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Detection of Johne's Disease on dairy farms using different qPCR target genes for *Mycobacterium avium* subsp. *paratuberculosis* in young stock

by

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A THESIS

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Abstract

Young stock can shed *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in feces, present antibody titers and transmit MAP to other young stock. However, most Johne's disease (JD) control programs do not include young stock in MAP testing strategies, which might be one of the reasons why only a few JD control programs were able to eradicate MAP. This study aimed to include young stock in a JD testing strategy and improve diagnostic tests. A literature review conducted reported that young stock can shed MAP as early as 4 mo of age. However, due to the chronic characteristic of the disease, it was considered important to improve current diagnostic tests and develop new tests, such as phage-based and metabolomics tests. A tentative inclusion of young stock in the MAP testing strategy was evaluated based on direct fecal qPCR and ELISA every 2 mo from animals between 2-12 mo of age. A sudden rise in MAP prevalence was detected at the second sampling, 2 mo after the start of the study. Although the high MAP prevalence was explained in part by the presence of MAP infections in the herd, it was not possible to explain the specificity of the ISMAP02 gene, which raises doubts about different *Mycobacterium* species being detected by the same assay. Furthermore, an in depth evaluation of the main MAP target genes for qPCR assays was proposed across different sample types and MAP concentrations. Overall, all MAP target genes were able to detect samples with high MAP concentration. IS900 and ISMAP02 consistently identified MAP in all sample types. However, the genes *mbtA*, *hspX* and F57 presented issues to detect samples with mid to low MAP concentrations.

Keywords: calves, cattle, diagnostic tests, *Mycobacterium avium* subsp. p*aratuberculosis*, Johne's disease

Preface

Three manuscripts are included in this MSc thesis. One manuscript has already been submitted for publication. Permission from all co-authors was obtained to include this manuscript in this thesis.

Chapter 2:

Martins, L., K. Orsel, R. Eshraghisamani, A. P. Koets, J. P. Bannantine, C. Ritter, D. F. Kelton, R. J. Whittington, M. F. Weber, A. Facciuolo, N. Dhand, K. Donat, S. Eisenberg, J. P. Kastelic, J. De Buck and H. W. Barkema. Advances in *Mycobacterium avium* subspecies *paratuberculosis* diagnostics and young stock testing may improve control of Johne's disease on dairy farms. *Manuscript submitted to Journal of Dairy Science as an Invited Review.*

Statement of Contribution

Chapter 2:

Larissa Martins (LM): delivered the objectives for the review, organized main research publications to be reviewed and drafted the manuscript with input from HB, KO and JDB.

Herman W. Barkema (HB): delivered additional objectives for the review, organized main research publications and worked on comments from external authors. HB also worked on all drafts of the manuscript.

Karin Orsel (KO): structured objectives, supported literature review, provided missing research publications and feedback on all drafts.

Jeroen De Buck (JDB): supported literature review, provided missing research publications, feedback on the drafts and expertise on new diagnostic tests for MAP detection in young stock.

John P. Kastelic (JK): provided expertise on research editing and missing topics for the review.

Razieh Eshraghisamani (RE): contributed with editing, inclusion of missing publications and expertise on new diagnostic tests for MAP detection in young stock.

Ad P. Koets, John P. Bannantine, Caroline Ritter, David F. Kelton, Richard J. Whittington, Maarten F. Weber, Antonio Facciuolo, Navneet Dhand, Karsten Donat and Susanne Eisenberg reviewed Chapter 2, contributed with editing, inclusion of missing publications, and provided additional expertise on recommendations on how producers can include young stock on JD testing strategies.

Chapter 3:

Larissa Martins (LM): delivered the objectives for the trial, validated diagnostic tests, enrolled the farm and coordinated all data collection.

Herman W. Barkema (HB): reviewed animal care protocol, supported study design and logistics of data collection and worked on all drafts of the manuscript.

Karin Orsel (KO): structured objectives, reviewed animal care protocol, supported design and logistics of field work and worked on all drafts of the manuscript.

Jeroen De Buck (JDB): supported the validation of diagnostic tests, design and logistics of field work and worked on all drafts of the manuscript.

Samita Shrestha (SS): supported the validation of diagnostic tests and tested all fecal and blood samples.

Julia Bodaneze (JB): provided expertise on study design and data collection on farm.

Berina Traub (BT): provided expertise on study design and data collection on farm.

Chapter 4:

Larissa Martins (LM): delivered the objectives for the study, validated diagnostic tests, designed and tested all plasmids for qPCR quantification. Larissa also enrolled the farms and coordinated all data collection.

Herman W. Barkema (HB): supported study design and logistics of data collection and worked on all drafts of the manuscript.

Karin Orsel (KO): structured objectives, supported study design and logistics of field work and worked on all drafts of the manuscript.

Jeroen De Buck (JDB): supported the validation of diagnostic tests and plasmid development for qPCR quantififcation, design and logistics of field work and worked on all drafts of the manuscript.

Samita Shrestha (SS): supported the validation of diagnostic tests, plasmid development. Samita also tested all environmental samples.

Ana Hernández Reyes (AHR): supported plasmid development, testing of standard curves and qPCR quantification expertise.

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Dedication

To all dairy producers and cows!

I really enjoyed working with you all and I will be back soon!

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List of abbreviations

Abbreviation	Definition
AF	Acid-fast
d	Day(s)
ELISA	Enzyme-linked immunosorbent assay
h	hour(s)
IAC	Internal Amplification Control
IFN-γ	Interferon gamma
IGRA	Interferon-gamma release assay
IL	Interleukin
JD	Johne's disease
LB/LA	Luria broth/agar
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
mo	Month(s)
µL	microliters
PI	Post infection
PPD	Purified protein derivative
qPCR	Quantitative polymerase chain reaction
Se	Sensitivity
Sp	Specificity
T	Time-point
TOPO cloning	Topoisomerase-based cloning

USDA	United States Department of Agriculture
wk	week(s)
yr	year(s)

Chapter 1: General Introduction

1.1 Bovine paratuberculosis

Paratuberculosis, commonly termed Johne's disease (JD), is an enteric disorder caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in wild and domesticated ruminants (Tiwari et al., 2006). Clinical signs include chronic diarrhea (Rathnaiah et al., 2017), weight loss and edema due to hypoproteinemia and protein losing enteropathy (Tiwari et al., 2006; Sweeney, 2011). However, most MAP infections in cattle remain subclinical, but cause reductions in milk production and fertility, and increased susceptibility to other infectious diseases such as mastitis (Pritchard et al., 2017).

Average herd-level MAP prevalence ranges from 20% in Europe (based on fecal culture, milk and serum ELISA; Nielsen and Toft, 2009) to 70% in United States (based on culture of environmental samples; Lombard et al., 2013), whereas in Canada the average herd-level prevalence was estimated at 46% (based on environmental culture; Corbett et al., 2018). The within-herd MAP prevalence is estimated to be between 2.6 and 5% based on fecal culture in United States (Lombard et al., 2006). Based on serology, the within-herd MAP prevalence was 7.1% in UK (Woodbine et al., 2009). However, serological information is not available for Canada.

1.1.1 Causative agent and host

MAP is a facultative intracellular, slow-growing, Gram-positive, acid-fast (AF) bacterium (Rathnaiah et al., 2017; Tiwari et al., 2006). Characteristics distinguishing MAP from other *Mycobacterium* species are the mycobactin requirement for growth (Rathnaiah et al., 2017) and the MAP target genes IS900 and F57 (Slana et al., 2008). In addition, ISMAP02 is another insertion element with multiple copies that is reported to be specific to MAP (Irenge et al., 2009).

After being ingested orally, MAP enters the intestinal wall, in the ileum region, through M cells in Peyer's patches (Momotani et al., 1988) and is engulfed by macrophages (Tessema et al., 2011). At this stage, there are no visible signs of an infection caused by MAP. Macrophages carrying the bacterium disseminate the infection to other lymph nodes where MAP can initiate inflammatory responses (Chiodini et al., 1984).

Research evaluating age susceptibility concluded that young stock are more prone to develop MAP infection, as the intestinal walls are more permeable (Sweeney, 1996), and due to the lower volume of the ruminal contents (Windsor and Whittington), which allows a higher MAP uptake and MAP infection. During the first few months of life, young stock are highly susceptible to MAP infection, with resistance being developed by 12 mo of age (Arsenault et al. 2014).

Natural hosts for MAP are domestic and wild ruminants, such as cattle, sheep, goats, red deer, cervids and camelids (Tiwari et al., 2006). MAP infection has been documented in wildlife, such as especially non-ruminants, including fox, weasel, crow, rat, wood mouse, rabbit, hare, and badger (Beard et al., 2001).

1.1.2 Routes of transmission

MAP is mainly transmitted fecal-orally. Within-herd transmission of MAP is through milk or colostrum, or through intrauterine or environmental transmission. Furthermore, between-herd transmission risk is associated with cattle purchases (usually without knowledge of MAP infection in the herd) as well as environmental contamination (Barkema et al., 2018a). Cow-to calf transmission is often due to MAP in milk or colostrum used to feed calves (Stabel et al., 2014; Steuer et al., 2020), or as a result of interactions between cow and calf after calving (Cashman et al., 2008). Intrauterine transmission of MAP should also be consider. For cows in a subclinical or clinical phase of MAP infection, 9 and 39% of their newborn calves, respectively, were infected with MAP (Whittington and Windsor, 2009).

Shedding of MAP in milk and feces contaminates the environment (Smith et al., 2011), with MAP being resistant to various environmental conditions. Survival of MAP strains in soil, in the presence or absence of sunlight, ranged from 1 to 16 wks (Eppleston et al., 2014), whereas MAP was viable for 55 wks in a dry, shaded environment (Whittington et al., 2004).

Calf-to-calf MAP transmission has been reported in experimental settings (Corbett et al., 2017). The basic reproduction ratio was estimated as 3.24 (1.41-7.41) new infections per MAP-infected calf; therefore, 1 MAP-infected calf infected approximately 3 non-infected pen mates (Corbett et al., 2019). Between-herd transmission is also reported. For example, the odds of MAP-positive environmental sample results was related to introduction of infected animals into the herd (Pillars et al., 2009). If cattle are in the subclinical stages of MAP infection at the time of introduction, they will already be shedding MAP into the environment (Rangel et al., 2015).

1.1.3 Economic impact of JD on dairy herds

Direct and indirect economic losses as a result of MAP infection are caused by decreased milk production, reduced feed conversion efficiency, early culling, increased predisposition to other diseases, and expenses related to veterinary costs and ultimately replacement (Hasonova and Pavlik, 2006; Garcia and Shalloo, 2015). A reduction in milk yield of 1.87 kg/d was reported in cows positive to MAP using fecal culture or PCR (McAloon et al., 2016). Recently, Ozsvari et al. (2020) reported a milk yield reduction of 4.6 kg in ELISA-positive compared to ELISA-negative cows, considering 305 d of milk production. Growth and slaughter value are also negatively affected by MAP infection (Roy et al., 2017). In Canada, 25% of MAP-positive animals had lower slaughter value (Tiwari et al., 2008), whereas Kudahl and Nielsen, (2009) reported 31% slaughter value reduction for MAP-positive animals (based on fecal culture). In addition, a study in Australian dairy herds reported losses of US$278 per year related to premature culling (Shephard et al., 2016).

Based on Markov Chain Monte Carlo modeling, economic losses for the dairy industry in United Sates were reported as US$198 million due to JD, whereas in Canada, economic losses were between US$17 and US$28 million (Rasmussen et al., 2021). In Europe, results from Germany and France reported economic losses of US$75 and US$56 million, respectively (Rasmussen et al., 2021). According to Rasmussen et al. (2021), differences in economic losses between countries or specific regions can be attributed to: 1) overall MAP prevalence; 2) within-herd MAP prevalence increasing or decreasing over time; 3) farm factors, such as milk production per cow and farm-gate prices; 4) influence of MAP infection on production losses due to various MAP prevalences; and 5) the dairy industry system of each country.

1.2 Diagnostics

Diagnostic tests to detect MAP-infected cattle are categorized based on methods to identify the pathogen based on bacterial culture of tissue or feces, or molecular tests. To detect host responses, various methods are used, including clinical signs, gross and microscopic pathology, as well as immunological diagnostic tests, e.g., ELISA and IGRA (Tiwari et al., 2006).

1.2.1 MAP culture of tissue and feces

Tissue culture is considered the gold standard for detection of MAP infection, especially in the following tissues: mid-jejunum, mid-jejunum lymph nodes, distal jejunum, mid-ileum, distal ileum, ileal lymph nodes, ileo-cecal valves, and ileo-cecal lymph nodes (Tiwari et al., 2006). As tissue samples can, in general, only be collected on dead animals, fecal samples are used for disease detection in live animals. Therefore, fecal culture is defined as the reference test in many studies (Leite et al., 2013; Rasool et al., 2017). Although fecal culture is now considered the reference test, the sensitivity (Se) is relatively low (38%) (Whitlock et al., 2000), and results are only available after 50 d due to the slow growth of MAP (Rathnaiah et al., 2017). Additionally, there are issues with contamination due to other microorganisms with faster growth (Whittington, 2009). Therefore, after confirmation of growth, morphologic evaluation and AF staining or PCR to detect the insertion sequences IS900 and F57 are necessary to confirm that growth is indeed MAP (Collins, 1996; Slana et al., 2008). Due to high costs of individual fecal culture, pooled fecal samples have been applied as an alternative surveillance method (McKenna et al., 2018). A 2018 study evaluated pools of 3, 5, 8, 10, and 15 cows; crude Se ranged from 39% for pools of 15 to 59% for pools of 10 animals (McKenna et al., 2018). Environmental

sampling is another strategy to detect MAP-positive herds (Lombard et al., 2006; Wolf et al., 2015a). When MAP culture methods were followed by F57 PCR to assess environmental samples, Se and specificity (Sp) were 38 and 100%, respectively (Corbett et al., 2018).

1.2.2 Direct detection by PCR

Rapid and accurate MAP detection is an important component of JD control programs (Clark et al., 2008). Based on issues with MAP culture as mentioned in Section 1.2.1, development of more efficient and sensitive diagnostic tests are needed to detect herd-level or within-herd prevalence of MAP (Whittington et al., 2000).

Use of fecal PCR or quantitative PCR without prior application of fecal culture would markedly decrease the time required for detection of MAP. The Se and Sp of direct fecal PCR were 70 and 100% in randomly-selected cows (Clark et al., 2008; Logar et al., 2012), respectively. In a comparison of 3 direct PCR kits on feces, Se ranged from 73.5 to 93%, whereas Sp ranged from 97 to 100% (Prendergast et al., 2018). Although studies reported good results using direct PCR to detect MAP, considerations regarding MAP-target genes and their number of copies in the MAP genome should be addressed, as MAP-target genes with higher copy numbers (e.g., target gene IS900) have a higher Se of MAP detection compared to other target genes (Clark et al., 2008). The majority of PCR and qPCR assays available use IS900 as their main target gene (Herthnek and Bölske, 2006) due to the multicopy characteristic (17 copies; Li et al., 2005). However, IS900-like genes are present in other Mycobacterium species, e.g., *Mycobacterium porcinum* (Taddei et al., 2008), *Mycobacterium cookii* (Englund et al., 2002) and *Mycobacterium scrofulaceum* (Cousins et al., 1999). Consequently, the IS900 target gene can yield false-positive results, when used as the main target gene for MAP detection. The

target gene F57 is also widely used for MAP detection. However, this gene has only a single copy in the MAP genome. Therefore, PCR using the F57 target gene is considered less sensitive than the IS900 target gene (Ricchi et al., 2016). Donaghy et al. (2011) reported the high Se of IS900 compared to the F57 target gene. The IS900 target gene detected as little as 1 MAP genome, whereas the target gene F57 needed at least 4 copies of the MAP genome, due to its single-copy status (Ellingson et al., 1998).

Although IS900 and F57 are widely used in MAP detection, it is important to address discrepancies in Sp of IS900 and Se of F57. Evaluation of other MAP-specific genes in various sample types are potential alternatives for more accurate MAP detection.

1.2.3 Gross and microscopic pathology

During the clinical stages of MAP infection, cattle generally present with emaciation, as well as fat atrophy and fluid accumulation (e.g., edema under the jaw). Macroscopic lesions are common in ileum, cecum, colon, and lymph nodes (OIE, 2014). However, not all affected cattle have meaningful gross lesions that are suggestive of MAP infection (Whittington et al., 2017).

Microscopic lesions associated with MAP infection included the presence of large macrophages with granular cytoplasm in the intestinal mucosa and submucosa, as well as lymphatics and mesenteric lymph nodes (OIE, 2014). Aggregations of macrophages may also include lymphocytes, eosinophils, and neutrophils (Tafti et al., 2000). Several scoring systems have been developed to classify these lesions. For instance, Perez et al. (1996) uses a scale that ranges from 1 – (mid focal), 2 – (focal) to 3a – (multifocal), and to 3b – (multifocal to diffuse, multibacillary) or 3c – (multifocal to diffuse, paucibacillary) based on the development and extension of inflammation.

1.2.4 Immune assays

Various ELISAs are widely used to detect immunological responses against MAP. However, due to the prolonged interval between MAP infection and a detectable immune response, the Se of ELISAs is compromised and false-negative results are common (Tiwari et al., 2006). Milk ELISA is not widely recommended, due to low Se (7 to 22%), attributed to variations in antibody titers, which are affected by milk production, parity and days in milk (Nielsen and Toft, 2008; Eisenberg et al., 2015; Laurin et al., 2017).

Interferon-gamma release assay (IGRA) detects early immune response against MAP infection. Efforts are underway to improve IGRA, especially regarding the ability of antigens to elicit a Th1 response and their potential to detect MAP in the early course of the disease and to identify the optimal time to use IGRA to maximize its diagnostic value (Mortier et al., 2014b; Hughes et al., 2017). The use of a cocktail of antigens and development of recombinant antigens to improve the Se of IGRA have also been proposed, as MAP-infected animals have variable responses to the same antigen (Hughes et al., 2017). Additional efforts to improve the assay were associated with T-cell stimulation with interleukin (IL), IL7 and IL12; this may increase duration of T-cell viable, as required for this test (Plain et al., 2012). Currently, an IGRA must be set up within 6-8 h after blood collection to obtain reliable results, based on viability of immune cells (Stabel, 1996; Jungersen et al., 2002).

Other considerations regarding IGRA are its inability to differentiate between latent and active MAP infections, as well as the numerous antigens shared between MAP and other mycobacterial species affecting test reliability and applicability (Nielsen and Toft, 2008; Mortier et al., 2014b).

1.3 Control of JD

1.3.1 Early efforts of JD control

Johne's disease control programs started in France in the 1920s using vaccination strategies, followed by the Netherlands in 1942, where cattle were tested using the Johnin allergy test and fecal culture. The majority of the subsequent JD control programs were based on vaccination (e.g., Iceland in the 1950s, Great Britain in 1964, Norway in 1967, and Cyprus in the 1970s). In addition, the United States also started a JD control program in several states based on different diagnostic tests and management strategies. Furthermore, the JD control program in Australia started in the 1990s and was based on animal movement control and immunological tests (Benedictus et al., 2000).

1.3.2 Control by testing and culling

Test and cull strategies to reduce MAP prevalence were often evaluated using disease transmission models (Marcé et al., 2010). A simulation model described by Collins and Morgan (1992) reported effects of test and culling strategies to reduce MAP prevalence. Diagnostic tests with Se > 70% reduced MAP prevalence to < 1% in 10 yrs. A simulation model described by Lu et al. (2010) reported a basic reproduction ratio < 1 using fecal culture and culling strategies.

Although simulation model studies reported testing and culling strategies as a potential method to reduce MAP prevalence, this strategy is not sufficient to eradicate MAP infections, as new MAP infections will occur due to residual MAP-positive animals in the herd as a

consequence of insufficient test Se, disease biology, and a lack of management strategies to prevent new MAP infections (Al-Mamun et al., 2017).

1.3.3 Control by testing and culling combined with management strategies

Control by testing and culling combined with management strategies was studied in both modeling and experimental settings. According to modeling, effectiveness of JD control programs was associated with adoption of management strategies. Groenendaal et al. (2002) reported a reduction of MAP prevalence and a financial advantage (US$ 29,196 over 20 yrs), based on net benefits minus costs, after improvements in heifer management, considering 50-cow dairy farm and increasing the herd size to 100 cows in 20 years. Furthermore, when improvements were associated with better heifer and calf management, economic benefit increased to US$ 43,917. Camanes et al. (2018) recently provided modeling information regarding the need to implement calf management to minimize calf contact with manure from adult cattle, in addition to test and cull actions to reduce MAP prevalence.

Collins et al. (2010) implemented a JD control program on 9 dairy herds in the United States following testing and culling strategies supported by management changes, including: individual calving pens; cow-calf separation within 24 h after calving; improvements in colostrum and milk management, separation of adult and young cattle, and culling of MAP-positive cattle and heifers from ELISA-positive cows. After 6 yrs of JD control, the MAP prevalence decreased from 11.6 to 5.6% based on serum ELISA (considering all 9 herds). In a JD control program on 105 dairy farms in Germany over 6 yrs, individual fecal samples were collected from cattle > 24 mo and subjected to culture followed by IS900 PCR. Management changes, such as control of incoming and outgoing animals from the herd, hygiene measures for

maternity and calf pens, and colostrum management were implemented in combination with test and culling strategies. The within-herd prevalence was reduced from 14.5 to 5.6% (Donat, 2016).

Moreover, some countries also adopted the JD control by testing and culling in addition to management strategies. For instance, the JD control program in Denmark, initiated in 2006, is a voluntary JD control program based on RA (risk assessment). Herds that join the program commit to test (milk ELISA) all milking cows four times annually. This program is funded by farmers interested in the program (Nielsen et al., 2007). Since 1998, the Netherlands has implemented a JD control program that focused on low risk of MAP transmission between herds through animal trade. Additionally, the country established a Milk Quality Assurance Program in 2006, which specifically targets the reduction of MAP concentration in milk (Gerathy et al., 2014; Weber and Schaik, 2007).

