantine et al. (2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP-gene241</td>
<td>Protein of unknown function produced by MAP</td>
<td>Recombinant</td>
<td>Immunoblot pos: 6/6 pos, 1/4 neg cattle</td>
<td>Specific compared to 9 mycobacteria species</td>
<td>Bannantine et al. (2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP-gene254</td>
<td>Protein of unknown function produced by MAP</td>
<td>Recombinant</td>
<td>Immunoblot pos: 5/6 pos, 4/6 neg cattle</td>
<td>Specific compared to 9 mycobacteria species</td>
<td>Bannantine et al. (2004)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: As in Table 1.
* Number of positive and negative animals extracted from available data.
A comparative study of different mycobacteria species genomes showed that the major differences are in the gene products constituting the cell wall and in particular genes encoding the PE and PPE proteins (Marri et al., 2006), which are unique to mycobacteria. PE and PPE proteins are named after a proline–glutaric acid (PE) motif and a proline–proline–glutamic acid (PPE) motif found in conserved domains near the N termini in these proteins. Their function remains unknown but members of the family have been linked to virulence (Li et al., 2005b). A recent genomic comparison study concluded that several PPE proteins were unique to MAP compared to other members of the *Mycobacterium avium* complex (Mackenzie et al., 2009). These proteins may therefore be useful antigen candidates in MAP specific diagnostic assays. Two PPE proteins expressed on the MAP cell wall are listed in Table 3, MAP1506 and MAP3420c (Newton et al., 2009). Recombinant MAP3420c showed positive immunoblot reaction with sera from four MAP faecal culture positive cows and did not react with sera from four faecal culture negative cows. Three of the faecal culture positive cows were confirmed tissue culture positive, whereas one faecal culture negative cow was tested tissue culture positive. Sera from the eight cows were also tested by three commercial ELISAs, but only one, two and four of the cows tested positive in each ELISA. The two PPE proteins could be promising diagnostic antigen candidates, however orthologous genes were found in *M. avium* subsp. *hominissuis* and MAA genomes (Newton et al., 2009) and if they are expressed in these species it would jeopardize the specificity.

### 4.3.3. Lipoprotein antigens

Lipids (Table 4) comprise a large part of the mycobacterial cell wall and, similar to cell wall antigens, many of these structures have demonstrated high seroreactivity as a consequence of their accessibility for antibodies and immune cells (Biet et al., 2008). In an early study lipoarabinomannan (LAM) was purified, analysed for chemical properties, and shown to react with serum from a MAP infected cow (Sugden et al., 1987). LAM is a highly immunodominant component of the MAP cell wall and has been widely used in serological assays for MAP diagnosis (Jark et al., 1997; Reichel et al., 1999). Antigen candidates LSP (Biet et al., 2008), Para-LP-01 (Eckstein et al., 2006) and lipopeptide PstA (Wu et al., 2009) were reported to be absent in MAA. The diagnostic value of these antigen candidates remains to be thoroughly validated with appropriate sample sizes.

### 4.3.4. Heat shock protein antigens

Hsps (Table 5) are highly conserved molecules produced by both eukaryotic and prokaryotic cells. Expression of Hsps is upregulated during cellular stress, such as heat stress in response to infection and inflammation (Wu et al., 2007). In addition to their chaperone function, these proteins have demonstrated to play an important role in activation and modulation of dendritic cells (Angelaar et al., 2002). Due to their conserved nature Hsps are likely to be immunogenic but are not ideal antigens for diagnostic purposes as cross reactivity with other bacteria is expected.

Five different Hsp antigen candidates are listed in Table 5: GroES, Hsp65K, Hsp70, MAP3840 and MAP3841. All five antigens were tested with relatively few sera, and as expected, none of them were reported to be MAP specific. One study focused on the interaction of Hsp70 and bovine antigen presenting cells investigating the use of recombinant Hsp70 as a tool to chaperone antigens into the cell to mediate a cytotoxic T-cell response initiated by presentation of antigens by MHC class I antigen presenting pathway (Angelaar et al., 2002). The results indicate that a Hsp70 receptor is present on bovine dendritic cells and macrophages and that Hsp70 may be useful as an adjuvant in vaccines, if not as the vaccine antigen itself (Koets et al., 2006).

### 4.3.5. Hypothetical proteins

Table 6 includes characterized hypothetical protein antigen candidates of unknown function or other antigen candidates of MAP. The majority of the listed antigen candidates are recombinant proteins. Several of these were reported from studies conducting large proteome analysis of MAP (Bannantine et al., 2004, 2008d; Hughes et al., 2008) and were validated with sera from few animals.

### 5. Future studies

In the pre-genomic era, the primary technique for discovery and characterization of novel MAP antigen candidates was immunoproteomics. Screening of expression libraries was employed in parallel but to a much lesser degree. With the completion of the MAP genome (Li et al., 2005a) proteomic approaches has opened up for more targeted strategies that will be exploited further in the future. Immunoproteomic approaches are based upon selection of antigen candidates by a combination of antigenicity and protein expression profiles (White et al., 1994). Proteins are resolved by two-dimensional (2D) gel-electrophoresis and interesting spots are identified by mass spectrometry followed by cloning, recombinant expression and ultimately testing for antigenicity with serology or CMI based assays (Bannantine and Paustian, 2006; Cumber and Whittington, 2009). Progress in proteomic techniques has resulted in high-resolution 2D electrophoresis proteomic maps comprising more than 1500 spots and combined with mass spectroscopy in the identification of several hundred expressed proteins (Leroy et al., 2007).

Comparison of the MAP genome against other mycobacteria genomes, including the highly homologous MAA genome, allowed the identification of coding sequences present only in MAP (Bannantine et al., 2002). Comparative studies revealed that less than 40 coding sequences are uniquely present in the MAP genome (Bannantine et al., 2002; Paustian et al., 2004). This relatively small number of unique coding sequences reflects the genetic similarity among members of the mycobacteria (Bannantine et al., 2003b).

Isolation and mass spectrometric identification of MHC binding peptides is a new approach that will allow the selection of a limited number of highly relevant proteins based upon the MAP genome sequence being publicly
available. As an alternative, the genome could be screened in silico in a non-biased way for epitopes that will be recognized by the bovine immune system (Lundegaard et al., 2007). This approach relies upon computer algorithms to identify T cell epitopes that bind the MHC II most strongly and promiscuously. In order for a protein to be recognized strongly in a diagnostic test over the entire course of infection it has to be constitutively and strongly expressed. Two recent studies demonstrated that protein expression of MAP differ from control conditions during starvation, hypoxia (Gumber and Whittington, 2009) and heat stress (Gumber and Whittington, 2009) in MAP strains originating from cattle and sheep. These results suggest that MAP expresses a diverse range of antigens throughout the course of infection during which the environmental conditions for the MAP bacteria change.

Regardless of which strategy we choose to follow there is currently no reliable way to predict diagnostic antigens and we are left with the task of screening each selected antigen for its potential, but in contrast to screening of the entire proteome it will be a doable task.

6. Summary

A number of MAP antigen candidates of different characteristic and species specificity have been identified and tested for immunogenicity to evaluate their diagnostic potential. Few of these antigens have been tested for CMI reactions that allow for early detection of MAP infected animals. Until now, no obvious antigen candidates for use in a CMI based diagnostic assay have been identified. However, with several mycobacteria genome sequences available along with new tools for in silico analysis and genomic techniques, specific and immunogenic antigens for paratuberculosis diagnosis should be expected soon. It is recommended that appropriate sample size is included for validation of future antigen candidates for diagnostic purposes. In addition, the use of a selected antigen cocktail for diagnostic application should be considered, to ensure that all infected animals are detected independent of disease status and MHC make-up.

