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Age and Dose Dependent Susceptibility to Mycobacterium avium subspecies paratuberculosis Infection in Dairy Calves

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Age and Dose Dependent Susceptibility to *Mycobacterium avium* subspecies *paratuberculosis*

Infection in Dairy Calves

by

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A THESIS

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Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease (JD), a chronic enteritis in ruminants. Control programs focus on prevention of infection of susceptible individuals: calves < 6 months of age. However, this age-cut-off for susceptibility is not well supported scientifically. Additionally, control programs struggle with low sensitivity of diagnostic tests in the early stages of JD.

The main objective of this thesis was to determine age-dependent susceptibility in dairy calves. Additionally, the course of immune responses as well as fecal shedding was assessed. Furthermore, gross lesions, histology and MAP-culture from tissues were used to confirm infection status of each calf, and to investigate age-dependent susceptibility.

Fifty calves were inoculated *per os* on 2 consecutive days at 2 weeks and 3, 6, 9, or 12 months. Within each age group calves received either a high (5×10^9 CFU) or low dose (5×10^7 CFU) of MAP. Six calves served as a negative control group. Serum, whole blood and fecal samples were collected regularly until necropsy at 17 months of age. Macroscopic and histological lesions were assessed and bacterial culture was performed on tissue samples.

Calves were successfully infected with MAP up to 1 year of age even with a low dose of MAP. Calves inoculated at 2 weeks, 3, or 6 months of age with a high dose of MAP had more severe necropsy lesions, were shedding MAP in feces more frequently, and had a stronger humoral and cellular immune response, than calves inoculated with a low dose. Shedding and humoral immune responses differed between individual calves and were detected in about half of the calves, which was more than anticipated. A dose-dependent cellular immune response was detected in each inoculated calf soon after inoculation using an interferon-gamma release assay and is therefore a good candidate test for early diagnosis. To conclude, calves are susceptible to

MAP infection up to 1 year of age and could be infectious to other calves. Keeping the infection pressure low on-farm could reduce the severity of JD. Early diagnosis of MAP-infection is possible and this could improve the potential to control JD on-farm.

Preface

The experimental infection trial described in this thesis was mainly a team-effort and also involved working together with experts on particular topics. This thesis consists of 5 manuscripts – 3 have been accepted for publication, 1 is currently under revision and 1 is ready for submission. For all 5 manuscripts, the first author was involved with study concept and design, inoculum preparation and inoculation procedure, collection and analysis of samples, performing necropsies, animal welfare and management, drafting the manuscript and statistical analysis of the results. This was all done under guidance of supervisor Dr. Jeroen De Buck and co-supervisor Dr. Herman Barkema. The supervisory committee contributed substantial knowledge, support and helped with development of the trial but also writing of the manuscripts. Additionally, veterinary pathologists such as Dr. Jan Bystrom and Dr. Oscar Illanes contributed to the pathology section of this project and the university veterinarian Dr. Greg Muench as well as Dr. Gordon Atkins helped with animal and health management. Also Dr. Robert Wolf aided in sample management and statistical analysis. Todd Wilson and Dr. Tolulope Sajobi collaborated on the statistical analysis of the manuscript included in Chapter 5. All co-authors provided critical review of the manuscripts and permission has been obtained from the publishing journals as well as all co-authors to reprint the manuscripts in this thesis.

Published manuscripts (Chapter 2) and manuscripts accepted for publication (Chapter 3&4):

- Mortier, R.A.R., Barkema, H.W., Bystrom, J.M., Illanes, O., Orsel, K., Wolf, R., Atkins, G., De Buck, J., 2013. Evaluation of age-dependent susceptibility in calves infected with two doses of *Mycobacterium avium* subspecies *paratuberculosis* using pathology and tissue culture. Vet Res 44, 94.

- Mortier, R.A.R., Barkema, H.W., Negron, M., Orsel, K., Wolf, R., de Buck, J., 2014. Antibody response early after experimental infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy calves. Journal of Dairy Science In press.

- Mortier, R.A.R., Barkema, H.W., Orsel, K., Wolf, R., de Buck, J., 2014. Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Veterinary Research In press.

Manuscripts under consideration (Chapter 5):

- Mortier, R.A.R., Barkema, H.W., Wilson, T.A., Sajobi, T.T., Wolf, R., de Buck, J., 2014. Dose-dependent interferon-gamma release in dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Veterinary immunology and immunopathology Submitted as short communication.

Chapter 6 will be used as a basis to write a case report for submission to BMC Veterinary Research:

- Mortier, R.A.R., Barkema, H.W., Orsel, K., Muench, G.P., Bystrom, J.M., de Buck, J., 2014. Longitudinal evaluation of diagnostics in young calves during subclinical and clinical paratuberculosis.

Additionally, the study presented in this thesis was the basis to more research projects. In a first study, an infection trial was performed using the same inoculum and laboratory procedures and the second study discusses gene expression profiling in calves inoculated with a high or low dose

of *Mycobacterium avium* subspecies *paratuberculosis*. Consequently, the following co-authored papers were published (but are not be included in this thesis):

- Eisenberg, S.W., Koets, A.P., Nielen, M., Heederik, D., Mortier, R.A.R., De Buck, J., Orsel, K., 2011. Intestinal infection following aerosol challenge of calves with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 42, 117.

- David, J., Barkema, H.W., Mortier, R.A.R., Ghosh, S., Guan le, L., De Buck, J., 2014. Gene expression profiling and putative biomarkers of calves 3 months after infection with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Immunol Immunopathol 160, 107-117.

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List of Abbreviations

µl	microliter
AJDI	Alberta Johne's disease Initiative
AUC	area under the curve
BCS	body condition score
BHI	brain heart infusion
CD	Crohn's disease
CFU	colony forming units
Ct	threshold cycle
ELISA	enzyme-linked immunosorbent assay
g	gram
HD	high dose
HE	Hematoxylin-Eosin
HPC	hexadecylpyridinium chloride
IFN-γ	interferon-gamma
IgG	immunoglobulin G
JD	Johne's disease
kGy	kiloGray
l	liter
LD	low dose
LN	lymph node
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
ml	milliliter
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPD	purified protein derivative
rcf	relative centrifugal force
RFLP	restriction fragment length polymorphism
S/P ratio	sample to positive ratio
SNP	single nucleotide polymorphism
Th1	T helper 1
Th2	T helper 2
ZN	Ziehl-Neelsen

CHAPTER 1. GENERAL INTRODUCTION

1.1. Pathogenesis and stages of MAP infection

The causative agent of Johne's disease (JD) is *Mycobacterium avium* subspecies *paratuberculosis* (MAP) a slow growing, acid-fast bacterium, dependent on mycobactin for its growth *in vitro* [1-3]. Typically, MAP infects domestic ruminants (i.e. sheep, goats and cattle) [2] and has been described as a major cause of economic losses for the dairy industry [4, 5]. Furthermore, MAP infection in wild ruminants has been reported worldwide, including bighorn sheep, bison, caribou, deer, and many more species [6-11]. Other domestic and non-domestic species, such as horses and birds, may passively shed MAP, but typically do not have symptoms of JD [2, 12]. Special attention in research has been given to rabbits, in which MAP infection causes subclinical disease and shedding of MAP onto pastures for ruminants [13, 14]. Consequently, these rabbits serve as potential reservoirs of infection, although in Chile, rabbits were found to shed MAP passively only [13-15].

It is noteworthy that MAP can be isolated from humans, and has been associated with Crohn's disease (CD); however, there are conflicting reports regarding the role of MAP in the pathogenesis of CD [16-19]. Nevertheless, CD is a multifactorial disease [19], and a potential zoonotic risk needs to be considered, thereby emphasizing the need to control JD in livestock.

The clinical progression of JD has been divided into four stages by Whitlock and Buergelt (1996) [20], according to presence or absence of symptoms, and detection with currently available diagnostics. These stages range from silent and subclinical infection (characterized by absence of symptoms, production losses and negative diagnostics) to clinical disease with wasting and diarrhea. These stages will be specified below in context of the underlying pathogenesis of MAP-infection. It is important to note that, for every cow with clinical signs on a

farm, 15 to 25 additional cases are present, of which only a small proportion will be detected using current diagnostics [20].

1.1.1. Stage I: 'silent' infection

In the "silent infection" stage, calves, and young stock (< 2 years of age) are infected with MAP but do not demonstrate any clinical signs [20]. Diagnostic tests are consistently negative; therefore, the only way to detect an infected calf is by histology or tissue culture [20].

Following oral ingestion, MAP is taken up by enterocytes or M-cells which are located in the epithelium of Peyer's patches [21]. These M-cells sample antigens in the intestinal lumen. The highest concentration of Peyer's patches is present in the ileum [20].On the M-cell surface, MAP binds to β1-integrins and forms a fibronectin bridge [22] and is transferred to subepithelial macrophages as soon as 5 to 20 hours after initial uptake [20, 23] or even sooner according to more recent research [24]. After subepithelial macrophages phagocytise MAP, the organism will start to hinder fusion of the phagosome and lysosome [25], continue to actively suppress macrophage function and consequently prevent an adequate cellular immune response, thereby ensuring its own intracellular survival [26]. Infected macrophages migrate to local lymph nodes [2, 27]; however, no immune response can be detected at this moment using current diagnostics [21]. If the animal manages to produce a sufficient cellular immune response necessary to kill intracellular pathogens [28], it might still clear the infection [29]. However, if MAP is not cleared, a host response will limit spread of MAP, although the organism will manage to proliferate slowly during this "silent infection stage" [29]. Histologically, small focal granulomatous lesions are present in the intestinal submucosa and regional lymph nodes [2, 30].

1.1.2. Stage II: subclinical disease

The subclinical stage can range from 6 months to several years in duration [29]. During the subclinical stage, the bacterial burden will increase in intestinal tissues and the cellular immune response will decrease [29]. Consequently, a switch from a cellular to a humoral immune response occurs [28, 31]. However, the latter is ineffective in coping with an intracellular pathogen [32], resulting in an increase of fecal shedding and antibody production [20]. However, subclinically affected adults are still without clinical signs of JD [20]. Although fecal culture can be negative in the early subclinical stage, but become positive later on. Shedding can be intermittent during this stage [20]. Additionally, if lactating, milk production will decrease [33-35]. Although dairy cattle are often removed from the herd for reasons apparently unrelated to JD (e.g. infertility and mastitis), it is often a MAP infection that predisposes to these conditions [20].

1.1.3. Stage III: clinical disease

Clinical disease usually begins 2-10 years after initial infection and typically lasts 3-4 months [20]. During this stage, the cellular immune response is absent, and a humoral immune response has taken over [28]. Typical clinical signs include gradual weight loss, diarrhea, and changes in serum biochemistry (hypoproteinemia) [20]. Diagnostic tests now clearly indicate a MAP infection: MAP can be detected in feces using fecal culture and MAP-specific antibodies can be detected using an enzyme-linked immunosorbent assay (ELISA). Histological lesions include diffuse multibacillary or paucibacillary lesions [30]; infiltration of large numbers of macrophages and epithelioid cells, granuloma formation, blunting and fusion of intestinal villi. The Ziehl-Neelsen (ZN) stain identifies the presence of mycobacteria in multibacillary cases

[30]. Granuloma formation and malformation of the villi will hinder uptake of nutrients from the intestinal lumen causing a protein-losing enteropathy [20].

Gross pathological changes of the intestinal mucosa begin as velvet-like thickening and progress to substantial thickening with rugose folds and enlarged regional lymph nodes [2]. Chronic enteritis, lymphangitis and mesenteric lymphadenopathy are typical macroscopic findings for MAP infection [36]. Even though the ileum is usually affected, lesions can extend into the colon, especially when clinical signs (diarrhea) are prominent [37]. These lesions are a consequence of unintentional damage caused by the host immune response [29].

1.1.4. Stage IV: advanced clinical disease

Advanced clinical stage (Stage IV) is characterized by a lethargic state, weakness, emaciation, clinically visible edema due to hypoproteinemia (bottle jaw), and diarrhea [20]. If the affected animal is not culled, it will die from dehydration and cachexia [20]. This stage is often not reached by infected animals, because they are either culled earlier in the disease process, or leave the herd for other reasons.

1.2. Importance of age at infection and infective dose

Early reports (1930-1975) of research in JD reported an age-dependent susceptibility to MAP infection; young calves were deemed susceptible to infection, whereas adults were considered more resistant [38-41]. Additionally, this age-dependent susceptibility to MAP infection was also reported in deer and sheep [42-44]. In a study including 12 calves, infected at birth, 3, and 6 months of age using IV and oral routes of infection, an age cut-off for susceptibility to MAP-infection was set at 6 months of age on the basis that calves inoculated at 6 months were more resistant to infection [41]. The results of these studies claiming age-dependent susceptibility

were included in a meta-analysis by Windsor and Whittington (2010) [45] and the authors concluded that younger animals were more susceptible to MAP infection compared to older individuals [45]. However, this review also concluded that these early studies were not comparable, because their experimental protocols differed substantially [45]. In a more recent meta-analysis, it was suggested that susceptibility of adults might have been missed because they were not included in experimental infection studies (due to assumed resistance) [46]. Consequently, although age dependency is not well documented, current herd-level control strategies focus on protecting young calves from exposure to MAP-infected manure and shedding dams [35, 45]. However, reports not included in the meta-analysis by Windsor and Whittington had contradictory results. In that regard, heifers introduced to a MAP-infected herd were subsequently infected, suggesting they were still susceptible to MAP infection [47]. Because this age-dependent susceptibility had not been well documented, it was identified as an important knowledge gap [48]. Clearly, elucidating age-related resistance is critical for development of effective control programs.

The infection dose to which susceptible animals are exposed is also of importance, as it has been suggested that cattle exposed to a higher dose progress to the clinical stage more rapidly [29, 49]. Additionally, heifers on high-prevalence farms start shedding earlier compared to those on low-prevalence farms [50]. Similarly, sheep exposed to a higher dose of MAP started shedding earlier and more frequently [44]. Even though it is complicated to determine the exact infection pressure present on-farm, the dose seems to affect the pathogenesis of MAP infection. In an experimental infection trial of sheep, lesions at necropsy were more severe in those inoculated with a higher dose of MAP [43]. Concerning infection pressure on-farm, the environment on dairy operations to which susceptible calves are exposed, was substantially contaminated with MAP posing an

actual risk for infection of susceptible calves [51, 52]. Infection can take place in young calves with a dose contained in 2 g of infected feces [35]. In studies by Eamens et al., who determined the dilution effect of pooled fecal samples, a high shedder was considered to have $> 2 \times 10^5$ MAP/gram (g) and low shedders $< 4\text{-}6 \times 10^4$ MAP/g [53, 54], providing an indication of the number of MAP present in feces. Experimental infection has been established with relatively low doses of MAP. A dose of 1.5×10^6 Colony Forming Units (CFU) reliably produced infection in young calves [55]. Regardless, the wide variety of challenge doses and methods to quantify inoculum employed in previous experiments makes comparisons difficult [45, 48]. Chiodini (1996) stated that the infectious dose for a calf likely contains $50 - 1000$ CFU [56]. However, this has not been confirmed experimentally, and the actual minimum infective dose for a calf remains unknown. However, it remains important, since it influences pathogenesis and how soon infected cattle become infectious.

1.3. Prevalence of MAP infection in dairy cattle in Canada

Development of prevention and control programs requires knowledge regarding the prevalence of MAP infection, risk factors, and corresponding financial losses [4]. In the Canadian dairy industry, these economic losses are estimated to be CDN$15 million annually [35], including infertility, mastitis, decreased milk production, reduced slaughter value, and premature culling [20, 34, 35].

It is noteworthy that MAP infection has been reported worldwide [4]. The prevalence of MAP infection in Canada was reviewed by Tiwari et al. (2006) and included the following reports. In Canada, true herd-level prevalence was estimated to be 27% based on serum ELISA (2 seropositive cows considered a herd infected) in Alberta [57]. A lower herd prevalence of 16.7%

(8.8 − 24.5%) was reported in Nova Scotia, Prince Edward Island and New Brunswick [58]. Saskatchewan had a herd-level seroprevalence of 24.3% (9.8 − 38.7%) [59], whereas Manitoba had a prevalence of 43.1% (24.9 − 61.4%) [60]. When pooled fecal cultures were used to increase sensitivity, the prevalence ranged from 28 to 57%, depending on the number of positive samples in the positive fecal pool [27, 57]. Furthermore, environmental sampling of manure storage areas and cow alleyways was recommended as a suitable alternative to sampling of single animals to determine herd-level prevalence [52]. A more recent study used environmental sampling and obtained an estimated true herd-level prevalence of 68% for Alberta and 76% for Saskatchewan [61]. The diagnostic test used is of primary importance in obtaining an accurate estimation of prevalence [4]; furthermore the actual prevalence is likely higher than reported [4, 27].

Seroprevalence at the animal level was 7% in Alberta [57], 4.5% in Manitoba [60], 2.7% in Saskatchewan [59], 2.9% in New Brunswick, 3.3% in Nova Scotia and only 1.3% in Prince Edward Island [27, 58]. These cow-level prevalences likely underestimate the true cow-level prevalence, due to the low sensitivity of ELISA [4]. In that regard, when tissue samples were cultured from individual cows in a New Brunswick abbatoir, the cow-level prevalence was 16.1% [62]. Because tissue culture is more sensitive than ELISA, this estimation of the prevalence is consequently a more accurate reflection of the true prevalence on an individual-animal level [27]. Notwithstanding underestimation due to low test sensitivity, clearly the prevalence of MAP infection is substantial.

1.4. Diagnostics

Diagnosis of MAP infection is problematic due to suboptimal test characteristics [63]. Additionally, fecal shedding and humoral immune responses are not present until the late subclinical and clinical stages of JD [20, 31]. Consequently, it is typically recommended to test cattle > 3 years of age when screening a herd for MAP infection [64]. Nevertheless, elucidating strengths and weaknesses of these diagnostics will aid in selecting the right test to use according to circumstances and goals.

1.4.1. MAP Culture from feces and tissues

Bacterial culture from feces or tissues confirms presence of live MAP and consequently infection. Tissue culture is considered the ideal gold standard for detection of MAP infection, because it can detect MAP prior to other diagnostics [20, 27]. However, tissue culture requires the death of the animal which is not always feasible or desired; consequently, fecal culture is more often used as a gold standard [27]. Culture of MAP from fecal samples is considered 100% specific when a positive test is reported [65], especially when confirmed with a Polymerase chain reaction (PCR)-detecting, MAP-specific genetic material, e.g. IS*900* or *F57* sequences [63, 66]. Sensitivity of fecal culture has been reported to be 70-74% in clinical and late subclinical stages; however, in the 'silent infection' and early subclinical stage, sensitivity is lower (23-29%)[63]. A false-positive result is only possible when an individual is passively shedding MAP but is not truly infected with MAP [67].

Culture of MAP is typically performed in three phases: (1) decontamination of fecal samples to eliminate commensal organisms; (2) incubation of MAP in broth or on agar (weeks to months); and (3) confirmation of the presence of MAP by detecting MAP-specific genetic material [68]. Fecal culture can be performed on individual or pooled fecal samples [69], but also from the

environment on dairy farms (e.g. manure storage area or cow alleyways) and is of particular interest in control programs [52]. Although there is some variation in culture protocols, a standardized procedure was proposed in 1991 [70]. Since then, improvements were made and again a variety of procedures are in use. For example, liquid culture is faster and more sensitive than agar [65, 71, 72], but it still takes weeks to culture MAP. Therefore, fecal culture is time consuming and has been regarded as expensive (US$16-19 per sample in the USA [73] or CDN$35-60 per sample in Canada [27]).

The same procedure can be used to culture MAP from intestinal tissues; however, the decontamination step can be reduced in time [65]. As a minimum, the ileum, ileocecal lymph node, and/or the ileal lymph node should be sampled [73], because the ileum is the location where initial uptake of MAP takes place and where most of the disease progression occurs [29]. In contrast, multiple studies have reported that sampling only these sites will underestimate the number of infected cows [74-76]. Therefore, it is recommended that other portions of the gastrointestinal tract with their corresponding lymph nodes, in addition to other locations in the body (e.g. liver, spleen, lungs, or supramammary lymph node) could be sampled to improve diagnostic sensitivity [37,75,76].

1.4.2. Direct PCR on fecal samples

Presence of MAP can also be confirmed by directly detecting MAP-specific genetic material (for example IS*900*, *F57*or/and IS*mav*2) in fecal material [77, 78]. However, fecal material can inhibit PCR reactions [27] and IS*900*(-like) sequences have also been identified in other mycobacteria [79-81], possibly creating false-negative or false-positive results, respectively. Sensitivity and specificity of the direct PCR is 30 and 99%, respectively [73].

1.4.3. Detection of a humoral immune response

During the late stages of JD (late subclinical and clinical stage), a humoral immune response characterized by the production of antibodies dominates [28]. An ELISA can be used to detect the presence of these MAP-specific antibodies in serum or milk. There are commercially available ELISAs as well as in-house developed ELISAs in which various antigen preparations have been used. Some antigen preparations include a mix of multiple proteins, for example filtration of a MAP culture containing secreted proteins by MAP or whole-cell lysates had immunogenic proteins with diagnostic potential [82]. Conversely, single proteins were also used, including lipoarabinomannan [83], PPE proteins [84, 85], cell surface proteins [86] and many more. Avidity and affinity of antibodies is an important factor for the success of ELISA but is not well documented for MAP-antigens [68]. The sensitivity of ELISA ranged from 7 to 94%, due to the use of different antigens, gold standards, and stages of JD [63]. For example, sensitivity of ELISA is lower in the 'silent infection' and subclinical stage compared to cattle in the clinical stage [63]. This low sensitivity makes ELISA more useful as a herd screening test for control purposes rather than for individual diagnosis of MAP infection [73]. As novel antigens are being identified, performance of ELISA could advance in the future [87]. The cost of ELISA per sample was estimated to be US$5 for a serum sample and $6 for a milk sample in the USA [73] or CDN$10 in Canada [27]. In addition, ELISA can also be performed on milk samples, which makes sampling of a dairy herd convenient, because milk is readily available from lactating cows. The sensitivity was reported to be 29-61% [63].

1.4.4. Detection of a cellular immune response

There are 2 methods to detect a cellular immune response: the intradermal johnin skin test and the interferon-gamma (IFN-γ) release assay [88]. The johnin skin test detects a delayed

hypersensitivity type IV reaction by formation of swelling after intradermal injection of a mycobacterial antigen (johnin) [88]. An IFN-γ release assay was developed for diagnosis of tuberculosis; however, this test has been successfully applied in paratuberculosis research as well [89]. The IFN-γ release assay is performed in 2 stages. First, whole blood is collected from the cow and peripheral blood mononuclear cells (PBMCs) are stimulated with MAP-specific antigens. Secondly, IFN-γ produced by the memory T-cells in response to antigen stimulation is detected using a sandwich ELISA [89]. This test is especially promising for detection of MAP infection soon after infection, because it targets the cellular immune response dominating during the 'silent infection' and subclinical stages [90]. However, some difficulties were experienced considering the interpretation of the IFN-γ release assay [88] and false positive reactions occurred [91], especially in calves < 15 months old [92]. Additionally, the issue of which antigen activates the memory T-cells best to evoke IFN-γ production is not well documented (as is the case for antigen preparations used for ELISA).

The sensitivity of the IFN-γ release assay is 13-85%; however, only 2 studies have evaluated test characteristics [63, 93, 94]. Little is known about the practical application of this IFN-γ release assay. One of the 2 studies concluded that this test could best be used for diagnosis of MAP infection in cattle 1-2 years old [94], consistent with knowledge on the cellular immune response early after infection [28, 31]. However, older animals have also tested positive on the IFN-γ release assay [94]. Therefore, interpretation of this diagnostic test is complicated. It is not known whether these animals were infected recently or if this is a longstanding cellular immune response or if this response fluctuates depending on the physiological state of the individual? Clearly, more information regarding the course of the cellular immune response and its interpretation would improve the use of this test.

1.5. Epidemiology and control programs

In the absence of an effective cure for JD, control programs are essential. It is well established that MAP is transmitted primarily through the fecal-oral route, from MAP-shedding individuals to the susceptible population [2, 27, 95]. Transmission takes place by ingestion of milk, water, or feed that is contaminated with MAP-containing feces [27]. Additionally, active shedding of MAP in milk [2, 95] and trans-placental transfer of MAP [95, 96] have been documented and are of particular importance in the clinical stage [97] when MAP is the most widely disseminated within the body of the infected animal. Presence of MAP was confirmed in semen from a bull in the subclinical stage of JD [98], making venereal transmission a possibility as well. Additionally, MAP can survive for up to 1 year in the environment [99], resulting in substantial contamination in the environment of a shedding animal.

Current JD control programs focus on prevention of the assumed most susceptible population (young calves) and reduce their exposure to any manure, and concurrently reduce the number of infected animals on a farm [35]. Individual testing aiming to screen a herd for MAP infection is typically done on cattle > 36 months of age [64], due to the assumption of low sensitivity of diagnostics in the 'silent infection' and early subclinical stage of JD.