1.3.4 Other approaches of JD control

Vaccination is an alternative to eradicate MAP. Recent results from the Australian ovine JD control program reported 70% vaccine coverage and the prevalence of lesions characteristic of JD at slaughter reduced from 2.4 to 0.8% in 9 years (Links et al., 2021). Although vaccines against MAP are effective in preventing clinical JD in dairy cattle, they are not as effective as the ovine vaccines. Also, differentiation of MAP-infected from vaccinated cattle is not possible, as the vaccines are not DIVA (differentiating infected from vaccinated animals). Also, JD vaccines can interfere with serological tests to detect *M. bovis* immune response, which can affect tuberculosis control programs (Patton et al., 2011). Finally, vaccination does not efficiently prevent MAP infection (Bastida and Juste, 2011). In summary, advancements to develop more

efficient and DIVA vaccines and to reduce cross-reactivity with *M. bovis* serological tests are necessary before implementation of vaccines to control new MAP infections in dairy herds.

Despite implementation of voluntary or mandatory JD control programs in many countries, only a few have been successful in achieving MAP eradication. Sweden and Norway stand out as the only countries to have achieved JD-free status since 2008 (Whittington et al., 2019). In Canada, regional JD control programs were in place, with high participation of producers and veterinarians. Despite many efforts in Canada, herd-level MAP prevalence remains high, at 46% (Corbett et al., 2018). A potential reason for the limited success of the JD control programs is the difficulty to sustain producer engagement with testing and management strategies (Barkema et al., 2018b). Another contributing factor might be the low Se of MAP diagnostic tests in low-prevalence herds, as well as exclusion of young stock from MAP testing strategies.

1.4 Transmission and infection of MAP in young stock

The subclinical stages of MAP infection in ruminants are related to intermittent MAP shedding in feces (Collins, 1996) and initial cell-mediated versus humoral responses, which acts as a protective effect and delays the detection of antibody titres based on ELISA. As a result, disease characteristics in calves greatly affect Se of fecal culture and ELISA (Vazquez et al., 2013).

Although the JD progression in calves results in a low Se for ELISA and fecal culture, observational studies detected fecal culture-positive results in naturally infected heifers, especially those > 7 mo. Wolf et al. (2015b) reported that 1.2% of calves or heifers were MAP-

positive before first calving in herds with at least 1 MAP-positive environmental sample with protocols using direct DNA extraction, followed by qPCR and TREK ESP culture (Thermofisher Scientific).

Shedding in calves were also detected in experimental infection studies with calves of various ages that were challenged with a variety of doses. Calves infected with a high dose of MAP presented the majority of MAP fecal shedding events within 2 mo after experimental infection (Mortier et al., 2014a; Corbett et al., 2018).

Each MAP-infected calf in group pens infected on average ~3 non-infected pen mates, whereas ~2% of calves were shedding MAP at any time (Corbett et al., 2017). Removing the MAP inoculation effect, the reproduction ratio associated with MAP transmission estimated with R_0 through feces was significantly > 1 (3.24; 1.41-7.41); therefore, MAP infections can result in outbreaks based on this transmission parameter (Corbett et al., 2019).

Based on previous studies regarding MAP early shedding and transmission between calves and the low Se of the current diagnostic tests for earlier stages of the disease, development of new diagnostic tests and surveillance strategies focused on calf transmission are necessary to detect positive calves in order to separate them from negative calves and therefore hasten progress in JD control and eradication programs.

1.5 Thesis rationale and summary

Although JD control programs have been implemented since the 1920s (Benedictus et al., 2000), reports of MAP eradication in dairy herds are scarce. Herd-level prevalence remains high in countries with developed dairy industries, including Canada (46%) and the United States

(70%) (Lombard et al., 2013; Corbett et al., 2018). Consequently, it is necessary to explore other alternatives to detect and control new MAP infections (Barkema et al., 2018a).

A direct qPCR assay with sufficient Se and Sp would be able to more efficiently identify cattle that are high MAP shedders and mid-low shedders, providing an economical alternative for MAP testing that would help producers make earlier and better decisions (Clark et al., 2008). The limited success of JD control programs to eradicate MAP infection in dairy herds could also be explained by the exclusion of young stock from the MAP testing strategy due to the low Se of current diagnostic tests. However, MAP shedding and transmission among calves appears to be important (Wolf et al., 2015b; Corbett et al., 2017), which likely causes new MAP infections. Consequently, evaluation and development of new testing strategies during early stages of MAP infection would support detection of MAP infection in calves.

This thesis, therefore, aims to include calves in a JD testing strategy and to improve diagnostic tests. Better understanding of why calves are not currently included in JD testing strategies and alternatives to MAP testing would be beneficial. In addition, evaluation of various qPCR target genes to improve Se and Sp of direct qPCR has potential to make MAP testing faster and cheaper.

Chapter 2 includes a literature review to evaluate advances in MAP diagnostics and young stock testing that have potential to improve control of JD on dairy farms.

In Chapter 3, the aims of the study were, to 1) explore the challenges of testing young stock for MAP, 2) examine the sudden rise in MAP prevalence observed in one farm, and 3) evaluate whether this sudden rise in qPCR positivity was the result of farm and/or laboratory

contamination with MAP, or presence of other *Mycobacterium* species cross-reacting with MAP qPCR targeting ISMAP02 gene. We hypothesized that ISMAP02 MAP-target gene is not specific to MAP, and that it might be associated with false-positive results on qPCR for MAP.

In Chapter 4, the aim of the study was to evaluate the accuracy of different MAP qPCR target genes to detect MAP in different sample types. We hypothesized that: 1) MAP-target genes correctly identify MAP-positive farms and animals if they have high MAP concentration or if they are negative; and 2) MAP-target genes misclassified MAP-positive farms or animals if they have mid-low MAP concentrations.

1.6 References

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CHAPTER 2. Advances in *Mycobacterium avium* subsp. *paratuberculosis* diagnostics and young stock testing may improve control of Johne's disease on dairy farms

2.1 Abstract

Many Johne's disease (JD) control programs have been implemented worldwide, but few have successfully eradicated *Mycobacterium avium* subsp. *paratuberculosis* (MAP). This limited success may be partly due to exclusion of young stock from most MAP testing strategies. Young stock (calves and replacement heifers or bulls) can shed MAP in feces and have antibody titers, as confirmed in experimentally or naturally infected cattle. Furthermore, there is potential MAP transmission between young stock. Calves and heifers are often included in JD management strategies on dairy farms but often excluded from MAP testing due to the lag between infection and detection of MAP shedding and antibody titers with conventional diagnostic tests. We summarize evidence of MAP shedding early in the course of infection and discuss promising diagnostics, testing and management strategies to support inclusion of young stock in JD control programs. Improvements in fecal PCR, IGRA and ELISA enable earlier detection of MAP and specific early immune responses. For instance, studies on IGRA and ELISA have focused on evaluation of new antigens and optimal age of testing. There are new diagnostics, including phage-based tests to detect viable MAP and gene expression patterns and metabolomics to detect MAP-infected young stock. In addition, refinements in testing and management of calves and heifers may enable reductions in MAP prevalence. In summary, although transmission between young stock may cause new MAP infections, use of new diagnostic tests and testing strategies for young stock should improve JD control programs.

Key words: paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis*, new diagnostics, calves

2.2 Introduction

Johne's disease (JD) or paratuberculosis is a chronic granulomatous enteritis caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a slow-growing Gram-positive bacterium (Thorel et al., 1990). This disease is common in domestic and wild ruminants (Whittington et al., 2019). During subclinical stages, cattle infected with MAP may transmit the bacterium to herd mates by shedding MAP in feces or milk (Collins et al., 2010).

Herd-level prevalence of MAP depends on geographical location, the diagnostic test used, and interpretation of test results. Regardless, true herd-level MAP prevalence in most developed countries is likely > 50% of dairy herds, based on individual fecal culture, milk and serum ELISA of a subset of individual cows, or environmental sampling (Nielsen and Toft, 2009; Lombard et al., 2013). Reported average within-herd prevalence in MAP-infected herds ranged from 2.6 to 9.6%, based on fecal culture methodologies, geographical location and diagnostic test used (Lombard et al., 2006; Donat et al., 2014). Infected herds can, however, have a very low apparent and true MAP prevalence and no shedders, whereas herds with > 50% shedders have also been reported (Donat et al., 2014). Economic impacts of MAP infection on dairy farms include reduced milk production and fertility; increases in SCC, incidence of clinical mastitis (Wilson et al., 1995; Pritchard et al., 2017; Rossi et al., 2017), incidence of other infectious diseases, and mortality and cull rates (Ott et al., 1999; Hendrick et al., 2005; Lombard et al.,

2005; Raizman et al., 2009; Smith et al., 2010), but reduced slaughter value (Kudahl and Nielsen, 2009; Liang et al., 2017; Pritchard et al., 2017; Rossi et al., 2017).

Programs to control and eradicate JD have been implemented to reduce economic losses, improve animal welfare, and mitigate potential hazards of MAP to public health (Whittington et al., 2019). Overall, these programs aim(ed) to establish regions with low incidence of new MAP infections and ultimately achieve zero MAP prevalence (Geraghty et al., 2014; Barkema et al., 2018a). Components of JD programs include confirming clinical JD cases and estimating numbers of infected animals on farms or in specific regions. Further, most programs focus on preventing transmission of MAP through testing and culling sero- and fecal culture-positive cattle, and management practices such as separating dams from calves, preventing exposure of calves to feces of adult cows, and decreasing prevalence in bulk tank milk samples (Wolf et al., 2014; Barkema et al., 2018a). Consequently, diagnostic tests are core to detecting infected herds and infectious animals to take appropriate actions to achieve success in JD control and eradication programs. The testing strategy is usually accompanied by biosecurity measures to reduce the likelihood of new infections due to exposure of susceptible animals (particularly young stock) to infectious MAP-positive animals. Although formal JD control programs are present in 22 countries (Whittington et al., 2019), it has been very difficult to achieve zero MAP prevalence in a dairy herd through JD control programs that focus on eradication. Only 2 studies reported MAP eradication; in 2015, Norway reported the last MAP-infected animal, including eradication of MAP infection in goats, whereas Sweden was JD-free since 2008 (Whittington et al., 2019).

Potential reasons for limited success of JD control programs to achieve zero MAP prevalence are the lack of a diagnostic test with high Se and consequently high negative

predictive value of a MAP infection, especially in low-prevalence herds (McKenna et al., 2005), plus exclusion of young stock from MAP testing strategies due to costs and assumed low test Se in young calves. Young stock usually excrete MAP in feces less frequently than older cattle and typically do not have detectable MAP-specific antibody titers to antigens in available diagnostic tests, as they are in the early stages of MAP infection (Tiwari et al., 2006). Although detection of MAP in feces of calves and heifers using available testing methods is difficult due to intermittent low-dose shedding, on MAP-infected farms, up to 4% of young stock were identified as shedding MAP (Wolf et al., 2015). Furthermore, in high-prevalence dairy herds, the proportion of cattle with onset of shedding prior to 2 yrs of age was estimated at 20% (Weber et al., 2010). Although most current JD control and eradication programs exclude testing young stock, transmission of MAP between young stock can cause new MAP infections and hamper control strategies (van Roermund et al., 2007; Weber and Groenendaal, 2012; Corbett et al., 2017; Barkema et al., 2018a; Corbett et al., 2019). Including young stock in existing testing schemes as well as development of more sensitive diagnostic tests for early disease stages and new testing strategies would enhance detection of MAP-infected young stock and enable assessment of contributions of MAP-infected calves and heifers to the within-herd incidence and prevalence of MAP infection. The objective of this narrative review is to provide an overview of advances in diagnostics and management control strategies to support inclusion of young stock in effective testing strategies. Based on the evidence as it pertains to the delineated scope, we provide recommendations for dairy farmers, researchers, veterinarians, and other stakeholders that may improve JD control programs.

2.3 MAP shedding and transmission among young stock

Shedding patterns were defined in experimentally infected young stock. Cattle are highly susceptible to MAP infections until at least 1 year of age (Mortier et al., 2015; Windsor and Whittington, 2010). In a challenge experiment lasting until 18 mo of age, young stock experimentally exposed to MAP at 2 wks and 3 mo of age shed MAP more regularly, with stronger immune response and more severe lesions, compared to young stock inoculated at an older age (Mortier et al., 2014a; b). Additionally, young animals experimentally infected with a high dose of MAP shed frequently, especially 2 mo after experimental infection (Mortier et al., 2014a). This implies that exposure to a higher MAP concentration at a younger age can accelerate disease progression and calves will likely test MAP-positive at an earlier age (Bolton et al., 2011; Mortier et al., 2014a). Interestingly, as gamma-delta T-cells are key early responders to MAP infection of young stock, colostrum may promote gamma-delta T-cell production (Krueger et al., 2016).

The very nature of the early stages of MAP infection contributes to the low Se of current diagnostic tests (antibody ELISA and fecal culture), due to undetectable titers of MAP-specific serum antibodies (in contrast to humoral immunity in the gut (Facciuolo et al., 2016)) during early stages of infection, due to delays in humoral immunity and intermittent fecal shedding (Tiwari et al., 2006). Regardless, in a cross-sectional study, 2% of young stock in naturally infected dairy herds were fecal culture-positive and likely infectious (Wolf et al., 2015). Pens with young stock from 6-12 mo of age had higher odds of having MAP culture-positive environmental samples compared to pens with young stock < 3 mo of age (Wolf et al., 2015). Using an ESP II protocol for fecal culture, 2% of samples from young stock were MAP-positive

at any given moment, including 0.6, 4 and 3% of samples from young stock 0-6, 7-14 and 15-24 mo of age, respectively (Bolton et al., 2011). Similarly, 3% of 8 mo old young stock were MAP culture-positive based on Herrold's egg yolk solid culture medium (Antognoli et al., 2007). In these 3 studies, infectious young stock naturally infected with MAP was detected between 8 and 12 mo of age.

MAP shedding by MAP-positive young stock infects pen mates. Van Roermund et al. (2007) reported transmission of MAP between young stock at a reproduction ratio of 0.9 (0.1-3.2) new infections per MAP-infected calf (Table 2.8.1). More recently, Corbett et al. (2017) reported that each MAP-infected young stock housed in group pens infected on average 3 non-infected pen mates. Considering MAP transmission among young stock, the reproduction ratio associated with MAP transmission was significantly > 1 (3.2; 95% CI 1.4-7.4) (Corbett et al., 2019), highlighting the importance of MAP transmission between young stock. In total, 2% of young stock were shedding MAP at any given time, a shedding prevalence similar to that reported in commercial dairy herds (Antognoli et al., 2007; Bolton et al., 2011; Wolf et al., 2015) (Table 2.8.2).

2.4 Diagnostic tests to detect MAP-infected and infectious young stock

As indicated above, testing young stock for detection of MAP or an immune response to an infection with MAP has been challenging due to low Se, albeit not because of poor-quality tests but due to biology of the disease. Detection of new MAP infections in young stock therefore requires improvement of existing diagnostic tests or development of new tests with sufficiently high Se and Sp during early stages of MAP infection (Barkema et al., 2018a). This is challenging

as young stock only shed a low dose of MAP intermittently and development of antibodies is delayed after infection.

JD tests can be divided into those that detect MAP (direct tests) and those that test for a host response or biomarkers (indirect tests). In contrast to direct tests, results of an indirect test indicate whether or not at some point in time there has been contact between MAP and the host which has led to an immune (or biomarker) response. As immune responses in essence are aimed at eliminating pathogens and restoring homeostasis, a measurable effector response (T-cell or B-cell/Immunoglobulins) may lead to elimination of the pathogen and/or a level of control preventing the animal from becoming infectious. If these animals do not shed and therefore fail to become infectious, there is no need to remove them from the herd to achieve herd level eradication. Alternatively, the immune or biomarker response may not lead to elimination and control but rather the animal becomes infectious, transmitting disease. Currently we cannot distinguish these 2 options (Table 2.8.3).

In addition, these tests are most often evaluated using experimentally infected animals; however, these models commonly use high single-point exposures and likely do not mimic on-farm exposure (continuous low level exposure). Therefore, proper validation in controlled field setting is essential for correct interpretation of these tests (Table 2.8.3).

2.4.1 Direct tests (MAP detection)

2.4.1.1 Fecal culture

In experimental studies the first MAP-positive fecal results occurred within 6 mo after MAP inoculation, with a peak at 2 mo (Mortier et al., 2014a). In naturally MAP-infected animals, Bolton et al. (2011) detected MAP-positive young stock from 0 to 14 mo of age.

However, 83% of the MAP-positive young stock were detected in animals > 6 mo of age, with the majority of the samples from young stock that were 7 to 14 mo old. Fecal shedding is not only depending on age but also depends on infectious dose (Mortier et al., 2014a).

An important limitation is that the method used to process feces significantly reduces MAP bacterial load; therefore, it may miss animals at the threshold level of detection. For instance, culture protocols for MAP in sheep decreased the number of MAP detected by 2.7 and 3.0 log for feces and tissues, respectively (Reddacliff et al., 2003).

An additional consideration in performing manure-based diagnostic tests is viability of the MAP bacterium, especially as samples are archived at cold or ultra-cold temperatures before processing. According to Raizman et al. (2011), the bacterial load decreased 13% per month in samples stored at -18 and -70°C from high to moderate-low MAP concentrations and from moderate-low to negative MAP concentration. In that study, temperature and effects of thawing and refreezing times were not significant. Storage of fecal samples in -20°C was recommended in cases of no access to -80°C freezers (Raizman et al., 2011) but not encouraged as an alternative to -80°C. For field work (veterinarians and producers), it was recommended to keep fecal samples at 4°C for at $\leq$ 2 d, with more prolonged storage at -70°C, as storage at -20°C had negative effects (Khare et al., 2008). In addition, Se of DNA extraction from feces was higher with samples stored at -80°C versus at -20°C (Plain et al., 2014).

Pooling. Due to additional costs to include young stock in the JD testing strategies, potential testing alternatives should be considered. The implementation of pooled fecal samples from different animals (Bolton et al., 2011) or pooled samples from the same animal over time (Hines Ii et al., 2007; Mortier et al., 2014c) are potential testing strategies to reduce cost of testing. However, studies on optimal testing frequency and number of animals included in fecal

pools should be addressed. In addition, most studies detecting MAP in naturally infected young stock collected fecal samples at most twice from the same animals (Weber et al., 2010) (Table 2.8.2). However, based on experimental studies in young stock, shedding occurs very sporadically (Mortier et al., 2014a). As a result, more fecal samples from the same animal (e.g., at least every 2 mo) may be more efficient to detect MAP in young stock. To reduce costs of testing, direct DNA extraction from feces followed by qPCR could be adopted instead of culture methods before qPCR, although this might reduce Se.

Environmental sampling. Environmental sampling may indicate potential exposure and transmission risks in certain farm areas and or buildings, especially those where susceptible young stock are housed (Eisenberg et al., 2012). This strategy can be considered as an alternative for pooling strategies, and it is easy to perform in a cost-effective manner (Field et al., 2023).

2.4.1.2 Direct PCR on feces

The use of fecal PCR or quantitative PCR without prior application of fecal culture is a diagnostic tool that overcomes the prolonged interval to culture and detect MAP. Depending on the number of samples, direct DNA extraction from feces followed by PCR can be done in 1 day. Sensitivity and Sp of direct fecal PCR compared to fecal culture were reported as 70 and 100%, respectively (Clark et al., 2008; Logar et al., 2012). In an evaluation of 3 direct PCR kits on feces, Se ranged from 74 to 93%, whereas Sp ranged from 97 to 100% (Prendergast et al., 2018).

Considering the initial recommendation of testing young stock every 2 mo, this method could be an option to provide faster and reliable testing results to producers, as most culture protocols take 8 to 16 wks (Clark et al., 2008). In addition, based on low and intermittent

shedding of MAP in young stock, direct DNA extraction from feces would avoid losses of MAP due to pre-culture decontamination protocols (Reddacliff et al., 2003).

Although direct DNA extraction from feces might be more efficient to detect MAP in young stock, it is important to be aware of challenges related to PCR inhibition factors in feces and presence of other microorganisms, which can impact direct MAP detection (Acharya et al., 2017; Fock-Chow-Tho et al., 2017). Moreover, it is important to consider the PCR target gene used for MAP detection in young stock. Based on the low MAP shedding of young stock, MAP-specific genes, such as F57 may generate false-negative results due to the single copy number characteristic of this gene (Ellingson et al., 1998).

2.4.1.3 Phage technology as a potential diagnostic test for detecting MAP-shedding calves

Phage technology is a test that may be applicable under field conditions but requires further evaluation considering naturally MAP-infected young stock. A rapid phage-based diagnostic test was developed to detect MAP and *Mycobacterium bovis* (PBD Biotech, Suffolk, UK). In humans with tuberculosis, Se and Sp of this phage test were 73 and 94%, respectively (Verma et al., 2020). Diagnostic tests involving mycobacteriophages have been used to detect viable MAP in various sample types, including milk, infant formula, cheese, bovine blood, and feces (Botsaris et al., 2010, 2013, 2016; Foddai et al., 2011; Swift et al., 2016). Lytic mycobacteriophages replicate within viable MAP cells resulting cell lysis and release of MAP DNA that can be detected with conventional PCR (Swift and Rees, 2019). Success of the phage assay depends on MAP cell viability, as mycobacterial metabolism promotes better phage replication (Grant, 2021).

In cattle, the phage test was reported having a limit of detection of 10 cells for MAP and *M. bovis*, whereas Se was 87% (Swift et al., 2020). Viable MAP were identified in 27% of bulk milk samples (Foddai et al., 2021). The rapid version of the phage test, called Actiphage, does not require molten agar or *M. smegmatis* culture and can be done in 7 versus 48 hours (Swift et al., 2019). These protocols are faster, inexpensive to implement and do not require expensive equipment, facilitating knowledge transfer among laboratories (Jones et al., 2020; Foddai et al., 2021). Furthermore, a different rapid version was also developed using magnetic separation that uses the phage D29 mycobacteriophage-coated paramagnetic beads. This method can perform magnetic separation of MAP from milk samples.

Although phage-based methods have been applied to detect MAP in various sample types, rapid versions of phage-based tests have mostly been used for milk samples, as an alternative to milk ELISA-based tests to increase Se of MAP detection on milk samples (Foddai et al., 2021). However, phage-based methods need improvements before becoming part of Johne's disease surveillance. For instance, the phage D29 used in the test is not specific to MAP, and therefore false-positives can occur due to other mycobacterial species in the sample (O'Brien et al., 2018); however, that can be addressed by PCR confirmation. The applicability of phage D29 was successfully evaluated on 43 MAP strains (Foddai and Grant, 2017). In addition, other MAP strains should also be assessed to ensure phage D29 can be applied to a wide range of MAP strains. Furthermore, phages bind only viable MAP cells in exponential growth (David et al., 1980; Swift et al., 2014). Consequently, false-negative outcomes can occur when only non-viable MAP cells are present. In addition, exclusion of non-viable MAP cells by phage assays can underestimate total MAP CFUs, which can be an issue for JD control programs that often classify animals as low, mid or high shedders (Bates et al., 2019; Navarro-Gonzalez et al., 2019).