References


Novel antigens for detection of cell mediated immune responses to
*Mycobacterium avium* subsp. *paratuberculosis* infection in cattle

Heidi Mikkelsen, Claus Aagaard, Søren Saxmose Nielsen and Gregers Jungersen

Veterinary Immunology and Immunopathology (in press)
Novel antigens for detection of cell mediated immune responses to *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle

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Abstract
Paratuberculosis is a chronic infection of the intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Early stage MAP infection can be detected by measuring specific cell mediated immune responses, using the whole blood interferon-γ (IFN-γ) assay. Available IFN-γ assays use purified protein derivative of MAP (PPDj) which are complex antigen mixtures with low specificity. The objectives of this study were to evaluate immunogenicity and specificity of 14 novel recombinant antigens for use in the IFN-γ assay and to assess the consistency of IFN-γ responses. The study included blood samples from 26 heifers from a MAP infected herd, collected three times with four to five week intervals, and blood samples from 60 heifers of a non-infected herd collected once. Heifers of the non-infected herd were used to establish cut-off values for each antigen. The case definition was an animal with ≥2 positive tests for ≥4 antigens, resulting in 13 cases and 13 non-cases. IFN-γ levels of cases was higher compared to IFN-γ levels of non-cases (P<0.05). The results of the IFN-γ assay using PPDj did not correlate well with the results using the novel antigens. PPDj produced elevated IFN-γ responses of samples from both the non-infected and the MAP infected herd, indicating unspecific IFN-γ responses and showed low consistency. Three latency proteins, LATP-1, LATP-2 and LATP-3 gave positive IFN-γ tests that correlated very well with the case definition suggesting high immunogenicity. Three tested antigens, LATP-2, MAP-1 and MAP-2 have no homologue in the *M. avium* subsp. *avium* or *M. bovis* genome and could be promising diagnostic antigens, especially LATP-2 correlated highly with the case definition.
1. Introduction
Paratuberculosis is a chronic infection of the intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Infection of dairy cattle can lead to reduced animal health and welfare, and economic losses through decreased milk yield and premature culling (Ott et al., 1999). It is generally believed that cattle are primarily infected as calves, either while still in the uterus or as neonates by ingestion of faecal matter, milk or colostrum from infected cows in the herd (Sweeney, 1996).

Cattle are dependent on development of cell mediated immune (CMI) responses in the defence against intracellular MAP, because antibodies provide little or no protection. CMI responses are generated at an early stage and as the infection progresses from subclinical to clinical disease, CMI responses are replaced by strong humoral responses characterised by presence of antibodies (Stabel, 2000). For early identification of MAP infected animals it is possible to exploit the presence of CMI mediators such as interferon-γ (IFN-γ), which is a cytokine secreted by T-helper type 1 cells following activation by antigen presenting cells displaying MHC II: MAP antigen complexes on the surface.

The IFN-γ assay is a whole-blood proliferation assay, in which blood is cultured overnight with MAP antigens followed by collection of supernatant (Wood et al., 1989). IFN-γ levels in supernatants are then quantified by an IFN-γ specific ELISA. Johnin purified protein derivative (PPDj), which is a crude undefined extract of MAP antigens, are often used for culture in the IFN-γ assay (Jungersen et al., 2002). PPDj have been prepared from different strains in different laboratories and no standardised protocol has been established. In many cases there is no information regarding the history of the strains used while other stains were passaged continuously in the laboratory for more than 70 years (Semret et al., 2006). PPDj are known to cross-react with environmental mycobacteria such as *Mycobacterium avium* subsp. *avium* (MAA) leading to low specificity of the IFN-γ assay. Identification of well-defined and MAP specific antigens, which are both highly immunogenic and specific in IFN-γ assay, is needed.

Relatively few antigens have been tested for their ability to recall CMI responses against MAP (Mikkelsen et al., 2011). These include secreted antigens (Olsen et al., 2005; Shin et al., 2005; Rosseels et al., 2006), cell wall and membrane antigens (Nagata et al., 2005), lipoproteins (Huntley et al., 2005), heat shock proteins (Koets et al., 1999) and a number of hypothetical proteins that are predicted to be expressed but lack experimental evidence. In this study, secreted proteins, latency proteins, antigens of the group early secretory antigenic target 6 (ESAT-6) and other antigens were tested. Secreted proteins are expected to be highly immunogenic due to their presence in the extracellular environment where they are likely to encounter sensitized immune cells and previous studies have tested antigens of the Antigen 85 Complex for implementation in diagnostic assays or vaccines (Shin et al., 2005; Rosseels et al., 2006; Park et al., 2008). Antigens of the Ag85 Complex are highly conserved and found in all mycobacteria species (Rosseels et al., 2006) which render them
less useful for diagnostic purposes. Antigens of the ESAT-6 family, are known to induce early specific IFN-γ responses of *M. tuberculosis* infected guinea pigs and humans (van Pinxteren et al., 2000)) and have also been used for detection of *M. bovis* (van Pinxteren et al., 2000); (Aagaard et al., 2006) and MAP (Olsen et al., 2005). Latency proteins are known to be expressed during the latent stage of *M. tuberculosis* and are able to induce IFN-γ responses in vitro and hence may contribute to controlling latent *M. tuberculosis* infection (Leyten et al., 2006).

The ideal diagnostic MAP antigen should be immunogenic, specific and recognised only by infected animals from the early subclinical stages of infection and throughout different stages of infection. In practice, an optimised IFN-γ assay may require a cocktail of antigens due to the range of heterogeneous MHC molecules of out-bred animals and due to differential expression patterns of MAP bacteria (Radosevich et al., 2007).

The aim of this study was to evaluate immunogenicity and specificity of 14 novel recombinant antigens, Ag85B and PPDj, and to assess the consistency of IFN-γ responses. The study included single blood samples of 60 heifers from a MAP non-infected herd and repeated samples from 26 heifers from a MAP infected herd collected three times with four to five week intervals.

2. Material and methods

2.1. Selection, cloning and purification of antigens

Fourteen potential MAP antigens and the well known antigen 85B (Ag85B) were selected for this study (Table 1). Selection was either based on absence from the genomes of MAA (Semret et al., 2004; Wu et al., 2006) and *M. bovis* (Garnier et al., 2003), experimental knowledge from in vitro and in vivo expression studies with *M. tuberculosis* or immune recognition of orthologue *M. tuberculosis* proteins in mice. Three of the antigens (ESAP-2, ESAP-3 and ESAP-4) were synthesised as 20 mer peptides with 10 amino acid overlaps (Eurofins MWG Operon, Ebersberg, Germany) and tested for recognition as peptide pools, one pool for each antigen. The remaining 12 antigens were produced and tested as recombinant proteins.

Genes encoding these proteins were amplified from MAP chromosomal DNA (strain 441499, a kind gift from Dr. Peter Willemsen, Central Veterinary Institute, Lelystad, the Netherlands) by PCR using 12 gene specific primer sets and the iProof HF master mix™ according to the manufacturer instructions (Bio-Rad, Hercules, CA). The cycling conditions were: 10 sec @ 98° C followed by 60 sec @ 72° C, repeated 30 times. The resulting PCR products were cloned into expression vector pDest17 (Invitrogen, Taastrup, Denmark) by a two-step recombination using the Gateway™ system as recommended (Invitrogen).
All proteins were expressed in *Escherichia coli* BL21 AI and the vector encoded His-tag fused to the N-terminus was exploited for purification. *E. coli* cultures harbouring MAP protein expression vectors were grown to OD$_{600}$ ~ 0.6 (mid-log phase) in 6 L LB medium containing 100 µg/mL ampicillin after which recombinant protein expression was induced by addition of 0.02% (w/v) arabinose for 4 hours. Bacteria were pelleted and the outer membrane lysed using mild detergents (B-PER, Bacterial Protein Extraction Reagent) according to the manufacturer (Pierce, Rockford, IL, USA). All recombinant proteins were found as insoluble inclusion bodies and therefore purified after the same procedure. Inclusion bodies were washed three times in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% deoxycholic acid and dissolved in 8 M urea, 100 mM Na$_2$PO$_4$, 10 mM Tris-HCl pH 8.0 (buffer A). After clearing by centrifugation soluble protein was applied and bound to a 10 mL metal affinity column (TalonTM, Clontech, Mountain View, CA, USA) pre-equilibrated in buffer A. Bound protein was washed 5 times alternating between 10 mM Tris-HCl pH 8.0, 60 % isopropanol, and 50 mM NaH$_2$PO$_4$ pH 8.0 before being eluted in buffer A pH 4.5 supplemented with 200 mM imidazole in 5 mL fractions. All protein containing fractions were analysed on Coomassie blue stained SDS-PAGE gels and fractions with the highest amount of recombinant protein relative to contaminants were collected and pooled. Protein pools were dialysed against 3 M urea, 10 mM Tris-HCl pH 8.5 and then applied to an anion-exchange column (HiTrap MonoQTM, Pharmacia, Uppsala, Sweden) and eluted using a linear gradient from 0 to 0.5 M NaCl over 10 column volumes before being dialysed against 25 mM NaH$_2$PO$_4$ pH 8.0, 10% glycerol, 150 mM NaCl, 0.05% Tween 20. Protein concentration and final yield was determined by a sensitive fluorescent based quantification kit (Invitrogen) or bicinchoninic