In 2010, Alberta Milk together with the Department of Production Animal Health of the University of Calgary started the Alberta Johne's Disease Initiative (AJDI) [5]. This control program involved collection of 6 environmental samples each year, completion of a risk assessment on-farm by the herd veterinarian and suggestions for changes in management strategies [5]. These suggested management strategies were directed towards purchasing cattle, prevention of infection of young calves by separating them from adult cattle and exposure to manure [100]. The AJDI follows a similar pattern as the control program in the USA, with the

exception that no individual testing is applied [5, 68]. Control programs worldwide were reviewed in *Paratuberculosis: Organism, Disease, Control* by Behr and Collins (2010) [68]. In summary, in addition to reducing contact with manure and preventing calves to be exposed, other control strategies such as testing and culling were useful in countries with a low prevalence or when the value of the individual animals is low, e.g. sheep and goats [68]. Vaccination is a useful tool in control programs for small ruminants as it reduces losses and clinical disease; however, it does not eradicate MAP infection [68]. Although vaccination in cattle interferes with tuberculosis testing and is, therefore, less applicable in control programs for dairy herds, it has been done in the USA, Australia and New Zealand [68]. Regardless, success of control programs is hindered by the several potential transmission routes, prolonged persistence of MAP in the environment, low sensitivity of diagnostic tests used for herd screening, and the wide range of host species involved (discussed above) [35]. It was stated that: "no herd in the USA has completely eradicated JD, even over 25 years of excellent management, semi-annual testing and culling of most positive cows" [68]. However, control programs have reported to be successful in reducing the prevalence of MAP on a herd level [101]. It is believed to be worthwhile to continue to make improvements to current control strategies.

1.6. Hypotheses

An age-related resistance to MAP inoculation occurs, and a higher dose of MAP is required to infect older animals.

When calves are inoculated at a younger age or/and with a higher inoculation dose, diagnostic tests (fecal shedding, ELISA, IFN-γ release assay) used for MAP detection become positive earlier after experimental MAP infection.

1.7. Objectives of the study

The main objective of the studies reported in this thesis was to determine age- and dose

dependent susceptibility in dairy calves in order to have a more reliable basis for susceptibility in

control programs. Additionally, the onset and course of cellular (using the IFN-γ release assay)

and humoral (using ELISA) immune responses, as well as fecal shedding, will be assessed to

determine when diagnostic tests become positive so that they can be used more reliably for

individual animal or herd level testing. The infection status of each calf will be confirmed using

gross lesions, histology and MAP culture from numerous tissues to ensure accuracy in

conclusions regarding susceptibility.

1.8. Outline of thesis

In order to address the objectives, a clinical infection trial was performed. Fifty calves were

inoculated with a high or a low dose of MAP at 5 ages and one extra group served as a control

group. In separate chapters, findings on the diagnostics in these calves will be discussed. Chapter

2 will present a conclusion on age- and dose-dependent susceptibility based on results from MAP

culture from tissues, because this is considered to be the gold standard for detection of MAP

infection and therefore the most conclusive test to answer this objective. In addition, histology

and gross lesions were assessed and related to tissue culture. Chapter 3 discusses the effect of

age and dose at inoculation on antibody responses detected with ELISA early after inoculation.

Fecal shedding and differences in shedding patterns according to age and dose at inoculation are

presented in Chapter 4. As a last diagnostic test, the use of IFN-γ release assay as a measure of

the cellular immune response early after inoculation is discussed in Chapter 5 and the effect of

age and dose on the onset and course of the cellular immune response were evaluated. Lastly, in

Chapter 6, a case report is included about 2 calves who presented unexpectedly with clinical signs of JD during the trial and which gave us an opportunity to compare diagnostic profiles between asymptomatic and clinical calves.

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CHAPTER 2: EVALUATION OF AGE-DEPENDENT SUSCEPTIBILITY IN CALVES INFECTED WITH TWO DOSES OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* USING PATHOLOGY AND TISSUE CULTURE

2.1. Abstract

The longstanding assumption that calves greater than 6 months of age are more resistant to *Mycobacterium avium* subspecies *paratuberculosis (*MAP) infection has recently been challenged. In order to elucidate this, a challenge experiment was performed to evaluate age- and dose-dependent susceptibility to MAP infection in dairy calves. Fifty-six calves from MAP-negative dams were randomly allocated to 10 MAP challenge groups (5 animals per group) and a negative control group (6 calves). Calves were inoculated orally on 2 consecutive days at 5 ages: 2 weeks and 3, 6, 9 or 12 months. Within each age group 5 calves received either a high – or low - dose of 5×10^9 CFU or 5×10^7 CFU, respectively. All calves were euthanized at 17 months of age. Macroscopic and histological lesions were assessed and bacterial culture was done on numerous tissue samples. Within all 5 age groups, calves were successfully infected with either dose of MAP. Calves inoculated at < 6 months usually had more culture-positive tissue locations and higher histological lesion scores. Furthermore, those infected with a high dose had more severe scores for histologic and macroscopic lesions as well as more culture-positive tissue locations compared to calves infected with a low dose. In conclusion, calves up to 1 year of age were susceptible to MAP challenge and a high infective dose produced more severe lesions than a low dose.

2.2. Introduction

Paratuberculosis or Johne's disease (JD) is a chronic enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Clinically affected animals have chronic, non-treatable diarrhea and wasting [1]. The major effects are reduced milk yield [2, 3], premature culling and reduced slaughter value [4].

In cattle, JD has an incubation period ranging from 2 to 10 year [5]. In most cases, MAP is transmitted by the fecal-oral route [1]; in infected herds, calves are likely exposed to manure from mature cattle that shed the bacteria in their feces and to contaminated water, feed, or milk [6]. In Eastern Canada and Maine, the prevalence of MAP infection was 16.1% based on a systematic random sample of cattle at an abattoir [7].

Several studies claim cattle were infected as calves but become more resistant to the infection as they get older [8-11]. However, interpretation and comparison of the results of these studies is hindered by the low number of animals per experiment, the variety of inoculation doses and routes of inoculation used, incomplete information regarding animal housing and the variation in diagnostics used to confirm infection [12]. For this reason, proposed international guidelines were designed in order to standardize and simplify interpretation of challenge experiments [13].

It has also been suggested that calves become more resistant as of 6 months of age [11, 12]. As a result, current herd level control strategies focus on reducing calf exposure to all manure by recommending hygienic measures such as removing young calves from their dams to prevent transmission of MAP [6, 12].

The basis for this age-dependent susceptibility has not been well documented and was recently identified as an important knowledge gap [13]. Clearly, correct understanding of age-related resistance is critical for development of effective control programs.

Because this technique can identify infection earlier than any other diagnostic test, MAP culture from gastro-intestinal tissues is considered to be the gold standard for detection of MAP infection [1] and was used to confirm infection status [14] in multiple studies.

The objective of the present study was to evaluate age- and dose-dependent susceptibility to MAP infection in dairy calves by the use of tissue culture and macroscopic and microscopic evaluation of tissues.

2.3. Materials and Methods

2.3.1. Calves

Fifty-six Holstein-Friesian bull calves were purchased from 16 Alberta (Canada) dairy farms. These 16 herds were selected from 24 southern Alberta dairy herds tested to estimate the prevalence of MAP infection. Fecal and serum samples were collected from all 2nd lactation and older cows and individual milk samples were obtained simultaneously with the fecal and serum sampling through the milk recording agency CanWest DHI (Guelph, ON, Canada). To minimize the risk of including calves that had acquired intra-uterine MAP infection, calves were collected only from the 16 herds that yielded negative pooled (n=5) fecal samples (decontaminated and prepared for culture according to manufacturer's instructions; para-JEM®, TREK Diagnostic systems, OH, USA) and had a within-herd seroprevalence < 5% (IDEXX Paratuberculosis Ab Test; IDEXX Laboratories Inc, Westbrook, ME, USA).

Only calves from heifers or second parity cows and born on-farm in the presence of the research team were included in the study; contact (licking, suckling) with the dam or environment was prevented. Additional fecal and serum samples were collected from the dam within 2 weeks after calving and tested with fecal culture and serum ELISA, respectively. Only calves from dams negative for both tests were included in the study. In addition, a precolostral serum sample was collected from each calf and tested for presence of Bovine Viral Diarrhea Virus which would indicate persistently infected calves (only calves with negative test results were used).

2.3.2. Nutrition, health and husbandry

The calves were transported to the research facility and fed 6 l of gamma-irradiated colostrum (collected from fecal culture- and ELISA-negative herds) within 6 hours after birth. To ensure colostrum would not contain any live MAP bacteria, this colostrum was treated with gamma irradiation with a minimum dose of 10 kGy per pail (containing 17L of colostrum) using a Cobalt-60 source (McMaster Nuclear Reactor, Hamilton, ON, Canada) [15]. This was followed by milk replacer and calf starter grain without antimicrobial additives and high-quality hay, as Eisenberg *et al.* described [16]. After weaning at 7 weeks of age, calves were fed ad libitum hay and water and supplemented with concentrates to guarantee a balanced diet.

Calves were dehorned under local anesthesia using a cauterizing iron and they were surgically castrated after administration of sedation and local anesthesia. Calves were monitored for 17 months, after which they were euthanized and necropsied.

The calves were housed in a biosecurity level 2 housing facility. This facility included 33 individual custom built housing units with waterproof liners to contain any leakage. One individual housing unit consisted of the pen containing the calf and a marked zone, in which a dedicated boot dip, boots, coveralls and gloves were provided for each individual housing unit. Calves were contained in small pens until 4 months of age after which they were individually transferred to a large animal facility – maintaining the individual housing unit set up. Personnel were trained in strict biosafety and isolation protocols to avoid transmitting MAP between calves and health status was monitored and recorded daily by clinical inspection. Animal care protocols M09083 and M09050 were approved by the Health Sciences Animal Care Committee of the University of Calgary and procedures were conducted in compliance to these protocols.

2.3.3. Study design

The 56 calves were randomly allocated to 5 age groups and 2 dose groups within each age group. Calves were inoculated with MAP at 5 ages (2 weeks, 3 months, 6 months, 9 months and 12 months). Six calves housed in the same conditions were not inoculated (negative controls). Within each of the 5 age groups containing 10 calves, 5 calves were infected with a high dose (HD) of MAP and 5 with a low dose (LD) of MAP.

The research facility allowed housing for a maximum of 33 calves individually at a time. Consequently, the first 33 calves equally representing all age and dose groups, as well as 3 controls were included. The experiment was then repeated with 23 calves, including 3 control calves, also equally representing all age and dose groups.

2.3.4. Inoculum

A virulent cattle type MAP strain isolated from a clinical Alberta JD case (Cow 69) was used for inoculation. This isolate has an identical *Bam*HI, *Pvu*II and *Pst*I IS*900* − restriction fragment length polymorphism (RFLP) profile to the reference strain K10 (data not shown), the strain type recommended for experimental challenge trials [13]. Two doses of inoculum were used, a HD of 5 x 10^9 CFU given on 2 consecutive days (= 5 times the recommended standard bovine challenge dose [13]) and a LD of 5 x 10^7 CFU also given on 2 consecutive days (= 10 times higher than the lowest confirmed and consistent infectious dose for young calves [14]).

Inoculum was prepared and cultured in 7H9 broth and quantified using the pelleted wet weight method, as well as quantitative PCR as described by Eisenberg *et al*. [16]. Before each inoculation, one tube containing an identical aliquot of MAP cells was taken out of the -80°C freezer and resuspended in 350 ml of 7H9 broth. The culture was incubated for exactly 7 days at 37°C in a shaking incubator. In this period, the inoculum was tested for contamination. Right

before inoculation, a 50 ml volume was prepared for the HD inoculation group and a 100-fold dilution was created for the LD inoculation group. The inoculum was placed in a syringe and expelled at the root of the tongue.

2.3.5. Necropsies

Calves were euthanized at 17 months of age by intravenous injection of barbiturate (Euthanyl Forte®, DIN 00241326, Bimeda-MTC Animal Health Inc., ON, Canada) and necropsies were performed immediately. No other ruminants were allowed in the pathology room during necropsies and the necropsy room and tables were thoroughly cleaned and disinfected before and after each necropsy.

Twenty-one tissue samples were collected from each calf. For each tissue sample, a new set of disinfected instruments and clean gloves were used (to prevent cross contamination). Intestinal tissue sample locations were marked and isolated (zip ties) to prevent movement of intestinal contents. Lymph nodes (LN) were sampled before opening and sampling intestinal tissue to prevent cross contamination.

Macroscopic lesions were assessed at necropsy by a veterinary pathologist, who was blinded to the inoculation status of the calves. Macroscopic lesions were scored, based on previous studies [1, 17-22], to the following categories: 0 = no macroscopic changes; 1 = one enlarged or edematous LN of the small intestine or liver; 2 = multiple enlarged and edematous mesenteric LN and/or hyperemia of the ileocaecal valve; 3 = enlarged mesenteric LN and/or mild to moderate thickening of ileal or jejunal mucosa; and 4 = enlarged mesenteric LN and severe thickening and corrugation of the ileal, jejunal and colon mucosa.

Gastro-intestinal tissue samples were collected from the duodenum, jejunum (mid and distal), ileum (proximal, mid and distal), ileocaecal valve, caecum, colon (spiral colon and transverse

colon) and rectum. Lymph nodes were sampled on locations corresponding with the gastro-intestinal tract samples (except for the spiral colon, transverse colon and rectum). Additionally, the hepatic LN, tonsil, retropharyngeal LN and the superficial inguinal LN were sampled. From the 23 calves included in the second replicate, additional samples were collected (kidney, liver and spleen). Special consideration was given to evaluation of a specific subset of sampling locations: ileocaecal valve, ileocaecal LN and distal ileum. These tissue sites were considered particularly important because these were previously described as the most reliable sampling sites for MAP diagnosis [18, 23, 24] and these sites would provide the most reliable detection of successful infection.

From 4 specific tissue sites (ileocaecal valve, ileocaecal LN, ileal LN and distal ileum), samples were placed in a labeled cassette, immersed in 10% neutral buffered formalin solution (VWR International, Inc., Edmonton, AB, Canada) and routinely processed for histological assessment. Samples were embedded in paraffin, sectioned and stained with Hematoxylin-Eosin (HE) as well as Ziehl-Neelsen (ZN) by Prairie Diagnostic Services (Saskatoon, SK, Canada). Slides were examined by light microscopy and scored for paratuberculosis-associated histological lesions according to González et al. [25] (0 = no lesions; 1 = focal lesions; 2 = multifocal lesions; and 3 = diffuse lymphocytic, multibacillary or intermediate lesions) by an experienced veterinary pathologist who was blinded to the inoculation status of the calves.

Intestinal samples were rinsed with phosphate buffered saline (PBS) to remove intestinal content and the mucosa was scraped off the intestinal wall using microscope slides. Fat was trimmed from the LN and these were homogenized in a Stomacher® (Stomacher® 80 Biomaster, Seward Laboratory Systems Inc., Bohemia, NY, USA) and stored at -80°C until cultured.

2.3.6. Tissue Culture

From each tissue sample, 2 g was added to a sterile polyethylene stomacher bag with 5 ml of PBS and homogenized in a Stomacher®. The sample was then added to 20 ml of 0.6% hexadecylpyridinium chloride (HPC) in half strength Brain Heart Infusion (BHI). After incubation (3 hours at 37°C), tubes were centrifuged at 1700 rcf for 20 minutes. Further processing was performed according to manufacturer's recommendations (para-JEM®, TREK Diagnostic Systems). Tissue culture results were assessed in categories. Calves were assigned to the following categories: 0 = no positive tissues; 1 = 1-3 tissues positive; 2 = 4-6 tissues positive and 3 = more than 6 tissues positive.

2.3.7. DNA extraction and real-time qPCR using F57

From these liquid tissue cultures, DNA was extracted as described by Forde *et al*. [26]. Next, real-time PCR targeting the *F57* region was performed as described by Slana *et al.* [27] and based on Forde *et al.* [28]. Samples with amplification curves with a threshold cycle below 40 were considered positive.

2.3.8. Data analyses

Differences in distributions of tissue culture results, macroscopic and microscopic lesions between age and dose groups were evaluated using Chi-square and Fisher's exact tests. For comparisons between the HD and LD groups or between specific age groups, one-sided testing was used. Agreement between the three diagnostics used was calculated with a linearly weighted kappa coefficient [29]. Analyses were performed using STATA 11.0 (StataCorp LP, College Station, TX, USA). A P-value ≤ 0.05 was considered significant.

2.4. Results

2.4.1. Tissue culture

Twenty-eight of the 50 (56%) inoculated calves had at least one MAP-positive tissue (Table 2-1). One control calf had one culture-positive tissue (duodenal LN), whereas another control calf had two culture-positive tissues (ileal LN and ileocaecal LN).

Positive tissue culture results were present in calves of all age and dose groups. The proportion of calves with at least one MAP-positive tissue culture, was equal (14 calves of 25; 56%) between the LD and the HD calves ($P = 1.00$). However, all 5 calves with ≥ 4 culture-positive tissues were inoculated with a HD ($P = 0.03$). The proportion of calves with at least one culture-positive tissue was similar in the 5 age groups, ranging from 40-70% ($P = 0.82$) and the proportion of tissue culture-positive calves did not decrease with increasing age at inoculation (Table 2-1). However, all 5 calves with ≥ 4 culture-positive tissues were inoculated at ≤ 6 months of age ($P = 0.07$).

2.4.2. Tissue locations

All tissue locations were MAP culture-positive in at least one calf, except for the kidney, although no location was MAP culture-positive in all inoculated calves (Figure 2-1). The proportion of positive tissue sites ranged from 0% (kidney) to 16% (mid ileum and ileocaecal valve); most sampling sites in this trial were culture-positive in < 10% of the MAP- inoculated calves (Figure 2-1).

A minimum of 10 tissue locations was necessary to identify all culture-positive calves. Any extra samples did not increase the number of calves detected (Figure 2-2). Using the subset of tissue locations most frequently used to diagnose MAP infection, ileocaecal valve, ileocaecal LN and

distal ileum, 8 (31%) of the 26 calves with at least one MAP-positive tissue culture were detected. All of these 8 calves were MAP culture-positive for the ileocaecal valve and from these, 4 also had a positive culture for the distal ileum, whereas 3 had a positive culture for the ileocaecal LN.

By the end of the trial, two HD calves inoculated at 2 weeks of age had clinical symptoms of JD; these calves both had 18 of 24 culture-positive tissue locations.

2.4.3. Macroscopic lesions

Macroscopic lesions were present in calves in all age and dose groups (Table 2-2). Thirty-one (62%) of 50 inoculated calves had macroscopic lesions, whereas no gross lesions were detected in the control calves ($P = 0.005$). Of the 25 calves inoculated with a HD, 16 (64%) of 25 had a macroscopic lesion score > 2 compared to 9 (36%) of the 25 LD calves ($P = 0.04$). The proportion of calves with macroscopic lesions differed among age groups (Table 2-2; $P = 0.03$). However, more calves inoculated at 12 months of age had macroscopic lesions compared to 9-month inoculated calves ($P = 0.03$).

Both of the 2 HD calves inoculated at 2 weeks of age that had clinical symptoms of JD had severe (category 4) macroscopic lesions.

2.4.4. Histology

Histological lesions in samples of the ileocaecal valve, ileocaecal LN, ileal LN and distal ileum were present in calves in all age and dose groups (Table 2-3). Forty-two (84%) of 50 MAP-inoculated calves had histological lesions which, with the exception of 2 animals, were classified as mild and focal within affected tissues. Subtle and focal lesions (occasional tiny clusters of

epithelioid ZN-negative macrophages) were also detected in 4 of 6 control calves. Twenty (80%) calves in the LD and 22 (88%) calves in the HD groups had lesions ($P = 0.35$).

Six (12%) of 50 inoculated calves had extensive histological lesions (scores 2 or 3; Table 2-3). These extensive histological lesions were only present in calves in the 2-week group and 3-month group ($P = 0.002$), but not in the other age groups. Also, most calves (5 of 6) with histology score 2 or 3 were inoculated with a HD. The 6 calves with extensive histological lesions were also the only calves that were ZN-positive. The 2 calves in the 3-month HD group had lesions in 3 of 4 tissues; both were positive in the ileal LN with the ZN stain. The two non-clinical 2-week calves had lesions in 3 of 4 tissues; one had a ZN-positive ileocaecal valve, and the second calf was ZN-positive in 3 of 4 tissues.

Both HD calves inoculated at 2 weeks of age that had clinical symptoms of JD had severe histological lesions in all 4 tissues and were strongly ZN-positive in all 4 tissues.

2.4.5. Tissue culture, macroscopy and histology combined

Forty-three (86%) of 50 MAP-inoculated calves were positive on at least one diagnostic test, represented in all age and dose groups (Table 2-4), whereas 2 control calves were also positive on one of the 3 tests (with a histology score ≥ 2 considered positive). Twenty-two (44%) calves in the LD and 21 (42%) in the HD groups were identified with either tissue culture, histology (score > 2) or macroscopy ($P = 1.00$). All 5 age groups had calves positive on at least one of 3 tests, ranging from 70 to 100% ($P = 0.12$).

2.4.6. Association between tissue culture, macroscopic lesions and histology

The highest agreement was found between histopathology findings and tissue culture (80%) (Table 2-5), but kappa values were all <0.2 indicating slight agreement between the 3 diagnostic methods.

2.5. Discussion

In all age and dose groups at least one calf had a positive sample for tissue culture, macroscopic lesions or histology. Calves inoculated at a younger age had higher scores for histology and tissue culture, while calves inoculated at an older age were more severely affected macroscopically. Also, only a slight agreement was present between the three diagnostic methods used. Furthermore, a low proportion of tissue sites was culture-positive and multiple tissues were needed to identify a calf as infected.

Based on a previous study [12], the proportion of successfully infected calves was expected to be 75% in calves < 6 months of age, 50% of calves 6 to 12 months of age, and 20% of cattle > 12 months of age. Based on that study, it would only be possible to successfully infect older calves with a HD [12]. In the present study, in all age groups, inoculated from 2 weeks to 12 months, a high proportion of animals became infected, even with a LD. Similarly, when 1 to 2-year-old cattle grazed on pasture previously grazed by MAP-infected cattle, these yearlings also became MAP-infected [30]. Susceptibility of adult animals might have been missed in a previous trial, as adults were not included due to their expected resistance to MAP infection [31].

Calves inoculated at 6 months or younger had more culture-positive tissue locations and slightly more prominent histological lesions. The follow-up period of calves inoculated at 12 months of age was 5 months, whereas calves inoculated at 2 weeks were necropsied 16.5 months after

inoculation. The observed difference in severity could, therefore, also be due to differences among groups in follow-up. As expected, calves infected with a HD had higher histological and macroscopic lesion scores, as well as more culture-positive tissue locations compared to calves infected with a LD.

No age-dependent susceptibility to MAP infection was detected in a small ruminant study [32]. The resulting guidelines for small ruminant control programs pragmatically suggest to "keep in mind that while young animals are the most susceptible to infection, small ruminants can be infected as adults and may succumb to JD at any age". This is in agreement with findings in deer [33], and it can be concluded that older ruminants are still susceptible to MAP infection.

In the absence of an absolute age-related resistance, other mechanisms should be considered that explain the observed variability in individual host response to MAP infection. In cattle, genetic susceptibility markers have been identified [34-38]. Consequently, marker-assisted breeding might be implemented in JD prevention and control strategies [38]. Genetic susceptibility traits have also been found in deer; some breeds of red deer vary in resistance/susceptibility [39]. Based on these previous findings, we speculated that the genetic variation related to the regulation of the cellular immune response could be responsible for observed differences in host responses.

Remarkably, calves at all ages were successfully infected with either a HD or a LD of MAP. However, calves given a HD of MAP had more culture-positive tissue locations as well as slightly more prominent macroscopic and microscopic lesions. Furthermore, it was noteworthy that the two calves with the highest number of positive tissue locations and the highest scoring histological and macroscopical lesions were inoculated with a HD. This dose dependency, also

reported in sheep [40], emphasizes the importance of keeping the infection pressure low - to minimize transmission.

In this study, tissue culture, histology and gross lesions results did not always match on an individual animal level. However, there was a positive association between tissue culture and the relative severity of histological findings consistent with a previous study [41]. Also, deer as well as cattle with macroscopic lesions generally have a higher likelihood of culture-positive tissue, but MAP infection could still be detected in animals with macroscopically normal tissues [41-43], as was also the case in the present study. If tissue culture is combined with histopathology, detection of MAP infection is improved [41]. Furthermore, tissue culture was a far superior diagnostic tool to the use of ZN acid fast stain, as previously described [44]. However, in contrast to Brady *et al*. [21], MAP was not detected in all grossly affected tissues in this study, nor was MAP detected in all tissues with histological lesions. That MAP was absent in gross and histological lesions could have been due to a lesion not caused by MAP, or too few MAP were present in that site and hence not successfully cultured, or cure could have occurred.

Dissemination of MAP is usually not expected until the clinical stage [45]; however MAP was distributed in clinically normal animals [21]. Consequently, it was suggested that restricting tissue sampling to the ileum and ileocaecal LN would decrease detection of many infected animals [46] and routine culture should be extended from the gastroinstestinal tract to other LN and tissues. [47]. Furthermore, Buergelt [18] and Condron [48] both suggested that MAP was disseminated in subclinical infections [46] and we concluded that dissemination of MAP soon after infection (during the subclinical stage) might be much more widespread than commonly believed.