While promising to detect MAP-infected animals, test characteristics of phage technology in young animals are unknown and need to be defined through field and experimental studies based on comparisons among IGRA, ELISA and fecal PCR (Table 2.8.3). Additionally, regarding blood samples, phage technology is promising and may provide more sensitive detection of MAP in blood samples and perhaps new perspectives regarding MAP bacteremia, a transient event that can happen during any stage of MAP infection (Table 2.8.3; Dennis et al., 2008).

2.4.2 Indirect tests

2.4.2.1 Interferon-gamma release assay (IGRA)

There are several reports that early cell-mediated immune responses can be used to detect MAP infection (Jenvey et al., 2021; Krueger et al., 2016; Baquero and Plattner, 2016. Mortier et al. (2014c) detected higher predicted interferon-gamma secretion by blood leukocytes from 2 to 4 mo of age, with a higher interferon-gamma response 4 mo after MAP infection (Table 2.8.1). Stronger cellular immune responses were also detected 2 mo after MAP inoculation in an earlier study (Stabel et al., 2003). In a Danish longitudinal study, MAP-infected animals were nearly all at least once IGRA-positive as young stock (Huda et al., 2003). However, IGRA also had many false-positive animals (i.e., low Sp as an indicator for infection). Although the cellular immune response tests have the potential to detect early exposure to MAP, these tests do not detect infection and certainly not infectiousness but rather test for exposure to MAP.

The IGRA has been used in JD diagnostics for more than 2 decades (Kalis et al., 2003). It is a very time-consuming assay, with specific conditions while transporting and processing samples, complicating field use (Mortier et al., 2014c). However, as IGRA is the only test with

potential to detect an animal that has been exposed to MAP and potentially infected, efforts continue to improve test performance and criteria to interpret test results. Studies have focused on the ability of antigens to elicit a Th1 response, their potential to detect MAP early in the course of the disease, and to identify the optimal time to use IGRA to maximize its diagnostic value (Mortier et al., 2014c; Hughes et al., 2017). Interpretation criteria and test characteristics of IGRA were recently improved. For this, IGRA was done in 3 dairy herds (1 MAP-infected, 1 MAP-free, and 1 with bovine tuberculosis) using bovine PPD, avium PPD and 3 new PPD johnins. The IGRA results were evaluated based on 12 new interpretive criteria. Two interpretive criteria, based on a comparison of optical density results among various PPD, had Se ranging from 81 to 100%, whereas Sp ranged from 92 to 100% (Corneli et al., 2021). Recombinant MAP antigens were also evaluated based on a 20-h IGRA; the best antigen identified 39% of confirmed MAP-infected animals, whereas ELISA and fecal culture identified only 3.0 and 1.7%, respectively, of MAP-infected calves (Dernivoix et al., 2017).

The use of a cocktail of antigens and development of recombinant antigens to improve Se of IGRA has also been proposed, as MAP-infected animals have various responses to the same antigen (Hughes et al., 2017). In an evaluation of a cocktail of recombinant antigens in goats based on MAP recombinant PPD, there was no IFN-γ response after stimulation with *Mycobacterium bovis* peptide cocktails ESAT6-CFP10, Rv3020c, and Rv3615c, which might further improve test Sp (Köhler et al., 2021). Additional efforts to improve the assay were associated with T-cell stimulation with interleukins (IL), specifically IL7 and IL12 that may prolong viability of T-cells (Plain et al., 2012). The current IGRA requires conducting the test within 6-8 h after blood collection to obtain reliable results, due to short-duration viability of immune cells (Stabel, 1996; Jungersen et al., 2002). However, a technological advance uses

recombinant IL-12 as a preservative, extending the deadline to perform the assay (Mikkelsen et al., 2012). Simultaneous measurement of IFN-γ and IL-2 could increase assay Se in active stages of the disease (Burrells et al., 1999). Consequently, although previous research reported technical issues regarding applicability of the IGRA and interpretation of test results, there has been research to improve test characteristics of the IGRA and its applicability. Although test characteristics reported by Corneli et al. (2021) seem acceptable, they would be insufficient in a JD control program. For example, at a prevalence of 2% and a Se of 90% and a Sp of 96%, there would be 10 and 4% of calves false-negative and false-positive, respectively, based on a 1-time test. Serial testing strategies would likely increase Se, but due to the less than perfect Sp, this also leads to the question what needs to be done with single time-positive young stock. Exploring IGRA as repeated testing, Huda et al. (2004) observed that in cattle 1-2 yrs of age, IGRA performed better with repeated testing. Obvious disadvantages of serial testing are increased costs of testing and delays in identifying and culling infectious calves, which could result in more penmates being infected with MAP.

In summary, studies considering multiple samplings in young stock over time would predict onset of MAP exposure and subsequently MAP infection to maximize use of IGRA. However, sample collection, transportation and processing remain challenging.

2.4.2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Using a variety of ELISAs, experimentally MAP-infected young stock were detected 2.5 to 4 mo after MAP infection (Koets et al., 2001; Bannantine et al., 2008) (Table 2.8.1). The most recent study reported 42% of experimentally MAP-infected young stock as ELISA-positive, considering various age groups (2 wks to 12 mo of age) (Mortier et al., 2014b). Experimentally

MAP-infected young stock at 1 year of age had detectable antibody titers indicative of immune response to MAP infection within 4.5 mo after MAP exposure (Mortier et al., 2014b). Studies evaluating age of testing using ELISA assays on naturally infected young stock are not available. The majority of research studies on shedding patterns or detection of MAP-specific antibodies of young stock are experimental studies. Little is known regarding\ minimal infectious dose, age and pattern of MAP shedding and immune response in naturally infected animals as well as detection capacity of potential new diagnostic tests in young animals (Table 2.8.3). Consequently, more field studies including multiple testing in the same animals at least every 2 mo are essential to determine the incidence of new infections and provide the best recommendation in terms of diagnostic tests and testing strategies.

Humoral immunity early in life has been reported, with MAP-specific antibody titers detected from 10 to 17 mo of age in experimental settings (Lepper et al., 1989). Using commercially available ELISA, an early humoral immune response was identified 4.5 mo after young stock were experimentally infected at 12 mo of age (Mortier et al., 2014b). Similarly, non-commercially available direct ELISAs using lipoarabinomannan as the antigen detected MAP-specific antibodies 4.5 mo after experimental MAP infection (Waters et al., 2003). Bannantine et al. (2008) detected MAP-specific serum antibodies to recombinant MAP proteins 70 d after experimental MAP infection, whereas antibody responses to stress-associated recombinant MAP proteins were detected in young stock 30 wks after MAP infection (Kawaji et al., 2012) (Table 2.8.1). Investigation of humoral immune responses in calves after vaccination with a modified live MAP strain (Neoparasec, Rhone-Merieux, Lyon, France) resulted in antibody detection between 8 and 16 wks after immunization (Köhler et al., 2001). Furthermore, an in-house IgG2 ELISA assay detected a MAP-specific antibody response 160 d after experimental infection with

MAP (Fernández et al., 2019). Humoral immune tests can be used to determine whether a calf had an immune response to MAP; they are, however, not a good proxy for "infectiousness" in calves (Whittington et al., 2017).

Although young stock on MAP-infected dairy farms are relatively commonly shedding this bacterium (Wolf et al., 2015), serological assays are not usually recommended to test young stock, as early stages of MAP infection are characterized by a prolonged incubation period in which many animals do not develop MAP-specific antibodies that are detectable in blood (McKenna et al., 2006). In an experimental study using the IDEXX ELISA (IDEXX Laboratories Inc., Westbrook, ME), MAP-specific antibodies were detected when in only 42% of MAP-inoculated young stock, particularly in those infected with a low dose of MAP (Mortier et al., 2014b). Se ranged from 7 to 100%, whereas Sp ranged from 7 to 94% (Nielsen and Toft, 2008), depending on disease stage. It remains to be determined whether MAP-infected animals indeed have low or undetectable titres of MAP-specific antibodies in blood during the early stages of infection or whether failure to detect these antibodies is due to poor test antigens or reagents used in commercial ELISAs.

Efforts have been made to improve Se of ELISAs for use in young stock. For example, recombinant MAP proteins MAP1027c, MAP1339, MAP1588c, MAP1589c and MAP2411 were evaluated by ELISA during 30 d after MAP infection of young stock. Antibody responses were detected 2 wks post-inoculation, and 87.5% of inoculated young stock responded to at least 1 of the 5 recombinant proteins (Kawaji et al., 2012). Employing 2-step fractionation to capture MAP secreted proteins, novel antigens were identified for use in an ELISA-based format. These novel proteins were MAP-specific and reacted with serum of animals shedding MAP in low concentrations (Facciuolo et al., 2013). In addition, recombinant 20.8-kDa MAP protein was

used in old and young stock, with Se and Sp of 73.3 and 98.3% respectively (Goswami et al., 2017). As a result, recombinant proteins can be an adequate indicator of early MAP infection. In an investigation of the ability of ELISA to detect specific biomarkers associated with patent or latent forms of MAP infection, the best biomarker was ABCA13, detected with an ABCA13-based ELISA, with Se and Sp of 69 and 87%, respectively (Blanco Vázquez et al., 2020).

To more definitively identify MAP antigens that are detected at the earliest disease stage, a set of stratified serum samples representing all disease stages is needed, along with a catalog of purified MAP antigens to screen. However, obtaining stratified serum samples is not trivial as it requires a detailed health history of each donor cow, including results from multiple commercial ELISA tests, fecal culture and PCR tests, and IFN-gamma tests. These well-characterized serum samples were obtained and applied in a study that successfully identified 18 antigens for serological diagnosis of early-stage disease, 26 antigens detected during middle stages, and 15 late-stage antigens of Johne's disease (Li et al., 2019). The 18 early detected antigens were not recognized by antibodies as the disease progressed to advance stages, implying a dynamic humoral immune response as disease progresses. However, these early detected antigens can be multiplexed into a bead assay to obtain increased Se, as reported (Li et al., 2017).

Detection of IgG isotype-specific ELISA has also been promising. Regarding the use of an ELISA to detect IgG2 antibodies in MAP-infected young stock experimentally infected with MAP, an isotype-specific ELISA, based on a protoplasmic JD antigen (PPA) as capture antigen and a monoclonal anti-bovine IgG2, detected MAP infections more often than the common ELISA (Fernández et al., 2019). However, there is a need for larger sample sizes and more applicable techniques.

To the authors' knowledge no improvement of test characteristics was done using commercial ELISAs (Table 2.8.3). Rather than improving within-house ELISAs or developing ELISAs targeting new antigens, perhaps the focus should be commercially available tests.

2.4.2.3 Host-based gene expression analysis

Another potential improvement in early diagnosis of MAP infection has been associated with transcriptional characterization (van den Esker and Koets, 2019), i.e., evaluation of host RNA transcripts (Jenvey et al., 2019). Some studies have evaluated early stages of MAP infection based on gene expression profile, miRNA, protein, and metabolites that may differ between MAP-negative and positive calves and cows (David et al., 2014a, 2014b; Park et al., 2018; Gupta et al., 2018; Karuppusamy et al., 2018; Tata et al., 2021).

Transcriptomic analysis in young animals experimentally infected by MAP identified multiple genes differently expressed compared to control young stock in the dorsal mandibular, ventral mandibular, dorsal parotid, and ventral parotid salivary glands (Mallikarjunappa et al., 2019). Additionally, Farrell et al. (2015) compared sera from MAP-infected and negative control young stock pre-infection and 6 mo post-infection. Increased levels of miRNA-205 (2-fold) and a decrease miRNA-432 (2-fold) were observed in both groups, which suggested changes in circulating miRNA profiles due to aging or disease development. In a subsequent study from the same group on longitudinal miRNA profiling of young stock, these changes were attributed to ageing (Shaughnessy et al., 2015). Furthermore, levels of miRNA can be associated with disease severity based on canonical discrimination analysis of 20 miRNAs (Gupta et al., 2018).

Studies on gene expression profiles at varying levels of infection were also reported. Biomarkers from calves were compared among high- and low-dose and negative control animals

3, 6 and 9 mo after experimental MAP infection. Considering negative control young stock as a reference, after 3 mo of infection, MAP-infected young stock had differences in gene expression. For instance, in MAP-infected young stock, genes *CD46*, *ICOS* and *CEP350* were upregulated, whereas genes *CTLA4*, *YARS*, and *PARVB* were downregulated (David et al., 2014a). Both *CD22* and *IL6ST* were differentially expressed in ELISA-positive animals, implying a role in early antibody response. *PARVB* and *YARS* may also be promising biomarkers for early MAP infection as both were upregulated in calves infected with low- or high-dose MAP (David et al., 2014b).

Gene expression patterns were evaluated in older cattle as a potential diagnostic test or to evaluate MAP survival during subclinical stages (Park et al., 2017, 2018). Genes *Hp*, *Serpinel* and *Tfrc* had good ability to differentiate MAP-infected and non-infected cattle based on fecal PCR and ELISA tests (Park et al., 2017). Furthermore, another study considering various MAP infection stages of MAP-positive young stock reported expression of different genes (Park et al., 2018). Cytokines-associated with Th17 responses were downregulated, whereas *IL-17* was upregulated in the later stages of MAP infection. Malvisi et al. (2020) evaluated transcriptomic data to differentiate exposed versus non-exposed cattle based on ELISA, from 4-5 yrs of age. Five genes differentiated exposed ELISA-negative from healthy and exposed cattle in both MAP-positive and negative herds. Purdie et al. (2019), based on gene expression profiles in Merino sheep, provided an alternative to detect MAP-positive animals. Several genes were differentially regulated, especially S100 calcium binding, lysozyme function, MHC class I and class II and T-cell receptor. Evaluation of gene expression factors in MAP-infected animals was also evaluated in animals with focal and diffuse lesions in gut tissues (Alonso-Hearn et al., 2019). Subclinical detection of MAP at earlier stages should be improved by analysing host gene expression and pathways involved in progression of MAP infection (Table 2.8.3)

2.4.2.4 Metabolites as biomarkers for MAP infection in calves

Metabolomics integrates transcriptome and proteome analyses and has potential as a sensitive method to detect MAP infection in calves. Recently, a prospective study evaluated metabolomic markers using serum from heifers and cows. These markers were associated with MAP-infected and infectious heifers and cows using mass spectrometry (DART-HRMS). Isobutyrate, dimethylethanolamine, palmitic acid, and rhamnitol concentrations were higher in MAP-infected heifers and cows compared to MAP-negative heifers and cows (Tata et al., 2021).

Differences in the metabolic profile were detected in experimentally MAP-infected young stock before common tests were diagnostic (De Buck et al., 2014). For instance, glucose acetate, dimethyl sulfone, 3-hydroxybutyrate and methanol concentrations were lower in MAP-infected young stock compared to non-infected young stock. Furthermore, there were no differences in metabolic profiles of young stock infected with a low- versus high-dose of MAP (De Buck et al., 2014). In evaluations of metabolomic profiles of experimentally infected and control young stock between 2 wks and 19 mo of age, there was a rapid increase of fatty acids and some n-3 PUFA within 1 month and subsequent increase of n-6 PUFA and COX products, implying roles in colonization and immune modulation, respectively (Taylor et al., 2022). Assessment of metabolomics in naturally MAP-infected young stock from 1 to 19 mo of age also indicated an elevation of leukotriene B4, bicyclo prostaglandin E2 (bicyclo PGE2), itaconic acid, 2-hydroxyglutaric acid and N6-acetyl-L-lysin which could have potential as MAP diagnostic biomarkers (Taylor et al., 2021). Further, the fatty acids 8, 11, 14-eicosatrienoic acid and cis-8, 11, 14, 17-eicosatetraenoic acid had varying concentrations in young stock from 1 to 19 mo of age compared to control groups, which could be also used as a potential diagnostic biomarker

(Taylor et al., 2022). A German group focused on volatile organic substances for MAP diagnosis of MAP, similar to metabolomic testing (Küntzel et al., 2019; Weber et al., 2021).

Based on the small scale of these investigations, further studies are necessary to evaluate these promising diagnostic tests in more animals, especially in naturally MAP-infected young stock, to evaluate whether these technologies could be included as a testing strategy (Table 2.8.3). In addition, it is important to ensure that various metabolomic profiles are specific to MAP infections and not associated with infections caused by other pathogens or more general indicators of infectious disease or poor body condition. Additionally, these profiles need to be specific to animals with active MAP infections and also to exclude animals that were exposed to MAP but MAP failed to establish a persistent infection.

2.4.2.5 Microbiota composition

The microbiota composition of MAP-infected animals differed from non-infected animals, and it can be used as an additional diagnostic test (e.g., Umanets et al., 2021; Lee et al., 2023; Matthews et al., 2023). In addition, when comparing microbiota data in longitudinal sample sets, there were indications that that microbiome profiling of test-positive young stock could be analyzed statistically to predict future shedding status (Umanets et al., 2021). This could be used in management decision making especially on endemic farms with higher prevalence to rank animals for culling if for instance the prediction indicates that certain calves are more likely to progress to super shedder state or clinical outcome.

Overall, these studies were promising for detection of MAP infection in young stock. However, they were all experimental and, similar to gene expression analysis and metabolomics,

field studies are needed to determine if differences in microbiota are specific to MAP infection (Table 2.8.3).

2.5 Including calves in JD control

Simply culling MAP test-positive animals is not sufficient to stop new MAP infections from occurring, nor is it sufficient to stop MAP transmission within a herd. Management strategies are necessary to limit new MAP infections (Al-Mamun et al., 2017). Similarly, adoption of management strategies alone is also not sufficient and needs to be accompanied by testing and culling to reduce MAP prevalence (Groenendaal et al., 2002). A modeling approach by Camanes et al. (2018) recently supported implementation of young stock management in addition to test and cull to effectively reduce MAP prevalence. Although calves and heifers are often included in management strategies of JD control programs, more research is needed to understand the role of naturally MAP-infected young stock in MAP transmission and expected reductions in within-herd MAP prevalence if MAP test-positive young stock were culled. It is very likely housing circumstances of the calves, e.g., individual versus group pens, stocking density, bedding type and frequency of changing bedding, affect MAP shedding and risk of exposure (Table 2.8.3). Nutrition may also have a role as calves on a lower plane of nutrition are more likely to suckle one another (Lidfors and Isberg, 2003), and thus potentially ingest MAP-contaminated manure that may be on the calf being suckled (Table 2.8.3). Finally, in addition to environmental contamination, the role of bioaerosol formation needs attention (Table 2.8.3; Eisenberg et al., 2010, 2011).

The pathogenesis of MAP infection is neither linear nor at a constant rate and some infected (and even diseased) animals are capable of recovery (i.e., reverse pathogenesis) as

demonstrated in longitudinal studies involving surgical biopsies (Begg et al., 2018; Dennis et al., 2011). Animals exposed to MAP that are resistant would be good to retain rather than cull. However, we have no way to identify them at an early age and distinguish them from early-infected animals destined to succumb to the infection (Table 2.8.3). Perhaps this will be a future role of gene expression and metabolomic profiling.

The majority of JD control and eradication programs only test lactating and dry cows. Young stock are the most susceptible to infection, but they are only the subject of management strategies. It would, however, be very advantageous if young stock could be reliably tested. First, MAP-infected young stock could be identified early in the course of MAP infection and also infectious young stock could be identified. Secondly, identification of MAP-positive young stock would support evaluation of the success of management strategies. However, Se of the current diagnostic tests in the earlier stages of the disease to identify MAP-infected and particularly infectious young stock housed in commercial dairy herds has been too low. Therefore, development of new diagnostic tests, or modifying existing test, and new surveillance strategies are necessary to detect MAP-positive young stock.

The status of a particular herd will dictate what needs to and can be done with calves that are detected as MAP-infected or infectious. If there is a commitment to decreasing prevalence of MAP infection, priority should be given to segregate and cull infectious (i.e., MAP-shedding) calves. Feces of calves identified as MAP-infected but that are (currently) not infectious should at a minimum be tested relatively frequently to detect whether they have become a shedder. Ideally, they neither calve nor enter the lactating herd. Although on average these calves grow less than non-infected calves (Roy et al., 2017), fattening and slaughter may be an option.

2.5.1 In utero transmission

Proportion of calves that are reported to be infected *in utero* from a MAP-infected dam ranges between 4 and 15% (Whittington and Windsor, 2009; Mitchell et al., 2015). This percentage is higher when the dam has clinical JD but may be lower in herds with a low within-herd MAP prevalence (Adaska and Whitlock, 2012).

Based on both *in utero* and colostral transmission, there have been recommendations to cull all calves born from known MAP-infected dams, and from sisters of known MAP-infected dams (because the dam may have infected all siblings, and thence progeny of siblings) (Rossiter and Burhans, 1996). It is, however, not known what the fate is of *in utero* infected calves: e.g., will they grow as well as not infected calves, and will they become infectious (i.e., MAP shedder) (Table 2.8.3).

2.5.2 Reducing contact between newborn calf and dam

Young stock are usually included in disease control strategies on dairy farms, including JD management. Contact between the dam and the calf is often regarded as a risk factor for MAP infection; an assumption that is biologically very plausible. Early separation of calves and their dams soon after birth reduced the odds of animals to become MAP-infected by 55% (Vass-Bognár et al., 2022). Also, preventive management measures to separate young stock from adult cattle remain economically important (Weber et al., 2012). However, a recent systematic review of 14 studies using a variety of testing strategies demonstrated that there is little evidence for this claim and the authors concluded that the association between early cow-calf separation and risk for MAP infection of the calf is unsubstantiated (Beaver et al., 2019). However, reducing calf

contact with the dam and the calving environment can be accomplished by placing the calf in a feed tub or other unit where the cow and calf are 'together' (Behr and Collins, 2010).