### Table 1. Description of antigens used in this study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Homology in MAA (%)</th>
<th>Homology in <em>M. bovis</em> (%)</th>
<th>Protein size (amino acids)</th>
<th>Product of <em>Mycobacterium avium</em> subsp. <em>paratuberculosis</em></th>
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<tbody>
<tr>
<td>Ag8SB</td>
<td>99</td>
<td>85</td>
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<td>Secreted antigen 85B</td>
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<tr>
<td>ESAP-1</td>
<td>100</td>
<td>76</td>
<td>100</td>
<td>Hypothetical conserved protein, ESAT-6 like protein</td>
</tr>
<tr>
<td>ESAP-2</td>
<td>100</td>
<td>87</td>
<td>98</td>
<td>Hypothetical ESAT-6 like protein</td>
</tr>
<tr>
<td>ESAP-3</td>
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<td>80</td>
<td>96</td>
<td>Hypothetical protein, ESAT-6 like protein</td>
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<td>ESAP-4</td>
<td>94</td>
<td>79</td>
<td>105</td>
<td>Hypothetical protein, ESAT-6 like protein</td>
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<td>LATP-1</td>
<td>99</td>
<td>80</td>
<td>163</td>
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<td>NP</td>
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<td>Putative phiRv2 prophage integrase (latency protein)</td>
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<td>LATP-3</td>
<td>98</td>
<td>66</td>
<td>324</td>
<td>Hypothetical conserved protein (latency protein)</td>
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<td>LATP-4</td>
<td>100</td>
<td>71</td>
<td>146</td>
<td>Heat shock protein (latency protein)</td>
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<td>84</td>
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<td>83</td>
<td>300</td>
<td>Secreted protein</td>
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<td>Secreted hypothetical protein, putative cutinase</td>
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<tr>
<td>SECP-3</td>
<td>98</td>
<td>82</td>
<td>373</td>
<td>Secreted alanine dehydrogenase</td>
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</table>

NP: not present in genome

Gene annotation according to JCVI (J. Craig Venter Institute, Rockville, MD, USA)
acid assay (Pierce, Rockford, IL, USA) according to the manufacturer instructions, in both cases using BSA as standard.

2.2. Herds and animals
Two Danish dairy cattle herds were included in the study. A presumed non-infected herd, and a herd in which MAP infection had been confirmed by cultivation of MAP. The non-infected dairy herd was selected for the study based on the results of the Danish paratuberculosis control programme. Herds included in the control programme were tested four times annually for MAP antibodies in milk (Nielsen, 2002). The estimated true prevalence in the non-infected herd, based on cow-level milk antibody ELISA recordings, was equal or close to zero at the 17 herd screenings performed in the period September 2006 through January 2011. The few test positive animals had low ELISA values and all tested negative at the following tests. At the time of sample collection, April 2008, the non-infected herd consisted of 352 Danish Jersey cattle, including 173 cows and 177 heifers, and did not have any milk antibody ELISA reactors among tested cows in the three previous herd screenings. Blood samples were collected from 60 heifers 12-27 months of age (mean 18.3 ±3.4 months).

Furthermore, a dairy herd, that was confirmed MAP infected by culture and by clinical cases of paratuberculosis, was selected for inclusion in the study. The herd was included in a study in 2003, including 125 cows of which nine (7%) were serum antibody positive and 62 (50%) were IFN-γ positive. In addition, faecal samples were collected from 24 cows and five (21%) cows were confirmed to be faecal culture positive. The infected herd was also included in the Danish paratuberculosis control programme and was tested four times annually for MAP antibodies in milk. During the period April 2006 through December 2010, the estimated true prevalence and apparent prevalence among milking cows were in the range 5-20%. The prevalence of MAP in the herd was not expected to be 100% and some heifers may not have been exposed to MAP, but infection pressure was expected to be high due to the long history of paratuberculosis problems. The herd consisted of 396 Danish Holstein (black and white) cattle, including 193 cows and 199 young stock at the first sample collection in February 2009. Blood samples were collected three times with four and five week intervals from the same 30 heifers 10-21 months of age (mean 15.0 ±2.9 months) at the first sampling.

2.3. Proliferation assay and IFN-γ ELISA
Blood samples were collected in heparinised vacutainer tubes and immediately stored in thermal boxes to prevent the blood from exposure to temperature fluctuations during sample collection and transportation. Culture of blood with antigens was initiated within 8 to 12 hours of sampling. In each well of a 48-well culture plate (Greiner Bio-one, Heidelberg, Germany), 0.5 ml whole blood was cultured with either 50 µl antigen, positive or negative control solution separately for 20-22 hours at 37°C in 5% CO₂. Antigen solutions were 10 µg/ml PPDj (prepared in 1975 based on the strain Promise, collection no. MNC1053,
confirmed to be MAP by IS900 PCR, National Veterinary Institute, Copenhagen, Denmark), 1 μg/ml Antigen 85B (Ag85B) and 1 μg/ml of one of 14 novel recombinant antigens: ESAP-1, ESAP-2, ESAP-3, ESAP-4, LATP-1, LATP-2, LATP-3, LATP-4, SECP-1, SECP-2, SECP-3, MAP1, MAP-2 and MAP-3. As a positive control 1 μg/ml of superantigen Staphylococcal enterotoxin B (SEB) was used and as a negative control phosphate buffered saline (PBS) was used in parallel cultures. All antigens and the positive control were diluted in PBS.

Following overnight culture, the culture plates were centrifuged and the supernatants collected and stored below -20°C until further analysis. The antigen specific IFN-γ production in supernatants was determined by an in-house monoclonal sandwich ELISA as described in detail previously (Mikkelsen et al., 2009). The level of IFN-γ (pg/ml) was calculated using linear regression on log-log transformed readings from the two-fold dilution series of a reference standard with known IFN-γ concentration.

2.4. Interpretation and statistical analysis
Samples were excluded if the IFN-γ response to SEB stimulation was lower than 1000 pg/ml or the IFN-γ response to PBS was higher than 150 pg/ml. Based on these exclusion criteria, from the MAP infected herd one heifer (no. 17) was excluded at first sampling, two heifers (no. 17 and 19) were excluded at second sampling and three heifers (no. 17, 20 and 21) were excluded at the third sampling. In total, 4 animals were excluded from the study, resulting in a final study group of 26 animals at three sample dates. Heifer 17 that was excluded at all sample dates, had extremely elevated IFN-γ levels against all antigens and PBS, probably caused by presence of a large abscess on her hind leg observed at the third sampling.

IFN-γ responses to each of the 14 novel antigens, Ag85B and PPDj in the MAP non-infected herd were used to calculate cut-off values (listed in Figure 1 above IFN-γ results of uninfected animals) for each antigen. Samples from the non-infected herd should fulfil the same requirements as samples from the infected herd but no samples were excluded. The IFN-γ response to PBS was subtracted from the IFN-γ response to antigen of each animal and cut-off values was calculated for each antigen as the 95 percentile of this corrected IFN-γ response.

Prior to further calculations, the IFN-γ response to PBS was subtracted from the IFN-response to each antigen of each animal from the infected herd. If this corrected IFN-γ response was below 1, the value was set at 1. Based on cut-off values for each antigen, the number of positive tests at the three sample days was calculated for each animal from the MAP infected herd. The case definition (Nielsen et al., 2011) was an animal with ≥ 2 positive tests for ≥ 4 antigens. For each antigen, IFN-γ levels were compared in samples from individual animals from infected and non-infected herds using the non-parametric Mann-Whitney test (GraphPad Prism version 4.00, GraphPad Software, La Jolla, CA, USA). Similarly, IFN-γ levels of cases were compared to non-cases. Youden index and 95% confidence
intervals was calculated for each antigen. Youden index is a measure of test performance, and take both immunogenicity and diagnostic specificity into account (Youden, 1950).

