2018-05-10

Mycobacterium avium subsp. paratuberculosis: herd prevalence and calf-to-calf transmission

Corbett, Caroline Susan

UNIVERSITY OF CALGARY

Mycobacterium avium subsp. *paratuberculosis*: Herd prevalence and calf-to-calf transmission

by

Caroline Susan Corbett

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN VETERINARY MEDICAL SCIENCES

CALGARY, ALBERTA

MAY, 2018

Abstract

Johne's disease results in a progressive chronic enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), leading to economic losses among dairy producers worldwide. In the absence of an effective vaccine to prevent infection and treatment for infected animals, control is primarily based on decreasing the number of new transmissions within a herd. Control programs have been implemented in countries and regions around the world; however, comparisons among prevalence estimates of difference regions and control programs are difficult and unreliable due to different tests used to identify infected animals and herds. Therefore, the first objectives of this thesis were to elucidate the influences of environmental sample characteristics on the outcome status of a herd, and to estimate the prevalence of MAP based on 2 environmental samples (and 3 environmental samples when including young stock) in Canada. Six environmental samples were collected twice, 3.5 years apart, from 148 dairy farms to determine whether difference in prevalence between sampling periods were associated with herd size and sample characteristics. All environmental samples regardless of type, had decreased odds of testing positive in the second sampling, and the largest herds had increased odds of testing positive than smaller herds at both sample periods. Across 4 regions (10 provinces) in Canada, 2 environmental samples, one from the lactating cow area and one the manure storage, were collected from 362 dairy farms, with an additional sample collected from breeding age heifers. Prevalence was lowest among tie-stall herds, in herds ≤ 100 cows, and in Québec; and although breeding age heifer samples did not affect prevalence estimates, they provided additional evidence that young stock are shedding MAP on farm. Therefore, the second objectives of this thesis were to determine

the extent to which calf-to-calf transmission occurs among group-housed penmates, and to quantify the amount of fecal shedding that occurs among these infectious calves. An experimental transmission study was conducted, where 32 newborn calves were grouped into 7 experimental groups of 4, consisting of 2 inoculated (IN), and 2 contact exposed (CE) calves, and 1 control pen with 4 non-exposed calves. Calves were group-housed for 3 months, during which fecal, blood and environmental samples were collected frequently. The based reproduction ratio (R_0) was estimated as a parameter of transmission of MAP infection using a final size (FS) model with a susceptible-infected-recovered (SIR) model based on ELISA and tissue culture. In addition, transmission rate parameter (β) was estimated using a GLM with a susceptible-infected-susceptible (SIS) model based on fecal culture during group housing. Throughout group housing, all IN and CE calves had MAP-positive fecal samples, and although there was a difference between frequency of shedding, there was no difference between the quantities of MAP shed in feces. All IN calves had positive MAP-tissue samples, and 7 (50%) of CE calves had positive tissue samples. Based on fecal shedding, the basic reproduction ratio R_0 for CE calves (R_0^{CE}) was 3.24 (95% CI: 1.14, 7.41). R_0^{I} (based on interferon-γ results from blood samples) was 0.90 (95% CI: 0.24, 2.59), and R_0^{T} (based on tissue) was 1.36 (95% CI: 0.45, 3.94). Additionally, the effects of freezing on the ability to identify MAP in tissue samples were found to be minor; however, there may be a greater effect for CE calves that should be considered when freezing tissue samples. In conclusion, environmental samples characteristics did not influence the infection status of a herd, and collecting 2 environmental samples could be used to estimate prevalence and compare differences among regions. Shedding calves transmit infection to fellow penmates;

therefore, future control programs should consider monitoring and testing of young stock to further decrease new transmissions on farm.

Preface

This thesis consists of six manuscripts, three which have been accepted for publication, and three which are currently under review. The following manuscripts are included in this thesis:

Chapter 2

Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, J.P., Barkema, H.W. (submitted 2018). Environmental sample characteristics and herd size associated with decrease in herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis*. J Dairy Sci, submitted.

Chapter 3

Corbett, C.S., Naqvi, S.A., Bauman, C. A., De Buck, J., Orsel, K., Uehlinger, F., Kelton, D.F., Barkema H.W. (submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci, submitted.

Chapter 4

Corbett, C.S., De Buck, J., Orsel, K, Barkema, H.W., 2017. Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48, 27.

Chapter 5

Corbett, C.S., de Jong, M.C.M., Orsel, K., De Buck, J., Barkema, H.W. (submitted 2017). Quantifying transmission of *Mycobacterium avium* subsp. *paratuberculosis* among group-housed dairy calves. Vet Res, submitted.

Chapter 6

Corbett, C.S., Barkema, H.W., De Buck., J. 2018. Quantifying fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* from calves after experimental infection and exposure. J Dairy Sci 101, 1-10.