There is a recent tendency to keep young stock housed together before weaning, in response to development of new technologies, e.g., automatic milk feeders (Barkema et al., 2015). Also, early separation of young stock from their dams is challenged from an animal welfare perspective (Meagher et al., 2019) by many members of the public (reviewed by Placzek et al., 2021). Therefore, the dairy industry is in the challenging position to navigate its efforts for MAP prevention with considerations regarding animal welfare and public perception. In addition, there is a trend in which a growing number of dairy farms are moving away from current practices towards more extensive, nature inclusive forms of farming with a very different view on management and hygiene (Placzek et al., 2021). There are also some regulatory changes in the EU and elsewhere that will impact this, including the new Canadian Code of Practice for the Care and Handling of Dairy Cattle (NFACC, 2023). If early separation from the dam is not practiced, it will become even more important to detect MAP infection in young stock early in the course of MAP infection and to evaluate whether management practices to prevent MAP transmission of young stock are working (Table 2.8.3).

2.5.3 Minimize MAP exposure in calf housing

Immediate calf removal should not be regarded as a substitute for other management strategies, including appropriate hygiene in the maternity area (Beaver et al., 2019). Young stock should be born in a designated clean area for calving that is dry and free of manure to reduce exposure to MAP (and other pathogens) during calving (McKenna et al., 2006).

It remains important to prevent point exposure of calves to feces or feces contaminated feed or fomites containing a high MAP load. However, on endemic farms, there is a consistent MAP contamination of the environment, in part driven by MAP-containing bioaerosols that cause continuous exposure of calves and young stock to (low doses of) MAP (Eisenberg et al., 2010, 2011). Current management strategies do not take this into account, but it may contribute to a lack of success of hygiene-based management strategies. In addition, bioaerosols originating from feces of infectious calves may further complicate MAP control due to early life infections, even in well-managed calf rearing facilities (Eisenberg et al., 2012). It is currently not known whether these chronic low dose exposures are as important as preventing high-dose, single time point infections, as mimicked by experimental oral infections (Table 2.8.3). Therefore, environmental sampling of calf housing should be explored as a part of a diagnostic strategy similar to pooled fecal samples.

2.5.4 MAP-free colostrum, milk and water

In a 1-year longitudinal study, feeding young stock milk replacer and UHT pasteurized milk significantly reduced MAP burden in their tissues compared to raw milk (that may be contaminated by MAP) or copper-treated milk (Steuer et al., 2021). Clean water, devoid of manure, should be provided for young stock. When introducing new animals to the herd, producers should be aware of the risk to introduce a new source of MAP infection (Steuer et al., 2021). Discarding colostrum from MAP-positive cows significantly decreased the odds of animals becoming MAP-infected (Vass-Bognár et al., 2022).

2.5.5 Testing

In a JD control program, objectives of testing young stock are to: 1) determine effects of control in their dams to prevent MAP infection *in utero* and through colostrum; 2) detect potential MAP transmission between young stock; and 3) identify MAP-infected and particularly infectious young stock. It is important to recognize implications of various tests, particularly direct versus indirect tests mean, and also what action can/should be taken in response to specific test outcomes, based on objectives of the control program (Table 2.8.4).

Efficient reduction of new MAP infections in young stock requires improvements in Se and Sp of existing diagnostic tests, plus development of new tests and efficient testing strategies to detect MAP-infected young stock (Table 2.8.3). In non-classical farming systems as well as in case of lack of results of management measures, the need for active protection of young stock, e.g., through vaccination strategies, would be important, despite a lack of current options (Table 2.8.3).

Age. Inclusion of young stock to establish these objectives is limited by the age that young stock can be included in most MAP testing strategies. However, in both experimental and naturally infected young stock, MAP shedding occurred from 7 to 14 mo of age (Bolton et al., 2011). Based on ELISA, the age range that antibody titers were detected in young stock ranged from 2.5 to 4.5 mo after MAP exposure (Koets et al., 2001; Bannantine et al., 2008; Mortier et al., 2014b). We should therefore reconsider age recommendations for testing.

Frequency of testing. In addition to optimal age at testing, it is important to consider testing frequency, especially due to intermittent MAP shedding and delayed onset of detectable immune response in young stock. In experimental studies, fecal samples were collected twice monthly, whereas blood samples for ELISA were collected once monthly, from 2 mo after MAP

exposure to necropsy at 18 mo of age (Mortier et al., 2014b; a). Considering naturally infected young stock, Bolton et al. (2011) collected samples from young stock every 4 mo, with 8 data collection points per herd. Herd size and prevalence of MAP-positive animals in the herd, as an indicator of average infectious dose, may affect age at testing. Larger herds have higher odds of being MAP-infected (Pillars et al., 2009) and if the MAP prevalence is high, young stock may become infected with a higher dose of MAP and consequently may shed MAP more frequently. Based on experimentally MAP-infected young stock, MAP shedding occurred more frequently in young stock inoculated between 2 wks and 3 mo with a high dose of MAP (Mortier et al., 2014a). The same pattern was also observed in naturally infected animals. On farms with high MAP prevalence, young stock shed MAP more frequently (Weber et al., 2010). Detection of MAP-positive young stock increased in herds that had > 10% within-herd MAP prevalence (Bolton et al., 2011) and proportion of MAP shedding in young stock increased from 1 to 20% in dairy herds with an apparent within-adult herd MAP prevalence < 5% to > 20%, respectively (Weber et al., 2010). This may also be explained by improvements in greater Se of MAP detection in herds with high MAP prevalence (Tavornpanich et al., 2008). Although a test frequency of 2 mo would be best for detection of MAP-infected of infectious calves, it is ambitious, labor-intensive and expensive. On farms that are less determined to control MAP infection, acceptance and applicability is expected to be low. Therefore, more research is needed to produce evidence-based recommendations on testing frequencies for naturally MAP-infected young stock (Table 2.8.3).

Diagnostic test alternatives may overcome issues with current diagnostic tests to detect MAP early in the course of the disease. IGRA is a widely evaluated testing alternative. Based on monthly evaluation, MAP testing of young stock at 4 mo after MAP infection was recommended

(Table 2.8.1) (Mortier et al., 2014c), As the actual time of infection is unknown under field conditions, it may be worthwhile to test young stock every 2 mo. However, IGRA is able to detect only MAP exposure in young stock (Jungersen et al., 2012) and it is unknown whether calves designated as MAP-exposed calves based on IGRA and other indirect tests are always truly MAP-infected under field conditions (Table 2.8.3). Additionally, testing every 2 mo may be too costly for most farms.

Regarding testing young stock, there are still many questions, including: 1) How can new methods be used in routine diagnostics; 2) What sample has to be collected and how complicated and costly are the laboratory procedures?; and 3) Can it be performed automatically or is it work intensive? Answers to these questions are not currently available.

2.5.6 Management

It is well known that to avoid new MAP infections, young stock should be separated from their dams as early as possible and consume only colostrum and milk free of MAP (Behr and Collins, 2010). When introducing new animals to the herd, producers should be aware of the risk in the source for MAP infection (Steuer et al., 2021). Early separation of calves and their dams soon after birth reduced the odds of animals to become MAP-infected by 55% (Vass-Bognár et al., 2022). Additionally, discarding colostrum from MAP-positive cows significantly decreased the odds of animals becoming MAP-positive (Vass-Bognár et al., 2022).

2.5.7 Testing strategies

Objectives of testing young stock in a JD control program are to: 1) determine the effect of control in their dams to prevent MAP infection *in utero* and through colostrum; 2) detect potential MAP transmission between young stock; and 3) identify MAP-infected young stock.

Age. Currently, inclusion of young stock to establish these objectives is limited because of the age that young stock can be included in MAP testing strategies. In naturally MAP-infected animals, Bolton et al. (2011) detected MAP-positive young stock from 0 to 14 mo of age. However, 83% of the MAP-positive young stock were detected in animals > 6 mo of age, with the majority of the samples from young stock between 7 to 14 mo old. In experimental studies the first MAP-positive fecal results occurred within 6 mo after MAP inoculation with a peak at 2 mo (Mortier et al., 2014a). Using a variety of ELISAs, experimentally MAP-infected young stock were detected 2.5 to 4 mo after MAP infection (Koets et al., 2001; Bannantine et al., 2008) (Table 2.8.1). The most recent study reported 42% of experimentally MAP-infected young stock as ELISA-positive, considering various age groups (2 wk to 12 mo old). Experimentally MAP-infected young stock at 1 y of age old had an immune response within 4.5 mo after MAP exposure (Mortier et al., 2014b). Studies evaluating the age of testing using ELISA assays on naturally infected young stock are not available. The majority of research studies on shedding patterns of young stock are experimental studies. Little is known regarding the age and pattern of MAP shedding in naturally infected animals as well as detection capacity of potential new diagnostic tests in young animals. Consequently, more field studies including multiple testing in the same animals at least every 2 mo are essential to determine the incidence of new infections, to provide the best recommendation in terms of diagnostic tests and testing strategy.

Frequency of testing. In addition to best age at testing, it is important to consider frequency of testing, especially due to intermittent MAP shedding and immune response in

young stock. Experimental studies collected fecal samples twice monthly, whereas blood samples for ELISA were collected once monthly from 2 mo after MAP exposure to necropsy (Mortier et al., 2014b; a). Considering naturally infected young stock, Bolton et al. (2011) collected samples from young stock every 4 mo, with 8 data collection points per herd. Herd size and prevalence of MAP-positive animals in the herd might affect age at testing. Larger herds have higher odds of being MAP-infected (Pillars et al., 2009) and if the MAP prevalence is high, the young stock might become infected with a higher dose of MAP and consequently young stock might shed MAP more frequently. Mortier et al. (2014a) based on experimentally MAP-infected young stock reported MAP shedding more frequently in young stock inoculated between 2 wk and 3 mo with a high dose of MAP. The same pattern was also observed in naturally infected animals. On farms with high MAP prevalence, young stock shed MAP more frequently (Weber et al., 2010). Detection of MAP-positive young stock increased in herds with within-herd MAP prevalence > 10% (Bolton et al., 2011) and the proportion of MAP shedding in young stock increased from 1 to 20% in dairy herds with an apparent within-adult herd MAP prevalence < 5% to > 20%, respectively (Weber et al., 2010). This might also be explained by improvements in accuracy of MAP detection in high MAP prevalence herds (Tavornpanich et al., 2008). However, more research is needed to produce evidence-based recommendations on testing frequencies for naturally MAP-infected young stock.

Pooling. Due to additional costs to include young stock in the testing strategy, pooled fecal samples in young stock (Bolton et al., 2011) or temporally pooled samples from the same animal over time (Hines Ii et al., 2007; Mortier et al., 2014c) are alternatives to be explored in terms of test characteristics, optimal testing frequency, and number of animals included in fecal pools or number of fecal samples from the same young stock in the fecal pool. In addition, most

studies detecting MAP in naturally infected young stock collected fecal samples at most twice from the same animals (Weber et al., 2010) (Table 2.8.2). However, based on experimental studies in young stock, shedding occurs very sporadically (Mortier et al., 2014a). As a result, more fecal samples from the same animal (e.g., at least every 2 mo) may be more efficient to detect MAP in young stock. To reduce costs of testing, direct DNA extraction from feces followed by qPCR could be adopted instead of culture methods before qPCR.

Diagnostic test alternatives may overcome issues with current diagnostic tests to detect MAP early in the course of the disease. IGRA is a widely evaluated testing alternative. Mortier et al. (2014c), after monthly evaluation, recommended MAP testing of young stock 4 mo after MAP infection (Table 2.8.1). As the actual time of infection in the field is unknown, it may be worthwhile to test young stock every 2 mo. However, IGRA is able to detect only MAP exposure in young stock (Jungersen et al., 2012) and it is unknown whether calves designated as MAP-exposed calves based on IGRA are always truly MAP-infected under field conditions. Studies considering multiple samplings in young stock over time would predict the onset of MAP exposure and subsequently MAP infection to maximize use of IGRA. However, issues with IGRA regarding sample collection, transportation and processing previously mentioned remain challenges. Phage technology is another test highly applicable under field conditions that requires further evaluation considering MAP naturally infected young stock.

2.6 Conclusions

JD has profound and deleterious health and financial impacts on the dairy industry. Slow progress towards MAP eradication may be associated with transmission between young stock

that is currently not addressed by JD control programs. Producers should be aware that MAP-infected young stock can shed MAP as early as 4 mo after infection and transmit MAP to pen mates. Therefore, due to JD biology, low Se associated with commercially available diagnostic tests demands improvements to our existing test platforms and emphasizes the need for new diagnostic tests. Small-scale studies have assessed new promising diagnostic tests, e.g., phage technology, genomics and metabolomics; however, these need to be replicated for further validation and confirmation as well as explored for automated application. Furthermore, improvements in diagnostic tests should include an evaluation of the best age for use in young stock. Ideally, this would be before heifers are bred, so that test-negative animals can be used as replacements and positives delegated for fattening and slaughter. More efficient management strategies, e.g., separation of MAP-shedding young stock, should be considered. In summary, new MAP infections may be associated with transmission of MAP between young stock. A combination of better diagnostic tests, testing and management strategies could reduce MAP prevalence on dairy herds.

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Table 2.1. Testing strategies recommendations of studies based on experimental M*ycobacterium avium* subspecies *paratuberculosis* (MAP) infected young stock

Manuscript	Diagnostic tests[1]	Testing strategy	Results	Recommendations
Corbett et al., 2017	Fecal and tissue culture; F57 qPCR, IDEXX ELISA and IGRA	Feces - Group housing: daily fecal sampling until 14 d after inoculation followed by fecal sampling 3 times/week; After 3 mo of group housing: weekly fecal sample collection Blood – weekly	ELISA: negative for all calves Inoculated calves: all MAP-positive for fecal and tissue culture; IFN-γ[2] detected after 43 d PI[3] Control exposed calves: all MAP-positive for fecal culture; 50% MAP-positive on tissue culture; 36% positive IFN-γ response	Calf-to-calf transmission should be evaluated in JD control programs
Mortier et al., 2014a	Fecal and tissue culture; IS900 PCR	Sample collection: before inoculation, once a week PI for 30 d and once a month until necropsy Calves were followed from 5 to 17 mo according to their age at inoculation	64% shed MAP especially after 2 mo PI; frequency of shedding associated with MAP concentration at inoculation; first shedding event took longer to happen calves inoculated at older ages; frequency of MAP shedding associated with number of tissue MAP-positive tissue areas	Susceptibility of calves until 12 mo should be considered in JD control programs
Mortier et al., 2014b	IDEXX ELISA	Samples were collected before MAP infection and monthly PI	Calves with high MAP dose: 42% of ELISA positive calves; antibodies were detected earlier (4.5 mo PI)	Use of ELISA as screening in young stock might be useful. However, the low MAP concentration at exposure might not be enough to generate detectable antibody responses
Mortier et al., 2014c	IGRA	Samples were collected before MAP infection and monthly PI	Calves with high MAP dose: earlier and stronger IFN-γ response, which was higher at 4 mo after exposure	Testing of calves for IGRA every 4 mo is an optimal test interval to detect new MAP infections
Kawaji et al., 2012	Trial 1: fecal culture and ELISA; Trial 2: fecal culture, qPCR, ELISA	Trial 1: samples were collected before and in 30 wks PI Trial 2: MAP inoculation 2-8 wks old; Samples were collected every 2-4 wks	Antibodies against 5 recombinant antigen preparations (MAP1027c, MAP1339, MAP1588c, MAP1589c and MAP2411) were detected only in infected calves around 2 wks PI	No practical recommendations; suggested new studies in animals at various MAP infection stages

Santema et al., 2012	Fecal culture, MAP Hsp70 protein ELISA, Bovigam IGRA	Vaccinated and non-vaccinated groups. Blood and fecal samples were collected every 3 wks until necropsy	Peak of MAP shedding at 130 d post-challenge; only one contact animal (non-vaccinated) was MAP-positive at necropsy	MAP transmission was not fully observed; MAP-positive calves had little influence on MAP transmission
Subharat et al., 2012	Fecal culture, Pourquier ELISA and Bovigam IGRA	Fecal samples: between 2-3 mo Blood samples: periodically	MAP shedding: majority of animals 2-4 mo PI; reduction of MAP-positive tissue locations and lesion scores, which was negatively associated with interleukin-10	Initial MAP infection progression followed by infection control between 7-15 mo. Consider the variable course of MAP infection in JD control programs
Eisenberg et al., 2011	Fecal culture, IS900 PCR, Pourquier ELISA and Bovigam IGRA	Four groups of calves inoculated by: transtracheal inoculation, nasal aspiration of aerolized MAP, oral inoculation, and control Sampling strategy varied according to following events: MAP shedding before infection, passive MAP shedding PI, MAP shedding in multiple time points, environmental contamination, antibody production and MAP detection in tissue	MAP shedding in fecal samples was not detected; tissue culture detected MAP-positive calves; IFN-γ response was detected 12 wks PI	Lungs are one of the entry routes for MAP; transmission of MAP by bioaerosols should be considered in JD control programs
Bannantine et al., 2008	Dot blot assays	In vivo hypothetical proteins, metabolic proteins and cell envelop proteins, known antigens and unique proteins	Antibody activity considering different antigens in multiple time points starting at 70 d PI	At least 2 antigens might be able to support MAP early detection
Van Roermund et al., 2007	Fecal and tissue culture, IS900 PCR, Pourquier ELISA	Blood and fecal samples were collected every 2 wks	Cow-calf transmission: all calves excreted MAP Calf-to-calf transmission: 4 MAP-positive calves infected 2 MAP-negative calves	MAP-positive calves are able to shed MAP and infect other calves in a short period PI
Waters et al., 2003	Fecal and tissue culture, IS900 PCR, Bovigam IGRA, nitric oxide assay, lipoarabinomannan-ELISA, IDEXX ELISA	Details were not provided	Positive MAP tissue culture, IFN-γ and nitric oxide responses were higher compared to pre-challenge values and started at 194 d PI; the lipoarabinomannan-ELISA detect the first positive result at 134 d PI	MAP-specific ELISA detected early infection before the IDEXX ELISA

McDonald et al., 1999	Fecal and tissue culture, Parachek ELISA, Bovigam IGRA	0 – 18 mo PI: blood and fecal samples were collected monthly 19 mo PI - euthanasia: testing every 2 mo	Confirmed by fecal culture: 6 orally infected and 1 naturally infected calf; Confirmed by positive ELISA: one orally infected, one naturally infected and 3 vaccinated calves; All calves were IGRA-positive	Detection of MAP-infected calves still a challenge when using fecal culture, ELISA and IGRA

[1]qPCR = quantitative polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; IGRA = interferon-gamma release assay

[2]IFN-γ = interferon-gamma

[3]PI = post infection

Table 2.2. Testing strategies and recommendations of studies based on naturally *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infected young stock

Reference	Diagnostic tests[1]	Testing strategy	Results	Recommendations
Wolf et al., 2015	Direct DNA extraction of individual fecal samples; culture of a subset of individual feces and environmental samples; IS900 and F57 PCR	Cross sectional study; Fecal samples were collected from 2606 young stock on 18 MAP-infected dairy farms	8, 1.2 and 2% of young stock were positive on IS900 qPCR, F57 qPCR and bacterial culture, respectively. Farms with MAP culture-positive environmental samples had higher odds of MAP-positive young stock detection by IS900 qPCR	MAP shedding by young stock supports the evidence that young stock is associated with MAP transmission
Bolton et al., 2011	Fecal culture, IS900 PCR	Fecal samples were collected at 4-month intervals with a maximum of 8 visits per herd over 28 mo of sample collection	83% of MAP-positive samples were detected in heifer older than 6 mo, with 52% of MAP-positive samples from heifers between 7-14 mo	Fecal sample collection from 7-14 month old heifers might be a good target age to detect MAP-positive calves
Weber et al., 2010	Individual culture, AF staining, IS900 PCR	Fecal samples were collected in a varied interval from 18,979 female Holstein-Friesian cattle in 353 Dutch herds	MAP shedding in young stock is associated with apparent MAP prevalence	Significant proportion of young stock shedding MAP; consider young stock shedding in JD control programs
Antognoli et al., 2007	MAP culture, IS900 PCR, intradermal skin test, Bovigam IGRA, Herdcheck *M. paratuberculosis* ELISA	Intradermal skin test, IGRA and ELISA at 2, 4, 6 and 8 mo Fecal samples collected at 8 mo.	3% of the calves shed MAP at 8 mo. No agreement was observed between tests	Detection of MAP infection in calves might be improved through the use of multiple tests combined

[1]PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; IGRA = interferon-gamma release assay

Table 2.3. Most important knowledge gaps for including young stock in control of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections

Area	Knowledge gap
Genetics	- Factors determining susceptibility of calves to MAP infection - Frequency of resistance to MAP infection and genetic determinants of resistance - Determinants of cure of MAP infection - Genetic markers
Housing	- Role of chronic low dose exposures like aerosols or contamination of bedding - Transmission of MAP among calves in different housing systems - Role of bedding and bedding frequency in MAP transmission - Nutrition and transmission of MAP infection
Testing	- Improve test characteristics (Se and Sp) of diagnostic tests for use in young stock - Test characteristics of phage technology in feces and blood - Test characteristics of potential new diagnostic tests in young animals - Identification of calves that are genetically resistant to MAP infection - Do indirect tests indicate exposure to MAP or also infection and/or being infectious? - Differentiation of passive and active MAP shedding - Validation of indirect tests in controlled field setting - Age and pattern of MAP shedding in naturally infected young stock - Transmission of MAP between naturally infected young stock - Immune response in naturally infected animals - Host gene expression and pathways involved in progression of MAP infection - Evaluation of metabolic signature of MAP infection in larger studies - Evaluation of differences in microbiota in field studies
Transmission	- Minimal infectious dose - Incidence of *in utero* transmission in low MAP prevalence herds - Fate of *in utero* infected calves - Development of a vaccine that prevents MAP shedding of young stock
Economics	- Economic impact of test and cull of test-positive young stock - Barriers and motivators for producers to cull or not MAP-positive young stock

Table 2.4. Implementation of testing young stock in a dairy herd controlling Mycobacterium avium subsp. paratuberculosis (MAP)

Type of test	Potential meaning of positive result in terms of MAP case definition terminology	Possible action following test	[2]Final action
Fecal culture and [1]PCR confirmation	Infected with MAP	Isolate calf, repeat test in 1 month	Cull if 2nd test also positive
Direct fecal DNA extraction and PCR confirmation	Infected with MAP	Isolate calf, repeat test in 1 month to distinguish passive shedding	Cull if 2nd test also positive
Serum ELISA	Exposed to MAP	Perform fecal culture or direct fecal DNA extraction and PCR	Cull if 2nd test also positive
Interferon-gamma release assay	Exposed to MAP	Perform fecal culture or direct fecal DNA extraction and PCR	Cull if 2nd test also positive

[1]PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

[2]If second test is negative, calves should be kept in the herd and retested

CHAPTER 3. Exploring an unexpected rise in within-herd *Mycobacterium avium* subsp. *paratuberculosis* prevalence based on ISMAP02 qPCR

3.1 Abstract

Johne's disease (JD) is a chronic inflammatory intestinal disease affecting domestic and wild ruminants. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) damages the intestinal lining, which leads to diarrhea, poor nutrient absorption, weight loss and submandibular edema. Although there is evidence of young stock shedding of MAP in the early stages of MAP infection, there is currently no agreement on how to effectively detect MAP-infected young stock in terms of target age, diagnostic tests and testing frequency. The aims of the study were, therefore to 1) explore the challenges of testing young stock for MAP, 2) examine the sudden rise in MAP prevalence observed in one farm, and 3) evaluate whether this sudden rise in qPCR positivity was the result of farm and/or laboratory contamination with MAP, or presence of other *Mycobacterium* species cross-reacting with MAP qPCR targeting ISMAP02 gene. Initially, the farm enrolled in this study presented MAP-positive environmental samples. Fecal and blood samples were collected every 2 mo from animals <12 mo and every 6 mo from animals >12 mo of age for direct fecal qPCR targeting ISMAP02 gene and ELISA. At the start of the study, the within-herd level MAP prevalence was 2%. However, at the second sampling, 2 mo in the study, the prevalence in young stock increased to 62% and it remained high throughout timepoints 3 and 4. Issues with cross-contamination during sample collection and laboratory practices were evaluated and excluded. The identification of other *Mycobacterium* species being identified by ISMAP02 target gene were evaluated by different methods, such as PCR, qPCR and TOPO

cloning to increase the quality of sequencing results. Solid culture to observe growth of other *Mycobacterium* species was also implemented. However, all these methods were not successful. As a result, based on MAP-positive environmental samples and individual fecal samples by F57 MAP-target gene, there is enough evidence that MAP is present on the farm. However, the sudden rise in MAP prevalence based on the ISMAP02 target gene could not be explained by the presence of other *Mycobacterium* species.