2.5. Definitions
The following definitions were used in the present paper. Consistency was the stability of the IFN-γ test performed with samples from the same animals at different days. A test positive animal had ≥ 2 positive tests against a given antigen. Diagnostic specificity of a test was the proportion of test negative animals among animals defined as non-cases. Immunogenicity was the ability of an antigen to induce an immune response and here, immunogenicity was calculated as the proportion of test positive animals of the animals defined as cases.
3. Results

IFN-γ responses of animals from both the non-infected herd and the infected herd are shown in Figure 1. Animals of the infected herd were divided into cases (black points) and non-cases (open circles) and IFN-γ levels of cases were significant higher (P<0.05) than IFN-γ levels of non-cases. Figure 1 also presents IFN-γ results of the non-infected herd (grey points). The infected and non-infected herd showed significant (P<0.05) different IFN-γ responses to the novel antigens. IFN-γ responses of the negative herd were used to calculate cut-off values for each of the 14 novel antigens, Ag85B and PPDj. These cut-off values are listed below the x-axis for each antigen in Figure 1. Cut-off values were in the range 3 to 105 pg/ml IFN-γ for the novel antigens and 226 pg/ml IFN-γ for PPDj.

![Figure 1](image)

Fig. 1. IFN-γ responses (pg/ml) of heifers from a *Mycobacterium avium* subsp. *paratuberculosis* infected cattle herd against novel recombinant antigens, recombinant Ag85B and PPDj. Antigens is divided into A) ESAT-6 family members, B) latency proteins, C) secreted proteins and D) other antigens. Black points and closed circles represent the average IFN-γ level of three sample days for individual animal (n=26). Black points represent animals considered cases (n=13) and open circles represent animals considered non-cases (n=13) according to the case definition: an animal was considered a case if the animal had ≥ 2 positive tests for ≥ 4 antigens. Grey points represent the non-infected herd, from which samples were collected once (n=60). Median IFN-γ response (pg/ml) is marked by a horizontal line and cut-off values for each antigen is listed below the x-axis. Cut-off values were calculated as the 95 percentile of IFN-γ responses in the non-infected herd. All IFN-γ responses were calculated as IFN-γ response against antigen subtracted IFN-γ response against PBS for each animal.
Median IFN-γ response of the infected heifers to each antigen is listed in Table 2, including values for lower and upper quartile. The median IFN-γ response was highest for PPDj with 216 pg/ml, but the majority of antigens had median IFN-γ responses below 20 pg/ml.

<table>
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<tr>
<th>Antigens</th>
<th>Lower quartile</th>
<th>Median</th>
<th>Upper quartile</th>
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<td>65</td>
</tr>
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<td>ESAP-4</td>
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<td>48</td>
</tr>
<tr>
<td>Latency proteins</td>
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<tr>
<td>LATP-1</td>
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<td>16</td>
<td>106</td>
</tr>
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<td>83</td>
</tr>
<tr>
<td>LATP-4</td>
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</tr>
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<td>1</td>
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<td>78</td>
</tr>
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<td>Ag85B</td>
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<tr>
<td>Other antigens</td>
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<td></td>
<td></td>
</tr>
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<td>1</td>
<td>21</td>
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<tr>
<td>PPDj</td>
<td>43</td>
<td>216</td>
<td>1129</td>
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</tbody>
</table>

IFN-γ responses (pg/ml) was based on 78 samples from 26 cattle sampled three times with 4 and 5 week interval from a *Mycobacterium avium* subsp. *paratuberculosis* infected herd. Responses were calculated as IFN-γ response against antigen subtracted IFN-γ response against PBS.

The number of positive IFN-γ tests, for each antigen at the three sample days stratified by animal, is listed in Table 3. According to the case definition, 13 heifers were considered cases, as they had ≥ 2 positive tests for ≥ 4 antigens. Cases were distributed as: five heifers tested IFN-γ positive against 15 antigens, one heifer tested positive against 12 antigens, two heifers tested positive against 10 antigens, one heifer tested positive against 8 antigens, one heifer tested positive against 6 antigens, one heifer tested positive against five antigens and the remaining two cases tested positive against four antigens.

The results of IFN-γ testing using PPDj did not correlate well with the results of IFN-γ testing using the other antigens. Of 17 animals that were IFN-γ positive to PPDj, five were non-cases
Table 3. Number of positive IFN-γ tests for each antigen stratified by animal (n=26). Total number of IFN-γ tests are summarised horizontally at animal level (excluding PPDj) and laterally at antigen level. Number of antigens with ≥2 positive tests (excluding PPDj) are summarised horizontally at animal level to define cases (C) and non-cases (NC). Number of antigens with ≥2 positive tests are summarised laterally at antigen level.

<table>
<thead>
<tr>
<th>Animal lab no.</th>
<th>Age at first sampling (months)</th>
<th>Case def.</th>
<th>ESAT-6 family members</th>
<th>Latency proteins</th>
<th>Secreted proteins</th>
<th>Other antigens</th>
<th>Total No. of test pos (≥2)</th>
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<tr>
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<td>8 5 11 10 11 11 9 13</td>
<td>8 8 15 17</td>
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</table>
and one case did not test IFN-γ positive to PPDj. In addition, stimulation with PPDj recalled high IFN-γ responses in blood samples from both the infected and non-infected herd.

Five animals (no. 3, 8, 10, 16 and 29) could be considered high-reactors as they had two or more positive tests against all novel antigens in the panel, and had three positive tests against most antigens. It is noteworthy that of these five high-reacting animals, animal no. 29 did not test IFN-γ positive to PPDj at all, while the remaining four animals had two positive tests, but none had three positive IFN-γ tests against PPDj.

In Table 4 the immunogenicity, diagnostic specificity, Youden indices and consistency of each antigen are listed.

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Immunogenicity</th>
<th>Diagnostic specificity</th>
<th>Youden</th>
<th>95% CI of Youden</th>
<th>Consistency</th>
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<td>ESAT-6 family members</td>
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<td>1.00</td>
<td>0.62</td>
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<td>0.69</td>
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<td>0.92</td>
<td>0.54</td>
<td>0.24; 0.84</td>
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<td>PPDj</td>
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<td>0.62</td>
<td>0.54</td>
<td>0.24; 0.84</td>
<td>4</td>
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</table>

\(^a\)Immunogenicity \((I)\) was calculated as the proportion of test positive animals of the animals defined as cases.\(^b\) Specificity \((S)\) was calculated as the proportion of test negative animals of the animals defined as non-cases.\(^c\) Youden index was calculated as \(I+S-1\), with 95% confidence interval (95% CI).\(^d\) Consistency is presented as the number of animals with 3 and 0 positive tests to each antigen.

Four members of the ESAT-6 family were assessed: ESAP-1, ESAP-2, ESAP-3 and ESAP-4, for their use as MAP diagnostic antigens. In general, this antigen group detected fewer animals as IFN-γ positive compared to the other antigen groups, which is reflected in low immunogenicity, but with a moderately high diagnostic specificity. The group of latency
proteins gave positive IFN-γ tests that correlated well with the case definition and
differentiation between cases and non-cases were highly significant for this group of
antigens. Three latency antigens, LATP-1, LATP-2 and LATP-3 had highest Youden values of
0.85. The group of secreted proteins also differentiated IFN-γ responses in cases from non-
cases, but a few non-cases produced positive IFN-γ responses against SECP-2 resulting in
diagnostic specificity of 0.77 and generally Youden indices was lower for secreted proteins
compared to latency proteins. In the present study, Ag 85B designated by Ag85B, was
included as a positive control, and immunogenicity was determined to 0.77, however
diagnostic specificity was also 0.77, which result in a low Youden index of 0.54. A Youden
index of 0.5 suggests that the diagnostic test has no value. Three of the tested antigens,
LATP-2, MAP-1 and MAP-2 have no homology to MAA and they all gave differentiated IFN-γ
responses of cases compared to non-cases with diagnostic specificity of 1.00.