Although tissue culture is considered the gold standard and capable of detecting a MAP-infected animal before other diagnostic tests [1], only half (56%) of challenged calves were tissue culture-positive in this study. There are other reports of low detection rates of MAP in infected tissues [46], especially when the number of tissue samples is limited. This low detection rate of infected animals may lead to underestimation of the true prevalence of MAP infection as well as underestimation of test agreement when using tissue culture as a gold standard. Furthermore, restricting tissue samples to ileum and ileocaecal LN will also underestimate the number of infected animals not yet expressing clinical signs [46, 47].

Statistical analyses could have been more meaningful if a logistic regression with interaction between dose and age could have been performed for each diagnostic test used. However, even though this was a study of considerable size, the number of calves in each category was too low to make this analysis possible. Another asset that would have added to this study is quantification of MAP-bacteria in the tissues by means of the time-to-positive signals. However, this was not done because the interpretation of the pressure curves by the liquid culture system is optimized for fecal culture and failed to perform well on cultured tissues.

Two of 6 non-inoculated calves had positive tissue culture results and 4 control calves had focal histological lesions. Perhaps presence of one focal lesion was a non-specific inflammatory response not due to MAP challenge, and may correspond to small clusters of what are known as "garbage" macrophages [25], especially because these lesions were found in the paracortex of the LN only. In one control calf, MAP was detected from the 2 LN that contained focal lesions microscopically, whereas another calf with a focal lesion in the ileocaecal LN was shedding at 3 time points during the trial as well, suggesting a true infection. A false-positive PCR result is unlikely as the MAP-specific *F57* target was used [27]. Even though all calves in this study were

housed individually, and strict biosecurity measures were applied to avoid cross-contamination, perhaps MAP was transferred from the infected calves to the control calves. Recently, dust has been suggested as a means of transmission for MAP [49-52]. Although the hay fed to the calves may have been contaminated with MAP, this was considered unlikely, as the hay fed was harvested from fields not grazed by cattle for several years. *In utero* infection is a possibility [53], despite considerable efforts to use calves with the lowest probability of an intrauterine infection. Although every effort was done to prevent contamination from calf to calf, it is also possible that these calves acquired MAP infection during the experiment. Due to insufficient DNA present after *F57* PCR, it was unfortunately not possible to genotype the isolates from control calves and gain knowledge on the source of infection. If more stringent criteria were used to determine infection status using tissue culture and histology based on the results from the control animals, only calves infected at or < 6 months of age with a HD were successfully infected with MAP (Tables 2-1 and 2-3). However, the shorter follow-up period of the 9 and 12 month infection group might lead to an underestimation of infection in these animals.

Based on the results of the present study, we conclude that JD prevention and control programs should emphasize lowering MAP infection pressure, as it was proven that a lower infection dose resulted in less severe lesions. Furthermore, since cattle up to at least 1 year of age are susceptible to MAP infection, prevention of infection should include calves of all ages.

2.6. References

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Tissue culture category*	Control	Low dose					High dose				
		2 wk	3 mo	6 mo	9 mo	12 mo	2 wk	3 mo	6 mo	9 mo	12 mo
0	4	4	2	2	1	2	1	2	4	3	1
1	2	1	3	3	4	3	1	2		2	4
2							1	1	1		
3							2				

Table 2-1. Detection of MAP in calves by culture of tissues per age and dose group.

*0 = no tissue locations culture-positive; 1 = 1-3 tissue locations culture-positive; 2 = 4-6 tissue locations culture-positive; 3 = > 6 tissues culture-positive. mo = months; wk = weeks

Macroscopic lesions*	Control	Low dose					High dose				
		2 wk	3 mo	6 mo	9 mo	12 mo	2 wk	3 mo	6 mo	9 mo	12 mo
0	6	1	1	3	5	2		1	2	3	1
1		1	1	1					1		
2			1				1				
3		3	2	1		3	2	4	2	2	4
4							2				

Table 2-2. Macroscopic lesions after MAP challenge in calves (according to age and dose groups).

*0 = no macroscopic changes; 1 = one enlarged or edematous lymph node of the small intestine or liver; 2 = multiple enlarged and edematous mesenteric lymph nodes and/or hyperemia of the ileocaecal valve; 3 = enlarged mesenteric lymph node(s) and/or mild to moderate thickening of ileal or jejunal mucosa; 4 = enlarged mesenteric lymph node(s) and severe thickening and corrugation of the ileal, jejunal and colon mucosa.

mo = months; wk = weeks.

Histology*	Control	Low dose					High dose				
		2 wk	3 mo	6 mo	9 mo	12 mo	2 wk	3 mo	6 mo	9 mo	12 mo
0	2	1	1		3					2	1
1	4	3	4	5	2	5	2	3	5	3	4
2		1					1	2			
3							2				

Table 2-3. Number of calves per histology category in each age and dose group.

*0 = no lesions; 1 = focal lesions; 2 = multifocal lesions; 3 = diffuse lymphocytic, multibacillary or intermediate lesions.

mo = months; wk = weeks.

Positive*	Control	Low dose					High dose				
		2 wk	3 mo	6 mo	9 mo	12 mo	2 wk	3 mo	6 mo	9 mo	12 mo
0	4	1		1	1				2	2	
1	2	4	5	4	4	5	5	5	3	3	5

Table 2-4: Number of calves positive on either MAP tissue culture, histology or macroscopy in each age and dose group.

*Tissue culture or histology (score >2) or macroscopic score.

mo = months; wk = weeks

	Agreement	Kappa	95% CI of kappa
Macroscopy – tissue culture	66%	0.08	-0.06 - 0.14
Macroscopy – histology	70%	0.17	0.13 - 0.24
Histology – tissue culture	80%	0.19	-0.11 - 0.27

Table 2-5. Linearly weighted kappa coefficients between histology, macroscopic lesions and MAP tissue culture.

CI = confidence interval
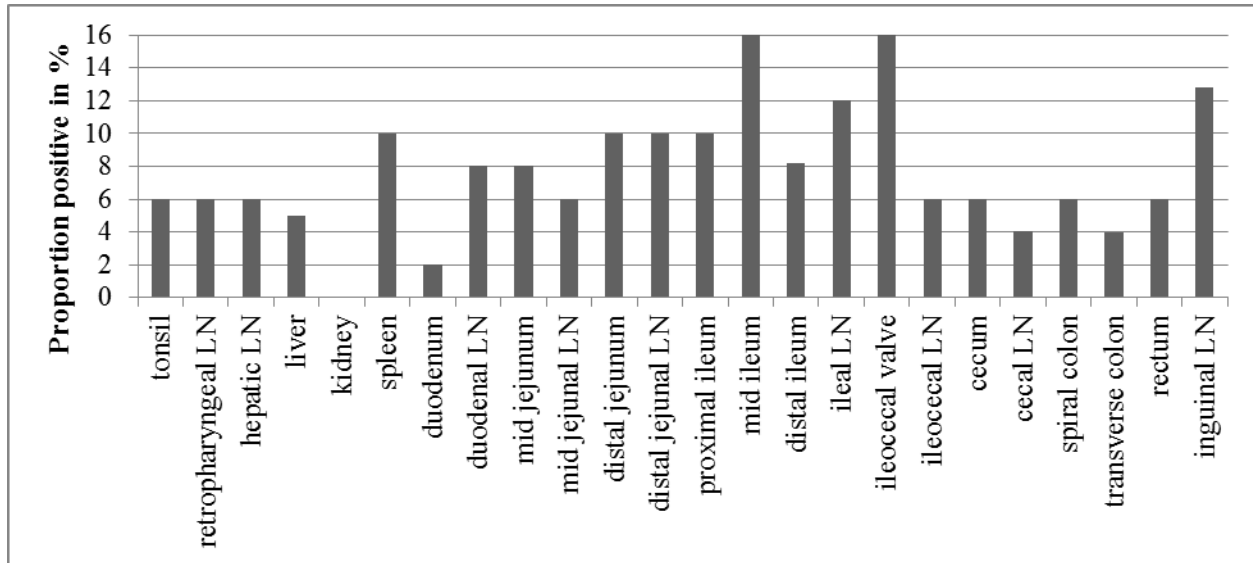
Figure 2-1. Detection of MAP infection in calves by culture of tissues per tissue location.

The y-axis displays the proportion that one particular tissue is positive over all calves and the x-

axis each tissue location sampled. LN = lymph node
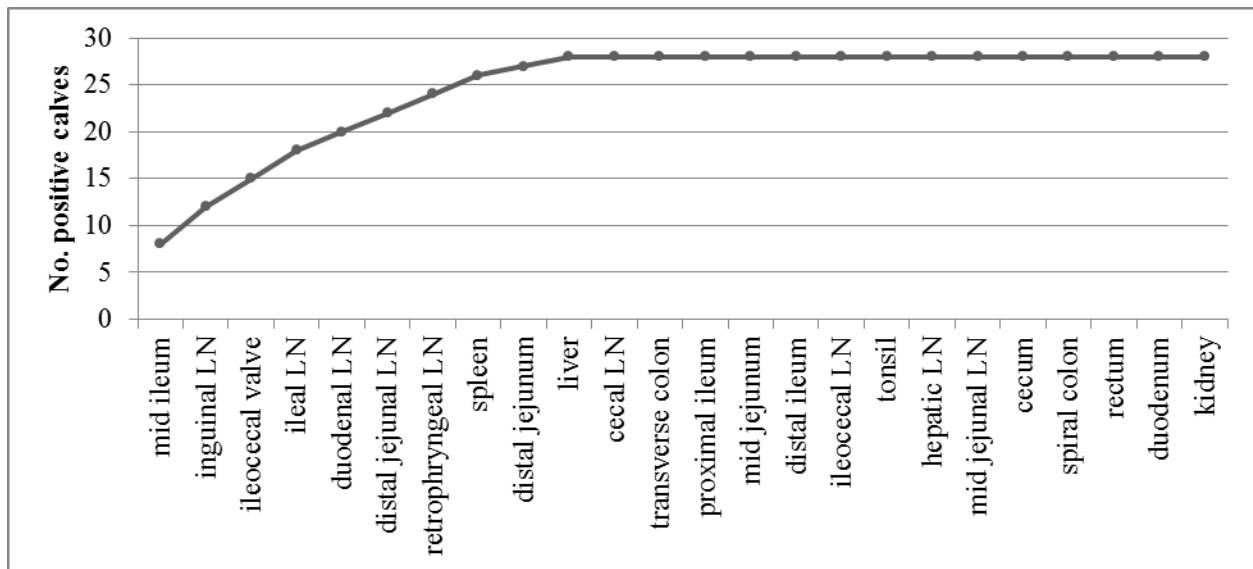
Figure 2-2. Number of calves detected as MAP culture-positive per additional tissue location sampled.

Tissue locations are ordered on the x-axis starting with the location that detected the most MAP-infected calves (y-axis) to the tissue location that yielded the least number of calves positive. LN = lymph node

CHAPTER 3: ANTIBODY RESPONSE EARLY AFTER EXPERIMENTAL INFECTION WITH *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN DAIRY CALVES

3.1. Abstract

Serological testing in the early stages of Johne's disease (JD) has been successful using specific antigens and in-house enzyme-linked immunosorbant assays (ELISA). However, the use of a commercial ELISA has not been evaluated shortly after *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection, nor has it been determined whether this serological response is age- or dose-dependent. Fifty-six calves were randomly allocated to challenge groups (5 per group) and a negative control group. Calves were inoculated orally on 2 consecutive days at 2 weeks or at 3, 6, 9 or 12 months. Within each age group, 5 calves received either a high or low dose of MAP. Using a commercial ELISA, antibody responses were detected in 42% of the inoculated calves and were present in all age and dose groups (except for the 6-month low-dose group). Antibody response profiles differed among individual calves; there were persistent as well as peak and bimodal peak responses. Calves inoculated at 12 months were ELISA-positive within 4.5 months after inoculation, whereas those inoculated at younger ages took longer to become ELISA-positive. Furthermore, calves inoculated with a high dose of MAP more often became ELISA-positive than low dose calves when inoculated at a younger age. In conclusion, a dose-dependent antibody response was detected by ELISA in a larger proportion of calves than expected soon after inoculation.

3.2. Introduction

Johne's disease (JD) is a chronic enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [1]. Consequences of infection are production losses such as reduced milk yield [2, 3], but also premature culling and reduced slaughter value [4]. The

incubation period for JD in cattle ranges from 2 to 10 years [5]; the most common clinical signs are chronic, non-treatable diarrhea and wasting [1].

Infection with MAP is initially controlled by a T helper 1 (Th1) immune response, which is a cellular immune response [6]. At approximately the onset of clinical symptoms, this response shifts to a T helper 2 (Th2) immune response which is characterized by production of antibodies [6]. Because of this long incubation period [7], dairy cows are most likely to become enzyme-linked immunosorbant assay (ELISA)-positive years after initial MAP exposure [8]. Although humoral immunity can be manifest as early as 10 to 17 months after infection, but testing before 2 years of age is typically not recommended [9]. However, recent studies are contradicting these statements. For example, immunoglobulin G (IgG) against purified MAP antigen was reported to detect subclinically infected cattle [10]. An ELISA using lipoarabinomannan as antigen detected antibodies starting at approximately 4 months after challenge; furthermore, using immunoblot analysis, antibodies were detected as early as 2 weeks after challenge [11]. Additionally, using specific antigens, an antibody response was detected as early as 70 days after MAP exposure [12].

Among tests for screening dairy herds, ELISA is the most widely used. Specificity and sensitivity ranged from 7 to 100% and 7 to 94%, respectively [13]. This wide range in reported test characteristics was attributed to using various ELISAs with specific antigen preparations and "gold standards," stage of disease, and age distribution of tested animals [13]. Additionally, ELISA has a higher sensitivity in frequently shedding cows compared to low shedders [14]. Therefore, an ELISA performs best in older animals in high-prevalence herds [15]. Calves shed MAP for a 6-month interval right after infection [16] in which they could also test ELISA-positive. However, no large scale, longitudinal experimental infection experiment has apparently

been done to determine the production of MAP-specific antibodies using a commercial ELISA under varying inoculation conditions. The objective of the current study was to use a commercially available ELISA to identify differences in humoral immune responses early after inoculation, in calves experimentally infected with 2 doses of MAP at 5 ages.

3.3. Materials and Methods

3.3.1. Herds and calves

Study design and sample collection have already been described (Chapter 2). Briefly, calves were collected from low-prevalence herds (< 5% seropositive) in Southern Alberta (Canada) and included in the study when born in the presence of a member of our research team. All dams were negative on a MAP ELISA (IDEXX Paratuberculosis Ab Test; IDEXX Laboratories Inc, Westbrook, ME, USA) and fecal culture. Furthermore, all calves in this study tested negative for Bovine Viral Diarrhea virus-antigen (samples processed by Feedlot Health Management Services, Okotoks, AB, Canada).

Upon arrival at the research facility, calves were fed 6 l of gamma-irradiated colostrum within 6 hours after birth. The colostrum used in this study was collected from ELISA-negative herds to ensure the absence of MAP-specific antibodies. This colostrum was then treated with gamma irradiation with a minimum dose of 10kGy per pail (containing 17 l of colostrum) using a Cobalt-60 source (McMaster Nuclear Reactor, Hamilton, ON, Canada) [17] to ensure it did not contain any live MAP bacteria. This was followed by milk replacer (GROBER nutrition milk replacer, Airdrie, AB, Canada) and calf starter grain (without antimicrobial additives) (Feedrite, Winnipeg, MB, Canada) and high-quality hay.

Calves were individually housed under stringent biosecurity conditions. The calves were monitored until 17 months of age (+ or – 2 weeks). Consequently, calves inoculated at 2 weeks or at 3, 6, 9, or 12 months were followed for 17, 14, 11, 8, or 5 months after inoculation, respectively. Health status was monitored on a daily basis by clinical inspection. At 17 months of age, euthanasia and necropsy examinations were performed, including assessment of gross and histological lesions, as well as tissue culture (Chapter 2). Animal care protocols M09083 and M09050 were reviewed and approved by the Health Sciences Animal Care Committee of the University of Calgary and procedures were conducted in compliance to these protocols.

3.3.2. Study design

Upon arrival at the research facility, 50 calves were randomly allocated to 5 groups that would be orally challenged at various ages (2 weeks or 3, 6, 9 and 12 months). An additional 6 calves housed in the same conditions were not inoculated and served as negative controls. Within each of the 5 age groups containing 10 calves, 5 calves were inoculated orally with a high dose (HD) of MAP and 5 calves were inoculated with a low dose (LD) of MAP.

The maximum capacity of the research facility was 33 calves housed individually. Consequently, the first 33 calves equally representing all age and dose groups, as well as 3 controls were included. The experiment was then repeated with 23 calves, including 3 control calves, also equally representing all age and dose groups.

3.3.3. Inoculum

Inoculum preparation has been described (Chapter 2). Briefly, a virulent cattle type MAP strain isolated from a clinical Alberta JD case (Cow 69) was used for inoculation. This isolate has an identical *Bam*HI, *Pvu*II and *Pst*I IS*900* – RFLP profile as the reference strain K10 [18]. Calves

were challenged orally on 2 consecutive days, with either a HD of 5 x 10^9 colony forming units (CFU) or 5 x 10^7 CFU (LD). The inoculum was prepared in one batch and cultured in 7H9 broth. It was quantified using the pelleted wet weight method as well as quantitative PCR, after which it was stored at -80 until used in the trial. Before each inoculation, one tube containing an identical aliquot of MAP cells was taken out of the -80°C freezer and resuspended in 350 ml of 7H9 broth. The culture was incubated for exactly 7 days at 37°C in a shaking incubator. In this period, the inoculum was tested for contamination. Immediately prior to inoculation, a 50 ml volume was prepared for the HD inoculation group and a 100-fold dilution was created for the LD inoculation group. The inoculum was placed in a syringe and expelled at the root of the tongue.

3.3.4. Sampling and ELISA

Serum samples were collected prior to inoculation. During the first month after inoculation, serum samples were collected weekly and from the second month to necropsy, serum samples were taken monthly.

Serum samples were analysed for MAP-specific antibodies using the IDEXX Paratuberculosis Ab Test (IDEXX Laboratories Inc, Westbrook, ME, USA) according to the manufacturer's instructions. However, 1 modification was made regarding interpretation of the test. Results of the test sample were expressed as a proportion to the positive control, corrected for the negative control (S/P ratio), thereby eliminating inter-plate variation. An S/P ratio of 60 was considered ELISA-positive. According to manufacturer's description, an S/P ratio of 60-70 should be considered as 'suspicious' when testing an animal in a herd a single time. However, because calves in this study were experimentally infected, the authors considered suspicious calves as positive.

Observed antibody profile types in this study were described as 'persistent response,' 'peak response,' and 'bimodal peak response' [19].

3.3.5. Statistical analyses

All statistical analyses were performed using STATA 11 (StataCorpLP, College Station, TX, USA). A *P*-value < 0.05 was considered significant. Tissue culture results, macroscopic and microscopic lesions were assigned to categories (Chapter 2). The ELISA results were categorized as follows: 0 = no ELISA-positive samples; 1 = ELISA-positive at least once after inoculation and before necropsy. Differences in distributions of tissue culture results, macroscopic and microscopic lesions between ELISA were evaluated using Chi-square and Fisher's exact tests.

The magnitude of the serological response was analyzed using the area under the curve (AUC) of the S/P ratio for the first 4.5 months after inoculation; this was done to ensure the same number of observations for each group (the last group was inoculated at 12 months of age and all calves were euthanized as 17 months of age). The AUC was compared across age and dose groups using the Kruskal-Wallis test [20] to identify differences in antibody responses between age and dose groups. To correct for multiple comparisons, a *P*-value of 0.003 was implemented using the following formula: $\alpha = \alpha/(2*(\kappa-1))$, where α is the significance and κ the number of observations [20]. To evaluate development of MAP antibodies over a longer interval (9 months), a separate analysis was conducted excluding calves inoculated at 9 and 12 months of age. The adjusted *P*-value for this analysis was 0.025.

3.4. Results

A humoral immune response was detected at least once during the entire follow-up period in 21 (42%) of the 50 MAP-inoculated calves (Table 3-1, Figure 3-1). All control calves remained ELISA-negative during the course of the trial. Calves inoculated at 2 weeks or at 3, 6, 9 or 12 months were followed for 17, 14, 11, 8 and 5 months after inoculation, respectively, except for 2 calves from the 2-week-HD group that developed clinical symptoms indicative of JD and were euthanized at 16 months of age.

There were ELISA-positive calves in all age and dose groups except the 6-month-LD group (Figure 3-1). The greatest number of calves with at least 1 positive sample was in the 2-week inoculation group (6 of 10 calves positive), with 4 of 5 (80%) calves of the HD group ELISA-positive compared to 2 of 5 (40%) from the LD group (Table 3-1). Twelve of 25 (48%) calves inoculated with a HD of MAP were ELISA-positive at least once, compared to 9 of 25 (36%) inoculated with a LD of MAP (positive at least once; Table 3-1; $P = 0.39$).

Starting at 4 months after inoculation, at any sampling, HD calves were more frequently seropositive compared to LD calves (apparent from Figures 3-1 & 3-2). This was predominantly caused by the calves inoculated at 2 weeks and 3 months of age (Figure 3-1).

The 'persistent response' was observed 5 times (for example 3 times in the 2 week HD group; Figure 3-1). The 'peak response' was observed 7 times (for example in the 6 month HD group; Figure 3-1), whereas a 'bimodal peak response' was observed once in the 3 month HD group (Figure 3-1). In 7 cases, it was unclear which response profile the calf would develop, due to completion of the trial (for example in the 2 week LD group; Figure 3-1).

Calves with at least 1 positive ELISA sample had no higher or lower chance than ELISA-negative calves of being positive grossly, histologically or on tissue culture ($P = 0.20$, 0.29 and

0.35, respectively). The 2 calves with clinical symptoms of JD both became ELISA-positive at 4 and 5 months after inoculation. These 2 calves also had several positive tissue locations and high histology and macroscopy scores ($P = 0.003$, 0.001 and 0.002, respectively; Table 3-2).

When comparing the groups for the first 4.5 months after inoculation, calves inoculated at 12 months with a HD or a LD had a larger AUC of the ELISA S/P ratio, indicating a stronger initial serological response, than the 2-week LD group ($P = 0.001$ and 0.0004, respectively). The other groups were not different when compared to the 2-week LD group (lowest *P*-value = 0.004; the adjusted *P*-value for significance for these multiple comparisons was 0.003). After exclusion of the 9- and 12-month inoculation groups and analyzing the AUC for a 9-month interval, the 2-week HD, the 3-month HD and the 6-month HD calves had a larger AUC than the 2-week LD group ($P = 0.001$; 0.004; 0.006, respectively; the adjusted *P*-value for significance for these multiple comparisons is 0.025).

3.5. Discussion

A serological response against MAP was detected in all age and dose groups, except for calves infected at 6 months of age with a LD. Calves inoculated with a HD had a larger AUC and thus a stronger antibody response, particularly when inoculated at a younger age (2 weeks or 3 or 6 months). Moreover, the humoral immune response started before 4.5 months after inoculation in calves inoculated at an older age (12 months).

An antibody response was detected in calves early after inoculation (Figure 3-1), consistent with other studies [10, 12]; however, it was noteworthy that the previous studies did not use a commercially available ELISA. To increase consistency and applicability, it is desirable to use an ELISA that is readily available and validated, and used in commercial labs that are available

to practitioners and producers. Although a serological response was detected in only 42% of the orally inoculated calves, it was noteworthy that more calves tested positive than expected. This can complement knowledge suggesting that ELISA is most useful in naturally exposed adults [8, 9, 13, 15]; using ELISA might also be valuable in cattle younger than 2 years.

The humoral immune response was clearly dose-dependent; in calves inoculated at 2 weeks, 3 or 6 months fewer LD than HD calves became ELISA-positive. Consequently, calves exposed to a LD of MAP on farm might not mount a detectable antibody response. These findings were consistent with previous reports that that earlier development of a humoral immune response is consistent with a higher infection dose of MAP [11, 21]. An inoculation dose was selected for this study that would mimic ingestion of MAP occurring naturally on a farm. Infection occurs in young calves with a dose contained in 2 g of infected feces [7]. However, the number of CFUs present in these 2 g remains to be determined. A dose of 1.5×10^6 CFU reliably produced infection in young calves [23], based on which a LD of 5 x 10^7 CFU was chosen. A HD that was a 100-fold higher, 5 x 10^9 CFU, was then selected based on the assumption that a larger dose would be needed to infect older animals; furthermore this dose was previously recommended [18] for experimental infection studies. In this study, 2 calves inoculated with a HD of MAP at 2 weeks of age had clinical symptoms of JD. The question remains whether these calves were inherently more susceptible to infection than the other 3 calves in the 2-week HD group. This early manifestation of clinical symptoms is atypical of JD [18] and could indicate the challenge dose was too high. On the other hand, 20% of heifers on high prevalence herds were shedding before 2 years of age [23], indicating that even young animals are shedding under high infection pressure and clinical disease in young animals might be underreported because differential

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diagnoses such as parasitic infections and other causes of diarrhea seem to be considered before JD.