3.2 Introduction

Johne's disease (JD) is a persistent and progressive inflammatory intestinal disease affecting both domestic and wild ruminants (Li et al., 2005). The causative agent of JD, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a facultative intracellular, slow growing, Gram-positive and acid-fast (AF) bacterium (Rathnaiah et al., 2017; Tiwari et al., 2006). MAP damages the intestinal lining of infected ruminants, leading to diarrhea, poor nutrient absorption, weight loss and submandibular edema during final stages (Tiwari et al., 2006). The herd-level MAP prevalence ranges from 20 to 70% in Europe and in the United States, respectively (Nielsen and Toft, 2009; Lombard et al., 2013), whereas the herd-level prevalence in Canada based on fecal culture and qPCR was estimated at 46% (Corbett et al., 2018). The within-herd MAP prevalence ranges from 2.6% in the United States based on fecal culture to 7.1% in the UK based on serology tests (Lombard et al., 2006; Woodbine et al., 2009). Therefore, JD is present in most countries, on a high percentage of farms, but with a relatively low average within-herd prevalence; however, it is associated with significant economic loss.

The limited success of JD control programs in achieving zero MAP prevalence can be attributed to several factors (Barkema et al., 2018). One is the low sensitivity (Se) of MAP diagnostic tests on farms with a low MAP prevalence which results not only in a low predictive value of a positive test (because of the low prevalence), but also in a low predictive value of a negative test (Dohoo, 2003). It is therefore very difficult to eradicate MAP infection for a dairy herd.

Culture of feces followed by qPCR is widely used to detect MAP-positive animals. Most PCR and qPCR assays use IS900 as their primary target gene (Herthnek and Bölske, 2006), primarily due to having 17 copies of this gene in the MAP genome (Li et al., 2005). However, IS900 target gene can also be detected in other *Mycobacterium* species (Taddei et al., 2008; Englund et al., 2002; Cousins et al., 1999), reducing its specificity (Sp). To overcome this issue, other more MAP-specific genes have been used. For instance, the F57 MAP-target gene is highly specific to MAP. However, the F57 MAP-target gene only has one copy in the MAP genome, limiting the Se of MAP detection using this gene (Slana et al., 2008). To address the limitations of IS900 and F57, the ISMAP02 MAP-target gene has been proposed as an alternative target, having 6 copies in the MAP genome and being very specific to MAP (Irenge et al., 2009). However, Park et al. (2018) questioned the Sp of ISMAP02 to MAP because *Mycobacterium heraklionense* also has ISMAP02 insertion sequences.

A second factor that limits the success of JD control programs is the exclusion of young stock from MAP testing strategies, as MAP-infected young stock excretes MAP less frequently in their feces than older cattle, and they also may not yet have detectable antibody titers, making it difficult to detect MAP infection in these age groups. However, recent research has demonstrated that young stock donnot just shed but also transmit MAP to other calves based on

experimental studies (Mortier et al., 2014; Corbett et al., 2017). Studies based on naturally MAP-infected young stock also proved that young stock shed MAP, such as reported by Wolf et al. (2015) who reported 2% of young stock shed MAP on previously confirmed MAP-positive dairy farms, as confirmed by fecal culture followed by PCR confirmation.

Although there is evidence that young stock can shed MAP early in the infection, there is currently no consensus on how to effectively test young stock for MAP, in terms of target age, best diagnostic test and testing frequency. Therefore, a control study was implemented in Alberta to evaluate the efficacy of including young stock in JD control programs. In this study, on one MAP-positive dairy farm, young stock was sampled every 2 mo to identify all MAP-infected young stock. The original within-herd MAP prevalence was estimated at 2% but increased in 2 mo to 62% based on direct fecal qPCR targeting the ISMAP02 gene. The sudden rise in MAP prevalence was very unexpected, as the prolonged time from MAP infection to infectious stage is estimated to be at least 2 mo after experimental MAP infection (Mortier et al., 2014). We, therefore, explored whether this sudden increase in prevalence of qPCR positivity was the result of farm and/or laboratory contamination with MAP, or presence of other Mycobacterial species cross-reacting with MAP qPCR targeting ISMAP02 gene.

3.3 Material and Methods

This study was approved by the University of Calgary Animal Care Committee (Protocol AC20-0103) and Research Ethics Board (Protocol REB20-1430) and complied with the Canadian Council on Animal Care guidelines.

3.3.1 Animals

Cows, heifers and calves that were part of this study were housed at a commercial dairy farm in Alberta that participated in the JD Control and Eradication Program. The farm had 140 cattle in total, was willing to take measures to eradicate MAP infection, and had proper facilities for animal handling during sample collection. Initially, only environmental samples were collected to identify the farms enrollment eligibility. Environmental samples were collected from lactating cow pens and manure storage areas, based on previous studies that reported higher odds to detect MAP-positive samples in these two areas (Corbett et al., 2018). Samples were collected using sterile sample spoons and fecal containers. On each container, 4 spoons from random areas were included. All 6 environmental samples were MAP-positive based on ISMAP02 target-gene. The herd was visited from November 2021 to June 2022 with 2 mo intervals.

3.3.2 Housing and management

In this herd, calves remained in individual pens until 2 mo of age when they were weaned and moved to group pens. Colostrum and milk were provided from their dams during the first 4 milkings without distinction whether they were MAP-positive or negative until the start of the project. After, calves drank milk replacer or milk from a high somatic cell count cow. Most of the time, young stock remained separated from adult cows, except pregnant heifers that were moved to maternity pens with adult dry cows 2 mo before calving.

3.3.3 Sample collection

Once MAP was detected using environmental samples, animals were sampled at 4 timepoints, with young stock (animals ≤ 12 mo of age) being sampled at all 4 timepoints, while

older animals (animals ≥ 13 mo of age) were sampled only at T1 and T4 (Table 3.8.1). Blood and fecal samples were collected in both animal categories. Blood samples for serum ELISA were collected in 10 ml serum blood collection tubes (red; BD Vacutainer, Mississauga, ON, Canada) from the coccygeal vein and fecal samples directly from the rectum with a clean shoulder length glove.

3.3.3.1 Biosecurity

Due to the high number of animals to be sampled and to minimize risk of transmitting pathogens from older to younger animals, older animals and young stock were sampled on different days. However, to minimize risk of contamination between herds, only animals from one farm were sampled on a given day. After each farm visit, boots were cleaned and disinfected with bleach, whereas coveralls were washed on heavy duty cycle in commercial washer before sampling a different herd.

3.3.3.2 Sample collection

To collect fecal samples, a new arm-length glove was used for each animal. This approach aimed to prevent any cross-contamination between samples from different animals and to reduce the risk of the sample collector's arms becoming contaminated, which could potentially affect other samples. Samples were transferred from the gloves in sterile containers and maintained in clean coolers with ice packs.

3.3.4 Laboratory analyses

3.3.4.1 Serum ELISA

Serum separation was performed after centrifugation of whole blood at 1,200 x *g* for 20 min. Serum samples were stored at -20°C until further analysis, using the IDEXX Paratuberculosis Ab test for MAP-specific antibodies (IDEXX Laboratories Inc., Westbrook, ME, USA).

3.3.4.2 Environmental and fecal samples

Environmental samples were only collected at enrollment (T0). Environmental and fecal samples were collected individually and transported to our laboratory at the University of Calgary and stored at 4°C until further analysis for a maximum of 7 d. Individual fecal samples were organized in pools of 10 individual fecal samples. Aliquots of 0.3 g from each environmental and individual fecal samples were transferred to tubes prefilled with PBS. A MagMax total nucleic acid isolation kit (Applied Biosystems, Carlsbad, CA, USA) was used for direct DNA extraction. A qPCR targeting the ISMAP02 genomic target and an internal amplification control (IAC) was performed, with primers, probes and IAC sequence following recommendations provided by Slana et al. (2008) and Irenge et al. (2009). For both pooled and individual fecal samples as well as environmental samples, a Ct value cut off 35 was established with < 35 indicating that a sample is MAP-positive.

3.3.4.3 Laboratory practices to avoid cross-contamination

Samples from only one farm were handled at the same time. To prepare fecal pools, the bench surface was disinfected with bleach 10% and alcohol 70%, and a single wooden stick and weighing boat was used per sample; gloves were changed between 2 to 3 pools. However, if gloves were contaminated with feces, they were changed immediately. Further, DNA extractions

and qPCRs were always done using positive and negative controls, and no-template controls. If negative controls were positive, all DNA extraction kits and supporting materials were changed, the protocol was reviewed, and the DNA extraction from all samples was repeated. If no-template controls were positive, all master mix reactions and supporting materials were substituted to new ones and qPCR was repeated. However, if negative and no-template control were positive in the qPCR reaction, both materials from DNA extraction and master mix were changed to new ones and the full DNA extraction and qPCR were repeated. In addition, if positive controls were out of the expected Ct value range, it was concluded that the DNA extraction was not done efficiently, so the DNA extraction and qPCR for all samples were repeated.

3.3.4.4 Other *Mycobacterium* species cross-reacting with ISMAP02 qPCR

Approaches to evaluate the potential presence of other *Mycobacterium* species were explored to explain the sudden rise in qPCR positivity as presented in flowchart (Fig 3.9.1.).

a) Sequencing from PCR and qPCR products

DNA extracted from feces from positive calves based on qPCR targeting ISMAP02 gene were submitted to PCR and qPCR followed by sequencing. Initially a single PCR or qPCR reaction was purified and submitted to sequencing. Further, 5 PCR or 5 qPCR reactions were pooled together, purified (QIAquick PCR purification kit; Qiagen, Toronto, ON, Canada) and submitted to sequencing. The PCR and qPCR conditions were pre-denaturation 50°C for 2 min, followed by denaturation at 95°C for 20 sec, and 40 cycles of denaturation at 95°C for 3 sec, annealing at 60°C for 30 sec, followed by extension at 95°C for 1 min, and a final step of 72°C for 5 min. All sets of samples were included in agarose gels for electrophoresis of DNA samples.

b) TOPO cloning of a single or pooled sample DNA followed by sequencing

The same DNA templates from step a) were used in step b). The PCR product was obtained in the same way as step a) and it was submitted to TOPO cloning procedure (Invitrogen, Life technologies, Carlsbad, CA, USA). TOPO cloning contained 1 µL of purified PCR product, 1 µL of salt solution, 1 µL of pCR-2.1 TOPO vector and 3 µL of sterile water. The reaction was incubated for 25 min at room temperature. All 6 µL of the TOPO cloning reaction was transferred to DN5α cells and incubated with 900 µL of Super Optimal broth with Catabolite repression (SOC media) for 1.5 h at 37 °C. The cells were subsequently plated on LB agar plates with 100 ug/ml of ampicillin (Sigma-Aldrich) with 1 mg/ml of X-gal (Sigma-Aldrich) and incubated at 37°C for 16 h. The vector inserted into a competent cell can be transformed and grown in the presence of X-gal. Cells producing white colonies were transformed with the vectors containing the recombinant DNA of interest. When cells were transformed with vectors containing the recombinant DNA, while blue cells were transformed with vectors with non-recombinant plasmids. White colonies were selected for plasmid extraction using QIAprep Spin Miniprep kit (Qiagen) and sequencing.

Due to the low success observed in the molecular methods, culture methods were done to increase the DNA concentration to increase the success rate of steps a and b for further sequencing.

c) DNA extraction and qPCR on fecal samples from timepoints 2 and 3

Previously the issue on this farm was detected only qPCR targeting ISMAP02 gene from the second timepoint. As a result, DNA extraction and qPCR (section 3.3.4.2 and Appendix 6) targeting IS900 and F57 genes was also done to evaluate if the young stock was also positive for these genes.

d) Culture of individual fecal samples on 7H11 slants

To perform the culture protocols, only calves that were ISMAP02-positive at least 3 time-points were included. From each calf, only one fecal sample was selected to be part of the culture. The single fecal sample with the lowest Ct value was selected as representative of each calf.

For the culture, fecal samples were submitted to a decontamination protocol (Stabel, 1997). Briefly, 2 g of feces from each young stock was suspended in 35 ml of distilled water and mixed by vortex for 5 sec and left for 30 min at room temperature. Further, 5 ml of supernatant was mixed with 25 ml of half strength Brain Heart Infusion (BHI) with 0.9% hexadecylpyridinium chloride (HPC) broth and incubated at 37°C for 24 h. After 24 h, samples were centrifuged at 3,000 g for 20 min and the supernatant was discarded. The pellet was resuspended with 1 ml of BHI, 1 ml of distilled water and 1 ml of antibiotic solution (TREK para-JEM®; TREK Diagnostic Systems, Cleveland, OH, USA). Samples were incubated at 37°C for 24 h. Next, 150 µL of the mixture was inoculated in 7H11 slants supplemented with 10% OADC (Oleic Albumin Dextrose Catalase, BD), 2 g/L of Mycobactin J (Allied Monitor Inc., Fayette, MO, USA) and 0.4% glycerol (Fisher Scientific, Fair Lawn, NJ, USA).

Growth of colonies was observed for 8-16 wks. To determine if colonies were MAP or other *Mycobacterium* species, colonies were transferred to 7H9 broth supplemented with 10% OADC (Oleic Albumin Dextrose Catalase, BD), 2 g/L of Mycobactin J (Allied Monitor Inc., Fayette, MO, USA) and 0.4% glycerol (Fisher Scientific, Fair Lawn, NJ, USA). Once growth was also observed on 7H9, Ziehl Neesen (acid-fast) staining was performed. The fixed smear was placed on a staining rack and covered with carbol fuchsin for 3 min over a heat plate (approximately 60°C). Then, it was rinsed with bacteria-free pure water and decolorized with

alcohol until no more color ran from the smear. The smear was rinsed again with pure water, followed by the application of a counter stain for 2 min. Finally, pure water was used and the ample was left it to dry.

Red-stained AF rods were suggestive of *Mycobacterium* species. Colonies with a positive AF staining result, were submitted to DNA extraction using the DNeasy Blood and Tissue kit (Qiagen, Toronto, ON, Canada). Further, qPCR targeting F57 and ISMAP02 genes were performed as well as sequencing.

e) Culture of individual fecal samples on TREK ESP II

The same set of samples were submitted to TREK ESP II (TREK para-JEM®; TREK Diagnostic Systems, Cleveland, OH, USA) culture protocol with subsequent DNA extraction (Forde et al., 2012) followed by F57, IS900 and ISMAP02-specific qPCR.

3.4 Results

3.4.1 Environmental samples

All 6 environmental samples were positive by qPCR on the ISMAP02 MAP-target gene, whereas 5 of the 6 of samples were F57-positive. The Ct value for each sample for both genes is presented in Table 3.8. The Ct value for ISMAP02 ranged from 30.1 to 33.3, while the Ct value for F57 ranged from 33.6 to 37.4 (Table 3.8).

3.4.2 Prevalence results

The initial dataset consisted of 146 unique animals evaluated at different timepoints according to their age (Table 3.3). Fifteen, 95 and 36 animals were tested only 1, 2 or 3 times, respectively.

Table 3.3 represents the MAP prevalence at 4 time points for each animal category based on direct fecal qPCR and ELISA. At the first time point, the MAP prevalence based on direct fecal qPCR was 2% in cows >24 mo, whereas the prevalence was zero for the other age categories. The first MAP-prevalence result was 2% in animals >24 mo and were due to 2 MAP-positive cows (>24 mo). One of these cows had clinical signs of MAP infection. It was recommended to withhold the second cow from breeding followed by culling at end of lactation.

However, at the second visit, the MAP prevalence based on direct fecal qPCR increased to 62% in animals of 2 - 12 mo of age. Over time, MAP prevalence based on direct fecal qPCR decreases but remains high, considering the chronic characteristic of MAP infection. The ELISA results remained low throughout the 4 time points, with zero prevalence in the young stock at T1, T3 and T4 and one individual testing MAP-positive at T2. Prevalence for older cows was 7.6 and 12.5% at T1 and T4, respectively (Table 3.3).

MAP-positive calves were stratified by age considering each time point to evaluate potential MAP transmission in specific locations or age groups (Fig. 3.2). Results were based on direct fecal qPCR. At T1 there were no MAP-positive young stock. However, at T2, there were MAP-positive young stock in all age categories, with the majority being detected as MAP-positive between 8 and 12 mo of age. At T3, prevalence of MAP-positive young stock decreased from 62 to 52%, which reflects in a reduction of MAP-positive young stock in almost all age categories, except for an increase of MAP-positive young stock between 5-7 mo of age, which means that calves that were previously between 2-4 mo of age were still shedding MAP. At T4,

there was an increase in prevalence of MAP infection of calves between 8-10 mo of age, whereas in the other categories the number of MAP-positive calves decreased.

3.4.3 Temporal MAP shedding

In Table 3.8.4, animals were organized by decreasing age, from older to younger. Overall, most calves shed MAP at any of the time points.

3.4.5 Presence of other *Mycobacterium* species cross-reacting with MAP qPCR

Out of calves that were included at all 4 timepoints, 18 were MAP-positive on at least 3 timepoints. Sequencing of PCR and qPCR products did not yield any results that could be evaluated due to low MAP concentration. The same set of samples did not yield bands on agarose gel that could be used for gel purification and sequencing. TOPO cloning resulted in white colonies; however, only poor sequencing results were obtained. Although the colonies were white, large and isolated, which is characteristic of successful cloning, they did not have the right inserts, as only vector sequence was obtained. To improve the TOPO cloning technique, it was recommended by the manufacturer (Thermofisher Scientific Baltics UAB) to purify the PCR products before adding to the TOPO cloning reaction. However, purified and non-purified products were used, and it did not yield meaningful results. In addition to sequencing, qPCR targeting IS900 and F57 was obtained from timepoints 2 and 3 in addition to ISMAP02 results.

It was recommended by the manufacturer (Thermofisher Scientific Baltics UAB) to use a 1:1 to 2:1 molar ratio, starting with a 1:1 of PCR product:TOPO vector, as the TOPO cloning efficiency decreases significantly in the ratio of PCR product:TOPO vector is < 0.1:1 or >5:1. As a result, the concentration of the PCR product were adjusted before proceeding to TOPO cloning.

In addition to issue, the PCR product amount were adjusted, and a series of pool PCR products was done compared to a single PCR product. Although the pool PCR products loaded colonies, the sequencing results were not meaningful.

Prevalence of IS900-positive samples was 45 and 53% at timepoints 2 and 3, respectively, whereas any young stock was positive when F57 target gene was used (Table 3.6). Discrepancies between genes ISMAP02 and IS900 were detected in 9 samples, where ISMAP02 detected lower Ct values compared to IS900 (Table 3.7).

3.4.6 Culture approaches to explore the sudden rise in qPCR positivity

MAP-negative calves (n = 5) and MAP-positive calves, on at least 3 time-points (n = 19), were selected for solid and liquid culture methods (Table 3.8). The TREK ESP II culture resulted in 42% (8/19) of MAP-positive calves based on IS900 qPCR target gene, whereas the genes ISMAP02 and F57 were identified in 21% of MAP-positive samples (4/19) (Table 3.8).

Solid culture on 7H11 agar slants yielded 37 colonies, which were transferred to 7H9 broth before being AF stained. All colonies were AF negative, which indicates the absence or low concentration of AF positive / MAP bacteria in the fecal samples.

3.5 Discussion

The study is part of an effort to identify possible causes of a sudden rise in MAP prevalence on a dairy farm. We aimed to investigate possible issues with farm and laboratory cross-contamination while collecting and processing samples, as well as to determine the presence of other *Mycobacterium* species being identified by the ISMAPO2-target gene. ISMAP02-positive

calves were also positive by IS900 and F57 MAP-target genes; however, this could not explain the high prevalence on this farm.

The initial presence of MAP-positive animals was expected due to the high number of MAP-positive environmental samples (N=5/6) based on F57 MAP-target gene, which is specific to MAP (Herthnek and Bölske, 2006). According to Raizman et al. (2004) there is a correlation between the presence of MAP-positive environmental samples and the within-herd MAP prevalence. The study reported that MAP-positive environmental samples collected from lactating cows and manure storage areas were related to 53 to 73% of MAP-positive pools detected on farm.