Consistency of IFN-γ responses to antigens is presented as the number of animals with three
and zero positive tests for each antigen. The ESAT-6 family member ESAP-4 showed the
highest consistency, as 18 animals had zero positive tests against this antigen, followed by
13 animals that had zero positive tests against antigens MAP-1 and MAP-2. Consistency of
IFN-γ responses to PPDj was low, as 9 animals had zero positive tests against PPDj while only
four animals had three positive tests.

4. Discussion
This is the first report of large scale testing of novel recombinant MAP antigens for CMI
based diagnosis using blood samples from MAP non-infected and infected cattle herds. As a
group, latency antigens gave highly promising results with immunogenicity of 0.85 and
diagnostic specificity of 1.00 for three antigens encoded by LATP-1, LATP-2 and LATP-3.
Youden indices were 0.85 for these three antigens, whereas for comparison PPDj were 0.54.
IFN-γ levels of the non-infected herd were in general very low, as can be seen on Figure 1
and the presumption that the herd is non-infected may very well be correct. The cut-off
value of PPDj (226 pg/ml) was more than twice the value of ESAP-2 (105 pg/ml), which was
highest of the remaining antigens, and suggesting that PPDj is highly immunogenic but of
limited value for diagnostic purposes because specificity is low. Fourteen of the antigens had
cut-off values below 50 pg/ml IFN-γ, only ESAP-2 and PPDj had higher cut-off values. These
cut-off values were calculated based on IFN-γ responses of the non-infected herd, but in
reality the level of detection for the IFN-γ ELISA is likely to be set higher than 3 pg/ml IFN-γ,
which was the calculated cut-off value for ESAP-4.

The true MAP status of individual animals from the infected herd was unknown, which
prevented calculations of diagnostic sensitivity and specificity. In an infected herd, there
could be three populations of animals: 1) non-exposed and consequently non-infected; 2)
exposed but non-infected either because they were non-susceptible or because infection
had been eliminated; and 3) exposed and infected. The infection pressure in this particular
herd was high and therefore it may be assumed that all heifers had been exposed to MAP. Evaluation of immunogenicity would have to be evaluated in an exposed population, and evaluation of specificity should preferably be done in the same population as immunogenicity and consequently sensitivity (Nielsen et al., 2011). Therefore, a case was defined based on results of multiple antigens: animals with ≥ 2 positive tests for ≥ 4 antigens. This provided an opportunity to evaluate both immunogenicity and specificity in an exposed population.

IFN-γ responses against PPDj did not correlate well with responses to the other antigens, and PPDj gave high IFN-γ responses of animals from the non-infected herd. These results support that an IFN-γ assay protocol using PPDj for culture may be of low specificity due to cross-reaction with environmental mycobacteria. The top nine animals in Table 3 had 8 to 15 tests positive and were all in age group 15 to 17 months old which suggest, that they may represent a birth cohort, infected with MAP in the same period of high MAP prevalence or poor management. Alternatively, the high IFN-γ responses could be due to the nature of the immune response, presuming that cattle in this age group are particular good IFN-γ responders. The duration of the IFN-γ response is not known. Therefore, young animals with lower reactions could simply be in earlier stages of infection.

The antigens encoded by SECP-2, MAP-3 and Ag85B gave two or more positive IFN-γ tests in several animals that were non-cases. These antigens may result in MAP unspecific IFN-γ production, since they are highly homologous to MAA proteins, or that they actually recall MAP specific IFN-γ responses that other antigens do not. Three of the tested antigens in the panel, LATP-2, MAP-1 and MAP-2 share no homology to MAA, and all gave differentiated IFN-γ test responses to cases compared to non-cases and could be useful antigens for the IFN-γ assay. However, whether these hypothetical proteins are actually expressed by MAP is unknown. The group of latency proteins may be promising diagnostic antigens as immunogenicity and diagnostic specificity was high and differentiation between cases and non-cases were highly significant for this group of antigens. The animals were young heifers (10-21 months of age at first sampling), and it is likely that these heifers are at an early and latent stage of MAP infection and hence, would produce IFN-γ responses against latent antigens.

Previous studies have shown that the PPD IFN-γ test can be applied specifically for animals older than 15 months, as samples from younger animals may be subject to non-specific false positive IFN-γ responses (Jungersen et al., 2002) probably produced by NK cells (Olsen et al., 2005) or γδ-T cells which are present high levels in young cattle (Baldwin et al., 2000). In the present study, a few animals younger than 15 months was included, as mean age were 18.3±3.4 months for the non-infected cattle and 15.0±2.9 months for cattle of the MAP infected herd at first sampling, and samples of these animals may be predisposed to false-positive reactions. However, for the majority of antigens the highest number of positive IFN-
y tests were observed on the second and third sampling (data of individual sample days are not presented).

To investigate the consistency of the IFN-γ response, samples were collected three times with four and five week interval from the MAP infected herd. We observed that IFN-γ responses did fluctuate in samples of the same animals between sample days, which emphasise the importance of repeated test for evaluation of antigen performance in the IFN-γ test. To interpret the results of the three sample days, we used a case definition that actually did differentiate animals into two groups with significant different IFN-γ responses, which confirm that this approach may be the best alternative available due to the lack of a reference test for MAP diagnosis. Diagnosis of MAP infected animals is often based on detection of MAP antibodies in milk by ELISA or by cultivation of bacteria from faces, however these methods are not applicable until years after infection (Nielsen and Toft, 2006). Heifers that are IFN-γ positive, do not necessarily become ELISA positive or faecal culture positive as cows (Mikkelson et al., 2009), consequently it is not possible to simply wait a few years for a true MAP diagnosis to evaluate test sensitivity and diagnostic specificity for the presented antigens.

In the present study, cattle of the infected herd were defined as cases or non-cases based on IFN-γ responses to the novel antigens. Despite homology searches showed that three of the novel antigens had no orthologues in the currently accessible mycobacterial genomes (including M. avium subsp. avium, M. bovis and M. marinum) we cannot rule out that some of the positive IFN-γ responses might be due to cross-reaction with environmental mycobacteria. When more genomes become available it may be possible to reduce the number of potential false-positives by including one or two antigens specific for the most common environmental mycobacteria. Alternatively, the animals would have to be killed and post-mortem histopathological evaluations done of up to 100 tissues per animal, which is required for correct diagnosis (Whitlock et al., 1996).

A general problem of previously published studies, concerning characterisation of antigens for CMI based MAP diagnosis, is that evaluation of antigens is often performed using samples from few animals. In some cases, the evaluation is performed using experimental infected animal of species different from the target species. Diagnostic test development and evaluations are optimally performed using samples from the target group, in this case young cattle exposed to MAP. A group of natural infected cattle may represent various stages of MAP infection and hence blood samples from such a group of animals may produce various levels of IFN-γ responses and even respond to different antigens due to heterogeneous MHC molecules. MAP express differentiated antigen patterns when the environmental conditions change (Gumber et al., 2009; Gumber and Whittington, 2009) and different strains of MAP show different antigen expression patterns (Radosевич et al., 2007). Based on this knowledge, we speculate that a cocktail of antigens is required to correctly diagnose MAP infected animals. To detect the animals defined as cases, eight of
the novel antigens (ESAP-3, LATP-1, LATP-2, LATP-3, LATP-4, MAP-2, MAP-3 and SECP-1) and Ag85B could be included in a cocktail. A cocktail of antigens should preferable include different antigen groups to detect all MAP infected animals at different stages of disease.

5. Conclusion
IFN-γ test results fluctuate between sample days and repeated blood samples may be necessary to establish the true level of IFN-γ against antigens of the individual animal. Differentiation of animals into cases and non-cases according to a case definition, made it possible to validate immunogenicity of antigens even though true infection status of the presumed non-infected herd and MAP infected herd was unknown. IFN-γ responses of cases compared to non-cases were significant different, but PPDj gave IFN-γ responses that did not correlate well with the case definition. Diagnostic specificity of the 14 novel antigens were generally high, especially three latency protein antigens showed high diagnostic specificity and combined with high immunogenicity they may be promising diagnostic antigens.