Chapter 7

Corbett, C.S., De Buck, J., Barkema, H.W. 2018. Effects of freezing on ability to detect *Mycobacterium avium* subsp. *paratuberculosis* from bovine tissues following culture. J Vet Diagn Invest, accepted.

Statement of work done

Collection of samples, and processing of samples for research reported in Chapter 2 was organized and performed in collaboration with Uliana Kanevets and Aaron Lucko at the University of Calgary and with Saskatchewan Milk and Saskatchewan Agriculture. Similarly, for Chapter 3, describing the Canadian prevalence of MAP infection, data collection, and processing of results was done in collaboration of Drs. David Kelton and Cathy Bauman of the University of Guelph and the staff of the National Dairy Study. For the four manuscripts dealing with results from the experimental transmission trial, Caroline Corbett was involved in study concept, design, inoculum preparation and procedure, collection and analysis of samples, statistical analyses of results and drafting manuscripts. All six of theses manuscripts were completed under the guidance of Herman Barkema and Jeroen De Buck. The supervisory committee contributed substantial knowledge and support regarding design, development and analysis of manuscripts. University veterinarian Dr. Greg Muench provided essential help with animal health and management, along with the staff at the Veterinary Sciences Research Station. Ali Naqvi assisted with statistical analysis of research reported in Chapters 2 and 3. Dr. Mart de

Jong from Wageningen University, NL, assisted with the quantitative modeling in Chapter 5. All co-authors provided critical review of the manuscripts, and permission has been obtained from publishing journals as well as all co-authors to reprint the manuscripts in this thesis.

The following manuscript that I co-authored while I was a PhD-student at the University of Calgary was not included in this thesis:

Barkema, H.W., K. Orsel, S. S. Nielsen, A. P. Koets, V. P. Rutten, J. P. Bannantine, G. P. Keefe, D. F. Kelton, S. J. Wells, R. J. Whittington, C. G. Mackintosh, E. J. Manning, M. F. Weber1, C. Heuer, T. Forde, C. Ritter, S. Roche, C. Corbett, R. Wolf, J. P. Kastelic, J. De Buck. 2018. Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subsp. *paratuberculosis* infection. Transbound Emerg Dis.

Acknowledgements

I would sincerely like to thank my supervisors Herman Barkema and Jeroen De Buck, for all of your continued support, encouragement, and dedication to excellent scientific work. I truly believe that I would not be the researcher I am today without your guidance through the many fields of scientific research. Thank you both for pushing me to strive for the best, and to continue working through mistakes, frustration and tough situations (especially in those early lab days). I have learned so much through your mentorship, and I consider myself very fortunate to have been one of your students.

My supervisory committee: Dr. Karin Orsel, Dr. Andre Buret, and Dr. John Kastelic. Thank you for your experience, knowledge contribution, and unwavering support as I found my way through the PhD program. Dr. Karin Orsel, thank you for always having an open door and providing new perspectives to the challenges I was facing. You always knew the right questions to ask to guide me to the solutions on my own. Dr. Andre Buret, I am so grateful you were able to join my committee, as your knowledge and experience came at such an important time. Thank you for asking the hard questions, and being so supportive as I worked to find their answers. I truly have grown as a researcher because of your patience and guidance. Dr. John Kastelic, your support and kind words helped me to get through some very difficult times. When I had lost faith in myself as a research scientist, your encouragement helped me to overcome these challenges. You not only helped me grow as a researcher, but helped me to embrace and understand myself as a person.

Of course, this work would not have been possible without the support of the dairy producers, and amazing Veterinary staff and husbandry staff at the Veterinary Science

Research Station. Dr. Greg Muench, thank you for your patience and dedication to the health and welfare of the calves in my research study. To all of the staff that helped with the care of the 32 calves, thank you for caring so much about these animals. I would also like to thank Charlotte Pickel, Laura Cain, Uliana Kanevets and Indiana Best for all of your help throughout the course of the trial. I could not have accomplished so much without you.

To my fellow grad students, friends who have become my family over the last 4 years, thank you. I consider myself so lucky to have met you during this journey, and I would not be where I am today without you. We stumbled through and figured this thing out together, and I am eternally grateful I was not alone. Thank you for being there for me always, laughing with me, crying with me, sometimes both at the same time. For late nights and early mornings, and allowing me to work through my brain thoughts to come up with solutions for both work, and life problems. You are all so special to me.

Last but not least, thank you to my family. Aaron Lucko, thank you for being my never-ending supporter and enthusiast. I am so lucky to have you in my life to debate hypothetical science fiction scenarios, process samples late into the evenings, escape on new adventures, work out scientific and person problems, and to just be goosey with. I would not be the person and researcher I am today without your love and encouragement. To my parents, thank you for cheering me on throughout this endeavour. To my Mom in particular, I have no words to thank you for your unconditional love and support. Thank you for being an inspiration to me.