Considering the within-herd MAP prevalence results, at the first whole herd sampling (T1), the MAP prevalence was 2% and in 2 mo went to 62% (T2). At T2 only animals between 2-12 mo of age were sampled. Based on experimental studies, the prolonged time from MAP infection to infectious stage is estimated to be at least 2 mo (Mortier et al., 2014a). As a result, the sudden rise in MAP prevalence is unexpected. In a cross-sectional study, only 2% of naturally infected young stock MAP-positive by fecal culture (Wolf et al., 2015). Similarly, another study reported that 2% of young stock tested were MAP-positive at any time, but of those that were MAP-positive 52% were between 7-14 mo of age (Bolton et al. 2011). The age range is in accordance with the present study, as the majority of MAP-positive calves were detected between 8-12 mo of age.

Although the MAP infection pressure on this farm is unknown, it was likely high, as evidenced by the detection of 5/6 MAP-positive environmental samples by qPCR targeting F57 gene and the occurrence of a clinical case detected at T1. This may explain why some calves were continuously shedding MAP. Additionally, young stock on farms with high MAP

prevalence shed MAP more frequently compared to young stock on farms with low MAP prevalence (Weber et al., 2010). Calves exposed to a high dose of MAP began shedding MAP sooner after MAP exposure (Van Roermund et al., 2007; Mortier et al., 2014). It is, therefore, possible that the presence of MAP-positive calves between 2-6 mo of age in this study was due to their exposure to a higher dose of MAP in the environment.

Housing and management were additional factors to be considered in this study. Young stock in this farm were housed in groups starting at 2 mo after birth and no biosecurity measures were implemented to prevent MAP transmission between calves. Although MAP transmission between calves was not evaluated on this farm, fecal-oral calf-to-calf transmission cannot be ruled out (Corbett et al., 2017), as well as MAP transmission by dust (Eisenberg et al., 2011). While the calves did not receive leftover feed from the older animals, it remains unclear whether the feed given to young stock was contaminated with MAP.

The high MAP prevalence detected in young stock could be attributed to passive shedding. However, experimental studies proved that passive shedding occurs after MAP inoculation and is not present one week later (Hines et al., 2007; Mortier et al., 2014). At this farm 8 calves shed MAP continuously between T2 to T4, which more likely indicates actual MAP infection than MAP passive shedding.

Although there is proof of the presence of MAP in the environment and a confirmed clinical case, the qPCR positive fecal samples presented a very high Ct value (between 30 to 34.9 Ct) based on ISMAP02 target gene. As a result, a confirmation using more specific MAP target genes, such as F57, was not possible due to the single copy number characteristic (Ellingson et al., 1998). In addition, as previous studies reported the presence of *Mycobacterium virginiense*

and *Mycobacterium nonchromogenicum* being detected by ISMAP02 (Park et al., 2018) is was important to further investigate the real cause of the outbreak.

Initially, issues with cross contamination during sample collection and laboratory practices were evaluated and excluded. Secondly, the identification of other potential *Mycobacterium* species being identified by ISMAP02 target gene were evaluated by different methods.

The sequencing of DNA directly extracted from feces was not successful. After performing PCR or qPCR to increase *Mycobacterium* copy numbers followed by product purification, the template concentration was too low to perform gel observation or sequencing. Even though the product concentration was adjusted by adding more template to the sequencing reaction, the results were still not meaningful, which is probably related to a poor quality of the original DNA sample. The second most common sequencing issue was a good quality data in the beginning to a suddenly not meaningful data, which might be a sign of secondary material in the template (DNA core). Regarding the TOPO cloning technique, potential colonies were obtained after performing the TOPO cloning methodology, which indicates high probability of successful cloning. However, after plasmid extraction, the plasmid did not contain the region of interest, which might be associated to cloning artifacts.

Further, based on the culture results from 19 MAP-positive calves by ISMAP02 gene from DNA directly extracted from feces, only a few samples were positive by ISMAP02, F57 and IS900 target genes. The samples were stored in -80 °C freezers from January 2022 to January 2023, when they were processed for culture. The probability that a sample decreases from high to mid-low MAP concentration and from mid-low to negative MAP concentration was 13.5% per month (Raizman et al., 2011). Considering all processed samples being mid-low

Mycobacterium load, there were chances of loss of viable MAP available for TREK ESP II liquid culture system. Although the liquid culture system confirmed the presence of truly MAP-positive young stock, due to the F57 qPCR positive results, this was previously confirmed by the environmental sample results. However, the results from TREK ESP II liquid culture system did not exclude the presence of other Mycobacterial species.

Despite these fecal samples were initially positive by qPCR targeting ISMAP02, they were mid-low shedding samples, and the *Mycobacterium* concentration was too low to yield positive culture results. Samples with higher Ct value (mid-low shedding animals) probably have lower odds of being identified as MAP-positive by solid culture, as solid culture presents approximately 2log MAP CFU lower compared to qPCR targeting F57 gene (Kralik et al., 2012), as molecular methods usually do not distinguish between dead and viable cells (Slana et al., 2008).

In the future, the issues with MAP-target genes should be addressed. Although IS900 and ISMAP02 can identify a higher proportion of MAP-positive animals that are mid-low shedding compared to the MAP-single copy genes, these genes are present in the genome of other *Mycobacterium* species ((Englund et al., 2002; Park et al., 2018). However, to date, there is no information on the proportion of other *Mycobacterium* species being identified by these genes. As a result, an overview of MAP-specific target genes and how they perform under different sample types, as well as accounting for the proportion of other mycobacterial species being identified by these genes should be addressed.

3.6 Conclusions

Based on MAP-positive environmental samples and young stock by F57 MAP-target gene, there was enough evidence that MAP is present on this farm. However, the sudden rise in MAP prevalence based on the ISMAP02 target gene could not be explained by MAP transmission and/or disease dynamics, and is therefore most likely due to other *Mycobacterium* species.

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Table 3.1. Animal category sampled at each timepoint on the farm enrolled in the study. Fecal samples for direct qPCR and blood samples for serum ELISA were collected from young stock (<12 mo) every 2 mo, whereas older animals (>12 mo) were sampled every 6 mo

Time-point	Date	Animal categories included
T1	November, 2021	• Young stock • Older animals
T2	January, 2022	• Young stock
T3	March, 2022	• Young stock
T4	June, 2022	• Young stock • Older animals

Table 3.2. Average qPCR Ct values of two target genes from environmental samples collected from lactating cow and manure storage areas on the farm enrolled in the study. The samples were used to assess the presence of MAP in the environment

Sample location	F57	ISMAP02
L1	36.9	30.4
L2	35.2	32.3
L3	33.6	30.1
MP1	35.8	31.6
MP2	34.5	33.1
MP3	37.4	33.3

L = lactating herd

MP = manure pit

Table 3.3. Within-herd MAP prevalence of the farm enrolled in the study based on direct fecal qPCR targeting ISMAPO2 and serum ELISA. Animals were stratified by ages between 2-12 mo, 13-24 mo and older than 24 mo

Timepoint	Age (mo)	Test (%; positive animals/ tested animals)	
		Direct fecal qPCR	ELISA
T1	2-12	0 (0/42)	0 (0/42)
	13-24[1]	0 (0/19)	0 (0/19)
	> 24	2 (2/89)	8 (7/92)
T2	2-12	62 (23/37)	3 (1/35)
	13-24	N/A (0/0)	N/A (0/0)
	> 24	N/A (0/0)	N/A (0/0)
T3	2-12	52 (14/27)	0 (0/29)
	13-24	N/A (0/0)	N/A (0/0)
	> 24	N/A (0/0)	N/A (0/0)
T4	2-12	43 (15/35)	0 (0/35)
	13-24	100 (31/31)	0 (0/31)
	> 24	32 (23/72)	13 (9/72)

[1]For animals older than 13 mo, samples were collected only two times (T1 and T4).

Table 3.4. MAP shedding status of calves based on 4 timepoints. Results presented are related to fecal samples collected individually from each calf (2-12 mo) at each timepoint and detected based on ISMAP02 qPCR test

ID	T1	T2	T3	T4
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30				
31				

32				
33				
34				
35				
36				
37				
38				
39				
40				
41				
42				
43				
44				
45				
46				
47				
48				
49				
50				
51				
52				
53				

Negative
Not tested
Positive

Table 3.5. qPCR results targeting IS900, ISMAP02 and F57 to evaluate when the sudden rise in MAP prevalence started. Samples included were fecal samples from timepoints 2 and 3 when only calves (2-12 mo) were included

Timepoint	qPCR target genes		
	IS900	ISMAP02	F57
2	45% (16/36)	28% (10/36)	0 (0/36)
3	53% (20/38)	34% (13/38)	0 (0/38)

Table 3.6. qPCR Ct values discrepancies between genes ISMAP02 and IS900 from Table 3.6. Samples included were fecal samples from timepoints 2 and 3 when only calves (2-12 mo) were included

Timepoint	Sample ID	qPCR target genes (Ct value)	
		ISMAP02	IS900
2	1	32.4	N/A
	2	33.16	N/A
	3	32.51	N/A
	4	30.79	N/A
	5	33.08	N/A
3	3	33.35	36.18
	6	33.67	N/A
	7	32.75	N/A
	8	34.53	N/A

Table 3.7. Liquid MAP culture results of MAP-positive calves based on direct fecal qPCR targeting ISMAP02.

Fecal samples were selected from calves that presented at least 3 MAP-positive results from the timepoints

Animal ID	[1]Average Ct value ISMAP02 between 3 visits	TREK ESP II qPCR IS900	TREK ESP II qPCR ISMAP02	TREK ESP II qPCR F57
1	31.7	0.0	0.0	0.0
2	32.4	0.0	0.0	0.0
3	31.6	19.08	22.1	23
4	31.7	0.0	0.0	0.0
5	32.4	0.0	0.0	0.0
6	32.7	0.0	0.0	0.0
7	32.0	0.0	0.0	0.0
8	32.1	0.0	0.0	0.0
9	31.1	30.16	34.8	34.5
10	30.9	0.0	0.0	0.0
11	31.4	34.8	0.0	0.0
12	33.0	17.5	20	21.8
13	31.9	37.8	0.0	0.0
14	31.0	31.8	0.0	0.0
15	31.9	35.44	0.0	0.0
16	35.3	0.0	0.0	0.0
17	33.9	19	22.4	21.9
18	34.4	0.0	0.0	0.0
19	34.4	0.0	0.0	0.0
20	0.0	0.0	0.0	0.0
21	0.0	0.0	0.0	0.0
22	0.0	0.0	0.0	0.0
23	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0

[1]Average Ct value ISMAP02 it is based on direct fecal qPCR

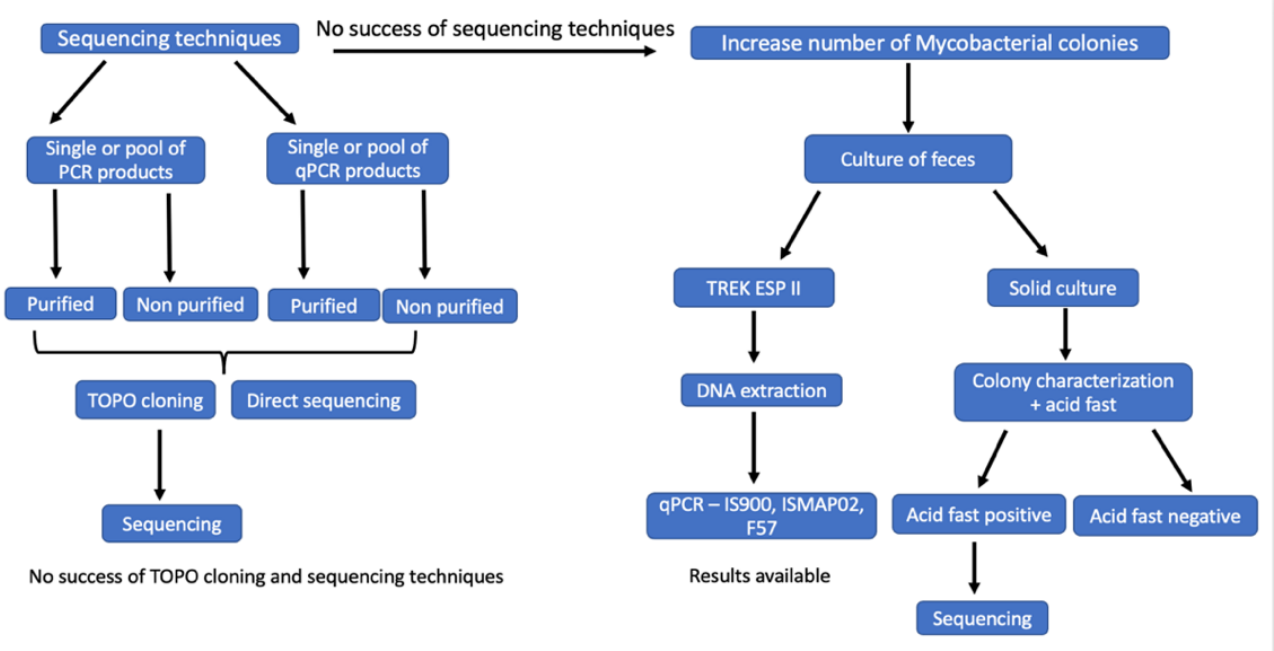

Figure 3.1. Approaches explored regarding the potential presence of other *Mycobacterium* species explaining the high prevalence of ISMAP02 PCR-positive fecal samples

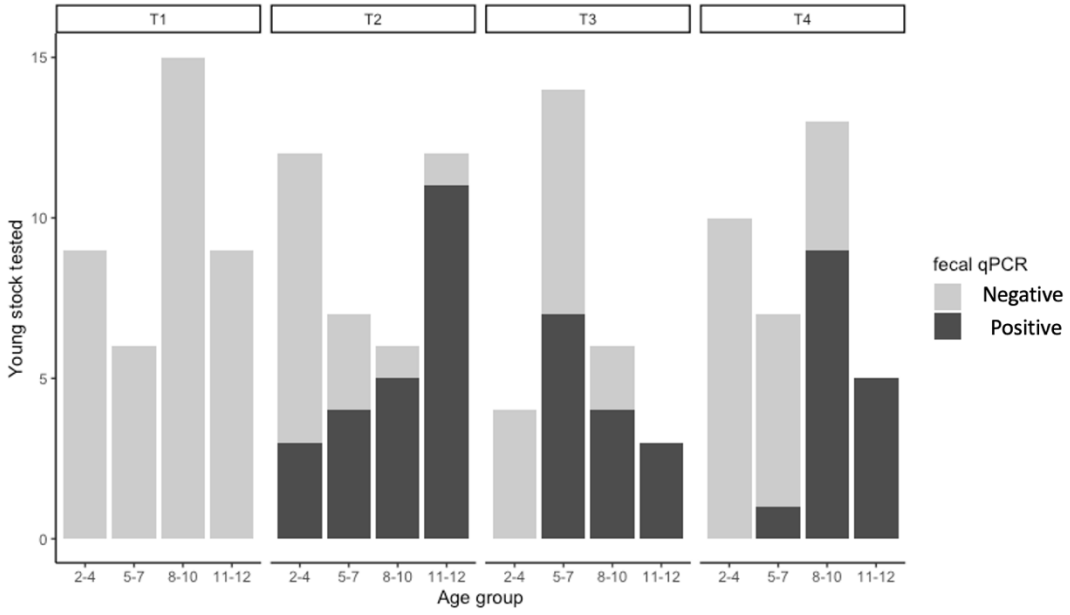

Figure 3.2. Number of ISMAP02-positive young stock stratified by age category and 4 timepoints. Fecal samples were collected individually from the farm with the sudden rise in MAP prevalence

CHAPTER 4. Accuracy of different *Mycobacterium avium* subsp. *paratuberculosis* target genes to detect MAP in different sample types

4.1 Abstract

Detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) based on culture methods is often challenging as the protocol is expensive due to specific materials required to stimulate growth of MAP and the 8-16 wks that the protocol takes to be completed (Clark et al., 2008). Direct MAP detection relying on efficient DNA extraction methods followed by qPCR is an attractive alternative. Improvement of the sensitivity and specificity of this method might come from an informed selection of the MAP target genes, as most single-copy MAP-specific genes are less efficient to detect MAP positive animals shedding low MAP concentration. The aim of the study was to evaluate the accuracy of different MAP qPCR target genes to detect MAP in different sample types. Genomic DNA of MAP, individual fecal samples, pools and environmental samples were obtained from different sources. For all sample types, DNA was extracted followed by qPCR targeting the following genes: IS900, F57, ISMAP02, *hspX*, *mbtA*-MAP217, MAP0865 and 251. All genes were able to detect MAP based on genomic DNA, individual and pool samples with high MAP shedding status. However, the single copy genes *hspX* and *mbtA* presented issues to detect MAP in mid to low level individual fecal samples and pools. Based on environmental samples collected from 24 farms, 42% of farms were positive by IS900, 20% by ISMAP02, 17% by F57, 8.3% by 865, 251 and *mbtA*, and 4% by *hspX*. IS900 and ISMAP02 lead to a higher percentage of MAP-positive farms compared to other target genes. Overall, all MAP target genes were able to detect samples with high MAP concentration. IS900

and ISMAP02 consistently identified MAP in all sample types. However, *mbtA*, *hspX* and F57 presented issues to detect samples with mid to low level MAP concentrations.

4.2 Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the cause of paratuberculosis, also known as Johne's disease (JD). MAP infection results in chronic granulomatous enteritis in domestic and wild ruminants (Li et al., 2005). Although several countries do not have surveillance programs in place (Fecteau, 2020), MAP-prevalence is still high, e.g. in Canada 46% of herds are MAP-positive (Corbett et al., 2018). Currently, MAP control in cattle relies on control programs focused on a combination of diagnostic tests, testing and management strategies and culling of MAP-positive animals.

Regarding MAP detection, although advancements in various tests have been made, culture-based methods are still considered the reference test when assessing sensitivity (Se) and specificity (Sp) of diagnostic tests (Dane et al., 2023). There are, however, several challenges associated with the culture and isolation of MAP. For instance, most culture methods available for MAP are expensive and require a long incubation period, usually between 8 to 16 wks. This lengthy incubation period can be further challenged by overgrowth with other microorganisms (Clark et al., 2008). Given the challenges associated with culture-based methods, it is important to refine existing options for MAP testing to more efficiently identify animals that are high or mid to low level MAP shedders and support JD control programs (Clark et al., 2008; Plain et al., 2014).

The rapid detection of MAP by PCR has been used as an alternative to overcome the issues with culture-based methods. Most studies have used the gene IS900 as the main MAP

target gene. Fang et al. (2002) achieved a Se and Sp of 93 and 92%, respectively, using DNA extraction from individual fecal samples followed by PCR targeting IS900 gene compared to HEYM culture. Khare et al. (2004) reported that they achieved a Se and Sp of 100% using immunomagnetic bead separation and bead-beating as the DNA extraction method compared to HEYM culture method. However, IS900-like sequences have been identified in other *Mycobacterium* species, such as *Mycobacterium porcinum* (Taddei et al., 2008), *Mycobacterium cookii* (Englund et al., 2002) and *Mycobacterium scrofulaceum* (Cousins et al., 1999), which raises concerns about the uniqueness of this gene to MAP. To address potential false-positive results, other MAP-specific target genes have been used, such as F57, *hspX*, ISMAP02, 251, 865, and *mbtA* (Brey et al., 2006; Slana et al., 2008; Irenge et al., 2009; de Kruijf et al., 2017). Although, F57, *hspX* and ISMAP02 have been used widely in studies, the remaining genes, such as 251, 865 and *mbtA* have been tested only on individual fecal samples. To date, no studies have evaluated and provided an overview of the ability of all these genes in detecting MAP based on different sample types, such as genomic DNA, individual, pooled and environmental samples. The objective of this study was, therefore, to evaluate the accuracy of different MAP – qPCR target genes to detect MAP in different sample types. We hypothesized that 1) all MAP-target genes correctly identify MAP-positive farms and animals if they have high MAP concentration on individual fecal, pooled fecal or environmental samples and correctly identify MAP-negative farms, 2) single copy MAP-target genes, such as F57, 865, 251*, mbtA* and *hspX* can misclassify MAP-positive farms or animals if they have mid to low MAP concentration, and 3) the genes IS900 and ISMAP02 correctly identify farms or animals if they have mid to low MAP concentration.

4.3 Material and Methods

4.3.1 Selection of MAP-specific genes

Specific qPCR primers for the genes in our the study were selected from previous publications (Table 4.8.1), except for a probe for *hsp*X which was newly designed using the Primer3 online tool (Thornton and Basu, 2011).

4.3.2 Plasmid standards and internal amplification controls

Plasmid standards and internal amplification control plasmids (IACs) for all 7 target genes for real time qPCR were constructed as described by Slana et al. (2008). Briefly, plasmid standards and IACs for each MAP-target gene were developed by cloning the PCR product into a pCR 2.1 cloning vector (Invitrogen, Life technologies, Carlsbad, CA, USA). Topoisomerase-based cloning (TOPO cloning) contained 1 µL of purified PCR product, 1 µL of salt solution, 1 µL of pCR-2.1 TOPO vector and 3 µL of sterile water. The reaction was incubated for 25 min at room temperature. Then, 6 µL of the TOPO cloning reaction was transferred to DN5α cells and they were incubated for 30 min on ice. After incubation, the cells were submitted to heat shock at 42°C for 90 sec and 2 min on ice and incubated with 900 µL of super optimal broth with Catabolite repression (SOC media) for 1.5 h at 37°C. The cells were subsequently plated on LB agar plates with 100 ug/ml of ampicillin (Sigma-Aldrich) with 1 mg/ml of X-gal (Sigma-Aldrich) and incubated at 37°C for 16 h. Colonies were evaluated based on the blue-white screening of the X-gal, and only successful colonies (white isolated colonies) were selected for plasmid extraction using QIAprep Spin Miniprep kit (Qiagen) and sequencing. After successful sequencing results were observed, the plasmid concentration was measured and the copy numbers were calculated (Slana et al., 2008).

A similar process was followed to develop the IACs for each MAP-target gene, with specific primer sequences for each of the 7 MAP-target genes flanking the potato (*Solanum tuberosum*) gene AtTS1 (AF483209). The same IAC probes were used for all IACs (Slana et al., 2008).