Acknowledgements
Technicians Abdellatif El Ghazi and Sardar Ahmad are acknowledged for their skilled laboratory assistance. This study was co-funded by the European Commission within the Sixth Framework Programme as part of the project ParaTBTools (contract no. 023106 (FOOD)), the Danish Cattle Federation, and University of Copenhagen.
References


Rosseels, V., Marche, S., Roupie, V., Govaerts, M., Godfroid, J., Walraven, K., Huygen, K., 2006. Members of the 30- to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of Mycobacterium
subsp. paratuberculosis are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. Infect. Immun. 74, 202-212.


Correlation of antigen-specific IFN-γ responses of fresh blood samples from *Mycobacterium avium* subsp. *paratuberculosis* infected cattle with responses of day-old samples co-cultured with IL-12 or anti-IL-10 antibodies.

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Abstract
Paratuberculosis is a chronic infection of the intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Early stage MAP infection can be detected by measuring cell-mediated immune responses using the interferon gamma (IFN-γ) assay. Whole blood samples are cultured overnight with specific MAP antigens followed by quantification of IFN-γ by ELISA. It is recommended that the time interval from sampling to culture does not exceed eight hours but addition of the co-stimulating cytokine interleukin 12 (IL-12) or anti-IL-10 antibodies to culture have been demonstrated to enhance IFN-γ responses of cultures stimulated with Johnin purified protein derivative (PPDj). Here we examined the correlation of IFN-γ production in response to PPDj and 15 recombinant antigens in day-old blood samples from a MAP infected herd with addition of either recombinant bovine IL-12 or anti-bovine IL-10 antibody with IFN-γ production in sample day samples. IFN-γ responses of sample day samples showed high correlation with responses to some antigens in day-old samples with addition of IL-12 or anti-IL-10 antibodies to cultures, indicating that day-old protocols can be applied as an alternative to the conventional IFN-γ protocol. Immunogenicity of the novel antigens was generally low for day-old samples. The most promising antigen using the day-old protocol with addition of IL-12 was latency protein LATP-2 as correlations, immunogenicity and diagnostic specificity collectively was high. The latency protein LATP-1 was the most promising antigen in the day-old protocol with addition of anti-IL-10 antibodies.
1. Introduction

Paratuberculosis is a chronic infection of the intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Chiodini et al., 1984). Infection with MAP leads to economic losses due to decreased milk yield and premature culling (Ott et al., 1999) and may reduce animal health and welfare. The prevalence of MAP in European cattle herds have been estimated to be >50% (Nielsen and Toft, 2009) and MAP is a worldwide problem. Early stage MAP infection is characterised by development of a cell-mediated immune (CMI) response, which cattle are dependent upon as antibodies that develop later in infection provide no protection against the intracellular MAP bacteria (Stabel, 2000). MAP specific CMI responses can be detected by the interferon gamma (IFN-γ) assay in which whole blood samples is cultured overnight with MAP antigens (Wood and Rothel, 1994) followed by quantification of produced IFN-γ by ELISA.

A major obstacle for the practical use of the IFN-assay is the recommended maximum eight hour time interval from sampling to culture. We have previously demonstrated the use of day-old blood samples in the IFN-γ assay (Jungersen et al., 2005; Mikkelsen et al., 2009). In this protocol, blood samples were cultured with antigens and co-stimulatory cytokine interleukin 12 (IL-12) within 20 hours of sample collection to rescue a weakened IFN-γ response. A previous study demonstrated that the IFN-γ response could be enhanced in by addition of anti-IL-10 antibodies to sample day peripheral blood mononuclear cells (PBMC) stimulated with Johnin purified protein derivative (PPDj) (Buza et al., 2004) and in the present study we applied this approach to day-old blood samples.

PPDj is a crude undefined extract of MAP antigens that are often used for culture in the IFN-γ assay (Jungersen et al., 2002). PPDj have been prepared from different MAP stains (Semret et al., 2006) and as a consequence the composition of antigens of these extracts may differ between laboratories. In addition, PPDj may cross-react with environmental mycobacteria such as the closely related *Mycobacterium avium* subsp. *avium* (MAA). Several MAP antigens have been characterised, but relatively few antigens have been tested for their ability to recall MAP specific CMI responses (Mikkelsen et al., 2011a) . We have previously assessed the immunogenicity and specificity of 14 novel recombinant antigens for culture including secreted proteins, latency proteins, antigens of the group early secretory target 6 (ESAT-6), other antigens and the well known Ag85B and PPDj on sample day samples (Mikkelsen et al., 2011b).

The first objective of this study was to determine correlation of IFN-γ responses of day-old samples with addition of either recombinant bovine IL-12 or addition of anti-bovine IL-10 antibody to IFN-γ responses of sample day samples. The second objective was to evaluate immunogenicity and specificity of 14 novel recombinant antigens, Ag85B and PPDj used for culture in the two day-old protocols.
2. Materials and methods

2.1. Selection, cloning and purification of recombinant antigens
Fourteen potential MAP antigens: ESAP-1, ESAP-2, ESAP-3, ESAP-4, LATP-1, LATP-2, LATP-3, LATP-4, MAP-1, MAP-2, MAP-3, SECP-1, SECP-2, SECP-3, and the well known Ag85B were selected, cloned and purified as described previously (Mikkelsen et al., 2011b).

2.2. Herds and animals
Two Danish dairy cattle herds were included in the study. A herd presumed to be non-infected and a herd in which MAP infection had been confirmed by cultivation of MAP. The non-infected dairy herd was selected for the study based on the results of the Danish paratuberculosis control programme. Animals included in the control programme were tested four times annually for MAP antibodies in milk (Nielsen, 2002). At the time of sample collection, April 2008, the non-infected herd consisted of 352 Danish Jersey cattle, including 173 cows and 177 heifers, and did not have any reactors among tested cows in the three previous herd screenings. Blood samples were collected from 60 heifers 12-27 months of age (mean 18.1 ±3.4 months). For the day-old samples with anti-IL-10 antibody protocol only blood samples from 40 of these heifers age 12-23 (mean 16.9 ±3.2 months) were used. From the time of blood sample collection this herd have had few isolated cases of animals with positive milk ELISA test results followed by negative test results and the herd is still assumed to be non-infected.

Furthermore, a dairy herd, that was confirmed MAP infected by culture and by clinical cases of paratuberculosis, was selected for inclusion in the study. The prevalence of MAP in the herd was not expected to be 100% and some heifers may not have been infected, but infection pressure was expected to be high due to a history of severe paratuberculosis problems and rearing of calves in close contact with housing of milking cows. The herd consisted of 396 Danish Holstein (black and white), including 193 cows and 199 young stock at the first sample collection in February 2009. Blood samples were collected three times with four and five week intervals from the same 30 heifers 10-21 months of age (mean 15.0 ±2.9 months) at the first sampling.

2.3. Proliferation assay and IFN-γ ELISA
Blood samples were collected in heparinised vaccutainer tubes and immediately stored in thermal boxes to prevent the blood from exposure to temperature fluctuations during sample collection and transportation. Separate tubes were used for blood samples for sample day cultures and day-old cultures. Sample day blood cultures was initiated within 6 hours of collection and blood samples for day-old cultures was kept at 5°C overnight before culture according to the protocol of day-old day samples described previously (Mikkelsen et al., 2009). To each well of a 48-well culture plate (Greiner Bio-one, Heidelberg, Germany) either 50 µl antigen, positive or negative control solution was added followed by 0.5 ml whole blood and cultured for 20-22 hours at 37°C in 5% CO₂. Antigen solutions were PPDj at 10 µg/ml (prepared in 1975 based on the American laboratory strain Promise, SSI collection
no. MNC1053, confirmed to be MAP by IS900 positive PCR, National Veterinary Institute, Copenhagen, Denmark), 1 μg/ml of Ag85B or one of 14 novel recombinant antigens: ESAP-1, ESAP-2, ESAP-3, ESAP-4, LATP-1, LATP-2, LATP-3, LATP-4, MAP-1, MAP-2, MAP-3, SECP-1, SECP-2 and SECP-3. As a positive control 1 μg/ml of superantigen Staphylococcal enterotoxin B (SEB) was used and as a negative control phosphate buffered saline (PBS) was used in parallel cultures. For day-old samples, antigen preparations were added either recombinant bovine IL-12 (a kind gift from Chris Howard and Jayne Hope, The Institute of Animal Health, Compton, UK) at 10 U/ml final culture concentration or mouse anti-bovine IL-10 antibody (MCA2110, AbD Serotec Oxford, UK) at 1 μg/ml final culture concentration. All antigens and the positive control were diluted in PBS.