In mouse models, it was observed that the antigen dose can direct the immune response towards a Th1 (very high and low antigen dose) or Th2 (intermediate-high antigen dose) response [24]. In a review on this topic, conflicting reports were presented on whether high- or low doses of antigen induce a Th1 or Th2 response [25]. In this review was also described how the antigen (bacteria versus parasites) can affect the generated immune response, as well as the availability of antigenic epitopes during T-cell priming [25]. It is noteworthy that in vitro experiments and murine systems using various pathogens have serious limitations for MAP infection trials in cattle and therefore additional research will be needed on this particular topic. Since MAP suppresses antigen presentation [26, 27], we inferred that a higher dose of MAP would cause more suppression. In contrast, in the present study, there was a greater antibody response in calves inoculated with a HD of MAP. Presumably, suppression was absent or ineffective, or perhaps overruled by other factors. Additionally, for unknown reasons, this difference in response between a HD and a LD of MAP disappeared when inoculated at 9 or 12 months of age. Also, in studies focussed on gene expression profiling, there were differences between infected and non-infected animals in Major Histocompatibility Complex gene modulation, playing a crucial role in antigen presentation to T-cells [27], which might provide insights into the dose-dependent humoral immune responses.

Calves inoculated at 2 weeks or 3 months of age had a lower AUC in the first 4.5 months after inoculation compared to the 12-month group. Therefore, calves inoculated at 12 months of age started producing antibodies sooner after inoculation compared to calves in the 2 week or 3 month groups. In the 12-month inoculation group, 1 calf even became seropositive at 2 weeks

after inoculation. This was in agreement with detection of an antibody response early after infection using specific antigens [19]. In an article discussing immune responses in young calves, it was stated that innate immunity is less efficient and a lower proportion of B-cells is present in the neonate compared to adult cattle [28]. This immature immune system in the neonate can explain the observed delayed humoral immune response in the current study in calves inoculated 2 weeks or 3 months of age. Additionally, presence of leukocytes in the colostrum ensures a faster cellular immune response [28, 29]. In this study, however, colostrum was gamma-irradiated and these maternal leukocytes were destroyed; therefore, the active humoral response could have been delayed for 2-3 weeks [29]. It was noteworthy that none of the calves in this study had failure of transfer of maternal immunity.

Calves with at least 1 positive ELISA sample did not have a higher or lower chance of being positive for gross lesions, histologically or on tissue culture. However, the 2 calves with clinical symptoms of JD that had severe necropsy lesions and positive tissue culture became ELISA-positive 4 and 5 months after inoculation and remained positive until necropsy. In previous reports, there was a low sensitivity of ELISA when using tissue culture as a gold standard (9-17%) [30]. Additionally, it was reported that ELISA performance varied with the various stages of JD. In clinically affected cattle, ELISA had a higher sensitivity [13] and thus a higher association can be expected between ELISA and necropsy lesions and tissue culture in cattle with MAP infection that has progressed to the clinical stage.

Antibody response profiles were identified to characterize antibody responses in experimentally infected calves [19]. The same 3 distinct types of antibody response profiles were observed in this study: transient "peak responses," "bimodal peak response," and "persistent responses" [19] throughout the different age and dose groups (Figure 3-1). Remarkably, there was great

individual variability within groups in responses observed in this study, consistent with previous reports [19, 31] as well as the transience in some of these responses. Not only the performance of the ELISA needs to be taken into account when screening a herd for MAP infection, but also differences in antibody response profile between individual animals.

MAP infection can be detected earlier than originally expected using a commercially available ELISA; therefore, screening young stock in combination with adult cattle might be useful in the context of JD control programs. In contrast, calves exposed to a LD of MAP, which could occur in low prevalence herds, might be missed using a herd screening ELISA. Additionally, transient responses and individual variations in antibody responses should be taken into account, because these might also lead to incorrect interpretation of an ELISA result.

3.6. References

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	2 weeks	3 months	6 months	9 months	12 months	Total
High dose	4 (80%)	3 (60%)	2 (40%)	2 (40%)	1 (20%)	13 (48%)
Low dose	2 (40%)	2 (40%)	0 (0%)	3 (60%)	2 (40%)	9 (36%)
Total	6 (60%)	5 (50%)	2 (20%)	5 (50%)	3 (30%)	

Table 3-1. Number and percentage of calves in each group with at least one seropositive result. Calves were assigned to 10 groups and inoculated with a high or low dose of *Mycobacterium avium* subspecies *paratuberculosis* and at 2 weeks or 3, 6, 9 or 12 months of age.

ELISA[4]	Tissue culture[1]				Histology[2]				Macroscopy[3]				
	0	1	2	3	0	1	2	3	0	1	2	3	4
0	13	14	2	0	5	22	2	0	12	2	2	13	0
1	9	9	1	2[5]	3	14	2	2[5]	7	2	0	10	2[5]

Table 3-2. Comparison of enzyme-linked immunosorbant assay (ELISA) and necropsy findings in 50 calves experimentally infected at 5 ages using 2 doses of *Mycobacterium avium* subspecies *paratuberculosis*.

[1]0 = no tissue locations culture-positive; 1 = 1-3 tissue locations culture-positive; 2 = 4-6 tissue locations culture-positive; 3 = > 6 tissues culture-positive.

[2]0 = no lesions; 1 = focal lesions; 2 = multifocal lesions; 3 = diffuse lymphocytic, multibacillary or intermediate lesions.

[3]0 = no macroscopic changes; 1 = one enlarged or edematous lymph node of the small intestine or liver; 2 = multiple enlarged and edematous mesenteric lymph nodes and/or hyperemia of the ileocaecal valve; 3 = enlarged mesenteric lymph node(s) and/or mild to moderate thickening of ileal or jejunal mucosa; 4 = enlarged mesenteric lymph node(s) and severe thickening and corrugation of the ileal, jejunal and colon mucosa.

[4]0 = no ELISA-positive samples; 1 = ELISA-positive at least once during the period after inoculation and before necropsy.

[5]these two calves had clinical signs of Johne's disease and were euthanized at 16 months of age.
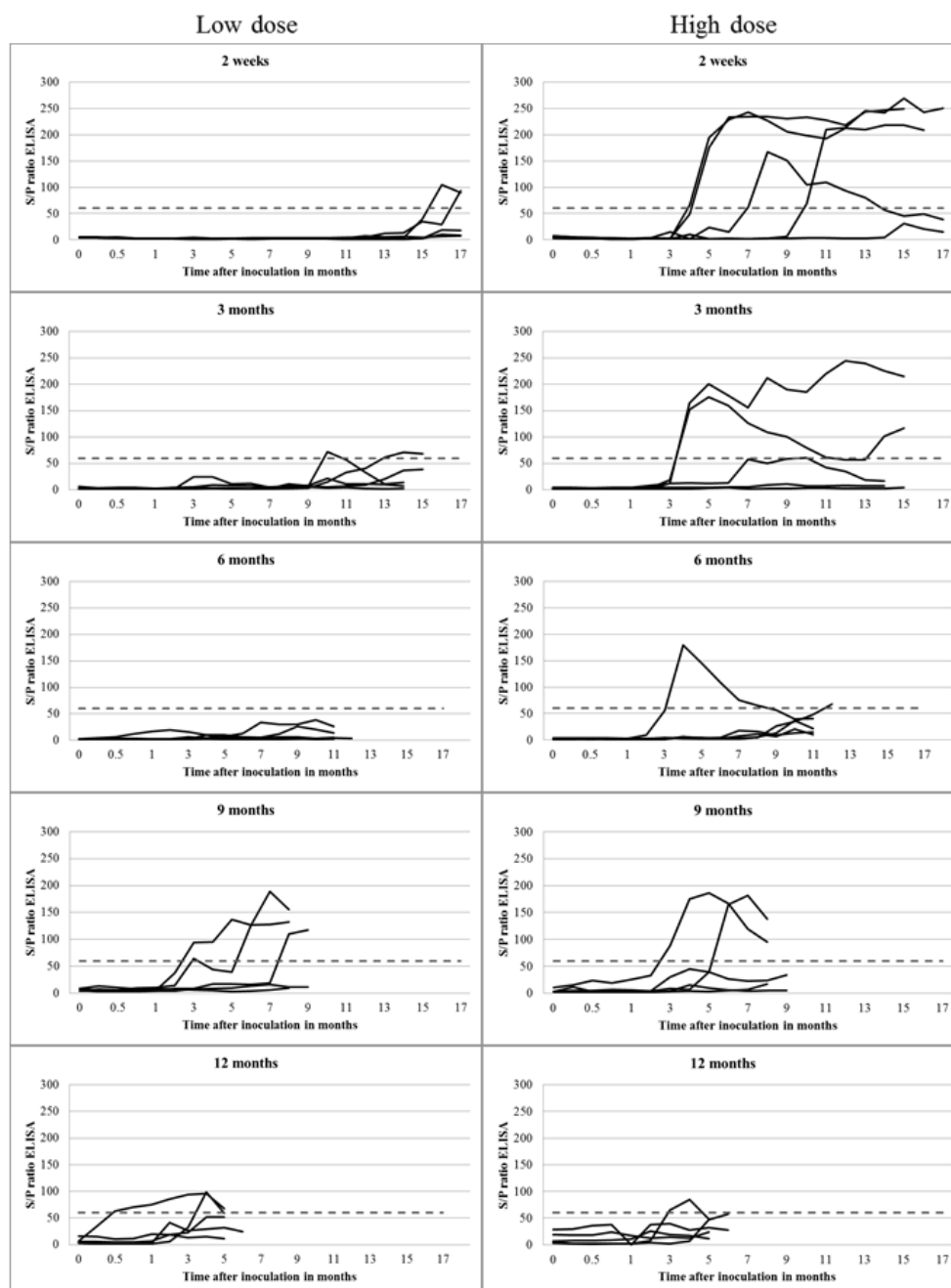
Figure 3-1. Course of antibody responses in 10 inoculation groups for each individual calf.

Calves were inoculated with a high or low dose of *Mycobacterium avium* subspecies

paratuberculosis and at 2 weeks or 3, 6, 9 or 12 months of age.

Samples with an ELISA cut-off > 60 (dashed line) are considered positive.
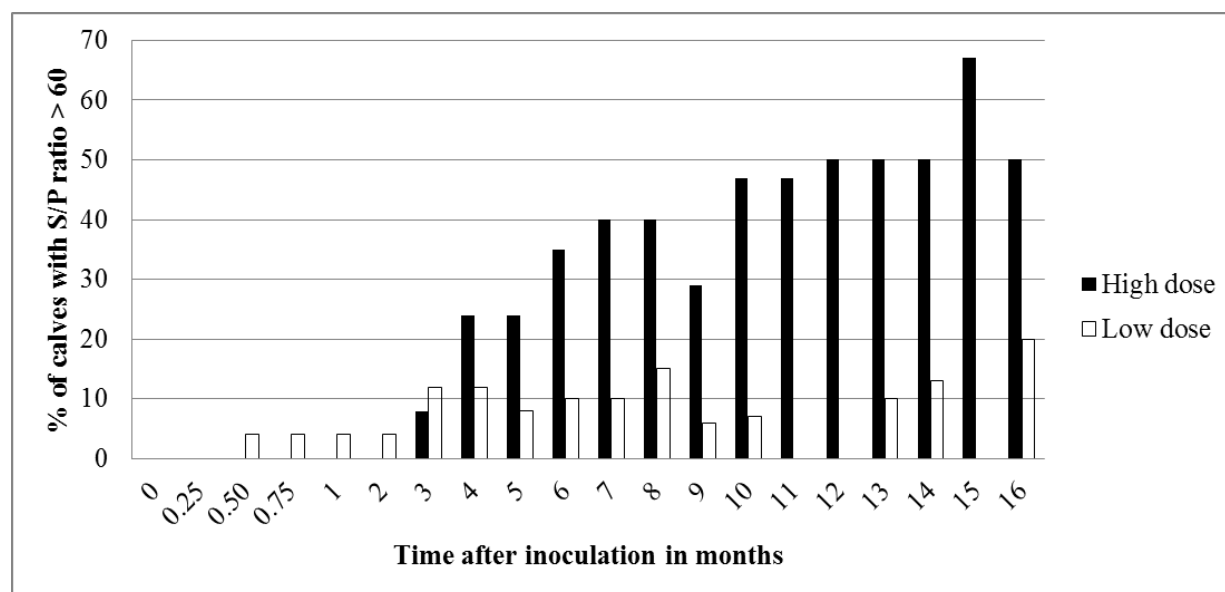
Figure 3-2. Percentage of calves with a positive antibody response in the high and low dose groups for every month after inoculation, including all 5 inoculation ages.

The proportion positive calves of a total number of calves at each time point (x-axis) is plotted for the HD as well and the LD group. Note: the number of calves used to calculate this proportion decreases as the time after inoculation increases (as calves inoculated at 2 weeks or at 3, 6, 9, or 12 months were followed for 17, 14, 11, 8 or 5 months after inoculation, respectively).

CHAPTER 4: SHEDDING PATTERNS OF DAIRY CALVES EXPERIMENTALLY

INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS*

4.1. Abstract

Although substantial fecal shedding is expected to start years after initial infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the potential for shedding by calves and therefore calf-to-calf transmission is underestimated in current Johne's disease (JD) control programs. Shedding patterns were determined in this study in experimentally infected calves.

Fifty calves were challenged at 2 weeks or at 3, 6, 9 or 12 months of age (6 calves served as a control group). In each age group, 5 calves were inoculated with a low and 5 with a high dose of MAP. Fecal culture was performed monthly until necropsy at 17 months of age.

Overall, 61% of inoculated calves, representing all age and dose groups, shed MAP in their feces at least once during the follow-up period. Although most calves shed sporadically, 4 calves in the 2-week and 3-month high dose groups shed at every sampling. In general, shedding peaked 2 months after inoculation. Calves inoculated at 2 weeks or 3 months with a high dose of MAP shed more frequently than those inoculated with a low dose. Calves shedding frequently had more culture-positive tissue locations and more severe gross and histological lesions at necropsy. In conclusion, calves inoculated up to 1 year of age shed MAP in their feces shortly after inoculation. Consequently, there is potential for MAP transfer between calves (especially if they are group housed) and therefore, JD control programs should consider young calves as a source of infection.

4.2. Introduction

Paratuberculosis is a chronic enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [1]. The disease is widespread in dairy herds worldwide and causes substantial economic losses [2, 3], due to reduced milk yield [4, 5], premature culling and

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reduced slaughter value [6, 7]. If not culled before clinical signs appear after a long incubation period (years) [8], cattle suffer from chronic, non-treatable diarrhea which leads to cachexia and ultimately culling or death [1]. The primary route of MAP transmission is fecal-oral, usually through ingestion of water, milk, or feed, contaminated by ruminants shedding MAP in their feces [1].

Poor manure management, a contaminated environment for calves, and contact with a shedding dam are the main sources of MAP infection on a farm [9-11]. Therefore, Johne's disease (JD) control programs involve 2 main objectives: reduce the number of infected animals that are shedding MAP, and prevent fecal-oral transmission by implementing best-hygiene practices for newborn calves [7]. Although research studies have described associations between management practices and the probability of cattle being infected with MAP [12-14], specific questions remain unanswered. In particular, the potential of calves shedding and contaminating the environment, as well as the risk of calf-to-calf transmission is largely overlooked in the current JD prevention and control programs. Furthermore, only 1 of 8 MAP modeling studies included calf-to-calf transmission [15, 16]. Even though most reports only claim transmission between adults and calves [17], recent reports suggest calves can be infected by other calves [18, 19]. These contradicting results can be explained by the delayed onset of clinical disease and the low sensitivity of diagnostic tests in the early stages after MAP infection [8]. Van Roermund et al. (2007) reported that calf-to-calf transmission occurred, and that contact with infectious calves increased the possibility of other calves being MAP-infected [19]. However, it is not known how often and when these calves are shedding in relation to initial infection. Consequently, there is a need for longitudinal studies determining how often infected calves shed MAP.

The objective of the current study was to determine shedding patterns in calves inoculated with 2 doses of MAP at 5 ages. Impact of age and dose at time of inoculation on shedding patterns, as well as on interval to first positive fecal culture, were assessed. Finally, the frequency of fecal shedding was related to the severity of tissue lesions in the same calves.

4.3. Materials and methods

4.3.1. Herds and calves

Study design and sample collection were described in detail in chapter 2. Briefly, male calves were collected from low MAP prevalence herds (<5% seropositive and <5% fecal culture-positive) in Southern Alberta (Canada) and included in the study when born in the presence of a member of our research team. All dams were MAP ELISA (IDEXX Paratuberculosis Ab Test; IDEXX Laboratories Inc, Westbrook, ME, USA) and fecal culture-negative.

Upon arrival at the research facility, calves were fed 6 l of gamma-irradiated colostrum within 6 hours after birth. The colostrum used in this study was collected from ELISA-negative herds. This was followed by milk replacer and calf starter grain (without antimicrobial additives) and high-quality hay. Calves were individually housed under stringent biosecurity conditions and no contact was possible between calves. Calves were monitored until 17 months of age ($+$ or $-$ 2 weeks). Consequently, calves inoculated at 2 weeks and 3, 6, 9, or 12 months were followed for 17, 14, 11, 8, and 5 months after inoculation, respectively. Health status was monitored on a daily basis by clinical inspection. At 17 months of age, euthanasia and necropsies were performed, including assessment for gross lesions, histology, and tissue culture (Chapter 2). Animal care protocols M09083 and M09050 were approved by the University of Calgary Health

Sciences Animal Care Committee and procedures were conducted in compliance to these protocols.

4.3.2. Study design and inoculum

Study design and preparation of inoculum were described in Chapter 2. Fifty calves were randomly allocated to 5 age groups (2 weeks, 3, 6, 9 and 12 months). Six calves housed in the same conditions were not inoculated (negative controls). In each of the 5 age groups, 5 calves were inoculated *per os* with a high dose (HD) of MAP and 5 calves were inoculated with a low dose (LD) of MAP. Because of logistics, this experiment was performed in 2 replicates. The first and second replicates included 33 and 23 calves, respectively, with all age and dose groups represented in both replicates.

A virulent cattle type MAP strain isolated from a clinical Alberta JD case (Cow 69) was used for inoculation. This isolate had an identical *Bam*HI, *Pvu*II and *Pst*I IS*900* − RFLP profile as the reference strain K10 recommended for use in infection trials [20]. Calves were challenged on 2 consecutive days, with either 5×10^9 CFU (HD) or 5×10^7 CFU (LD). The inoculum was prepared and cultured in 7H9 broth and quantified using the pelleted wet weight method. The quantification was confirmed using an in-house quantitative real-time PCR with a standard curve based on the 16*S* rRNA gene of *Mycobacterium smegmatis*, confirming the presence and the quantity of the 16*S* rRNA gene using primers p882 (5'-aggattagataccctggtag-3') and p1100 (5'-gctgacgacatccatgc-3').

4.3.3. Fecal sampling and culture

Fecal samples were collected from the rectum from each calf prior to inoculation. Samples collected 1-5 days after inoculation were pooled (maximum of 3 samples per pool), containing

samples from one calf collected over several days; this was an additional quality control measure to ensure viability of the inoculum, sensitivity of the fecal culture, and to confirm passive shedding of MAP [20, 21].

For the first 4 weeks after inoculation, rectal fecal samples were collected weekly; thereafter, fecal samples were collected monthly. To ensure age-matched control samples for each inoculation group, control calves were sampled twice per month.

All samples were processed using a modified TREK ESP II liquid culture system (TREK para-JEM®; TREK Diagnostic Systems, Cleveland, OH, USA) with subsequent IS*900* PCR on all samples. In more detail: From each fecal sample, 2 g was added to distilled water, mixed and allowed to settle for 30 minutes. Fecal samples were decontaminated according to manufacturer's instructions. First, 5 ml of the settled mixture was added to 25 ml of a 0.9% hexadecylpyridinium chloride (HPC) in half strength Brain Heart Infusion (BHI) solution and incubated overnight. Then, samples were centrifuged for 20 min at 3000 x g. The supernatant was discarded and the pellet was resuspended with a mixture of AS (ParaJem), water and full strength BHI. Tubes were incubated again for 24 hours at 37°C and added to the liquid culture medium (TREK para-JEM®; TREK Diagnostic Systems, Cleveland, OH, USA). After incubation for 48 days, MAP presence was confirmed by conventional PCR on culture medium targeting the IS*900* region. Extraction of DNA was done as described [22]. The IS*900* PCR procedure was modified from Vary et al. [23]; 5 µl of lysate was added to the described reaction mixture, containing 1.25 U Top Taq (Qiagen, Germantown, MD, USA), resulting in a reaction volume of 50 µL. Culture followed by PCR results were considered as a dichotomous outcome (MAP detected/not detected).

4.3.4. Statistical analyses

All statistical analyses were performed using STATA 11 (StataCorpLP, College Station, TX, USA), with a *P*-value < 0.10 considered significant.

To distinguish between frequent shedding calves and sporadic shedding calves, categories were assigned based on the observed data: 0 = non-shedding calves, 1 = calves shedding 1-4 times during the follow-up, and 2 = calves shedding > 4 times during the follow-up period. Gross lesions, microscopic lesions and tissue culture results at necropsy were assigned to categories as described (Chapter 2).

Differences in shedding between HD and LD calves, between age at inoculation, and among months after inoculation over time, as well as distributions of macroscopic and microscopic lesions and tissue culture results, between fecal shedding groups, were evaluated using Chi-square and Fisher's Exact tests. Interval from inoculation to the first positive fecal culture was plotted using a Kaplan-Meier graph; groups were compared using the logrank test of equality [24].

4.4. Results

4.4.1. Sporadic and frequent shedding

All pre-inoculation fecal samples were MAP culture-negative. Passive shedding was detected 1-5 days after inoculation. No calf was fecal culture-positive at 7 days after inoculation and as of 2 weeks after inoculation, shedding was considered active. All calves tested negative at 2 weeks after inoculation, except for Calf 4 inoculated at 2 weeks of age with a HD, which started shedding 2 weeks after inoculation (Figure 4-1). Two calves (4 and 5; Figure 4-1) from the 2-week-HD group developed clinical JD; these calves consistently remained fecal culture-positive

until they were euthanized (due to animal welfare concerns) at 16 months after inoculation (Figure 4-1).

In the control group that was followed until 17 months of age, 1 of 6 calves had 3 positive fecal cultures at 3.5, 4 and 7 months of age.

Shedding was detected at least once during the entire follow-up period in 32 (64%) of the 50 MAP-inoculated calves (Table 4-1, Figure 4-1) and occurred mostly sporadically in the shedding calves, with the exception of 4 calves that shed at most samplings Calves 3, 4, 5 and 11; Figure 4-1).

4.4.2. Impact of months after inoculation, dose, and age

Shedding peaked between inoculation and 6 months after inoculation, with the highest proportion (40%, 20 calves of 50) of calves shedding at 2 months after inoculation (P = 0.006; Figure 4-2).

Calves inoculated with a HD more frequently shed more than 4 times compared to calves inoculated with a LD (Figure 4-2) when inoculated at 2 weeks or 3 months of age (*P* = 0.04; Figure 4-2). Furthermore, all calves that shed more than 4 times were inoculated with a HD of MAP and at 2 weeks or 3 months of age. In groups inoculated at 6, 9, or 12 months of age, the proportion of calves that shed at least once was equal to non-shedding calves in those inoculated with either a HD or a LD of MAP (*P* = 1.00; Figure 4-1). Furthermore, in none of these groups did calves shed more than 4 times.

4.4.3. Association between frequency of shedding and necropsy observations

Overall, the distribution of tissue culture categories and gross lesion scores was not different between shedding and non-shedding calves (*P* = 0.90 and 0.19, respectively; Table 4-2).

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However, shedding calves had a higher histology score than non-shedding calves ($P = 0.08$). Conversely, when shedding frequency was taken into account, calves having > 4 fecal culture-positive samplings between inoculation and necropsy had more culture-positive tissue locations and more severe gross and histological lesions compared to less frequent shedders ($P = 0.03$, 0.013 and <0.001, respectively).

4.4.4. Interval inoculation to shedding

Interval to first positive fecal culture was not different between the HD and LD calves ($P = 0.25$); however, the interval between inoculation and first positive fecal culture increased with increasing age ($P = 0.07$; Figure 4-3).

4.5. Discussion

Calves in all 5 age groups and both dose groups shed MAP in their feces, and sporadic, intermittent as well as continuous shedding was detected. Calves inoculated at a young age with a HD shed MAP more frequently than LD calves. However, in older age groups (6, 9 and 12 months), this dose-dependent effect was no longer present. Although shedding usually started within 6 months after inoculation (and peaked at 2 months), calves inoculated at an older age usually started shedding later after inoculation. Finally, frequently shedding calves had more severe gross and histological lesions and more MAP culture-positive tissue locations.