4.3.3 Real time qPCR conditions

The reaction components were as follows: 2X of TaqMan Fast Advanced Master Mix (Thermofisher Scientific Baltics UAB), 10 pmol of the primers MAP-target geneF and MAP-target geneR, 10 pmol of the MAP-target geneP labelled with FAM, 10 pmol of the IACP labelled with Cy5, $5x10^1$ copies of MAP-specific target gene IAC plasmid, and 2 µL of DNA template, in a total volume of 20 µL. Amplification and fluorescence detection was performed on the CFX384 (Bio-rad laboratories, Applera's United States) instrument using 96-well PCR plates under the following conditions pre-denaturation 50°C for 2 min, followed by denaturation at 95°C for 20 sec, 40 cycles of denaturation at 95°C for 3 sec, annealing at 60°C for 30 sec, followed by extension at 95°C for 1 min, and a final step of 72°C for 5 min. For all genes, the qPCR cut off was determined as 37 Ct, except for ISMAP02 gene, which had a cut off as 35 Ct.

4.3.4 Determination of MAP cell number recovered from MAP culture by each target gene

A MAP isolate (MAP A1-157) from Middlebrook 7H9 (Difco, Livonia, MI, USA), with OADC enrichment supplemented with 2 mg of Mycobactin J, was centrifuged at 1,000 g for 2 min and the DNA extraction was done as described (Forde et al., 2012).

The supernatant served as the DNA template for the real-time qPCR targeting all 7 MAP-specific genes. Each sample was run in duplicate. Total number of MAP cells was determined

from each MAP-target gene based on a ten-fold serial dilution and the Ct value of each dilution. A comparison between the observed and predicted results was done by calculating the MAP copy numbers based on the DNA concentration from the MAP culture.

4.3.5 Evaluation of qPCR targeting different MAP-specific genes based on individual fecal samples

Individual fecal samples were provided by the Accredited Laboratory Program (USDA). Samples were known to be MAP-positive and MAP-negative by ISMAP02-target gene and divided in high level shedding (Ct between 20 and 30) and mid to low level shedding (Ct > 30) animals.

Aliquots of 0.3 g of each fecal sample were transferred to tubes containing 1X PBS. A MagMax total nucleic acid isolation kit (Applied Biosystems, Carlsbad, CA, USA) was used for DNA extraction. A qPCR targeting all 7 MAP target genes and an IAC were performed following real-time qPCR conditions (4.3.3). Results with high discrepancy compared to United States Department of Agriculture (USDA) initial results were submitted to a blind detection with two Master's degree students familiar with JD diagnostics.

4.3.6 Evaluation of qPCR targeting different MAP-specific genes based on individual pooled samples

Using a random sample selection, fecal samples from 26 dairy cows and young stock known to be high level shedding MAP-positive based on ISMAP02 target gene (Ct value between 20 and 30; n = 5 samples), MAP-positive mid to low level shedding (Ct between 31 and 35; n = 11 samples) and MAP-negative samples (Ct >35; n = 10 samples) were selected from

Alberta dairy farms and from previous experimental studies conducted at the University of Calgary. Aliquots of 0.3g of each fecal sample were transferred to tubes containing 1x PBS. A MagMax total nucleic acid isolation kit (Applied Biosystems, Carlsbad, CA, USA) was used for DNA extraction. A qPCR targeting only ISMAP02-target genes and an IAC were performed following real-time qPCR conditions (4.3.3). Based on the qPCR results, animals were classified into MAP-negative (Ct<35), MAP-positive high level shedding (Ct between 20 and 30) and mid to low level shedding (Ct between 30 and 35). Further, fecal samples were combined in pools of 10 animals going from MAP-negative pools, when only MAP-negative samples were included to MAP-positive pools mid to low level MAP shedding to MAP-positive pools high level MAP shedding.

Individual fecal samples were organized in groups of 10 individual fecal samples. Aliquots of 3 g from each pool were transferred to tubes prefilled with PBS. A MagMax total nucleic acid isolation kit (Applied Biosystems, Carlsbad, CA, USA) was used for DNA extraction. A qPCR targeting all 7 MAP-target genes and an IAC were performed following real time qPCR conditions (4.8.2).

4.3.7 Evaluation of qPCR targeting different MAP-specific genes in environmental samples

Environmental samples were collected in triplicate from lactating cow pens and manure storage areas from 24 dairy farms in Alberta. The location of sample collection was selected based on previous studies that reported higher odds to detect MAP-positive samples in lactating cow pens and manure storage areas (Corbett et al., 2018). Environmental samples were transported to our laboratory at the University of Calgary and stored at 4°C until further analysis.

4.4 Results

4.4.1 Determination of MAP cell number recovered from pure culture by each MAP-target gene

Number of MAP cells recovered from pure MAP culture based on direct DNA extraction followed by qPCR targeting all different 7 genes is presented in Table 4.8.3. All corresponding qPCR assays detected MAP under high concentration of MAP. The predicted results from qPCR differ up to 1 Ct value per gene, and for around 2 Ct values for the *hspX* gene between predicted and observed qPCR results (Table 4.8.3).

4.4.2 Evaluation of qPCR targeting different MAP-specific genes in individual fecal samples

Individual fecal samples provided by USDA were divided in high and mid to low level MAP shedding and MAP-negative based on Ct values obtained with the qPCR targeting the ISMAP02 gene. Overall, all genes correctly identified high level shedding MAP samples, while all MAP-target genes correctly identified the MAP-negative samples as negative (Table 4.8.4). However, mid to low level MAP-positive samples were not correctly identified by all target genes. qPCR results targeting the *mbtA* gene identified only 4/6 mid to low MAP-positive samples, whereas qPCR results targeting the *hspX* gene did not identify any of the mid to low level MAP-positive samples. Fecal samples classified as mid to low level MAP samples presented a lower concentration of MAP cells compared to samples classified as high level MAP samples (Fig. 4.9.1).

A comparison between two examiners demonstrated differences in the qPCR results for MAP-target genes IS900, 865 and *mbtA*. Both examiners had issues to identify MAP-positive mid to low level samples by *hspX* and issues to identify sample 4 as MAP-positive by all genes. In addition, examiner 1 misclassified 1 sample by the target gene *mbtA* as negative, whereas examiner 2 misclassified 2 samples as negative based on the gene *mbtA* and 1 sample was misclassified as negative by IS900 and 865 genes (Fig 4.9.2).

4.3.4 Evaluation of qPCR targeting different MAP-specific genes in pooled fecal samples

MAP copies detected in each pool based on all 7 MAP-target genes is presented in Figure 4.9.3. Overall, all genes detected MAP-positive pools if at least one high shedding MAP-positive sample was included in the pool. However, the identification of MAP-positive pools became challenging once the number of MAP-positive mid-low level shedding pools was increased in the pool composition. All MAP-positive mid-low level shedding pools were identified by IS900, ISMAP02 and 865 MAP-target genes, whereas 251, F57, mbtA and hspX identified 6, 4, 2 and zero MAP-positive mid-low pools out of 10 pools, respectively. All genes correctly identified the MAP-negative pool, which excluded issues with Sp (i.e. false-positive results).

4.3.7 Evaluation of qPCR targeting different MAP-specific genes in environmental samples

Most MAP-positive environmental samples were detected in manure storage areas for IS900 and ISMAP02 MAP-target genes, whereas for the single copy genes, samples collected in the lactating cow barn resulted in more MAP-positive samples (Table 4.8.5). The heatmap (Figure 4.9.4) provides an overview of MAP log copy numbers per gene. As expected, IS900 target genes presented higher log copy numbers compared to other MAP-target genes. Overall, the

difference was 1 log copy from IS900 to ISMAPO2 MAP-target genes and an additional 1 log copy difference from ISMAP02 to other single MAP-target genes. When results were classified in log of MAP cells/g the differences between multicopy and single copy genes were less than 1 MAP cell/g. However, when samples presented very low MAP cells/g being detected by multicopy genes, the samples were not detected as MAP-positive based on the single copy genes.

4.5 Discussion

The study aimed to evaluate the combined accuracy of direct DNA extraction followed by qPCR assays targeting 7 MAP genes applied to different sample types. To address issues associated with MAP culture, such as cost and long incubation periods (Clark et al., 2008), direct DNA extraction followed by qPCR was adopted as the preferred detection method in many veterinary diagnostic laboratories. To mitigate human error during DNA extraction and qPCR, and to avoid false-negative results, IAC plasmids were used as a control (Slana et al., 2008). When comparing 3 direct PCR kits on fecal samples, Se ranged from 73.5 to 93%, whereas Sp ranged from 97 to 100% (Prendergast et al., 2018).

Although differences in log copy numbers per gene were detected, the concentration of MAP, as indicated by log of MAP cells/g, observed among the genes were similar. However, when a low MAP concentration was detected based on IS900 and ISMAP02 target genes, MAP-positive results from the same samples based on the single copy MAP-target genes (F57, 865, 251, *mbtA* and *hspX*)were not detected.

Across all sample types, detection of higher MAP copy numbers was anticipated when MAP target genes with multiple copies in the MAP genome, such as IS900 and ISMAP02, were used.

For instance, a study involving fecal samples from cattle estimated the analytical Sp of IS900 and 251 target genes as 100% across 40 MAP isolates (Imirzalioglu et al., 2011) whereas the analytical Se of IS900 was 1 fg (1 Femtogram = 10^ -6 nanograms of genomic DNA) (Kawaji et al., 2007).

All 7 qPCR assays targeting MAP target genes successfully detected MAP genomic DNA. As expected, IS900 and ISMAP02 presented higher number of gene copies per genome than the single copy MAP-target genes as they have 15 and 6 copies in the MAP genome (Li et al., 2005). The single copy genes presented similar log copy numbers, ranging from 6.1 to 6.5 log copies, except for the gene *hspX*, which detected only 5 log copies/ml. The lower copy numbers detected by the MAP single copy genes can be attributed to their respective copy numbers in the MAP genome. For instance, Donaghy et al. (2011) reported higher Se of IS900 compared to F57 target gene. IS900 could detect as few as 1 MAP genome, whereas the F57 target gene required a minimum of 4 copies of the MAP genome due to its single copy nature (Ellingson et al., 1998).

In this study, issues were encountered with the genes *hspX* and *mbtA* across all sample types. Specifically, when examining individual fecal samples, all genes successfully identified MAP-positive samples with high shedding, whereas negative samples were correctly identified as MAP-negative. However, *mbtA* and *hspX* genes had issues to detect mid to low level MAP-positive individual samples. Although issues with *hspX* gene were observed in all sample types, it is worth noting that *hspX* is commonly used for MAP detection in the Vet Alert JD Real-time PCR (Tetracore, Inc., MD). Alinovi et al. (2009) reported a Se of 90% for qPCR using this kit. Nevertheless, the lower efficiency of *hspX* observed in this study may be attributed to issues on the probe design, as the Vet Alert kit was not used.

Another possible explanation for the lower efficiency of *hspX* and *mbtA* target genes is the size of the amplicons used. TaqMan-based amplicons are typically recommended to be within the range of 70 to 150 base pairs (Dooley et al., 2004). The *hspX* and *mbtA* genes were designed with larger amplicon sizes (211 and 307 base pairs, respectively). However, it is worth noting that other studies have reported similar results between *mbtA* gene and F57, which is inconsistent with the findings of this study (Krujif et al., 2017).

Overall, pools containing at least one fecal sample from a high shedding animal were successfully detected as MAP-positive by all MAP target genes. However, the F57, *mbtA* and *hspX* genes did not identify MAP in pools that consisted only of mid to low level MAP-positive and negative individual samples. The 865 gene outperformed the F57 gene in identifying MAP-positive pools, contrary to the expectation based on their similar Se on individual fecal samples in a previous study (Imirzalioglu et al., 2011). Studies have primarily assessed the Se and Sp of pools using culture followed by PCR targeting F57 or IS900 genes, examining various pool sizes (van Schaik et al., 2007; McKenna et al., 2018). In contrast, this study focused on fixed-sized pools (n=10 samples) using direct DNA extraction and qPCR targeting the 7 MAP target genes.

Among the sample types analyzed in this study, the environmental samples were the only ones with unknown MAP status. Only one of these samples tested positive for all MAP target genes (Fig. 4.9.4) whereas most of the positive samples were only positive by IS900 and ISMAP02 target genes. The lactating cow barn presented a higher prevalence of MAP-positive samples compared to manure storage areas, except for the IS900 and 251 genes (Table 4.8.5). Corbett et al. (2018) reported higher prevalence of MAP-positive samples from lactating cow areas compared to manure storage areas, which is in agreement with the present study.

The study provides an overview of the most used MAP target genes under different sample types. However, there were some limitations. For instance, only one environmental sample tested positive for all single copy genes, making it difficult to draw conclusions from this small subset. Although the sample size was limited, the general pattern of multicopy genes detecting more MAP mid to low level shedding samples was observed in all sample types. Therefore, further expansion of this study to encompass additional scenarios of composition of pooled fecal samples and explore more MAP-positive environmental samples from other dairy farms would be beneficial.

4.6 Conclusions

In general, all MAP target genes were able to detect samples with high MAP concentration and they presented similar concentration of MAP cells. Among them, IS900 and ISMAP02 consistently performed well in identifying MAP in all sample types. However, the *mbtA* and *hspX* genes had challenges to detect mid to low levels of MAP on individual fecal samples, while the F57, *mbtA* and *hspX* target genes identified less mid to low level MAP-positive pools.

Considering that the same DNA extraction method was applied to all sample types, the differences in MAP detection obtained between the genes can be attributed to their copy numbers in the MAP genome only when considering mid to low MAP positive samples and to qPCR issues related to primer design of the genes *mbtA* and *hspX* and probe design of the gene *hspX*.

4.7 References

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Table 4.1. MAP-target genes for used for qPCR on genomic DNA, individual, pooled and environmental samples

Target gene	Name	Type	Sequence 5' -> 3'	Location	Length of the product (bp)	Reference
IS900	IS900F	Forward	GATGGCCGAAGGAGATTG	94-111	145	Slana et al., 2008
	IS900R	Reverse	CACAACCACCTCCGTAACC	238-220		
	IS900P	Probe	6FAMATTGGATCGCTGTGTAAGGACACG-BHQ	158-182		
ISMAP02	ISMAP02F	Forward	CGCCAGGAACGCAAACAT	946-963	96	Irenge et al., 2009
	ISMAP02R	Reverse	GTGCAGGGTCGCTCTGATG	1023-1041		
	ISMAP02P	Probe	6FAMACTCCGCATCCAACAACTCACGCTG-BHQ	946-963		
F57	F57F	Forward	GCCCATTTCATCGATACCC	422-440	147	Slana et al., 2008
	F57R	Reverse	GTACCGAATGTTGTTGTCAC	568-549		
	F57P	Probe	6FAMCAATTCTCAGCTGCAACTCGAACACAC-BHQ	508-534		
865	865F	Forward	GCGCGGCCAGTATGGATATA	886453-886472	81	Imirzalioglu et al., 2011
	865R	Reverse	GACTCAACCCAACGAGCTCC	886534-886553		
	865P	Probe	6FAM-AGATGCCTCTCCGATGCTCGATGG-BHQ	886496-886519		
hspX	hspXF	Forward	CTCGTCGGCTTGCACCTG	2424510-2424527	211	Brey et al., 2006
	hspXR	Reverse	GACCGGCTATCTGTGGAAC	2424702-2424720		
	hspXP	Probe	6FAM-GATTCAGATACCACGACGGG-BHQ	2424554-2424573		
251	251F	Forward	GCAAGACGTTCATGGGAACT	3095973-3095992	165	Rajeev et al., 2005
	251R	Reverse	GCGTAACTCAGCGAACAACA	3095790-3095809		
	251P	Probe	6FAM-CTGACTTCACGATGCGGTTCTTC-BHQ	3095919-3095941		
mbtA-MAP2179	mbtAF	Forward	CTCCCGCAACTCGGTCAC	2422652-2422669	307	Kruijf et al., 2017
	mbtAR	Reverse	CACAGCCAGGTGTGAAAG	2422941-2422958		
	mbtAP	Probe	6FAM-ACGGTTCGAGAAGTCGATTCTACTTGCAC-BHQ	2422745-2422773		
IAC probe[1]	IACP	Probe	Cy5GGCTCTTCTATGTTTCTGACCTTGTTGGA-BHQ	2712-2826	152-154	Slana et al., 2008

[1]IAC: internal amplification control

Table 4.2. Pool composition designed to test qPCRs targeting all 7 genes

Pool composition			
Negative pools N= 1	Positive (N=6; mid to low shedding samples)	Positive (N=5; only high shedding samples)	Mixed pools (N=4)
10 x N[1]	1 x M[2], 9 x N	1 x H[3], 9 x N	4 x H, 1 x M, 5 x N
	2 x M, 8 x N	2 x H, 8 x N	3 x H, 2 x M, 5 x N
	3 x M, 7 x N	3 x H, 7 x N	2 x H, 3 x M, 5 x N
	4 x M, 6 x N	4 x H, 6 x N	1 x H, 4 x M, 5 x N
	5 x M, 5 x N	5 x H, 5 x N	
	10 x M		

[1]N: negative fecal samples (CT >35 or N/A)

[2]M: mid-low shedding fecal samples (CT between 31 and 35)

[3]H: high shedding fecal samples (CT between 20 and 30)

Table 4.3. Number of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) cells quantified by qPCR from MAP pure culture by each MAP-target gene. A comparison between predicted and observed qPCR results

Genes	Predicted		Observed	
	Copy numbers	Log copies	Copy numbers	Log copies
IS900	3.35E+08	8.52	1.98E+08	8.3
ISMAP02	1.34E+08	8.13	1.01E+07	7.0
F57	2.23E+07	7.35	1.24E+06	6.1
865	2.23E+07	7.35	3.75E+06	6.57
251	2.23E+07	7.35	1.87E+06	6.27
mbtA	2.23E+07	7.35	1.80E+06	6.25
hspX	2.23E+07	7.35	1.10E+05	5.04

Table 4.4. Number of MAP-positive individual samples provided by USDA and submitted to qPCR targeting all 7 genes. Results from animals shedding high MAP concentration and negative animals

Level of shedding	MAP-target gene						
	IS900	ISMAP02	F57	865	251	*mbtA*	*hspX*
High (n = 6)	6	6	6	6	6	6	6
Negative (n = 6)	0	0	0	0	0	0	0

Table 4.5. Percentage of MAP-positive environmental samples based on manure storage areas and lactating cow barn according to qPCR targeting 7 MAP-target genes

Environmental sample source	% of MAP-target genes (Number positive per location/Total number of positive samples)						
	IS900	ISMAP02	F57	865	251	*mbtA*	*hspX*
Manure storage areas	52 (12/23)	36.4 (4/11)	37.5 (3/8)	40 (2/5)	50 (2/4)	0 (0/4)	0 (0/4)
Lactating cow barn	48 (11/23)	64 (7/11)	75 (6/8)	60 (3/5)	50 (2/4)	100% (4/4)	100% (1/1)

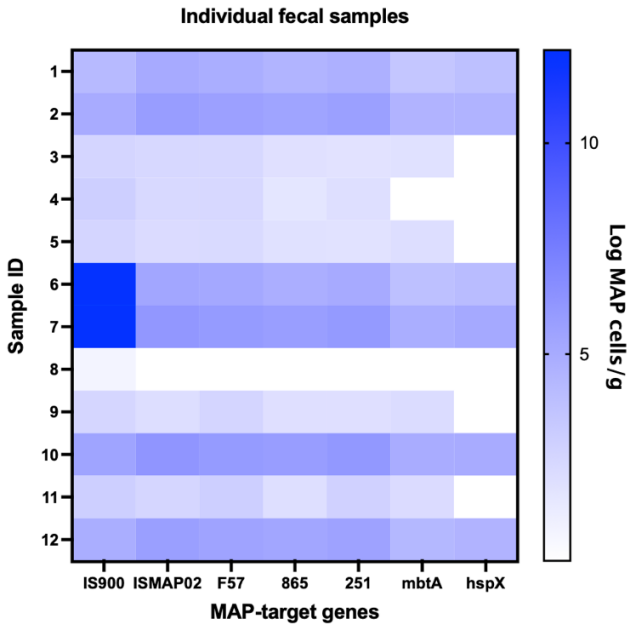

Figure 4.1. Heatmap of individual fecal samples and log of the number of MAP cells/g based on different qPCR assays targeting 7 MAP-target genes

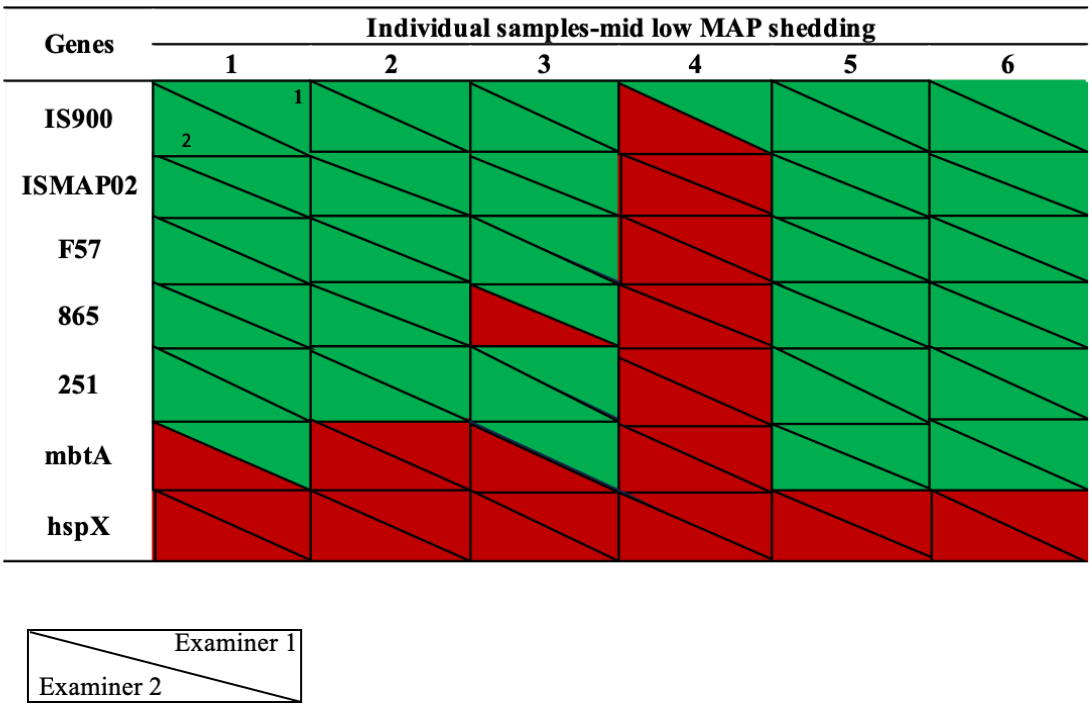

Figure 4.2. Comparison of MAP detection from mid to low individual fecal samples between 2 examiners.