Following 20 hour culture, the culture plates were centrifuged and the supernatants collected and stored below -20°C until further analysis. The antigen specific IFN-γ production in supernatants was determined by an in-house monoclonal sandwich ELISA as described previously (Mikkelsen et al., 2009). The level of IFN-γ (pg/ml) was calculated using linear regression on log-log transformed readings from the two-fold dilution series of a reference standard with known IFN-γ concentration.

2.4. Interpretation and statistical analysis
Samples were excluded if the IFN-γ response to SEB stimulation was lower than 1000 pg/ml or the IFN-γ response to PBS was higher than 150 pg/ml. Based on these exclusion criteria, four heifers (no. 17, 19, 20 and 21) were excluded from sample day samples, six heifers (no. 7, 12, 17, 19, 21 and 25) were excluded from day-old samples with IL-12 and five heifers (no. 4, 7, 17, 19, 21) were excluded from day-old samples with anti-IL-10 antibodies from the MAP infected herd. For correlation analysis of sample day samples compared to day-old samples with IL-12 seven heifers were excluded resulting in a final study group of 23 animals at the three samples day. For correlation analysis of sample day samples compared to day-old samples with anti-IL-10 antibodies six heifers were excluded resulting in a final study group of 24 animals at the three samples day.

IFN-γ responses to each of the 14 novel antigens, Ag85B and PPDj in the MAP non-infected herd were used to calculate cut-off values for each antigen. Samples from the non-infected herd should fulfil the same requirements as samples from the infected herd. From the day-old samples with IL-12 of the non-infected herd two animals were excluded resulting in 58 animals in the study group and from the day-old samples with anti-IL-10 antibodies of the non-infected herd three samples were excluded resulting in 37 animals in the study group. The IFN-γ response to PBS was extracted from the IFN-γ response to antigen of each animal and cut-off values was calculated for each antigen as the 95percentile of this corrected IFN-γ response. Prior to further calculations, the IFN-γ response to PBS was extracted from the IFN-γ response to each antigen of each animal from the infected herd. If this corrected IFN-γ response was below 1 pg/ml, the value was set to 1 pg/ml.
Correlations between IFN-γ results of sample day samples and day-old samples with IL-12 or anti-IL-10 antibodies was investigated by the non-parametric Spearman rank’s correlation (GraphPad Prism version 4.00, GraphPad Software, La Jolla, CA, USA) because data were non-normally distributed. Based on cut-off values for each antigen, the number of positive tests at the three sample days was calculated for each animal from the MAP infected herd. The case definition (Nielsen et al., 2011) was an animal that had had ≥ two positive tests for ≥ four antigens in sample day samples (Mikkelsen et al., 2011b). Hence, animals were defined as cases or non-cases based on the results of sample day samples and this case definition was used for both types of day-old samples. Youden index and confidence intervals was calculated for each antigen. Youden index is a measure of test performance, that take both immunogenicity (I) and diagnostic specificity (S) into account and is calculated as I+S-1 (Youden, 1950). For analysis of cases that were included in all three test types the study group consisted of nine animals.

2.5. Definitions
The following definitions were used in the present paper. Diagnostic specificity of a test was the proportion of test negative animals among animals defined as non-cases. Immunogenicity was the ability of an antigen to induce an immune response and here, immunogenicity was calculated as the proportion of test positive animals of the animals defined as cases.

3. Results
The number of positive IFN-γ tests for each heifer against each antigen is presented in supplementary Tables S1 and S2. Day-old samples with IL-12 included 10 cases and 13 non-cases and the day-old samples with anti-IL-10 antibodies included 11 cases and 13 non-cases. A heifer (no. 20) was excluded from sample day samples, although included in both day-old protocols this heifer could neither be classified as a case nor non-case. Generally, there were fewer test positive day-old samples with IL-12 compared to day-old samples with anti-IL-10 antibodies.

IFN-γ responses of the non-infected herd were used for calculation of cut-off values for each antigen (data not presented) and cut-off values for day-old samples with IL-12 were considerably higher (range 15-1176 pg/ml) than for day-old samples with anti-IL-10 antibodies (range 4-140 pg/ml) and reflect that IFN-γ responses of day-old samples with IL-12 were relatively higher in blood samples from the non-infected herd. The small difference between IFN-γ responses to antigens of infected and non-infected heifers using day-old samples with IL-12 was confirmed by statistical analysis as only two antigens; MAP-1 (P=0.002) and Ag85B (P=0.03), gave significant different IFN-γ responses. The difference between IFN-γ responses of infected and non-infected heifers was greater using day-old samples with anti-IL-10 antibodies, as nine of 14 novel antigens gave significant (P=0.05) different IFN-γ responses, whereas PPDj, Ag85B and five other antigens did not.
Correlations of sample day samples and day-old samples measured by Spearman’s $\rho$ presented in Table 1 were above or equal to 0.80 for three antigens (ESAP-2, ESAP-3 and Ag85B) tested with day-old samples with IL-12 and for four antigens (LATP-2, LATP-3, MAP-2 and MAP-3) tested with day-old samples with anti-IL-10 antibodies. Both day-old sample types gave correlations to sample day samples of the same range. PPDj that is used for the IFN-γ test by convention gave correlations of 0.78 between day-old samples with IL-12 and sample day samples and 0.75 between day-old samples with anti-IL-10 antibodies and sample day samples.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>IL-12</th>
<th>Anti-IL-10</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Spearman’s $\rho$</td>
<td>95% CI</td>
</tr>
<tr>
<td>ESAT-6 family</td>
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<td></td>
</tr>
<tr>
<td>ESAP-1</td>
<td>0.69</td>
<td>0.38;0.86</td>
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<tr>
<td>ESAP-2</td>
<td>0.85</td>
<td>0.67;0.94</td>
</tr>
<tr>
<td>ESAP-3</td>
<td>0.80</td>
<td>0.57;0.91</td>
</tr>
<tr>
<td>ESAP-4</td>
<td>0.58</td>
<td>0.20;0.80</td>
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<tr>
<td>Latency proteins</td>
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<td></td>
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<tr>
<td>LATP-1</td>
<td>0.72</td>
<td>0.43;0.88</td>
</tr>
<tr>
<td>LATP-2</td>
<td>0.71</td>
<td>0.40;0.87</td>
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<tr>
<td>LATP-3</td>
<td>0.61</td>
<td>0.25;0.82</td>
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<tr>
<td>LATP-4</td>
<td>0.72</td>
<td>0.43;0.88</td>
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<tr>
<td>Secreted proteins</td>
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<td>SECP-1</td>
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<td>SECP-2</td>
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<td>SECP-3</td>
<td>0.72</td>
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<tr>
<td>Ag85B</td>
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<tr>
<td>Other antigens</td>
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<tr>
<td>MAP-1</td>
<td>0.71</td>
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<tr>
<td>MAP-2</td>
<td>0.67</td>
<td>0.35;0.85</td>
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<td>MAP-3</td>
<td>0.79</td>
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</tr>
<tr>
<td>PPDj</td>
<td>0.78</td>
<td>0.53;0.90</td>
</tr>
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</table>

IFN-γ results are mean IFN-γ response to antigen of three blood samples from the same animal. Correlations are presented by Spearman rank’s correlations ($\rho$) with 95% confidence intervals (CI).