Even though older calves are still susceptible to MAP infection (Chapter 2), calves inoculated up to 3 months old in particular shed MAP more frequently, especially when inoculated with a HD of MAP. This was consistent with previous reports that when calves are exposed at a young age in particular to a HD of MAP, shedding and clinical signs of JD will develop sooner after infection [19, 25, 26]. However, it is unclear why this dose difference disappeared when calves

were inoculated at 6, 9 or 12 months of age. Although infection pressure under field conditions is unknown, infection pressure on a farm regularly having clinical JD cases is likely higher than a farm with only subclinical cases. Moreover, heifers on high-prevalence farms shed more often compared to heifers on low-prevalence farms [25]. Two doses of inoculum were used, a HD of 5 $\times 10^9$ CFU given on 2 consecutive days (5 times the recommended standard bovine challenge dose [20]) and a LD of 5×10^7 CFU also given on 2 consecutive days (10 times higher than the lowest confirmed and consistent infectious dose for young calves [27]). Even though a controlled infection trial cannot determine the bacterial burden on farm, we inferred that these two doses represented realistic infection pressures under field conditions. Calves inoculated at 2 weeks or 3 months with a HD of MAP shed more frequently, had more severe gross and histology lesions and more culture-positive tissue locations. Therefore, prevention of infection of young calves, in combination with lowering the infection pressure, remains an essential component of JD control programs.

Based on the general assumption that only younger calves are susceptible to MAP infection [26], older calves are rarely included in MAP challenge experiments [18]. However, in this experiment, it was noteworthy that calves inoculated at 6, 9, or 12 months became infected (Chapter 2), and 40% shed early (at 1 and 2 months after inoculation; Figure 4-1) after inoculation. Although this apparent lack of dose dependency in the 6-, 9- and 12-month inoculation groups was not expected *a priori*, susceptibility of calves up to at least 1 year of age, even with a low dose of MAP, should be considered in control programs.

Shedding was most frequent in the first 6 months after inoculation and peaked 2 months after inoculation. A meta-analysis of MAP challenge experiments concluded that the median time to first shedding was 3 months, whereas most shedding was detected within 6 months after

inoculation [18]. Other studies documenting early shedding that were not included in that meta-analysis confirmed its findings: shedding peaked at 2-4 months after oral inoculation in 4 calves receiving an oral dose of 10^{11}-10^{12} CFU of MAP [28] and in 20 calves inoculated orally with 10^8 CFU of MAP [29]. The peak in shedding probably depends on the dose of inoculation; in calves inoculated with a HD of MAP, the peak occurred sooner after inoculation [19]. Additionally, in a clinical trial involving 56 calves, this shedding peak was not reduced after Hsp70 vaccination, even though the candidate vaccine reduced shedding after this initial peak [30]. A peak in shedding shortly after inoculation also occurred in 4 white-tailed deer inoculated with an oral dose of 10^{10} CFU of MAP [31] and in a vaccination trial including 16 goats orally inoculated with 10^9 CFU of MAP [32]. Early shedding was also observed in 38 orally inoculated sheep (dose 10^7-10^8 CFU) [33]. Also, in a mouse model, a peak in fecal shedding occurred 4 months after inoculation, whereas only a low bacterial burden was detected in intestinal tissues [34]. At 5 months after inoculation, the number of bacteria in the tissues was still increasing progressively [34]. An early peak in shedding was in this case not consistent with the general assumption that high shedding is associated with a higher bacterial burden in the tissues. Clearly, there is an urgent need to elucidate mechanisms behind translocation of MAP to the intestinal lumen and subsequent shedding in feces. Unfortunately, the underlying cause of fecal shedding is currently unknown [35]. This peak did not coincide with age-related and developmental changes of the calves, for example weaning, as this peak occurred in all 5 inoculation age groups. Therefore, this peak was more likely due to temporal changes in host cells containing MAP. Previously, it was suggested that infected macrophages will emigrate from the mucosa into the intestinal lumen and consequently passed in the feces, consistent with intermittent shedding [36, 37]. Others have theorized that shedding of MAP comes from burst macrophages in the mucosa producing

extracellular bacteria, and if these bacteria remain extracellular and translocate to the lumen, this would explain increased shedding of MAP in feces [38]. However, none of these hypotheses explain a peak in shedding within the first 6 months, rather than a progressively increasing level of shedding throughout MAP infection. Understanding this shedding mechanism will likely explain this early peak in shedding.

Frequently shedding calves had more severe gross and histological lesions, and more culture-positive tissue locations. Dissemination of MAP in multiple organs was observed in high shedders [39, 40], corresponding with animals in a clinical stage of infection. Lesions at necropsy were more severe in calves that shed frequently. Even though only 2 of the 5 frequently shedding calves in this study had clinical signs, typically coinciding with consistent shedding and severe necropsy lesions [8], we expected that frequently shedding calves without clinical signs also would have more positive tissue locations and more severe gross and histological lesions. Additionally, it was reported that MAP can disseminate before appearance of clinical signs (Chapter 2, [40]) and cause positive tissue cultures. This could account for frequently shedding calves without clinical signs having more positive tissue locations and more severe gross and histological lesions compared to sporadically shedding calves. Presumably, clinical signs would have subsequently appeared in these frequently shedding calves if the study had not been completed at 17 months of age.

One of 6 non-inoculated calves had 3 positive fecal cultures (at 3.5, 4 and 7 months after inoculation) and was also positive on histology, suggesting a true infection with MAP. Even though all calves in this study were housed individually and strict biosecurity measures were applied to avoid cross-contamination, MAP may have been transferred from shedding calves to this control calf. This control calf was not housed adjacent to one of the calves that were

shedding constantly, but calf-to-calf transmission (via objects) could have occurred. Recently, dust has also been suggested as a means of transmission for MAP [41-44]. It is unlikely that the hay fed to the calves was contaminated with MAP, because the hay was harvested from fields not grazed by cattle for several years. *In utero* infection is a possibility [45], despite considerable efforts to use calves with minimal probability of an intrauterine infection. The MAP isolates recovered from the infected calves were found to be the same strain used for the inoculation. Based on whole-genome sequencing of over 100 Canadian MAP isolates, the inoculum strain belongs to a lineage that represents approximately 6% of isolates in Canada differing in 200 single nucleotide polymorphisms (SNPs) from the nearest lineage (Ahlstrom et al., unpublished). In addition, PCR amplification of a genomic region, including one of these lineage-specific SNPs (Ahlstrom et al., unpublished) was done on positive fecal culture samples selected from shedding calves representing all age and dose groups in the experiment. All sequences recovered from the fecal isolates shared the specific inoculum lineage SNP; because only 6% of isolates found in Alberta belong to this lineage, it can be concluded with confidence that the isolates were derived from inoculum strain Cow69. Unfortunately, this method was unsuccessful in amplifying the genomic region around this SNP for the shedding control calf and gaining insight regarding the source of infection was not possible. Additionally, whole-genome sequencing of MAP isolates recovered from the ileum and ileocecal valve of Calf 5 were in full agreement with the inoculum Cow69 genome.

Calves in the carrier/subclinical stage of JD may have shed MAP at low levels [46] and since sensitivity of fecal culture is relatively low (23 - 74%, depending on gold standard and definition used) [47], some shedding might have been missed in this study. Although more frequent sampling (weekly, daily) might have increased the detection rate of MAP in feces, this would

have been very costly. Additionally, quantification of MAP was not done because the levels of shedding were expected to be low [46] in most calves; consequently, quantitative techniques (e.g. direct qPCR) would not have provided much additional information.

Even though passive shedding could be mistaken for active shedding due to infection, there was no reason to believe this was the case. In this experiment, passive shedding was detected in the days immediately following oral inoculation of MAP, although calves subsequently tested negative 1 week after inoculation, consistent with previous reports [20, 21]. Therefore, shedding 2 weeks after inoculation was considered active shedding, as suggested [20].

In all MAP challenge experiments of younger calves [18, 19, 30], a high proportion of calves shed the bacteria relatively soon after infection. In this study, shedding was detected over the entire period of 16 months. Due to increased adoption of acidified milk feeding and automatic milk feeders, many dairy calves are group-housed both before and after weaning, with potential for calf-to-calf transmission. Therefore, as a next step, an experiment should be done in which inoculated calves are kept in groups with non-infected calves to confirm whether MAP is transferred between calves. Also, the presence of shedding of young stock on dairy farms should be determined. If these 2 steps confirm calf-to-calf transmission, JD control programs should be adjusted to include prevention of calf-to-calf transmission of MAP.

When minimizing exposure of calves to manure, this will benefit reduction of MAP infection as well as reduction of other fecal-orally transmitted diseases [2, 7]. Preventive measures for young calves should be extended to calves up to 1 year of age (based on this trial). However, naturally exposed heifers became infected [48], arguably all age references should be removed from control programs, because older calves are still susceptible to MAP infection and shedding as a

consequence. Reducing infection pressure in general is important to keep the infection dose low and thus reduce the effects of JD.

To conclude, calves inoculated with MAP up to 1 year of age shed MAP shortly after inoculation, with a peak 2 months after inoculation. Some calves inoculated with a HD shed continuously. This could result in contamination of the environment of calves, and when group-housed lead to calf-to-calf transmission. Prevention programs may be more effective if calves up to 1 year of age are considered both susceptible to MAP infection and a potential source of infection for other calves.

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	2 weeks	3 months	6 months	9 months	12 months	Total
Low dose	4* (80%)	4 (80%)	4 (80%)	2 (40%)	1(20%)	15 (60%)
High dose	5 (100%)	4 (80%)	3 (60%)	1 (20%)	4 (80%)	17 (68%)
Total	9 (90%)	8 (80%)	7 (70%)	3 (30%)	5 (50%)	

Table 4-1. Number and percentage of shedding calves inoculated with a high or low dose of *Mycobacterium avium* subspecies *paratuberculosis* at 5 ages.

*5 calves total in each group; 50 calves in total

Fecal culture[1]	Tissue culture[2]				Histology[3]				Macroscopy[4]				
	0	1	2	3	0	1	2	3	0	1	2	3	4
0	8†	9	1	0	6	11	1	0	10	0	2	8	0
1-4	13	13	1	0	2	24	1	0	9	4	0	12	0
> 4	1	1	1	2*	0	1	2	2*	0	0	0	3	2*

Table 4-2. Fecal culture results compared to tissue culture, histology and macroscopy determined at necropsy from 50 calves inoculated with *Mycobacterium avium* subspecies *paratuberculosis*.

†Number of calves assigned to each category; 50 in total.

[1]Number of fecal culture-positive samplings starting 2 weeks after inoculation until necropsy.

[2]0 = no tissue locations culture-positive; 1 = 1-3 tissue locations culture-positive; 2 = 4-6 tissue locations culture-positive; 3 = > 6 tissues culture-positive.

[3]0 = no lesions; 1 = focal lesions; 2 = multifocal lesions; 3 = diffuse lymphocytic, multibacillary or intermediate lesions.

[4]0 = no macroscopic changes; 1 = one enlarged or edematous lymph node of the small intestine or liver; 2 = multiple enlarged and edematous mesenteric lymph nodes and/or hyperemia of the ileocaecal valve; 3 = enlarged mesenteric lymph node(s) and/or mild to moderate thickening of ileal or jejunal mucosa; 4 = enlarged mesenteric lymph node(s) and severe thickening and corrugation of the ileal, jejunal and colon mucosa.

*These 2 calves had clinical signs of JD and were euthanized at 16 months of age.

Challenge group	Dose	Calf ID	G	H	T	Time after inoculation in months																		
						0	0.5	0.75	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2 weeks	High	1																							
		2																							
		3																							
		4																							*
		5																							*
	Low	6																							
		7																							
		8																							
		9																							
		10																							
3 months	High	11																							
		12																							
		13																							
		14																							
	Low	15																							
		16																							
		17																							
		18																							
		19																							
		20																							
6 months	High	21																							
		22																							
		23																							
		24																							
	Low	25																							
		26																							
		27																							
		28																							
		29																							
		30																							
9 months	High	31																							
		32																							
		33																							
		34																							
	Low	35																							
		36																							
		37																							
		38																							
		39																							
		40																							
12 months	High	41																							
		42																							
		43																							
		44																							
	Low	45																							
		46																							
		47																							
		48																							
		49																							
		50																							

Figure 4-1: Fecal culture results for individual calves per age and dose group.

A solid filled box indicates a positive fecal culture, a white box a negative culture and a box with a cross a missing sample. G = gross lesions, H = histology, and T = tissue culture at necropsy, boxes with shading indicate a positive sample. * = this calf developed clinical signs of JD.
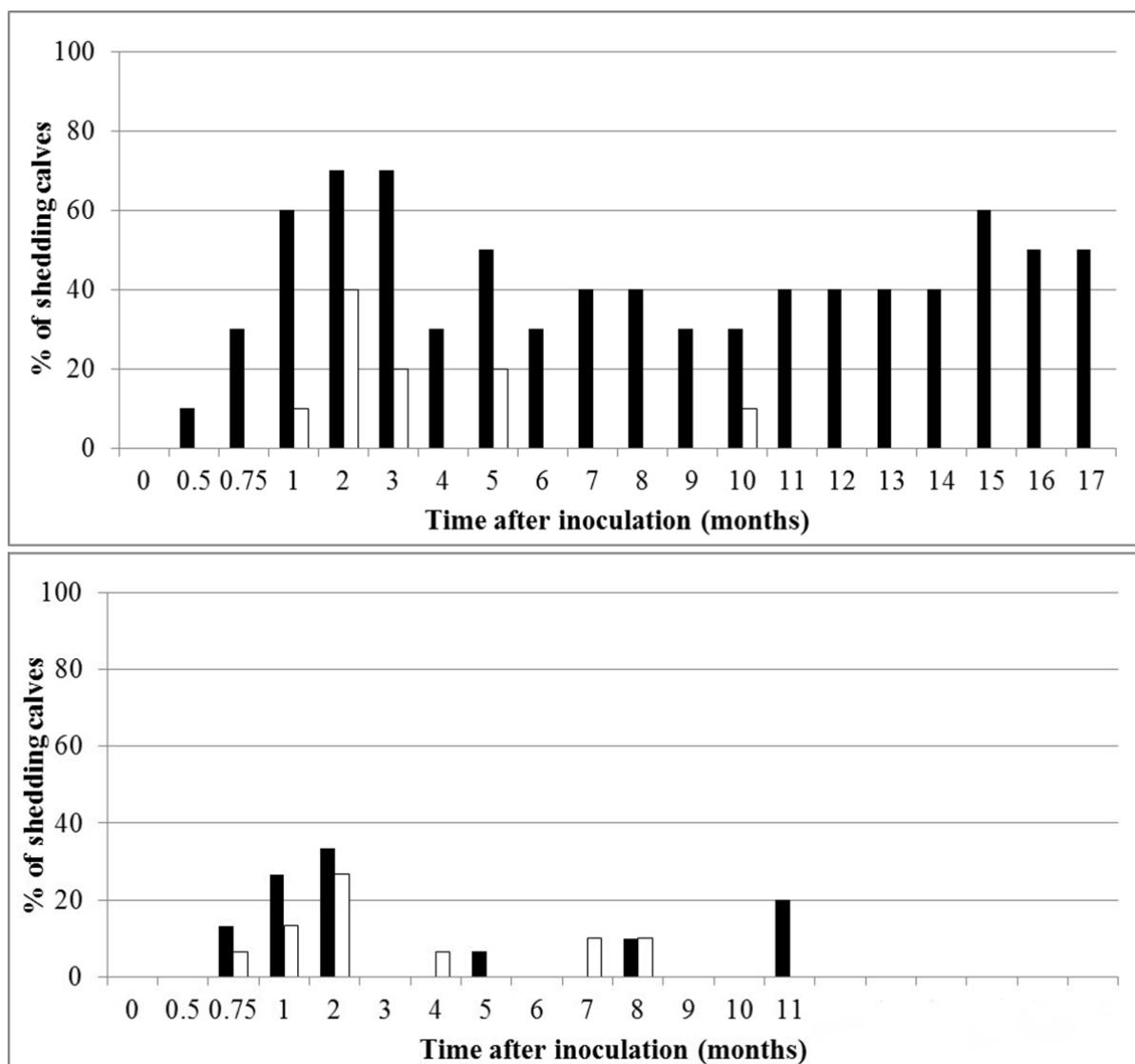
Figure 4-2: Percentage fecal culture-positive calves in the 2 dose groups for every month after inoculation with *Mycobacterium avium* subspecies *paratuberculosis*.

a) calves inoculated at 2 weeks or 3 months of age, and b) calves inoculated at 6, 9 or 12 months of age.

Solid dark bars represent calves inoculated with a HD of MAP and open white bars represent calves inoculated with a low dose of MAP.

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Note: the number of calves used to calculate this proportion decreases as the time after inoculation increases (calves inoculated at 2 weeks or 3, 6, 9 or 12 months were followed for 17, 14, 11, 8 and 5 months after inoculation, respectively).
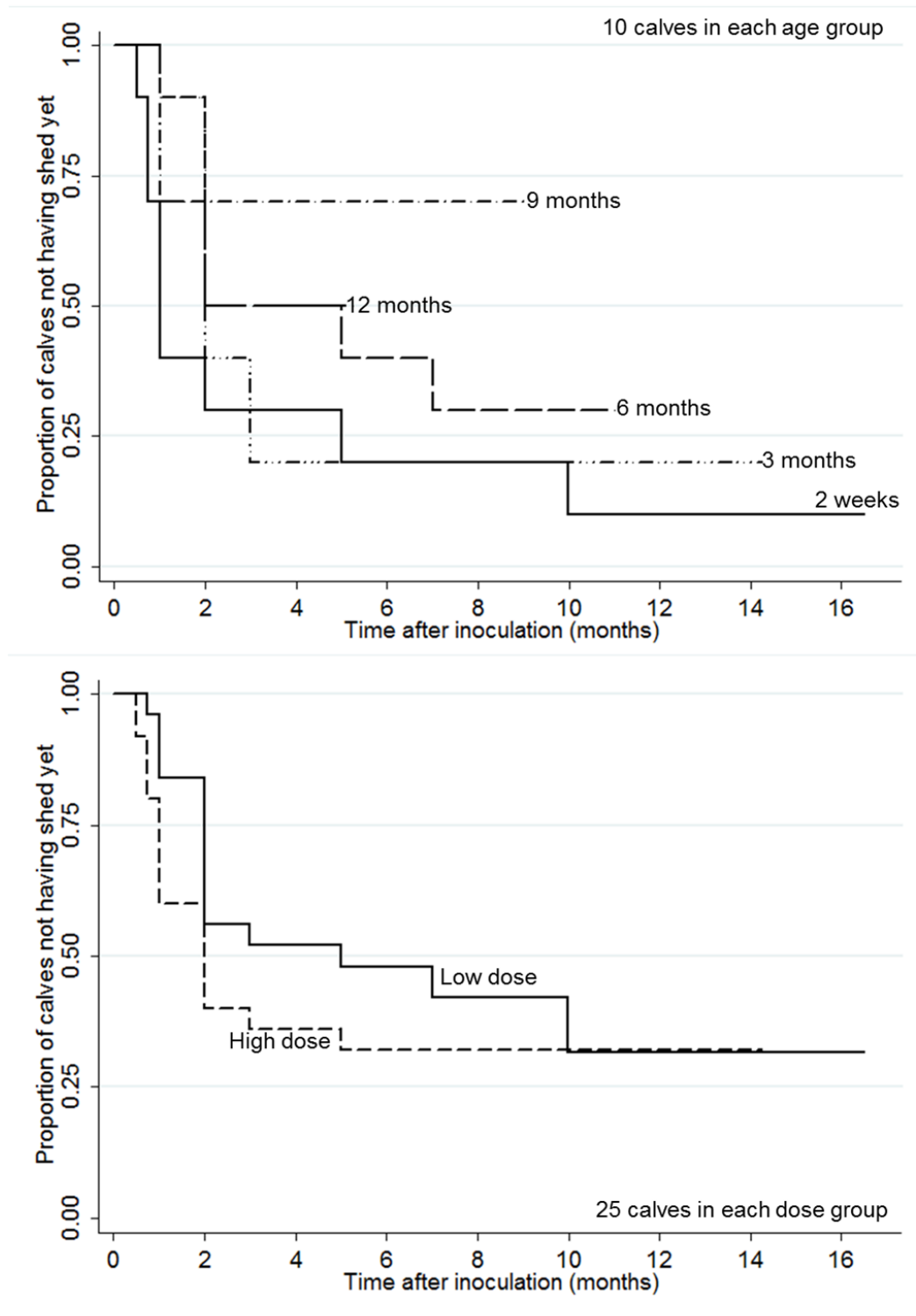
Figure 4-3: Kaplan-Meier curve for time to first positive fecal sample: a) per age group, and b) per dose group.

Proportion of calves not having shed yet are plotted for each month after inoculation; each curve represents all 10 calves each of the 5 age groups or all 25 calves in each dose group in a) and b), respectively.

CHAPTER 5. DOSE-DEPENDENT INTERFERON-GAMMA RELEASE IN DAIRY CALVES EXPERIMENTALLY INFECTED WITH *MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS*

5.1. Abstract

The interferon-gamma (IFN-γ) release assay is considered useful for diagnosis of subclinical paratuberculosis. However, interpretation can be subjective and complex; therefore, additional information regarding the course of the cellular immune response and effects of age and dose at infection would be helpful.

Thirty-three calves were randomly allocated to 10 challenge groups and a negative control group. Calves were inoculated orally at 2 weeks or at 3, 6, 9, or 12 months of age. Within each age group, calves received either a high or low dose of *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Monthly blood samples were collected, stimulated with johnin in vitro, and the subsequent release of IFN-γ measured.

Calves inoculated with a high dose had earlier and stronger IFN-γ responses than low-dose calves. Furthermore, calves inoculated at 2 weeks of age produced less IFN-γ compared to those inoculated later in life. The IFN-γ response peaked (on average) 4 months after exposure; therefore, this would be an optimal interval to test cattle for MAP infection (although the timing of field-based infections is unknown). Based on the IFN-γ response profile in all experimentally infected calves, the IFN-γ release assay could be a valuable diagnostic test early after infection.

5.2. Introduction

Johne's disease (JD) is a chronic enteritis of ruminants caused by MAP [1]. The disease is widespread in dairy herds worldwide and causes substantial economic losses [2, 3].

Diagnosis of JD is challenging, due to suboptimal test characteristics for fecal culture, serum and milk antibody ELISA particularly in the early stages of the disease [4]. Although these diagnostic tests are the most reliable during the clinical stage of JD [5, 6], detecting subclinical

paratuberculosis is more difficult. It is noteworthy that early after inoculation, a cellular immune response is present [7] which can be detected by an IFN-γ release assay, and was developed to diagnose tuberculosis [8, 9]. Despite suboptimal test characteristics (e.g. low sensitivity and specificity) [4, 10, 11] as well as false-positive reactions in infection trials [12] and in young animals [13], this test has the best potential to detect recent infections, because it targets the initial cellular immune response. Unfortunately, sensitivity and specificity were determined in suboptimal situations [4]. In that regard, the lack of knowledge regarding the best time to use an IFN-γ release assay has limited the diagnostic value of this test.

Very little is known about the effect of the inoculation dose on the IFN-γ response. In a proof of principle study, calves inoculated with a high dose (HD) of MAP had an earlier and stronger IFN-γ release response than calves inoculated with a low dose (LD) [14]. Therefore, infection dose and age at infection could affect IFN-γ response profiles. The objective of this study was to determine the course and onset of a cellular immune response using an IFN-γ release assay in calves experimentally infected at 5 ages with either a HD or LD of MAP.

5.3. Materials and Methods

5.3.1. Calves and inoculation

Study design and sample collection were as described (Chapter 2). IFN-γ response data were available for 30 of the 50 inoculated calves. Briefly, male calves were purchased from herds with a low MAP prevalence in Southern Alberta (Canada). All dams were negative on both a MAP ELISA (IDEXX Paratuberculosis Ab Test; IDEXX Laboratories Inc, Westbrook, ME, USA) and fecal culture (Chapter 2).

Calves were fed 6 l of gamma-irradiated colostrum collected from seronegative herds (to ensure absence of live bacteria and MAP antibodies) within 6 hours after birth. Colostrum was followed by milk replacer and calf starter grain (without antimicrobial additives) and high-quality hay. Calves were individually housed under stringent biosecurity conditions.

Thirty calves were randomly allocated to 5 age groups (2 weeks and 3, 6, 9, and 12 months) and 3 served as negative controls. At these ages, calves were orally inoculated on 2 consecutive days with either a HD (5×10^9 CFU) or a LD (5×10^7 CFU) of a MAP isolate originating from an Alberta clinical JD case (Cow 69; Chapter 2). The inoculum was cultured in 7H9 broth and quantified using the pelleted wet weight method as well as quantitative PCR [15]. The inoculum was placed in a syringe and expelled at the root of the tongue.

Calves were monitored for 17 months (+ or − 2 weeks), after which euthanasia and necropsy examinations were performed, including detailed assessments for gross and histological lesions, in addition to tissue culture (Chapter 2).

5.3.2. Blood sampling and IFN-γ release assay

Whole-blood samples were collected in heparinized tubes (BD Vacutainer, BD - Canada Mississauga, ON, Canada) prior to MAP inoculation and monthly thereafter; monthly samples were collected from the control calves. Samples were transported in an insulated container and the IFN-γ release assay was started within 6 hours after collection.