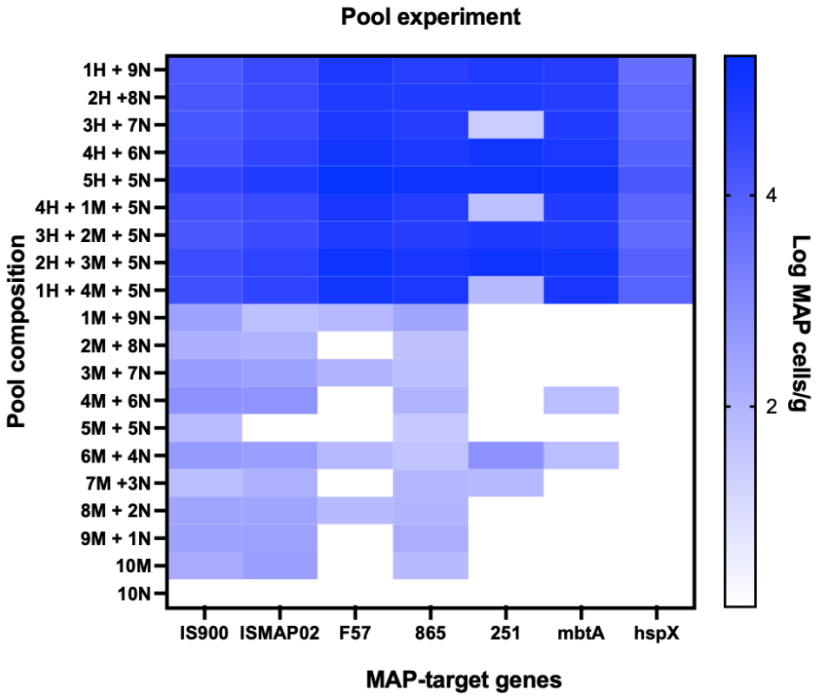

Figure 4.3. Heatmap of pool composition and log of MAP cells/g based on different qPCR assays targeting 7 MAP-target genes

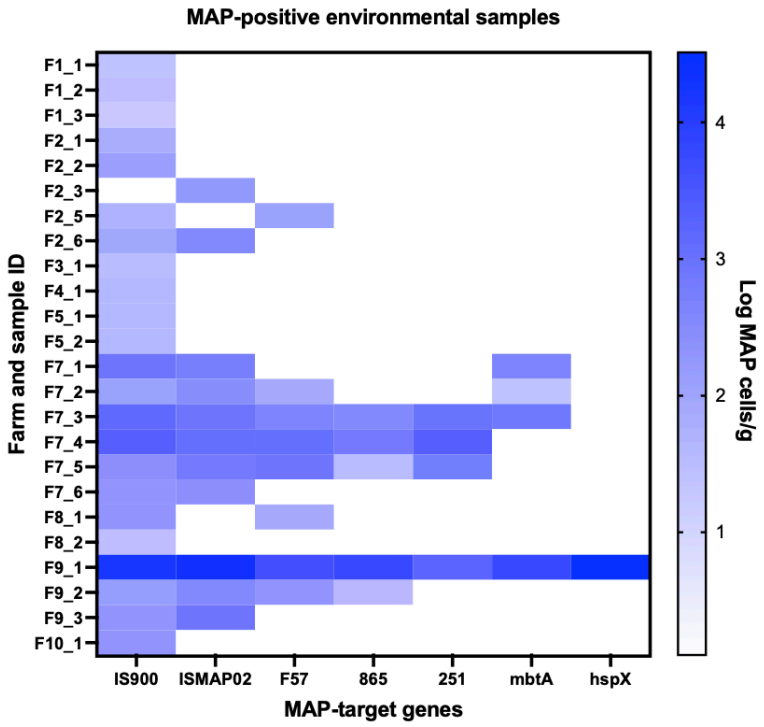

Figure 4.4. Heatmap related to log of MAP cells/g on environmental samples based on different qPCR assays targeting 7 MAP-target genes

CHAPTER 5. Summarizing discussion and future perspectives

5.1 Summary of results

The aims of this study were 1) to evaluate if inclusion of calves would improve the results of a JD testing strategy, and 2) to improve diagnostic MAP tests and evaluate their performance on commercial dairy farms. This study was supported by evidence that calves are able to shed MAP based on studies that focused on experimental and naturally infected calves (Mortier et al., 2014; Wolf et al., 2015) and also able to transmit MAP to other calves (Van Roermund et al.; Corbett et al., 2017).

Most JD control programs that have reported results include calves only as part of management strategies, such as biosecurity, and milk and colostrum management. In Chapter 2, we aimed to provide an overview of advances in diagnostics and management control strategies to support inclusion of young stock in effective testing strategies as part of JD control programs.

The recommendations from Chapter 2 were implemented in a JD control program focused on the inclusion of calves in the testing strategy (Chapter 3). The aim was to explore whether the sudden rise in qPCR positivity observed was the result of farm and/or laboratory contamination with MAP, or presence of other *Mycobacterium* species cross-reacting with MAP qPCR targeting ISMAP02 gene. To the best of our knowledge, this study is the first evaluation of including calves more actively in JD control programs, as part of testing strategies. Further, Chapter 4 was a deeper evaluation of the issues encountered in Chapter 3 due to MAP target genes used on qPCR assays. The aim was to evaluate the accuracy of different MAP qPCR target genes to detect MAP based on different sample types.

The research in Chapter 2 gathered evidence of MAP shedding and transmission among young stock based on culture of feces followed by PCR and ELISA assays. Experimental studies provided that young stock can shed MAP regular and present strong immune response when exposed to MAP at an early age (Mortier et al., 2014a; b). Based on naturally infected calves, a cross-sectional study detected 2% of young stock as MAP-positive by fecal culture followed by comfirmation with qPCR targeting F57 gene (Wolf et al., 2015). Chapter 2 also provides an overview of diagnostic tests that could be used to detect MAP and biomarkers that indicate MAP infection. Studies on ELISA have been focusing on the detection of recombinant proteins to improve sensitivity (Se) and specificity (Sp) of the test, whereas for IGRA most studies have been associated to the development of new antigens, optimization of the best time to use IGRA and changes in the interpretation criteria (Hughes et al., 2017, Dernivoix et al., 2017, Corneli et al., 2021). New diagnostic tests to improve MAP detection in young stock, such as host-based gene expression analysis, metabolic tests and phage based assays, were also described in Chapter 2.

Chapter 2 also provides an overview of recommendations to include young stock more actively on JD control programs based on management and tetsing strategies. Regarding management, although biosecurity, and milk and colostrum management are still essential to prevent new MAP infections among calves, it is very important to prevent transmission of MAP between calves, which might be an issue when addressing group housing of calves (Barkema et al., 2015). Considering experimental and naturally infected young stock, the optimal age for detecting MAP in young stock might be between 6-12 mo of age. However, there were no clear recommendations on the age and frequency of testing. Bolton et al., 2011 tested calves every 4 mo. Due to additional costs to include young stock in the JD testing strategy, pooled fecal

samples in young stock (Bolton et al., 2011) or temporally pooled samples from the same animal over time (Hines Ii et al., 2007; Mortier et al., 2014) were alternatives provided. The current study provided information that producers should be aware of MAP transmission between calves and alternatives for testing and testing strategies to support a reformulation of current JD control programs are needed.

Based on the evidence provided in Chapter 2, a tentative inclusion of calves in a JD control program was developed for the study described in Chapter 3. The farm enrolled in the study presented MAP-infected animals confirmed by MAP-positive environmental samples and MAP-positive calves by fecal culture followed by qPCR targeting the F57 gene, which is specific to MAP (Li et al., 2005; Slana et al., 2008). Although there was enough evidence of MAP infection, the MAP prevalence in young stock based on ISMAP02 target gene was higher with a more sudden increase compared to previous studies, which raised doubts regarding the MAP Sp of the ISMAP02 target gene. Sequencing and solid culture protocols were applied to evaluate the presence of other Mycobacterial species. However, the methods were not successful mainly due to the low MAP DNA concentration and presence of inhibitors on fecal samples.

Due to issues with the ISMAP02 target gene, the study described in Chapter 4 was designed to address these issues in a deeper level, to provide an overview of MAP target genes available and to compare their efficiency in detecting MAP between sample types. MAP qPCR target genes were tested on samples with high MAP concentration (genomic DNA from pure MAP culture) to samples with low MAP concentration (environmental samples). Across all sample types, the detection of higher MAP copy numbers was detected by IS900 and ISMAP02 MAP target genes, which was anticipated due to their higher number of copies in the MAP genome compared to single copy genes (Li et al., 2005). However, this difference was observed only on

mid to low level MAP-positive samples. When high level MAP-positive samples were evaluated, it was not possible to observe high differences in concentration of MAP between multi and single copy genes.

All 7 MAP-target genes detected MAP based on MAP culture (Chapter 4). In addition, all genes were able to detect MAP on samples with high MAP concentration, such as individual samples from high level shedding animals and pools with at least one high level shedding animal included in the pool. However, when mid-low level MAP-positive samples were evaluated, the single copy genes presented issues to detect MAP-positive samples, especially the genes *hspX* and *mbtA*. The issue might be explained by their amplicon size being larger than the TaqMan recommendation (Dooley et al., 2004), as they were designed with a larger amplicon (211 and 307 base pairs for hspX and mbtA, respectively). However, other studies reported adequate Se and Sp of the gene hspX (Alinovi et al., 2009), whereas de Kruijf et al. (2017) reported similar results between mbtA and F57 genes. However, these studies did not report MAP copy numbers or MAP cells present in the fecal samples. As a result, the studies did not evaluate the genes in different levels of MAP shedding from fecal samples. The challenge to identify mid-low level MAP-positive animals is also common in other diagnostic tests. For instance, Whitlock et al. (2000) detected only 38% of true MAP-positive cows by culture because they were detected as mid-low level shedding animals.

5.2 Study limitations

There were limitations in the studies reported in this thesis. In Chapter 2, we could not provide solid recommendations to producers based on new diagnostic tests. Most studies

regarding promising diagnostic tests are small trials involving small group of animals. In addition, recommendations on frequency of testing were only provided based on experimental trials as most studies based on naturally infected animals were cross-sectional studies.

In the study described in Chapter 3, it was not possible to satisfactorily explain the issues related to the sudden rise in MAP prevalence based on ISMAP02 target genes. Although issues with sample contamination on farm and in the lab was eliminated and the evidence that MAP on farm was confirmed, it was not possible to explain the cause of the sudden rise in MAP prevalence (from 0% in timepoint 1 to 62% in timepoint 2). Therefore, the study did not allow for the observation of other *Mycobacterium* species that were possibly present on the farm. In Chapter 4, the limitations are related mainly to the small sample size presented in all sample types. For instance, when environmental samples were evaluated, only one sample was positive for all 7 genes, which did not allow a proper analysis of the results. In addition, the individual fecal samples part of the pooled experiment originated from different sources. Mid-low level shedding fecal samples were provided from naturally infected animals, while feces of high-level shedding were provided from experimental studies involving young stock.

5.3 Future perspectives

Providing clear recommendations regarding new diagnostic tests age and frequency of testing would support producers to take better decisions for JD testing. Regarding diagnostic tests, phage-based tests, metabolomics and host-based gene expression studies are based on experimental studies with insufficient sample size and not readily implemented diagnostic testing

kits and protocols. The expansion of these studies for naturally infected young stock, considering the effects of a farm environment, could provide diagnostic alternatives to producers.

Chapters 3 and 4 provided issues of detecting MAP in calves and alternatives for MAP-target genes. Although several studies detected other *Mycobacterium* species being identified by IS900 and ISMAP02 (Englund et al., 2002; Park et al., 2018), there is not enough evidence of the proportion of other *Mycobacterium* species that could be present compared to MAP. As a result, whole genome sequencing studies to evaluate DNA from the environment and individual animals could support the evaluation of other *Mycobacterium* species.

Additional cost-effective diagnostic tests should be evaluated to include calves in the JD testing strategy more efficiently. As a result, direct DNA extraction from feces might be a possible alternative. However, it is important more studies on PCR inhibition factors that can affect MAP detection (Acharya et al., 2017; Fock-Chow-Tho et al., 2017).

5.4 References

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6. Appendix

6.1 Fecal qPCR protocol

6.1.1 Materials, reagents and calculations

Table 6.1. Materials and reagents required for DNA extraction

Materials	Reagents
Filter tips (20, 200, 1000 µL)	PBS
Micropipettes (20, 200, 1000 µL)	100% Isopropanol
Microcentrifuge tubes (2 mL, 1.5 mL)	Lysis binding solution
Serological pipettes and controller	Carrier NA
Bead tubes	Binding beads
Multichannel pipette	Lysis binding enhancer
Magnetic stand	Wash solution 1 and 2
Thermomixer	Nuclease-free water
Heat block	
Ice	
Centrifuge	

Table 6.2. Lysis binding solution calculation

Lysis binding solution	1 sample	# Samples	Total
Lysis binding solution	232 µL		
Carrier NA	3 µL		
Total	235 µL		

Table 6.3. Bead mix solution calculation

Bead mix	1 sample	# Samples	Total
Binding beads	10 µL		
Lysis/binding enhancer	10 µL		
Total	20 µL		

Table 6.4. Nuclease-free water and Isopropanol calculation

	1 sample	# Samples	Total
Nuclease-free water	50 µL		
Isopropanol	65 µL		

Wash solution 1: add 12 mL of 100% isopropanol to the original bottle.

Wash solution 2: add 32 mL of 100% ethanol to the original bottle.

Lysis/binding solution: Prepare solution inside the biosafety cabinet as calculated in the table above. Mix lysis binding solution by inverting the tube 10 times.

Bead Mix: Prepare bead mix inside the biosafety cabinet as calculated in the table above. Vortex the bead mix for few seconds and put on ice until use.

6.1.2 DNA extraction steps

Disruption of the samples

1. Label microcentrifuge tubes and bead tubes with sample IDs.

2. Fill 2 mL microcentrifuge tubes with 1 mL 1X PBS.

3. Weigh 0.3 g of feces and transfer into a microcentrifuge tube prefilled with 1mL PBS. Shake the tubes for 3 min in thermomixer at 1300 rpm.

4. Turn on the heat block and set the temperature to 65°C.

5. Centrifuge the sample-PBS mixture for 1 min at 100 g or allow sample to settle down for 20 min.

6. Aliquot required amount of elution buffer/ nuclease-free water for elution step and 100% isopropanol in microcentrifuge/centrifuge tubes as needed. Leave the elution buffer or water in heat block at 65°C.

7. Fill each bead tube with 235µL lysis binding solution.

8. Transfer 200µL of sample supernatant to the bead tubes pre-filled with lysis binding solution.

9. Bead beat the tubes for 5 min twice, leave the tubes on ice for 5 min between the bead beating cycles.

10. Transfer the tubes onto the heat block and leave for 10 min.

11. Centrifuge the tubes at 16000Xg for 6 min.

Purification of nucleic acid

12. Transfer 115µL of the supernatant into each well of 96-well plate.

13. Add 65 µL of isopropanol (100%) into each well with samples.

14. Shake the plate in the thermomixer for 1 min at 500 rpm, RT.

15. Add 20 µL of bead mix to each well with the sample and shake for 5 min in the thermomixer.

16. Put the plate on magnetic stand for 3-5 min and carefully discard the supernatant.

17. Wash the mixture with wash solution 1 by adding 150 µL of the wash solution with the multi-channel pipette. Shake the plate for 1 min at 500 rpm.

18. Put the plate on a magnetic stand for 1 min and discard the supernatant using multi-channel pipette.

19. Repeat steps 17 and 18 for the second washing step with wash solution 1.

20. Wash twice with wash solution 2 following the same steps as with wash solution 1 (steps 17, 18, and 19).

21. Take the lid off the plate and shake the plate on thermomixer at 500 rpm for 2 min to dry the bead.

• If there is remaining solution shake for another 1-2 min, do not exceed 5 min.

22. Add 50 µL of elution buffer then shake for 3 min at 500 rpm.

• Inspect the wells to ensure that the magnetics beads are uniformly resuspended in the elution buffer.

23. Put the plate on the magnetic stand for 1 min.

24. Transfer the supernatant to 1.5 mL tubes. Store the DNA in -20°C or -80°C freezer.

6.1.3 qPCR targeting ISMAP02

1. Thaw primers, probes, and plasmids on ice.

2. UV microcentrifuge tubes, 96-well plates, water and filter tips for 10-15 min.

3. Prepare the Mastermix solution in the Mastermix room by adding following components except IAC plasmid and DNA template.

Table 6.5. qPCR components for Mastermix

Components	Volume per sample	Total volume
Master Mix (TaqMan Fast Advanced)	10 µL	
Primer F (10 µm)	1 µL	
Primer R (10 µm)	1 µL	
Probe (10 µm)	1 µL	
IAC probe (10 µm)	1 µL	
IAC plasmid (50 copies)	2 µL	
Nuclease-free water	2 µL	
DNA template	2 µL	

Next steps will be conducted in template room.

5. Add required volume of IAC plasmid into the Mastermix solution in the template room.

6. Mix the solution by pipetting up and down and spin the tube briefly.

7. Transfer 18 µL of the Mastermix solution into individual well of 96-well plate.

8. Add 2 µL of template DNA into respective wells.

9. Seal the plate well.

10. Centrifuge the plate at 300g for 2 min at 4°C.

11. Load the plate into qPCR machine and run the following cycles.

1. 50°C, 2 min
2. 95°C, 20 sec
3. 95°C, 3 sec
4. 61°C, 30sec

} 40 cycles of step 3+4

5. 95°C, 1 min
6. 72°C, 5 min
7. 4°C

6.2 ISMAP02 and IAC plasmids

Primer sequences from ISMAP02 target gene were provided from Irenge et al., 2009. Plasmids and internal control plasmids were developed based on TOPO cloning as described in 3.3.4.4 section b). For plasmids, the PCR reaction for the PCR products was composed by MAP DNA from a pure culture and ISMAP02 primers. For IAC plasmids, the PCR was composed with ISMAP02 primers and instead of MAP DNA, a gBlock containing the ISMAP02 primers and a sequence of the potato (*Solanum tuberosum*) gene AtTS1 (AF483209) were used (sequence below).

CGCCAGGAACGCAAACATAGATTTACAGGACACTTCCTGTTAGAGAGGAAATACTTA

AGGCTCTTCTATGTTCTGACCTTGTTGGATTCCACACTTTCGACTATGCTCGACATTTC

CTTTCGTGTTGCAGCATCAGAGCGACCCTGCAC

The probe binds to green part. The only part that differs it is the red part, which is related to the forward and reverse primer from each gene of interest (F57, ISMAP02, IS900).

6.3 ISMAP02 validation

6.3.1 qPCR Ct cut off value

The Ct cut off value of the ISMAP02 target gene was determined based on a standard curve using plasmids in different concentrations as provided (Figure 6.3.1.1). Considering the results from the serial dilutions compared to the copy numbers, the Ct value was determined at 35 for ISMAP02 target gene (Table 6.3.1.1).

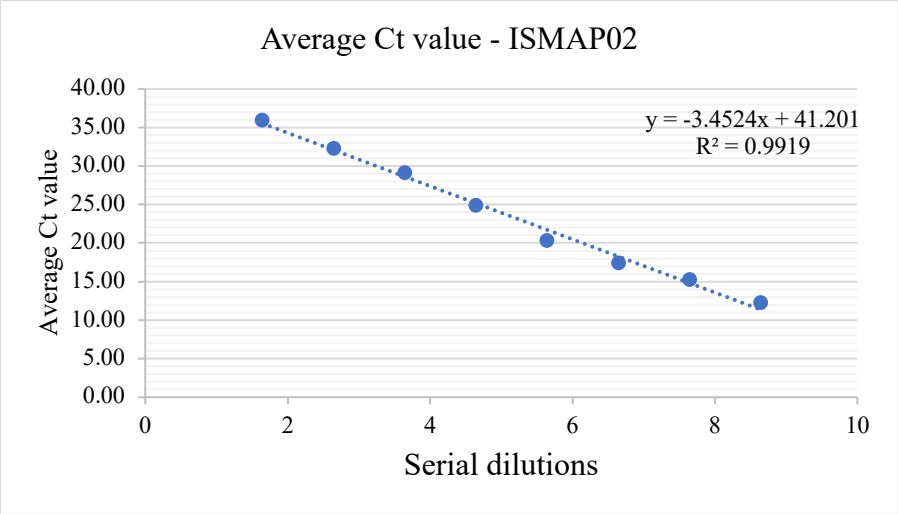

Figure 6.1. Standard curve using ISMAP02 target genes to determine Ct value cut off

Table 6.6. Standard curve to determine Ct value cut off decision

Standard	Copies	Ct value 1 FAM	Ct value 2 FAM	Average Ct value
1	4.41E+08	12.29	12.28	12.29
2	4.41E+07	15.29	15.22	15.26
3	4.41E+06	17.51	17.33	17.42
4	4.41E+05	20.39	20.33	20.36
5	4.41E+04	24.84	25.01	24.93
6	4.41E+03	28.95	29.28	29.12
7	4.41E+02	32.26	32.24	32.25
8	4.41E+01	36.15	35.67	35.91
9	4.41E+00	0	38.14	19.07
10	4.41E-01	0	0	0.00

6.3.2 USDA panel for ISMAP02 validation

Fecal samples previously known to be ISMAP02 positive and negative were provided by USDA to validate the lab internal protocols. Samples were submitted to all DNA extraction steps (6.1.2) and qPCR targeting ISMAP02 gene (6.1.3). Results comparing both USDA and our internal lab protocol is on Table 6.3.2.1.

Table 6.7. ISMAP02 validation based on fecal samples provided by USDA

Sample ID	USDA IMAP02 (Ct value)	ISMAP02 our qPCR
1	Negative	Negative
2	Negative	Negative
3	Negative	Negative
4	Negative	Negative
5	Negative	Negative
6	Negative	Negative
7	High (22.11)	Positive
8	High (22.3)	Positive
9	Low (34.4)	Positive
10	Low (31.6)	Positive
11	Low (34.6)	Positive
12	High (24.3)	Positive
13	High (23.6)	Positive
14	Low (36.8)	Negative
15	Low-mod (31.9)	Positive
16	High (20.7)	Positive
17	Low-mod (31.5)	Positive
18	High (22.4)	Positive