Figure 1 presents individual responses of nine animals defined as cases with mean antigen-specific IFN-γ responses of three sample days tested as sample day samples, day-old samples with IL-12 and day-old samples with anti-IL-10 antibodies. In general, the animals showed highest IFN-γ responses in day-old samples with IL-12 compared to the two other test types and many animals showed lowest IFN-γ responses in day-old samples with anti-IL-10 antibodies.
**Fig. 1.** IFN-γ responses (pg/ml) of heifers (n=9) from a *Mycobacterium avium* subsp. *paratuberculosis* infected herd against novel recombinant antigens, recombinant Ag85B and PPDj. Blood samples were cultured with antigens as day-old samples with addition of recombinant bovine IL-12, as sample day samples and as day-old samples with addition of anti-bovine IL-10 antibody. Antigens are grouped into: ESAT-6 family, latency proteins, secreted proteins and other antigens. Each line represent mean IFN-γ response of three blood sample days of a heifer that was defined as a case and show correlations between three test types.
Immunogenicity and diagnostic specificity of the 14 novel antigens, Ag85B and PPDj against day-old samples with either IL-12 or anti-IL-10 antibody is presented in Table 2.

Table 2. Immunogenicity (Imm.), diagnostic specificity (Diag. sp), Youden index and 95% confidence intervals of Youden of each antigen tested with day-old blood samples with addition of recombinant bovine IL-12 (n=24) or anti-bovine IL-10 antibody (n=25).

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Il-12</th>
<th>Anti-IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imm.</td>
<td>Diag. sp</td>
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<tr>
<td>ESAT-6 family</td>
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<td>ESAP-1</td>
<td>0.50</td>
<td>0.92</td>
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<td>ESAP-2</td>
<td>0.60</td>
<td>0.85</td>
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<td>ESAP-3</td>
<td>0.30</td>
<td>1.00</td>
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<td>ESAP-4</td>
<td>0.80</td>
<td>0.77</td>
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<td>Latency proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LATP-1</td>
<td>0.20</td>
<td>1.00</td>
</tr>
<tr>
<td>LATP-2</td>
<td>0.80</td>
<td>1.00</td>
</tr>
<tr>
<td>LATP-3</td>
<td>0.50</td>
<td>0.85</td>
</tr>
<tr>
<td>LATP-4</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Secreted proteins</td>
<td></td>
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</tr>
<tr>
<td>SECP-1</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>SECP-2</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>SECP-3</td>
<td>0.10</td>
<td>1.00</td>
</tr>
<tr>
<td>Ag85B</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Other antigens</td>
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<td></td>
</tr>
<tr>
<td>MAP-1</td>
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<td>1.00</td>
</tr>
<tr>
<td>MAP-2</td>
<td>0.70</td>
<td>0.92</td>
</tr>
<tr>
<td>MAP-3</td>
<td>0.50</td>
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</tr>
<tr>
<td>PPDj</td>
<td>0.20</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Immunogenicity (I) was calculated as the proportion of test positive animals of the animals defined as cases. The case definition was based on fresh blood samples. ** Specificity (S) was calculated as the proportion of test negative animals of the animals defined as non-cases. *** Youden index was calculated as I+S-1, with 95% confidence interval (95% CI)

Day-old samples with IL-12 resulted in a lower number (87) of IFN-γ positive tests compared to sample day samples (167) and day-old samples with anti-IL-10 antibodies (149). This was reflected in low immunogenicity, but high diagnostic specificity. However, Youden indices were low and only three antigens: ESAP-4, LATP-2 and MAP-2 had Youden indices significantly above 0.5, with the highest value of 0.80 by the latency antigen LATP-2. A Youden index of 0.5 suggests that the diagnostic test has no value. Day-old samples with anti-IL-10 antibodies resulted in more (149) IFN-γ positive tests, but immunogenicity and diagnostic specificity were generally low. Six antigens: ESAP-3, LATP-1, LATP-3, LATP-4, MAP-1 and SECP-3 had Youden indices above 0.5, with the highest value of 0.74 by the latency antigen LATP-1. PPDj gave low Youden indices of 0.20 and 0.29 using day-old protocols, due to very low immunogenicity in both protocols.

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4. Discussion
Correlations between IFN-γ responses of the sample day blood samples and of day-old blood samples were high for several antigens, and day-old samples stimulated with these antigens could be applied as an alternative to IFN-γ testing using fresh samples. PPDj is widely used for stimulation of blood in the IFN-γ test and IFN-γ results of both day-old protocols with PPDj and addition of either recombinant IL-12 (p=0.78) or anti-IL-10 antibody (p= 0.75) correlated reasonably well with IFN-γ results of sample day samples.

When antigens were tested with sample day samples, three latency proteins, LATP-1, LATP-2 and LATP-3 showed high immunogenicity and diagnostic specificity giving Youden indices of 0.85 (Mikkelsen et al., 2011b). As with sample day samples, latency proteins seem to be promising antigens in the day-old protocols, but the same antigens do not perform equally well. When correlation, immunogenicity and diagnostic specificity is considered collectively, the latency antigen LATP-2 seems to be the most promising antigen tested with day-old samples with IL-12, since correlation with sample day samples were 0.71 and Youden index was 0.80 for this antigen. For day-old samples with anti-IL-10 antibodies, the latency protein LATP-1 seems to be the most promising, since correlation with sample day samples were 0.76 and Youden index was 0.74.

Macrophages and dendritic cells are primary producers of the pleiotropic cytokine IL-12 that induce early differentiation of CD4+ T cells and hence favours CMI responses (Weiss and Souza, 2008; Mendez-Samperio, 2010). Addition of recombinant IL-12 induce IFN-γ production in vitro (Collins et al., 1999) and recombinant IL-12 have also been tested in vivo as an adjuvant to enhance CMI responses following vaccination against MAP, however the observed adjuvant effect was low (Uzonna et al., 2003). In the present study we demonstrated the ability of recombinant IL-12 and anti-IL-10 antibody to rescue specific T cell responses to MAP antigens.

Generally, the day-old protocol with IL-12 gave high test responses to antigens even in samples from the uninfected herd and this lead to high cut-off values for antigens, resulting in few test positive tests of the infected animals and thus low immunogenicity. IL-12 did not induce unspecific test-responses to the negative control PBS where the median IFN-γ responses of samples from the uninfected herd was low in both sample day samples ( 10 pg/ml) and day-old samples with IL-12 (9 pg/ml). This suggests that IL-12 can amplify even very small responses and that some assay optimization is needed.

IL-10 is an immunomodulatory cytokine produced by various cell types, including monocytes, macrophages and numerous T cell types and B lymphocytes. IL-10 is known to enhance antibody mediated (Th2) immune responses and suppress CMI (Th1) responses partly through inhibition of IL-12 production by macrophages (Moore et al., 2001; Weiss and Souza, 2008). Neutralisation of IL-10 leads to enhanced capacity of macrophages to kill MAP organisms, increased apoptosis of MAP infected bovine macrophages (Weiss et al., 2005)
and enhanced IFN-γ production of bovine whole blood stimulated with PPD (Buza et al., 2004). However, when IL-10 was neutralised in PPDj stimulated blood samples of MAP infected and exposed goats the IFN-γ production was increased in both groups and in unstimulated cells. The IL-10-producing cells were identified to be mainly monocytes and not regulatory T cells (Lybeck et al., 2009). In the present study, the IFN-γ response of PBS stimulated and IL-10 neutralised blood samples were low of both the infected and the non-infected herd, which indicate that IFN-γ is produced specifically in response to antigen stimulation and not unspecifically in response to PBS.

5. Conclusion
IFN-γ responses of blood samples cultured on the day of sampling showed high correlation ($\rho \geq 0.80$) with responses of day-old blood samples cultured with IL-12 and three antigens (Ag85B, ESAP-2 and ESAP-3) and with responses of day-old samples cultured with anti-IL-10 antibodies and four antigens (LATP-2, LATP-3, MAP-2 and MAP-3). PPDj showed lower correlations between sample day samples but both day-old protocols with either IL-12 or anti-IL-10 antibody including PPDj for culture could be an alternative to the conventional IFN-γ protocol. Immunogenicity of the novel antigens was generally low using day-old samples. For the day-old protocol with IL-12 the most promising antigen is latency protein LATP-2 as correlation with sample day samples was 0.71 and Youden index was 0.80 for this antigen. For the day-old protocol with anti-IL-10 antibodies the most promising antigen is latency protein LATP-1 as correlation with sample day samples were 0.76 and Youden index was 0.74.

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References


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