Whole blood (1.5 ml) was treated with 100 µl johnin [16] (0.3 mg/ml; Canadian Food Inspection Agency, Ottawa, ON, Canada), 100 µl of pokeweed mitogen (positive stimulation control; 0.3 mg/ml; Sigma-Aldrich Canada Co., Oakville, ON, Canada), or 100 µl PBS (negative stimulation control). All plates were incubated for 16 hours (overnight) at 37°C in a 5% CO_2 atmosphere and 500 µl of plasma was harvested and stored at −20°C until assayed. Plasma IFN-γ was determined

using the sandwich ELISA BOVIGAM® (Prionics, La Vista, NE, USA). The interpretation of the IFN-γ release assay was based on a previous algorithm [9]. If the optical density of the negative assay control was > 0.25, the difference between the positive and negative assay control was < 0.45, or the difference between the negative stimulation and negative assay control was > 0.2, observations were excluded for further data analysis because the assay was considered invalid. To correct for interplate variation, for the remaining observations %IFN-γ was calculated as follows: %IFN-γ = [(johnin-negative assay control)/(positive-negative assay control)] x 100 [9].

5.3.3. Statistical analysis

Statistical analyses of the antigen-specific immune responses were done using SAS (version 9.3, Cary, NC, USA). To normalize distribution, the natural logarithm of %IFN-γ (ln(%IFN-γ)) was used in all analyses and transferred to the geometric mean for presentation purposes. Differences in distributions of ln(%IFN-γ) at each month after inoculation between ages at inoculation or dose groups were evaluated with a repeated measures linear mixed effects (random intercept and trend) regression model, using backwards selection to determine the final model. The final model included time after inoculation, (time after inoculation)^2, (time after inoculation)^3, dose, and age at inoculation.

Differences in time-to-peak response among age and dose groups were evaluated using a proportional hazards model. Results were dichotomized using a cut-off described previously by calculating the average of presumed negatives (control calves) + 1.96 times the standard deviation [13].

5.4. Results and discussion

An IFN-γ response was confirmed in every calf inoculated with MAP and the course of IFN-γ production was similar for all calves. In addition, one of the control calves had a rapid increase in %IFN-γ just before euthanasia, comparable to MAP-exposed calves, suggesting an unintentional MAP infection. This calf also had histological lesions supporting this suspicion of infection (Chapter 2); it was however ELISA-negative during the entire experiment (Chapter 3). Pre-infection samples from all calves had a low %IFN-γ, with a geometric mean < 0 (Figure 5-1). This value increased rapidly 2-4 months after inoculation, with a peak at 4 months and a geometric mean %IFN-γ of 220. The %IFN-γ decreased steadily until 13 months after inoculation, after which it slowly increased (Figure 5-2). A peak at 2 months after inoculation has been described by [17]. The steady decrease after the initial peak can be explained by a recently published report suggesting that cows with a MAP infection are less capable of maintaining a protective immune response [18]. Consequently, the IFN-γ response decreased as the disease progressed. However, it is unclear why this response subsequently increased (13 months after inoculation).

The IFN-γ response was dose-dependent: the HD group had higher %IFN-γ than the LD group and the profiles of both groups followed a similar trend over the 17-month trial period (Figures 5-1&2; $P < 0.0001$). The IFN-γ response of HD and LD calves peaked 3 and 4 months after inoculation, respectively (Figure 5-1; $P = 0.003$). A dose-dependent cellular immune response was also reported in an experimental infection trial with lambs and adult ewes; in that regard, HD sheep had a strong response compared to no response in the LD groups [19]. Furthermore, there were distinct immune response profiles between calves inoculated with a HD or a LD of MAP directly in ileocaecal Peyer's patches [14]. The dose-dependent response seems important,

because the initial cellular immune response has been described as essential in controlling the progression of JD [7]. In contrast, it has been suggested in sheep that the intense cellular immune response could be causing disease symptoms instead of protection, as some animals became sick with severe paucibacillary lesions. Therefore, clinical symptoms and gross lesions are likely due to damage caused by the cellular immune response instead of the number of MAP present [20]. In the current study, an initial cellular immune response was generated by all calves inoculated with either dose of MAP. Consequently, the actual intensity of the cellular immune response could have affected the progression of JD more than the dose administered, even though the dose caused a discrepancy in the generated cellular immune response.

Calves inoculated at 2 weeks of age had a lower IFN-γ response compared to calves inoculated at 3, 6, 9, or 12 months of age (Figure 5-2; $P = 0.008$). Also in another study, there was an age-dependent response in a sheep infection trial, in which ewes had an earlier response than lambs [19]. This was not unexpected, because innate defense mechanisms decreased in very young calves, including: less complement activity, less neutrophil and macrophage activity, less interferon production, decreased natural killer function, and less dendritic cells [21]. This corresponded to the weaker IFN-γ response in the calves inoculated at 2 weeks of age compared to calves that were older at inoculation. In calves inoculated at 2 weeks, %IFN-γ increased from 2 months after inoculation onwards, as observed in the other age groups (Figure 5-1). Because these calves overall had a lower IFN-γ production, but the peak was reached at the same time after inoculation as in the other age groups, the onset of the cellular immune response was likely associated with a defined stage of the infection and was unaffected by the (im-)maturity of the immune response.

Some individual test results were inconsistent and unpredictable, adding to the previously described concerns regarding the interpretation and ease of use of the IFN-γ release assay as a diagnostic test [6, 9]. Disadvantages were the highly specific conditions under which to collect the samples and the requirement to store samples temporarily and transport fresh samples to the lab in variable weather conditions. In this study, a total of 68 observations were excluded based on the criteria described earlier [9]. The criteria were not met because the PBMCs were presumably not viable and therefore not capable of producing IFN-γ in response to antigen stimulation.

The test also requires immediate set-up (ideally within 6 hours after sampling, as cells are negatively affected when this period is > 24-48 hours [22]), laboratory processing, and centrifugation after overnight incubation (thereafter, samples can be stored until the IFN-γ assay is conducted). Therefore, the current version of the test was time-consuming and less applicable for field situations and rapid herd screening. Notwithstanding, the test was useful in infection trials, as the cellular immune response to MAP invasion seemed to play a crucial role in controlling infection [23] and could elucidate immune response mechanisms. Interpretation of the test varied from algorithms [9], to using a cut-off [13] or whether the %IFN-γ after stimulation with avian PPD exceeded stimulation with bovine PPD [12], or a combination of all described methods. Moreover, when a cut-off was used, this cut-off differed among laboratories [13, 19, 24] and even was different according to the antigens (johnin, avian PPD) used in the study by Jungersen et al. (2002) [13]. Consequently, the cut-offs described in Kalis et al. (2003) and Jungersen et al. (2002) were not applied in this study, and it was necessary to determine an in-house cut-off value. Furthermore, in an ideal situation, cattle on-farm would be tested approximately 4 months after inoculation, when the difference between infected and non-

infected animals is expected to be maximal. Naturally, this is not possible under field conditions, as the moment of natural infection is unknown.

That each calf had an increase in %IFN-γ value, but only 20 (67%) had 1 or more culture-positive tissue locations, 25 (83%) calves had histological lesions, and 19 (63.3%) had gross lesions (Chapter 2), could indicate that a positive IFN-γ release assay only recognized exposure to MAP. On the other hand, these necropsy techniques might be not sufficiently sensitive, as a cellular immune response 2-4 months after inoculation until necropsy also indicates continued exposure to MAP indicative of multiplication of MAP in tissues. In another study, the presence of bacteria in the tissues was confirmed in 36% of IFN-γ release assay-positive animals [25]. Perhaps initial IFN-γ increases observed in this study merely indicated exposure to MAP, or the IFN-γ production already had decreased as reported by Vazquez et al. (2013).

Calves inoculated with a HD had a stronger and earlier IFN-γ response than the LD calves. Newborn calves produced less IFN-γ compared to calves inoculated at an older age. The IFN-γ response peaked, on average, 4 months after exposure, making this the optimal time to test cattle for MAP infection. Obviously, under field conditions, it is not known when cattle were infected. Based on the IFN-γ response profile that occurred in all MAP-exposed calves in this study, we inferred that the IFN-γ release assay could be a valuable diagnostic test shortly after MAP-exposure.

5.5. References

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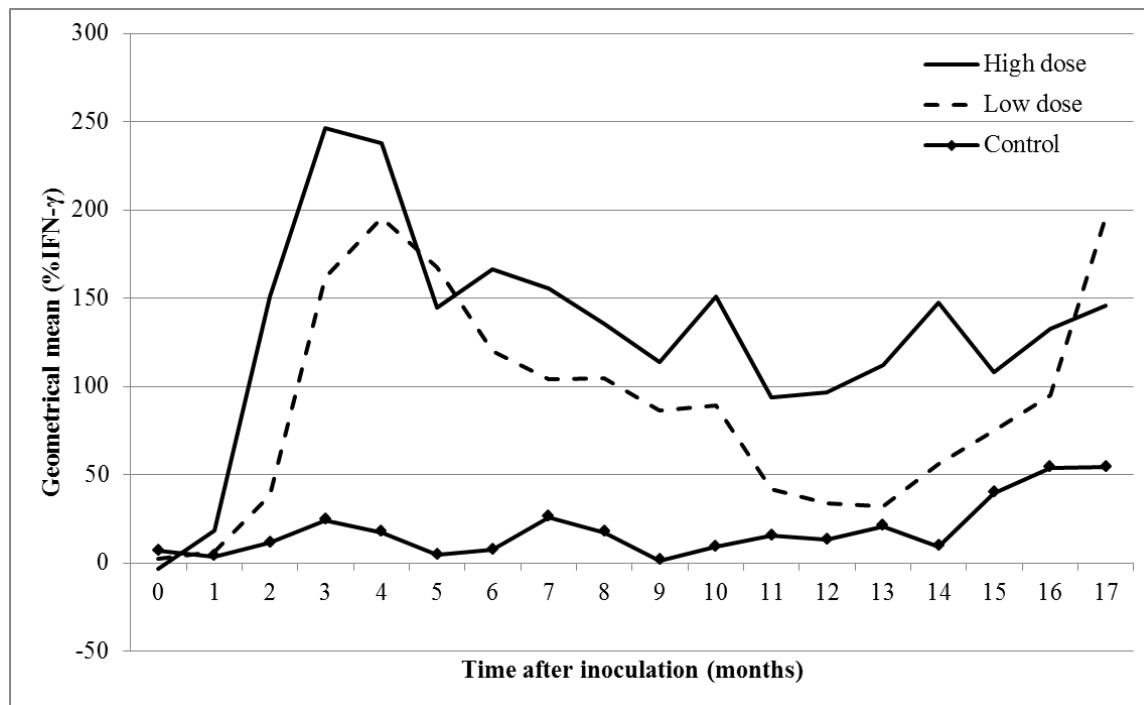
Figure 5-1. Geometric mean of %IFN-γ produced by peripheral blood mononuclear cells after stimulation with johnin in 30 male Holstein calves inoculated with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) at 5 different ages. Fifteen calves were inoculated with a HD of MAP and 15 with a low dose of MAP, whereas 3 calves served as a negative control group.
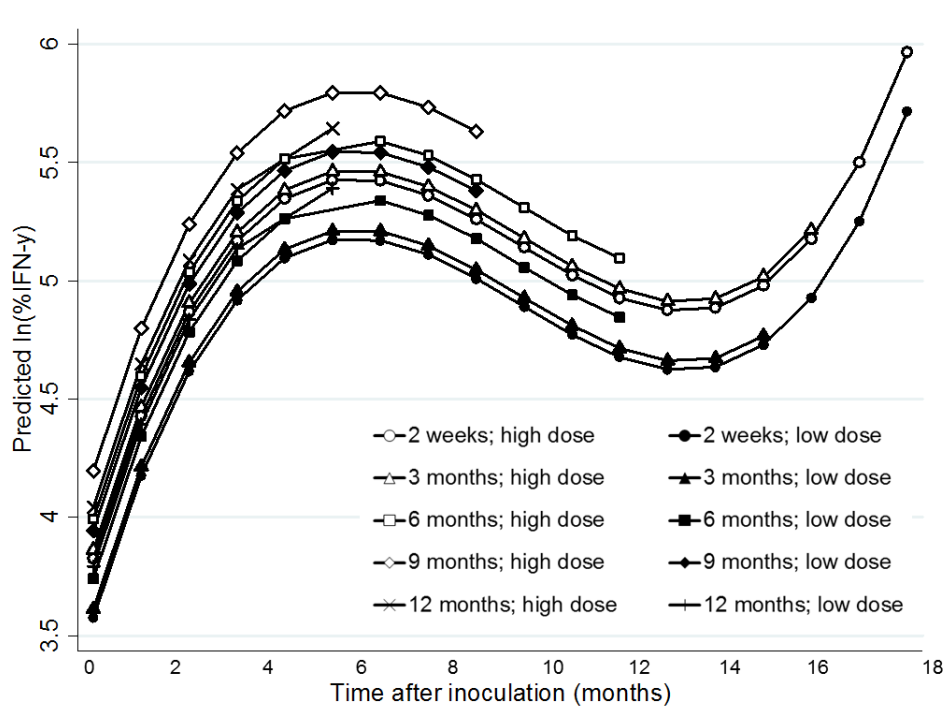
Figure 5-2. Predicted values of %IFN-γ using a repeated measures linear mixed effects (random intercept and trend) regression model: $E\big(\ln(J_{ij})\big|b_i\big) = (\beta_1 + b_{1i}) + (\beta_2 + b_{2i})(t_{ij} - 4.5) + \beta_3(t_{ij} - 4.5)^2 + \beta_4(t_{ij} - 4.5)^3 + \beta_5 dose_i + \beta_9 age_i$

CHAPTER 6. LONGITUDINAL EVALUATION OF DIAGNOSTICS IN YOUNG CALVES DURING SUBCLINICAL AND CLINICAL PARATUBERCULOSIS

6.1. Abstract

Commercial diagnostic tests for detecting *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection lack sensitivity, especially in the early stages of Johne's disease (JD). A case report is presented including 2 steers which were part of an experimental infection trial and developed clinical JD and provided a novel opportunity to identify the onset of positivity for routinely used diagnostic tests. Both calves were part of a group (n=5) inoculated orally at 2 weeks of age with a high dose of MAP. Whole blood, serum and feces were collected weekly during the first month after inoculation and thereafter monthly until necropsy at 16 or 17 months of age. Gross lesions and histology were assessed at necropsy and samples collected for tissue culture. Before clinical signs became apparent, these 2 calves were consistently MAP fecal culture-positive starting 2-3 weeks after inoculation, whereas antibody ELISA was positive as of 4-5 months after inoculation. From the other 3 calves in this group, 2 shed MAP intermittently, and 1 shed MAP persistently as of 11 months after inoculation; 1 was ELISA-negative, 1 had a transient response, and 1 was ELISA-positive as of 10 months after inoculation. In contrast, asymptomatic and clinical calves had increased IFN-γ production at 2-3 months after inoculation. At necropsy, all 3 asymptomatic calves were less severely affected compared to clinical calves, based on gross lesions, histology and tissue culture. In conclusion, these 2 steers exhibited clinical JD at a very young age. As of 15 months (shedding) and 1 year (ELISA) before the onset of clinical symptoms, these calves consistently tested positive, in contrast to those that were asymptomatic.

6.2. Introduction

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic enteritis mainly affecting ruminants and is endemic worldwide [1]. Cattle are usually

infected orally, through feces from a MAP-shedding animal, and when allowed to progress, JD has 4 stages, culminating in death of infected cattle [2, 3]. The "silent infection" stage is characterized by a lack of clinical signs and no diagnostic tests that reliably detect MAP infection [3]. In the (second) subclinical stage, cattle may start shedding MAP in their feces and have detectable anti-MAP antibodies. Also, milk production [4] and fertility may decline [3]. The clinical stage starts when symptoms such as weight loss, diarrhea are observed, reportedly from 2 up to 10 years after initial infection. Diagnostic tests will be positive in this stage and if affected animals are not culled this will quickly progress into the advanced clinical stage, in which the severity of the symptoms will further increase until the animal succumbs to dehydration and cachexia [3, 4].

Diagnosis of MAP infection is a challenge, especially in the early stages. Currently used diagnostics for MAP infection include serum ELISA, fecal culture and interferon-gamma (IFN-γ) release assay. In general, all these tests have relatively low sensitivity, although a wide range of values has been reported [5]. Post-mortem diagnostics include assessing macroscopic lesions, histopathology and bacterial culture of tissues. Typical macroscopic findings for MAP infection are chronic enteritis, chronic intestinal lymphangitis and mesenteric lymphadenopathy [6] and thickening of the ileal mucosa [3]. Histologically, the villi of the intestinal mucosa become shorter and thicker [4] and lesions in the intestinal mucosae contain macrophages filled with MAP bacteria as well as giant cells and granulomas [6, 7]. MAP can be visualized using the Ziehl-Neelsen (ZN) stain, which specifically stains acid-fast bacteria. Therefore, histology can indicate a mycobacterial infection, which is more specific than gross lesions only. MAP culture from intestinal tissue samples confirmed by PCR is considered to be the test with highest

sensitivity for detection of MAP infection [8] and is also considered the most specific test to identify MAP of the three post-mortem diagnostics.

During a large experimental infection trial, 2 unexpected cases of clinical JD occurred in steers that were only 16 months old. These 2 steers and 3 others which remained asymptomatic had received an identical oral inoculum at 2 weeks of age containing a relatively high dose of MAP. The aim of this case report was to compare the longitudinal diagnostic profile of the clinical calves to the diagnostic profile of the asymptomatic calves. Secondly, the onset of positivity for routinely used diagnostic tests was related to the 4 stages of JD.

6.3. Materials and methods

Five male Holstein-Friesian calves born from 1st or 2nd parity cows on 4 dairy farms located north of Calgary, Alberta, Canada, were collected as part of a larger study (Chapter 2). The 4 farms were identified as low MAP prevalent ($< 3\%$) by testing individual fecal, serum, and milk samples collected from cows > 36 months of age. Fecal samples were cultured by para-JEM automated MAP culturing (para-JEM®, TREK Diagnostic Systems, Cleveland, OH), whereas serum and milk samples were analyzed for MAP antibodies using the IDEXX Paratuberculosis Ab Test (IDEXX Laboratories Inc, Westbrook, ME). An S/P ratio > 60 was considered a positive result. Fecal and serum samples were collected from the dam within 2 weeks after calving; these samples were culture- and seronegative. All 5 calves were relocated at the research facility and fed 6 l of gamma-irradiated colostrum (Hamilton McMaster Nuclear Reactor, ON, Canada) (collected from MAP-seronegative herds to ensure absence of MAP-antibodies and gamma-irradiated to ensure no live bacteria were present), followed by milk replacer and calf starter grain without antimicrobial additives.

The 5 calves were inoculated orally at 2 weeks of age with a high dose (5×10^9 CFU on 2 consecutive days) of a virulent cattle-type MAP strain isolated from a clinical case in a dairy cow (Cow 69; Chapter 2). Six calves were included in the study as negative controls. All calves were dehorned under local anesthesia using a cauterizing iron and surgically castrated at 7 weeks of age (after sedation and local anesthesia).

Blood, serum and fecal samples were collected before inoculation and then weekly during the first month after inoculation; thereafter, these samples were collected once monthly until necropsy. The same samples were collected twice per month from the control calves. Serum and fecal samples were processed as described above.

The IFN-γ release assay was performed monthly by stimulating peripheral blood mononuclear cells (PBMCs) with avian protein purified derivative (PPD) (Prionics, La Vista, NE) and positive (pokeweed mitogen) (Sigma-Aldrich Canada Co., Oakville, ON, Canada) and negative (PBS) controls. The IFN-γ produced by the PBMC's after stimulation was measured with a commercial IFN-γ ELISA (Bovigam®, Prionics, La Vista, NE). The algorithm for interpretation of test results described by Kalis et al. (2003) was used to determine whether a sample was positive or negative [9]. This was also described in the previous chapter in more detail. It is noteworthy that Calf 1, 2, and 3 were included in the described population in Chapter 5, but Calf 4 and 5 were not, due to reasons explained in Chapter 5.

Euthanasia was performed in Calf 1-4 due to completion of the trial and in Calf 5 due to animal welfare concerns with an intravenous injection of barbiturates (Euthanyl Forte, pentobarbital sodium, Bimeda-MTC Animal Health, Cambridge, ON, Canada) and necropsy was performed immediately thereafter. Macroscopic lesions were assessed during necropsy by a veterinary pathologist (blinded to the inoculation status of the calves) and assigned to categories as

described (Chapter 2): 0 = no macroscopic changes; 1 = one enlarged or edematous lymph node (LN) of the small intestine or liver; 2 = multiple enlarged and edematous mesenteric LNs and/or hyperemia of the ileocaecal valve; 3 = enlarged mesenteric LN(s) and/or mild to moderate thickening of ileal or jejunal mucosa; and 4 = enlarged mesenteric LN(s) and severe thickening and corrugation of the ileal, jejunal and colon mucosa.

From 4 specific tissue sites (ileocaecal valve, ileocaecal LN, ileal LN and distal ileum), samples were placed in a labeled cassette, immersed in 10% neutral buffered formalin solution and routinely processed for histological assessment as described in Chapter 2. Samples were embedded in paraffin, sectioned and stained with Hematoxylin-Eosin (HE), as well as ZN by Prairie Diagnostic Services (Saskatoon, SK, Canada). Slides were examined by light microscopy by an experienced veterinary pathologist blinded to inoculation status. Calves were assigned to previously described categories [7]: 0 = no lesions; 1 = focal lesions; 2 = multifocal lesions; and 3 = diffuse lymphocytic, multibacillary or intermediate lesions.

Extensive tissue sampling was done and tissues were cultured for MAP, also using the para-JEM automated MAP culturing system (para-JEM®, TREK Diagnostic Systems, Cleveland, OH). Tissue samples collected were: duodenum, mid and terminal jejunum, proximal, mid, and terminal ileum, ileocaecal valve, cecum (and their corresponding lymph nodes), spiral colon, transverse colon, rectum, retropharyngeal LN, tonsil, inguinal LN, liver and hepatic LN, spleen and kidney. From each tissue sample, 2 g was added to a sterile polyethylene stomacher bag with 5 ml of PBS and homogenized in a Seward Stomacher 80 Biomaster (Seward USA, Davie, FL). The sample was then added to 20 ml of 0.6% hexadecylpyridinium chloride (HPC) in half-strength Brain Heart Infusion (BHI). After incubation (3 hours at 37°C), tubes were centrifuged at 1700 rcf for 20 minutes. Further processing was performed according to manufacturer's

recommendations (para-JEM®, TREK Diagnostic Systems, Cleveland, OH). From these liquid tissue cultures, DNA was extracted [10] and real-time PCR targeting the *F57* and IS*900* region was performed as described [11, 12]. Samples with amplification curves with a threshold cycle < 40 were considered positive. Tissue culture results were assessed in categories as described (Chapter 2). Calves were assigned to the following categories: 0 = no positive tissues; 1 = 1-3 tissues positive; 2 = 4-6 tissues positive; and 3 = more than 6 tissues positive.

6.4. Results

6.4.1. Calves with clinical signs

Two calves had clinical symptoms of JD. Calf 4 had a chronic presentation with deteriorating body condition starting 11.5 months after inoculation. Its body condition score (BCS) was 2.5 on a scale of 5 [13] and it continued to deteriorate until 14 months after inoculation (BCS: 2 on a scale of 5), despite being fed an improved diet. Two weeks later, diarrhea was noticed for the first time and remained intermittently present until euthanasia at 16 months after inoculation due to completion of the trial.

In contrast, Calf 5 had acute rather than chronic symptoms. When Calf 5 was 16 months old, it presented with severe abdominal pain, lack of appetite and diarrhea. Its body condition score was 2.5 on a scale of 5 [13], with a body temperature of 39.4°C. Based on clinical examination, rumen impaction/obstruction or mild peritonitis/hardware disease were differential diagnoses. The calf was given ceftiofur (Excede, Zoetis Canada, Kirkland, QC, Canada), meloxicam (Metacam, Boehringer Ingelheim Canada, Burlington, ON, Canada), a magnet, a vitamin and mineral supplement (Ketamalt, Bimeda-MTC Animal Health, Cambridge, ON, Canada),

electrolytes (V-Lytes, Vetoquinol Canada Inc., Lavaltrie, QC, Canada), mineral oil (Light mineral oil, Vetoquinol Canada Inc., Lavaltrie, QC, Canada) *per os*, and fluid (water) *per os*. Symptoms decreased 2 days later, but the calf deteriorated at day 3. White blood cell, hematocrit and platelet counts (days 4 and 7) were within normal ranges. On serum chemistry, there was hypoproteinemia (51 g/L, reference range 67-75), the A/G ratio was low (0.5, reference range 0.8-0.9), phosphorus was elevated 2.42 mmol/L (reference range 1.29-2.32), and calcium was just below the normal range 2.11 mmol/L (reference range 2.18-3.10). As symptoms did not improve, the steer was euthanized due to animal welfare concerns 7 days after the first symptoms occurred.

Both Calf 4 and 5 were positive for MAP-specific ELISA as of 5 and 4 months after inoculation, respectively, and the ELISA S/P ratio remained high every month until necropsy (Table 6-1). Fecal culture was initially positive at 2 and 3 weeks after inoculation for Calf 4 and 5, respectively, and remained positive until necropsy (Table 6-2). A cellular immune response was detected in both calves at 2 months after inoculation and the IFN-γ production remained high until 6 months after inoculation, after which it declined.

At necropsy, both calves appeared externally thin, although Calf 4 had adequate internal fat stores (381 kg, BCS: 2 on a scale of 5), whereas Calf 5 (394 kg, BCS: 2.5 on a scale of 5 [13]) had minimal internal fat stores. Both calves had mild hydroperitoneum. In Calf 5, there was diffuse serosal/mesenteric edema (most severe around the abomasum and spiral colon). In both steers, there was lymphadenopathy in lymph nodes adjacent to the ileum and jejunum. In Calf 4, the proximal ileum was thick with rugose mucosa (Figure 6-1), as well as the mid-ileum (albeit to a lesser degree). The distal ileum was mildly thickened, and less rugose, but had a granular mucosal surface. In contrast, Calf 5 only had mild mucosal roughening and thickening in the mid

ileum. In both calves, there was thickening and granularity in the spiral colon. Based on their gross lesions, both calves were assigned to gross lesion category 4 (Chapter 2). Furthermore, based on histology, both were classified as 'diffuse multibacillary lesions' (category 3) [7].

Most sampled tissues were MAP culture-positive, except for 6 tissues in Calf 4 and 5 tissues in Calf 5. For Calf 4, the 6 tissues that were not culture-positive were the liver, kidney, spleen, rectum, ileocaecal LN and duodenum. For Calf 5, the 5 tissues that were not culture-positive were the liver, kidney, spleen, distal jejunal LN, retropharyngeal LN, and duodenum. Consequently, both calves were assigned to tissue culture category 3 (Chapter 2).

Based on gross lesions, histological findings and tissue culture results, in combination with other diagnostic tests, both calves were regarded as having clinical JD.

6.4.2. Asymptomatic calves and control group

Three calves concurrently inoculated at the same age and with the same dose did not have clinical symptoms. One of these three calves (Calf 1) remained antibody ELISA-negative, one developed a transient response (Calf 2), and one (Calf 3) became ELISA-positive 10 months after inoculation (Table 6-1). The calf with the transient response (Calf 2) was ELISA-positive between 7 and 13 months after inoculation (Table 6-1).

Fecal shedding was confirmed in all three asymptomatic calves (Table 6-2); Calf 1 was fecal culture-positive twice at 1 and 3 months after inoculation; Calf 2 was positive 6 times at 1-5 and 11 months after inoculation; and Calf 3 was positive 13 times at 0.75-3, 5, 7-8, and 11-17 months after inoculation (Table 6-2).

An IFN-γ response was detected in Calf 1 and 2 as of 2 months after inoculation and in Calf 3 as of 3 months after inoculation.

Gross lesions were less severe than in the clinical cases (Table 6-3): Calf 1 was assigned to category 2 (multiple enlarged and edematous mesenteric lymph nodes and/or hyperemia of the ileocaecal valve), whereas Calf 2 and 3 were assigned to category 3 (enlarged mesenteric lymph node(s) and/or mild to moderate thickening of ileal or jejunal mucosa; Table 6-3). Compared to the clinical calves, all 3 calves were in a lower category for gross lesions, histology and tissue culture (0, 2 and 5 tissues were culture-positive; Table 6-3).

In the control group, no calves had a humoral immune response or gross lesions. One calf was shedding on 3 occasions. One control calf had one culture-positive tissue, whereas another control calf had two culture-positive tissues (ileal LN and ileocaecal LN). Subtle and focal lesions (occasional clusters of epithelioid ZN-negative macrophages) were also detected in 4 of 6 control calves (Chapter 2).

6.5. Discussion

Even though a recommended bovine challenge dose [14] of $5x10^9$ CFU/dose (100 mg wet weight) was given on two successive days at 2 weeks of age, 2 of 5 calves developed clinical JD at 16 months of age. Clinical disease is not typical at this age and might indicate that an excessively high dose was used for inoculation [14]. However, because 3 of the calves infected with the same dose and housed under similar conditions remained asymptomatic, we inferred that inherent individual differences, likely genetic, resulted in early clinical signs. Variation in time to progress to the clinical stage was described; sometimes the clinical stage is reached as fast as 6 months after first fecal shedding [4]. Differences between animals that do or do not progress to the clinical stage are critical in clarifying mechanisms of susceptibility/resistance to JD. A genetic basis for resistance/susceptibility to MAP infection based on differences in cellular

immune response has been described in deer [15]. Although the cellular immune response was not apparently different between clinical and asymptomatic calves, this was based solely on the IFN-γ release assay, which may not be the most appropriate tool to identify such a difference. A wide variety in genetic material has been observed in dairy cattle and has been related to MAP infection status [16]. Studies on genetic traits suggested that heritability of resistance to MAP infection can be up to 12% [17] and heritability of positive fecal culture ranged from 16-23% [18] and up to 27% in the most recent report [19]. Presence of antibodies had a heritability of 9-10% [20]. These lower values for antibody production could possibly be explained by a lower sensitivity of the ELISA and likely true heritability of MAP infection is higher than currently reported. Moreover, MAP infection status was correlated to candidate genes such as the NOD2/CARD15 gene [21] and the Toll-like Receptor 1 [22] and Toll-like Receptor 2 gene [23]. All of these genes are involved in the initial immune response which is suggested to be of importance in controlling MAP infection [24]. A true genetic effect on susceptibility can also be assumed in this case because environment, management and bacterial inoculum were similar for all 5 calves in this study.

This was apparently the first report to document progression of an experimental infection with MAP to clinical JD with monthly results from commercial diagnostic tests, ultimately combined with necropsy data (including gross and histological lesions and tissue culture). Due to the intense monitoring, it was possible to determine the exact duration of each stage of MAP infection for these 2 calves. In the "silent infection" stage (stage I), calves, heifers, and young stock up to 2 years of age are infected with MAP but do not show any signs [3]. Diagnostic tests are consistently negative (Table 6-4) and the only way to detect an infected calf is by histology or tissue culture (Table 6-3) [3]. In Calf 4 and 5 with clinical signs, this first stage lasted 2 and 3

weeks, respectively, followed by onset of fecal shedding (beginning of the subclinical stage). In these 2 calves, the "silent infection" stage was short, however, under field conditions, the interval from onset of infection to first detectable fecal shedding of MAP is variable (usually > 2 years) [4]. In the asymptomatic calves, the "silent infection stage" lasted 3-4 weeks, until the start of fecal shedding (Table 6-2), a similar timespan as the clinical calves. It was noteworthy that fecal shedding was consistently the first diagnostic test to be positive, even before the IFN-γ release assay, in all cases before or at 1 month after inoculation. This early shedding was described previously in experimental infections in goats and calves [25-28]. One of the asymptomatic calves, Calf 3, had a persistently positive ELISA response as of 10 months after inoculation (Table 6-1) and shed MAP persistently as of 11 months after inoculation (Table 6-2). Likely, if the duration of the trial had been longer, this calf could have become clinical as well.

Subclinical disease (Stage II) typically includes adult cattle still without visible signs of JD [3], although antibodies may be present and a cellular immune response detected [3] (Table 6-4). Fecal culture is positive or negative [3] (Table 6-4), and if lactating, milk production will decrease [29-31]. This subclinical stage can vary in duration from 6 months to several years [4]. The second stage lasted 11 and 14.5 months in Calf 4 and 5, respectively. In the asymptomatic calves, this stage lasted from the first fecal shedding (Table 6-2) to the end of the trial (the actual length of this stage could therefore not be determined in these asymptomatic calves). However, each asymptomatic calf had a distinct antibody response profile (negative, transient response and persistently positive; Table 6-1); furthermore, fecal shedding patterns in asymptomatic calves were also variable during this subclinical stage.

It is not known why differences were present in duration of the stages between asymptomatic and clinical calves. Probably, calves with early clinical signs failed in their initial cellular

immune response, even though such a difference was not detected with the IFN-γ release assay, as described in deer [15]. Consequently, invading MAP was not controlled and a higher bacterial burden was established in the intestines of the clinical calves. Because duration of the subclinical stage differed most between asymptomatic and clinical calves in this study, the motives for this difference should be found in the characteristics of the subclinical stage. Because cattle in the subclinical stage predominantly have a cellular immune response [32], likely a disruption in this cellular immune response is the culprit of progression of infection as suggested in the studies on genetic variability discussed above and in knowledge on immune responses in paratuberculosis [32, 33].

Clinical disease (Stage III) usually starts 2-10 years after infection; typical clinical signs include gradual weight loss, diarrhea, and changes in serum biochemistry [3]. Diagnostic tests now clearly indicate a MAP infection: positive fecal culture and ELISA (Table 6-4). This stage typically lasts 3-4 months [3]. In the two calves described in this study, this stage was observed for 4.5 months in Calf 4, but only 1 month in Calf 5. As the calves were euthanized at this point, the clinical stage was terminated prematurely and the actual duration of this stage could not be determined. It is believed that dissemination happens in the clinical stage [3], as must have occurred in these calves since most tissue samples were positive. However, in other calves included in this study, multiple tissue locations were positive in the absence of clinical symptoms (subclinical stage) indicating dissemination of MAP might occur prior to the clinical stage (Chapter 2, [4, 34]).

Advanced clinical stage (Stage IV) is characterized by a lethargic state, weakness, emaciation, clinically visible hypoproteinemia (bottle jaw), and diarrhea [3]. However, these clinical signs were not observed in the 2 calves described in this article.

Even though the 2 clinical calves described in this paper probably progressed through the stages of JD quicker than most cattle infected under field conditions, the approximate length of each stage of JD was determined. In all inoculated calves, the IFN-γ test was positive at 2 or 3 months after inoculation and the reaction diminished at 8-9 months after inoculation. Fecal shedding started in all calves before or at 1 month after inoculation and clinical calves became ELISA-positive 4-5 months after inoculation; this was the last test to become positive. It is noteworthy that, in this study, there were only 2 clinical cases after 5 calves were inoculated with a high dose at 2 weeks of age. However, no clinical disease was noted in calves inoculated at 2 weeks with a low dose of MAP or any other ages (3, 6, 9, or 12 months of age) with both doses of MAP in the same trial (Chapter 2). This was consistent with published studies suggesting that the rate of progression of JD is MAP-dose dependent, in addition to being dependent on age at infection [35].

The inoculation dose used in the infection trial was intended to mimic ingestion of MAP which may occur naturally from contact with infected dams, shedding animals and resulting environmental contamination. However, little is known about the exact number of MAP bacteria present in and around a typical MAP-infected dairy farm. Infection can be established in young calves with a dose contained in 2 g of feces [31]. Nevertheless, the number of CFUs present in these 2 g remains to be determined and is highly dependent on the infection status of the source animal. Consequently, the dose with which calves are generally infected on farm remains unknown.

Because a low number of bacteria in fecal samples was expected [25] after a fairly short follow-up period (16.5 months) in this trial, no quantification was done on fecal samples by a time-to

positive signal. Additionally, the pressure curves generated by the liquid culture system are optimized for fecal samples and did not perform well for tissue samples.

As discussed in more detail in Chapter 2, 2 of 6 non-inoculated calves had positive tissue culture results and 4 control calves had focal histological lesions. In one control calf, MAP was cultured from the same 2 lymph nodes that also contained histological lesions, whereas another calf with a focal lesion was shedding as well, suggesting a true infection. However unlikely, possible explanations for this are a false-positive PCR result, cross contamination from infected to control calves, transmission via dust [36-39] or hay, or *in utero* infection [40]. Even though calf-to-calf transmission was prevented at all times, these calves could have acquired MAP infection during the experiment. It was unfortunately not possible to determine the source of infection due to insufficient amounts of DNA present for genotyping. However, despite these results in the control calves, conclusions can be drawn concerning the diagnostic profiles of the calves inoculated at 2 weeks of age.

In conclusion, the 2 steers (16 months old) in this study were exceptional clinical JD cases. The silent infection stage lasted up to 1 month in clinical as well as asymptomatic calves. The subclinical stage was relatively short in the 2 clinical steers, but of unknown length in the asymptomatic calves. Diagnostic profiles clearly differed between clinical and asymptomatic calves: clinical calves were consistently positive on ELISA and fecal culture long before appearance of clinical signs, whereas asymptomatic calves had an intermittent shedding pattern and variable ELISA profiles. Whether this difference in diagnostic profile had a genetic basis remains to be confirmed.

6.6. References

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Interval PI[†] (months)	Asymptomatic calves			Calves with clinical signs	
	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5
0	-	-	-	-	-
0.25	-	-	-	-	-
0.5	-	-	-	-	-
0.75	-	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	+
5	-	-	-	+	+
6	-	-	-	+	+
7	-	+	-	+	+
8	-	+	-	+	+
9	-	+	-	+	+
10	-	+	+	+	+
11	-	+	+	+*	+
12	-	+	+	+*	+
13	-	+	+	+*	+
14	-	-	+	+*	+
15	-	-	+	+*	+
16	-	-	+		+*
17	-	-	+		

Table 6-1: Antibody ELISA results for all 5 calves inoculated at 2 weeks of age with

Mycobacterium avium subspecies *paratuberculosis*. An S/P ratio > 60 was considered positive.

* clinical symptoms

† post inoculation

Interval PI[†] (months)	Asymptomatic calves			Calves with clinical signs	
	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5
0	-	-	-	-	-
0.25	-	-	-	x[‡]	-
0.5	-	-	-	+	-
0.75	-	-	+	+	+
1	+	+	+	+	+
2	-	+	+	+	+
3	+	+	+	+	+
4	x[‡]	+	-	+	+
5	-	+	+	+	+
6	-	-	-	+	+
7	-	-	+	+	+
8	-	-	+	+	+
9	-	-	-	+	+
10	-	-	-	+	+
11	-	+	+	x[‡]*	+
12	-	-	+	+*	+
13	-	-	+	+*	+
14	-	-	+	+*	+
15	-	-	+	+*	+
16	-	-	+		+*
17	-	-	+		

Table 6-2: Fecal culture results for all 5 calves inoculated at 2 weeks of age with *Mycobacterium avium* subspecies *paratuberculosis*.

* clinical symptoms

† post inoculation

‡ missing sample

	Gross lesions category[1]	Histology category[2]	Tissue culture category[3]
Asymptomatic calves			
Calf 1	1	1	1
Calf 2	1	1	0
Calf 3	1	2	1
Calves with clinical signs			
Calf 4	4	3	3
Calf 5	4	3	3

Table 6-3: Severity of gross and histological lesions as well as tissue culture-positives in 5 calves inoculated at 2 weeks of age with *Mycobacterium avium* subspecies *paratuberculosis*.

[1]0 = no macroscopic changes; 1 = one enlarged or edematous lymph node of the small intestine or liver; 2 = multiple enlarged and edematous mesenteric lymph nodes and/or hyperemia of the ileocaecal valve; 3 = enlarged mesenteric lymph node(s) and/or mild to moderate thickening of ileal or jejunal mucosa; and 4 = enlarged mesenteric lymph node(s) and severe thickening and corrugation of the ileal, jejunal and colon mucosa.

[2]0 = no lesions; 1 = focal lesions; 2 = multifocal lesions; and 3 = diffuse lymphocytic, multibacillary or intermediate lesions.

[3]0 = no tissue locations culture-positive; 1 = 1-3 tissue locations culture-positive; 2 = 4-6 tissue locations culture-positive; and 3 = more than 6 tissues culture-positive.

	Silent infection stage	Subclinical stage	Clinical stage	Advanced clinical stage
IFN-γ test	-	+	+ or -	- (?)
ELISA	-	+	+	+
Fecal culture	-	+ or -	+	+
Macroscopic lesions	-	-	+	+
Histology	+	+	+	+
Tissue culture	+	+	+	+
Clinical signs	none	none	Weight loss Diarrhea	Emaciation, diarrhea, bottle jaw

Table 6-4: Overview of characteristics for each of the stages of JD based on Whitlock and Buergelt (1996), Sweeney (2011), and Behr and Collins (2010).
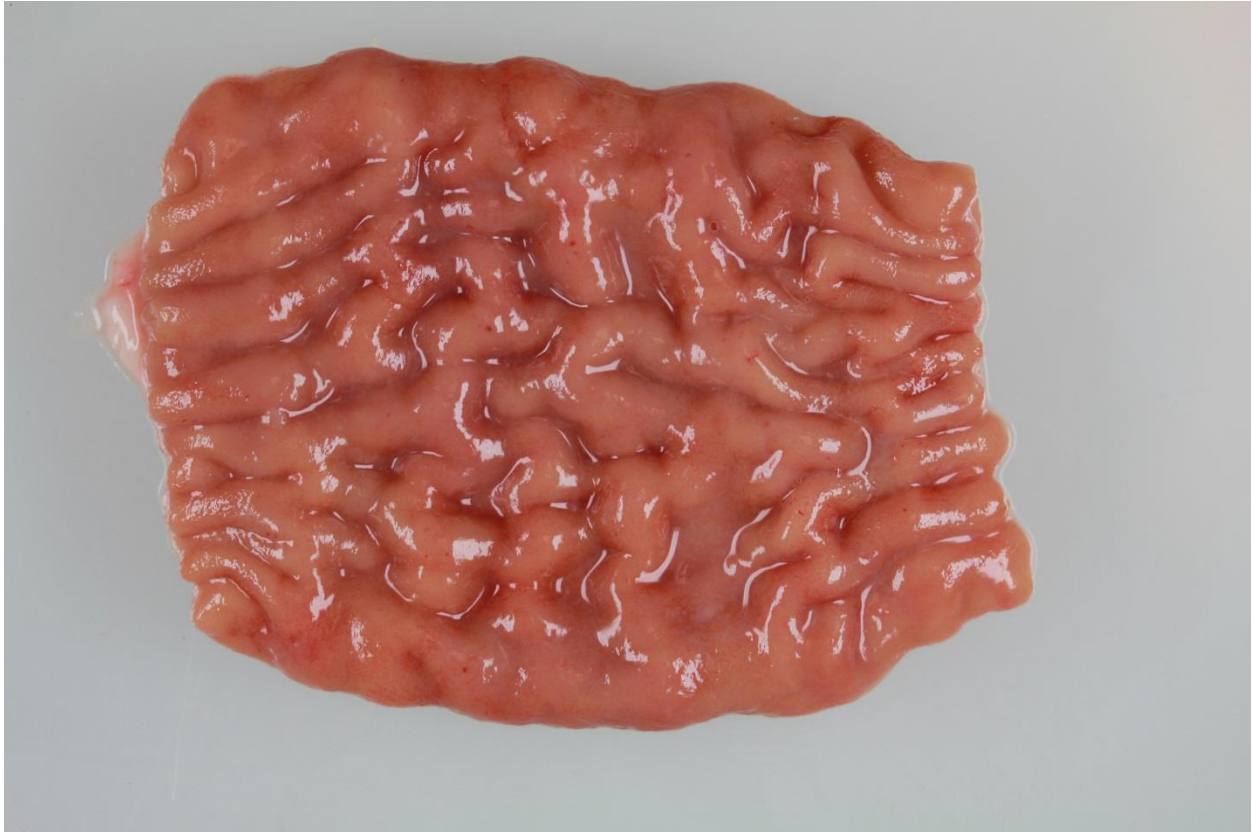
Figure 6-1: Corrugation and thickening in the proximal ileum of Calf 4.

CHAPTER 7: GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1. Introduction

The main objective of this thesis was to determine age- and dose-dependent susceptibility to MAP infection in dairy calves, in order to improve control programs. Additionally, the onset and course of cellular and humoral immune responses, as well as patterns in fecal shedding, were also assessed. Gross lesions, histology and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) culture from numerous tissue locations were used to confirm infection in each calf, susceptibility, and diagnostic test results.

Although unexpected based on previous research, at least some calves in all age groups were successfully infected with either a high dose (HD) or low dose (LD) of MAP. However, calves inoculated with a HD had more severe gross and histological lesions and more culture-positive tissue locations than those inoculated with a LD (Chapter 2). A dose-dependent antibody response was detected using a commercially available ELISA in a larger proportion of calves than expected soon after inoculation (Chapter 3). Shedding of MAP in feces was detected soon after inoculation, with a peak at 2 months after inoculation (Chapter 4). Shedding was dose-dependent when calves were inoculated at 2 weeks or 3 months of age, but this dose-dependency disappeared when older calves were inoculated (Chapter 4). Calves inoculated with a HD had earlier and stronger IFN-γ responses than LD calves. Furthermore, calves inoculated at 2 weeks of age produced less IFN-γ compared to those inoculated later in life. The IFN-γ response peaked (on average) 4 months after exposure (Chapter 5). In a final chapter, calves with clinical signs of JD had a distinct diagnostic profile from asymptomatic calves inoculated with the same dose and at the same age (Chapter 6). These findings will be discussed separately and in combination below.

7.2. Age-dependency

Antibodies, fecal shedding, and an IFN-γ response were detected in each age and dose group (except the 6 months-LD group which did not develop antibodies; Chapter 3-5). Furthermore, in all age and dose groups, at least one calf was positive for at least 1 of the following: tissue culture, macroscopic lesions, or histology (Chapter 2). Therefore, calves up to 1 year of age were successfully infected with MAP, even with a LD. Notwithstanding, there were some differences between the younger calves and older age groups based on necropsy lesions, immune responses and fecal shedding. Calves inoculated at 2 weeks and 3 months of age with a HD shed more frequently, had a stronger humoral immune response, and more severe lesions at necropsy than those inoculated at an older age or at the same age but with a LD. It was noteworthy that this dose dependency disappeared when older calves were inoculated. In that regard, a cellular immune response in calves inoculated at 2 weeks of age was less strong compared to calves inoculated at older ages (Chapter 5). If a strong cellular immune response is required to have a better protective effect towards MAP infection [1], this could explain why MAP was thriving (more ELISA and fecal-positives and more severe necropsy lesions) in these young calves. More severe gross lesions observed in older calves (Chapter 2) could be the result of a more pronounced cellular immune response, because inflammation can cause visible swelling of lymph nodes and thickening of ileal mucosa. At the same time, inflammation is more effective at keeping MAP under control and consequently there was less shedding and fewer tissue culture-positives. Conversely, follow-up in these older calves was shorter, and shedding might not have been detected yet. This is similar to a previous study in which less severe necropsy lesions were observed in sheep when infected as adults compared to when infected as lambs [2]. These findings were attributed to a more efficient peripheral immune response in adult ewes versus

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lambs [2]. Moreover, in the current study, the humoral immune response started before 4.5 months after inoculation in calves inoculated at 12 months compared to calves inoculated at 2 weeks and 3 months (Chapter 3). Therefore, we inferred, similar to ewes [2], that the peripheral immune response is more efficient in older cattle, yielding a stronger cellular and earlier humoral immune response. To conclude, it appeared that a strong initial cellular immune response is the key to controlling progression of JD, as described previously [1], possibly in combination with humoral immunity. Consequently, when infected at a young age when the immune system is still immature [3], lesions were generally more pronounced.

7.3. Dose-dependency

Overall, LD calves were less severely affected after inoculation with MAP, especially in the 2-week and 3-month groups. This pattern discrepancy was noted for all diagnostic tests: calves inoculated with a HD had more positive tissues and more severe histological lesions compared to LD calves when inoculated at < 6 months of age (Chapter 2). Gross lesions were dose-dependent in all age groups. Calves inoculated with a HD had a stronger antibody response, particularly if they were young (2 weeks or 3 or 6 months; Chapter 3) at inoculation. Calves inoculated at a young age with a HD shed MAP more frequently than LD calves (Chapter 4). In older age groups (6, 9 and 12 months), this dose-dependent effect was no longer present. The cellular immune response was more pronounced in HD calves compared to LD calves (Chapter 5).

Overall, we inferred that there is no clear switch from a cellular to a humoral immune response, but a lower cellular immune response at any time will facilitate more humoral immunity to act simultaneously as an extra measure of the immune system to try and keep MAP under control when the cellular response is dampened. It is noteworthy that MAP regulates its own survival by

altering the innate immune response, as was recently reviewed by Arsenault et al. (2014) [4]. The authors concluded that MAP actively suppresses an IFN-γ response, utilizing a variety of mechanisms [4]. However, a cellular immune response is necessary for elimination of MAP from the macrophages [5]. Additionally, production of Interleukin-10 by regulatory T-cells is increased in MAP-infected cattle, which also suppresses an effective IFN-γ response, again securing MAP proliferation in the macrophage [6]. Also, pro-inflammatory T-helper 17 responses are dampened in MAP infected cows, further disabling the cellular immune response [7]. As a consequence of this suppression of the cellular immune response, T helper 2 (Th2) responses prevail [6]. However, Th2 responses are ineffective in controlling MAP infection [5, 8], because antibodies are not effective in fighting intracellular pathogens [1]. Consequently, young calves infected with MAP are less able to respond to this infection (as described in Section 7.2), but when infected with a HD of MAP, the organisms might suppress the cellular immune response even more and JD will progress more quickly compared to older and immune-mature cattle who are more capable of a stronger cellular immune response.

For unknown reasons, the dose effect in antibody production and fecal shedding disappeared in older age groups (9 and 12 months of age; Chapters 3 & 4). If the initial cellular immune response determines the frequency of shedding or antibody response, also an effect of dose on antibody production or fecal shedding would have to be noted in the 9 and 12 month infection groups because the cellular immune response was stronger in the HD compared to LD calves in all age groups (Chapter 5). Despite a dose dependent cellular immune response in 9 and 12 month groups, an effect of dose was not observed in antibody production or fecal shedding. In another infection model, dose-dependent cellular and humoral immune responses were observed in a calf model [9]. Results from the latter study combined with those from the current study

indicate a strong influence of dose on immune responses and consequently on the progression of JD. However, the underlying mechanisms remain to be elucidated.

7.4. Early diagnosis

When screening a herd for paratuberculosis, it is advised to test cattle older than 36 months [10], assuming that calves get infected at a young age and go through carrier and subclinical stages during which diagnostics tests have a low sensitivity [11, 12]. The IFN-γ release assay was the only test in which all inoculated calves in the current study tested positive; furthermore the course of IFN-γ production was similar for all calves included in the first replicate of the trial (n = 30; Chapter 5). From the fact that each calf had an increase in %IFN-γ value, but only 20 (67%) had 1 or more culture-positive tissue locations, 25 (83%) calves had histological lesions, and 19 (63%) had gross lesions, we inferred that a positive IFN-γ release assay only indicated exposure to MAP or that tissue culture/histology was less sensitive than generally accepted [13, 14], as previously mentioned [15-17]. In the current study, fecal shedding was the first test to become positive starting at 2 weeks after inoculation (Figure 7-1) followed by IFN-γ release assay that peaked 4 months after inoculation in all calves. The antibody response differed between individual calves, with less than half of the calves testing positive and some of them having a transient response. Even though these transient responses could explain some ELISA results classified as 'false-positive' during herd screening, they also indicate that a commercial antibody test is capable of detecting MAP antibodies – when they are present. The amount of peripheral antibodies seems to increase and decrease over time, and likely depending on a varying equilibrium in the immune system, [18] steering towards or suppressing a humoral immune response. In this study, antibodies were detected in 42% of the calves (Chapter 3), the

IFN-γ release assay indicated a cellular immune response in all calves (Chapter 5), and fecal shedding was detected in 61% of the calves (Chapter 4). In particular, fecal shedding peaked between 1 and 6 months after inoculation (as described before [19]), whereas the cellular immune response peaked, on average, 4 months after inoculation. Overall, we inferred that the chance of detecting a MAP infected animal is highest within a 6-month period after infection, with fecal culture being the first test to become positive. Fecal shedding in young stock was also confirmed on-farm with the highest proportion of calves shedding prior to 6 months of age [20], consistent with observations in this study. After this initial peak, a balance between the cellular and humoral component is established and diagnostics remain negative, probably to become positive again in the later stages of JD [1, 8, 11]. Unfortunately, in this study the follow-up interval was not long enough to conclude whether infected cattle indeed test positive again in later stages of JD. Because 42% and 61% of the calves in this study were positive at least once during this trial for antibodies or fecal shedding, respectively, it seems useful to combine diagnostics when testing shortly after infection, as was suggested previously in McDonald et al. (1999) [15].

Two 16-month old steers in this study developed clinical signs of JD exceptionally early after inoculation (Chapter 6). Diagnostic profiles clearly differed between clinical and asymptomatic calves inoculated at 2 weeks of age with a HD: clinical calves were consistently ELISA and fecal culture-positive long before appearance of clinical signs, whereas asymptomatic calves had an intermittent shedding pattern (1 calf was shedding persistently towards the end of the trial) and variable ELISA profiles (Chapter 6). Because cattle < 36 months typically are not included in herd screening [10], their positivity on the mentioned diagnostics would likely have been missed.

Therefore, when screening a herd for JD, testing calves shortly after infection with a combination of ELISA and fecal culture could complement testing cattle > 36 months of age.

7.5. Diagnostic test agreement

Histopathological findings and tissue culture had the highest agreement (80%) of all test comparisons, but kappa values were all <0.2, indicating no more than a slight agreement between gross and histological lesions and MAP culture from tissues. Furthermore, a low proportion of tissue sites were culture-positive and multiple tissues were needed to identify a calf as infected (Chapter 2). Although each calf had an increase in %IFN-γ value, only about 70% had positive necropsy lesions or tissue culture, indicative of a positive IFN-γ release assay only recognizing exposure to MAP (Chapter 5). Calves with ≥ 1 positive ELISA sample did not have a higher or lower chance of being positive for gross lesions, histological lesions or tissue culture (Chapter 3). The distribution of tissue culture categories and gross lesion scores was not different between shedding and non-shedding calves. However, frequently shedding calves had more severe gross and histological lesions, and more culture-positive tissue locations (Chapter 4). There was a good overall agreement (84%) between serology and fecal culture results for each sample tested during the trial. However, the proportion of positive agreement was 36% and the proportion of negative agreement was 91%, meaning that the overall agreement between these 2 tests was mainly caused by an agreement on negative test results. This is also due to a relatively high specificity of ELISA and fecal culture in comparison to a low sensitivity. A kappa value of 0.27 indicates only fair agreement between serology and fecal culture. It needs to be noted that this is very likely an overestimation, again due to the 91% negative agreement.

Immune response profiles were determined for calves in this study, because test agreement between diagnostic tests for MAP infection seemed to be low. Three distinct types of immune

response profiles previously described in sheep [21], were observed in 30 calves inoculated in the current study (no IFN-γ data were available from the other 20 inoculated calves; Figure 7-2). The first profile is characterized by an initial increase in IFN-γ, followed by a switch of response towards a humoral immune response and then loss of the cellular immune response, has been described as the "classical switch profile" [21] (Figure 7-2A). This profile only occurred in 1 calf (3%), given a HD at 3 months of age. In the second "combined IFN-γ/antibody" [21] profile, IFN-γ and antibody responses increased simultaneously (Figure 7-2B&C); this occurred in 13 (43%) of 30 inoculated calves, with 2 of these 13 calves having a slightly delayed antibody response (Figure 7-2C). The third profile, "IFN-γ only" [21], was characterized by an IFN-γ response not followed by an antibody response during the follow-up period [21]. This profile was observed in most calves (47%, derived from all age and dose groups; Figure 7-2D). One calf had too many missing IFN-γ data and could not be classified. Despite the fact that only about half of the inoculated calves were used to determine these immune response profiles, missing IFN-γ data and differences in the length of follow-up period between age groups, some conclusions can be made. These immune response profiles did not depend on age (P = 0.48) or dose group (P = 0.66) and were not associated with the gross lesion score (P = 0.95), or the number of MAP-culture positive tissues at necropsy (P = 0.94). Secondly, the assumed [1] "classical switch profile" [21] was less common in our calves than expected – only a small proportion of inoculated calves had this profile during this study and a more open-minded approach towards immune responses after MAP infection needs to be considered. The shift from a cellular to a humoral immune response has been well documented [1], but may not be accurate and an antibody response can start independent of the cellular immune response during the course of the disease [8]. The immune response profiles were associated with the histology score

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(P = 0.04): calves with the "classical switch profile" had histology score of 2, whereas the "IFN-γ only" calves all had a histological score of 1, suggesting that when an antibody response is present, more severe histological lesions occurred. However, the analysis was underpowered because only a few animals were included in each category and results should be interpreted with caution. The immune response profile tended to be related to fecal shedding category ($P = 0.06$). Non-shedders had a "combined IFN-γ/antibody" or "IFN-γ only" profile, whereas frequent shedders had the "classical switch" or "combined IFN-γ/antibody". These findings suggest that a "classical switch" profile coincides with more frequent shedding and non-shedding with an "IFN-γ only" profile. However no final conclusion can be made since only 1 calf had the "classical switch" profile and therefore an issue of low power needs to be taken into account. Conversely, the "combined IFN-γ/antibody" occurred in shedding as well as non-shedding calves. It is possible that decline in IFN-γ observed in calves with the "classical switch" profile rather than the presence of antibodies determines the frequency of shedding in MAP-infected calves. Similarly, in humans with inflammatory bowel disease, MAP was detected in both healthy controls and Crohn's disease (CD) patients, but the healthy controls had a strong cellular immune response against MAP-specific antigens in contrast to CD patients [22]. Comparable to MAP infected cattle, these CD patients had a strong antibody response, which was not capable of controlling an intracellular infection [1, 22]. In sheep, multibacillary animals had a decreased ability to produce IFN-γ [21], again confirming the role of MAP to suppress cellular immune responses [4]. The same principle can be extrapolated to observations where subclinical cows have a strong MAP-specific cellular immune response, whereas clinical and non-infected cows do not. Consequently, the cellular immune response keeps a MAP infection under control and as

soon as the cellular response is disrupted, MAP proliferates and disease progresses [18], irrespective of the presence or absence of MAP-antibodies.

Shedding typically happened 'around' the IFN-γ peak. From the 30 calves from which serology, shedding and IFN-γ data were available, 16 (53%) were shedding during the observation period. The majority, 9 of 16 (56%) started shedding 1 to 3.25 months before the IFN-γ peak, 4 of 16 (25%) calves started shedding at the same moment as the IFN-γ peak and 3 calves (19%) started shedding shortly after this IFN-γ peak. Perhaps shedding and the cellular immune response are more closely related to the pathogenesis of MAP infection than to antibody production. Unfortunately, the underlying cause of fecal shedding is currently unknown [23]. It has been suggested that a migration of infected macrophages to the lumen results in shedding [24, 25] or extracellular MAP produced by burst macrophages caused shedding of MAP in feces [26]. It is noteworthy that these hypotheses link a cellular immune response to fecal shedding; therefore, understanding the interaction of shedding and cellular immune response could improve early diagnosis of MAP infection.

7.6. Potential sources of individual variation

Calves included in this clinical trial responded differently on diagnostic tests. In that regard, several antibody response profiles were observed (no response, transient response, persistent response [27]) and onset varied for each calf. Furthermore, fecal shedding patterns were different for each inoculated calf, as described in Chapter 4 (Figure 7-1). Most calves shed sporadically, 4 calves shed frequently, but almost 40% of calves did not shed at all during the follow-up period (Chapter 4). Moreover, calves had great variation in numbers of positive tissue locations: 22 calves were negative, 23 had 1-3 tissues MAP-culture positive, 3 calves had 4-6 tissues MAP-

culture positive and 2 calves had > 6 positive tissues (Chapter 2). In the 2 week-LD group, Calf 10 was negative for all used diagnostics (Figure 7-1), except the IFN-γ release assay, and 2 calves in the 2-week group inoculated with a HD presented with clinical signs of JD before the trial was completed (Chapter 6). Because this clinical trial was conducted in a controlled environment, the basis of the differences in responses between all calves included in this trial are likely genetic.

Knowledge on genetics of host susceptibility has recently been reviewed in detail in [28] and a brief summary will be provided here. A substantial heritability of susceptibility/resistance to MAP infection was indicated in this review of several studies [28]. Identified candidate genes of importance in resistance/susceptibility to MAP infection are the SLC11A1 gene in sheep [29], NOD2 in cattle [30], and Toll-like receptor 1 [28, 31]. Furthermore, whole-genome analysis in cattle indicated certain genotypes were associated with MAP infection [28, 32, 33]. These genes identified as possibly involved in resistance/susceptibility to MAP infection all have a role in the cellular immune response [28], confirming the importance of a cellular immune response to control MAP infection, as described in previous sections.

Following publication of this review, more studies on a genetic basis for susceptibility were published. Heritability for ELISA and fecal culture positivity in Jersey cattle was reported to be 8-27% [34], and for shedding up to 16-23% [35]. Concerning candidate genes, a single nucleotide polymorphism (SNP) in bovine Dectin-1 gene was related to ELISA-positivity [36] and new candidate genes are still being reported, for example CD209 and SP110 [37, 38]. In Crohn's disease, SNPs in candidate genes (Toll-like Receptor 4, Interleukin 10 Receptor A, NOD2) were also associated with presence of MAP [39]. Moreover, genome-wide analysis in cattle identified association between SNPs and MAP-infection (ELISA and shedding) [40] or to

positive milk ELISA [32]. In addition, a genetic basis for resistance/susceptibility to MAP infection based on differences in cellular immune response was recently described in deer [41]. Genes involved in cellular immune responses were differentially expressed in susceptible and resistant red deer [42]. Many more studies have reported an association between susceptibility to MAP infection and genotype. Although the cellular immune response was not apparently different between all inoculated calves in this study (Chapter 5), this was based solely on the IFN-γ release assay, which may not be the most appropriate tool to identify such a difference. More detailed research including multiple signalling molecules such as Tumor Necrosis Factor-α, Interleukin 10, in addition to IFN-γ as well as in vitro infection studies with MAP to identify differential gene expression profiles might give a more complete image of the cellular immune response. When taking together all findings on breed effects, heritability, candidate genes, and genome-wide association studies, and findings from this trial, there is strong indication that genetic variability is the basis for susceptibility to MAP infection and therefore a rationale to consider genetic improvement for controlling JD on-farm.

7.7. Limitations of the study

Even though this study included 56 cattle in total, a high number for a study of the described duration, only 5 calves were included in each age and dose group. In addition, the individual calves unexpectedly displayed substantial individual variation. This caused a non-normal distribution in the population and non-parametric tests had to be used. Consequently, statistical analyses were under-powered in most cases, making it more difficult to detect significant differences.

Even though stringent biosecurity measures were in force in the research barn, some control calves did have scattered positive test results. The question remains whether these calves indeed got infected due to insufficient separation of the calves, contamination of the hay, in utero, or whether false-positive reactions occurred due to imperfect diagnostic tests or laboratory error. Quantification of MAP burden in tissues and fecal samples would have provided important information on how many MAP bacteria are shed by recently infected calves (and to determine their infectiousness to other calves) as well as what the bacterial load in tissues was. Because calves were euthanized at different time-points after inoculation (16.5, 14, 11, 8 and 5 months for the 2 week, 3, 6, 9, or 12 month-groups, respectively) the number of MAP bacteria could have been characterized at each time point and contributed to knowledge on pathogenesis. In particular, the minimal infectious dose for a calf/older animal, as well as the infection pressure on a particular farm, remains unknown. Quantification of the amount of MAP shed after inoculation with a known dose at a known age could have been very informative. A quantitative PCR direct on fecal samples collected during this trial could be part of future research.

Due to the chronic nature of the disease, the 17-month follow-up was likely not long enough to assess effect of dose or age at infection is on disease progression. Since 2 calves became clinical, either they were genetically more susceptible or the inoculation dose was too high. Some other calves were shedding frequently and the question remains whether they would have developed clinical symptoms soon as well. Additionally, peaks in shedding and IFN-γ were observed followed by a period of negative test results. Therefore a longer follow-up would have revealed whether these diagnostics became positive later on again, whether clinical signs occurred for more animals included in the study and whether for example the 12-month group really had a different progression of JD compared to those inoculated at 2 weeks or 3 months of age.

7.8. Conclusions and Recommendations

Calves up to 1 year of age should be considered both susceptible to MAP infection and a potential source of infection for other calves; these are important considerations for JD control programs. Recently infected calves can shed MAP and could be infectious to other calves when kept in groups. Calves should be kept separately from each other as well as from adult cattle to prevent spread of MAP. Additionally, appropriate measures need to be taken to avoid transmission of infection between groups of animals and even between separated calves.

Even though this was an infection trial and additional work needs to confirm the findings in a field situation, suggestions can be made. Control programs should focus on lowering MAP infection pressure in general on-farm, because a lower infection dose resulted in less severe necropsy lesions, less shedding and less antibody responses especially in calves infected < 6 months of age. It is imperative to keep the infection pressure on a farm as low as reasonably achievable. Next to removing clinical JD cases and shedding individuals on the farm, overall hygiene is important and minimizing exposure to manure will reduce the infection pressure (this will benefit the control of all infectious diseases on-farm).

Infection with MAP can be detected earlier than originally expected using a commercially available ELISA, fecal shedding or an IFN-γ release assay. Screening young stock using a combination of diagnostics and in addition to adult cattle might improve diagnostic sensitivity on-farm.

Diagnostic test agreement is low, because positivity on a test depends on the stage of JD the tested animal is in. The cellular immune response seemed to be strongly involved in susceptibility/resistance and progression of MAP infection and future research could focus on this topic.

Genetic traits were suggested to play a role in individual differences observed in calves inoculated with MAP and future research should focus on susceptibility/resistance genes as a means to help control MAP infection.

7.9. Future directions

Future research should focus on determining the genetic basis for resistance/susceptibility to MAP infection, as genotype was suggested to have an effect on susceptibility to MAP infection and numerous previous research, that there is a genetic basis for susceptibility, which has considerable promise for controlling JD. The importance of host genetics on resistance/susceptibility was also identified as a knowledge gap in another report [43]. Infection trials are a good starting point for these, because potential confounders such as management and other influences are minimized in a controlled environment. Investigating candidate genes as well as whole genome analysis would be appropriate approaches to determine genetic differences between calves that remain asymptomatic versus clinical calves.

Due to increased adoption of acidified milk feeding and automatic milk feeders, many dairy calves are group-housed both before and after weaning, providing the potential calf-to-calf transmission, as reported [17]. Therefore, as a next step, an experiment should be done in which inoculated calves are kept in groups with non-infected calves to confirm this finding with a larger number of calves to ensure sufficient doses of MAP are being shed to infect other calves consistently and to investigate the occurrence of passive shedding when calves are kept in groups. Also, currently the actual occurrence of shedding of young stock on dairy farms is being determined [20]. If these 2 steps confirm calf-to-calf transmission, JD control programs should be adjusted to include prevention of calf-to-calf transmission of MAP. Additionally, it should be

assessed whether adding an environmental sample from calf group housing would aid in determining the infection status of a herd by environmental sampling.

Since a peak in shedding, substantial humoral immune responses and IFN-γ responses were detected in the calves in this study, it might be useful to focus research on these diagnostics in young stock on-farm. Catching these initial host responses before shedding starts could allow early diagnosis and a sooner intervention from control programs. By testing animals > 3 years of age as currently recommended, these animals have already contaminated the environment and spread MAP on the dairy operation; consequently the organism will not be controlled. Identifying these infected animals before or at the start of shedding would immensely increase the potential to control JD. To achieve this, more research is needed on early shedding in experimentally infected calves, but also on the cellular immune response and use of ELISA (commercial or in-house). As a next step, these diagnostics should be tested on young stock on a dairy operation, as is currently done for fecal shedding [20].

Even though not the focus of this study, a different intensity and course of IFN-γ responses were noted between johnin and avian purified protein derivative (PPD) used in the IFN-γ release assay. Additionally, out of curiosity, results obtained with the IDEXX ELISA presented in this study were attempted to be duplicated with a second commercial ELISA (ID Screen Paratuberculosis Indirect, IDvet, Grabels, France). This ELISA detected less antibody responses in general, possibly due to protocol differences but most likely related to the antigen used as a coating for the ELISA. Affinity and avidity of antibodies is not well defined for MAP antigens [28] and new antigens have been described and are being described currently. However, the optimal antigen, with the best diagnostic potential has not been identified and this would likely solve some of the issues of low sensitivity experienced with ELISA and the IFN-γ release assay.

The IFN-γ release assay is a promising diagnostic test, because it detects the early cellular immune response and was measurable in every tested calf in this study. However, issues are prominent when it comes to interpretation, ease of use and previously described false positive reactions as were also observed with different antigens in the current study. Further optimisation can be done as this test is applied in more different situations and the external validity of this test is increasing.

7.10. References

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Figure 7-1 (diagnostic test results table):

Challenge	Dose	Calf	G	H	T	0	0.5	0.75	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2 weeks	High	1	▨	▨	▨							X												
		2		▨	▨										●	●	●	●	●	●	●			
		3		▨	▨														●	●	●	●	●	●	●
		4		▨	▨								●	●	●	●	●	●	X	●	●	●	●	*
		5		▨	▨							●	●	●	●	●	●	●	●	●	●	●	●	*
	Low	6	▨	▨	▨																			●
		7		▨	▨																			
		8	▨	▨																				
		9	▨	▨																				●
		10																						
3 months	High	11	▨	▨	▨							●	●	●	●	●	●	●	●	●	●	●		
		12	▨	▨	▨																			
		13			▨							●	●	●	●	●	●	●	●			●		
		14	▨	▨																				
		15		▨	▨													●						
	Low	16	▨	▨	▨																			
		17	▨	▨																	●	●		
		18	▨	▨	▨																			
		19	▨	▨														●						
		20																						
6 months	High	21	▨	▨	▨														▨					
		22		▨																				
		23	▨	▨															●					
		24	▨				X																	
		25		▨								●	●	●	●	●								
	Low	26		▨																				
		27	▨	▨	▨																			
		28	▨	▨																				
		29	▨																					
		30	▨	▨	▨																			
9 months	High	31	▨							●	●	●	●	●										
		32	▨																					
		33	▨										●	●	●									
		34		▨																				
		35	▨																					
	Low	36		▨						●			●	●	●									
		37		▨						●	●	●	●	●	●									
		38		▨																				
		39	▨	▨											●	●								
		40	▨	▨																				
12 months	High	41	▨	▨	▨						●	●												
		42	▨	▨	▨																			
		43	▨	▨	▨																			
		44	▨		▨																			
		45		▨	▨																			
	Low	46	▨	▨	▨		●	●	●	●	●	●	●											
		47	▨	▨	▨							●												
		48	▨	▨	▨																			
		49	▨	▨	▨																			
		50	▨	▨																				

Figure 7-1. Diagnostic test results in 50 calves inoculated at 2 weeks, 3, 6, 9, or 12 months of age with either a high or a low dose of *Mycobacterium avium* subspecies *paratuberculosis*. Shading indicates a positive test result. G = gross lesions; H = histology; T = tissue culture A grey box indicates a fecal culture-positive, ● = ELISA-positive, X = a missing fecal sample; * = calf with clinical signs
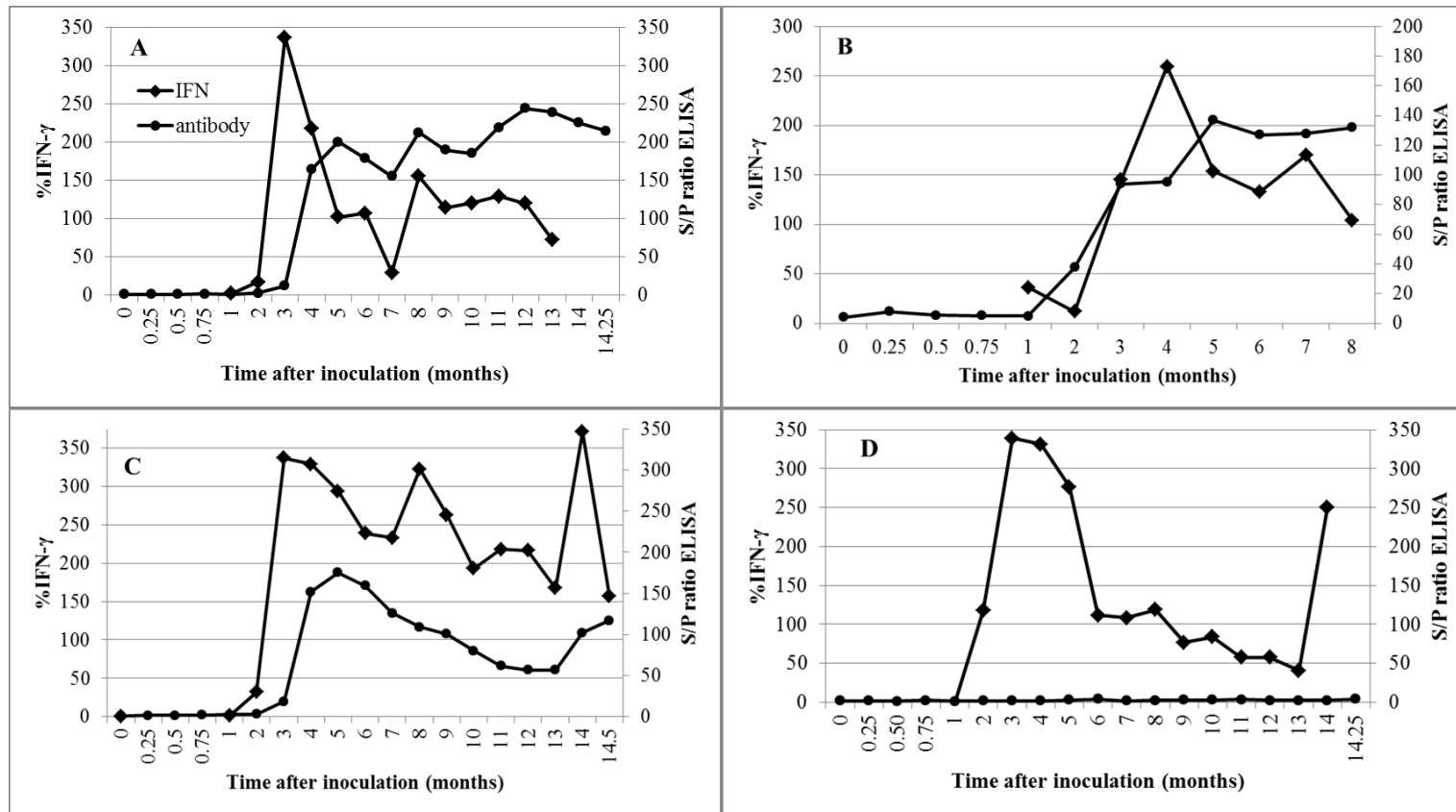
179

Figure 7-2. Immune response profiles as described by Begg et al. (2011): A "Classical switch profile"; B "Combined IFN-γ/antibody response"; C "Combined IFN-γ/antibody response", with a delayed onset of the antibody response; D "IFN- γ only".