To the calves whose lives were sacrificed for this research:

SteveRogers, TonyStark, Loki, BruceBanner, ScottSummers, Logan, Eric, CharlesXavier, Toad, Yoshi, Bowser, Luigi, Spock, Kirk, McCoy, Khan, Summer, Ghost, Shaggydog, Greywind, Arnold, Carlos, Ralphie, Tim, Moony, Padfoot, Wormtail, Prongs, Spike, Duckie, Littlefoot and Petrie.

Table of Contents

List of tables

List of figures

List of abbreviations

AJDI	Alberta Johne's Disease Initiative
β	Transmission rate
BAH	Breeding age heifer
BHI	Brain heart infusion
CAD$	Canadian dollar(s)
CE	Contact-exposed
CI	Confidence interval
CNDS	Canadian National Dairy Study
CT	Cycle threshold
d	Day(s)
DE	Direct extraction
ELISA	enzyme-linked immunosorbent assay
FS	Final size
GLM	Generalized linear model
HD	High dose
HPC	Hexadecylpyridinium chloride
I	Infectious
IAC	Internal amplification control
IN	Inoculated
INF-γ	Interferon-gamma
JD	Johne's disease
LAC	Lactating cow
LD	Low dose
LN	Lymph node
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MD	Moderate dose
mo	Month(s)
N	Total number of animals
PCR	Polymerase chain reaction

qPCR	Quantitative polymerase chain reaction
R_0	Reproduction ratio
S	Susceptible
Se	Sensitivity
SIR	Susceptible-infected-recovered
SIS	Susceptible-infected-susceptible
Sp	Specificity
T_{IN}	Infectious period of inoculated calves
T_{CE}	Infectious period of contact exposed calves
US$	United Stated dollar(s)
wk	Week(s)
yr	Year(s)

CHAPTER 1. GENERAL INTRODUCTION

1.1 Johne's disease and MAP infection

Johne's disease (JD) is a chronic, progressive, inflammatory disease in the small intestine of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is well established that MAP infection is widespread in cattle and causes great economic losses to dairy producers worldwide, due to lower productivity, increased risk of culling and decreased slaughter value [38]. It is estimated that these losses due to JD annually cost CAD$15 million in Canada [8] and US$ 200-250 million in the United States [9]. In addition to economic costs, there is also evidence of a potential association between MAP and Crohn's disease, a common and debilitating chronic enteritis of humans, leading to concern that it may be pathogenic to humans, thereby, increasing the need to control this disease [4, 52].

Clinical onset of JD is characterized by chronic, intermittent, non-treatable diarrhea and wasting, despite having a good appetite [8, 61] . In the subclinical phase, cows have decreased milk production, increased risk of being culled and a decreased slaughter value [8]. The main route of MAP transmission is considered to be fecal-oral from infectious adults to susceptible young calves through ingestion of contaminated material, such as contaminated feed, contaminated milk, or oral contact with manure containing MAP [60]; however, other routes such as intrauterine infection [74] and possibly inhalation of contaminated dust are possible [19]. An infection of MAP may be present in an animal for months or years before clinical signs appear. During this time, they are infectious when intermittently shedding MAP into the environment, with the potential to infect many cows or calves in the herd before detection and identification of infected individuals can be made. In an effort to control the spread of MAP, most dairy producers

implement the best possible hygiene management practices in order to decrease transmission via the fecal-oral route.

The bacterium MAP targets mucosa-associated lymphoid tissue in the gastrointestinal tract, making its way through the M-cells in the Peyer's patches and subsequently being phagocytosed by macrophages [21]. MAP is an intracellular bacterium, surviving and multiplying inside macrophages, inducing a cellular response in nearby lymph nodes [1, 10]. This subclinical stage of infection can persist for years before developing into clinical disease (which only manifests in a small proportion of MAP-infected animals). One of the early responses to the intracellular infection by MAP is a type 1 response by T cells, characterized by the production of interferon gamma (IFN-γ) [1, 15]. This response is persistent throughout infection indicating that once MAP has established itself it is able to inhibit INF-γ dependent clearance by the immune system of the cow [1]. At the onset of an unknown trigger, possibly stress during lactation or parturition, there is a shift from a type 1 immune response to type 2 response typically coinciding with the onset of clinical disease [3, 15, 59]. This shift is characterised by production of antibodies and inflammation of the gut which leads to clinical symptoms of the disease [58]. Over time, larger portions of the gastrointestinal tract become infected, leading to a chronic inflammatory response as a result of the immune system responding to the infection [21].

1.2 Diagnostics

One of the difficulties associated with control of JD and transmission of MAP is the ability to accurately detect infected animals and as a result, to reliably confirm negative herds. Diagnostic tests rely on the ability to either detect MAP in fecal or tissue samples, or through detection of the host's immune response with either INF-γ or antibody ELISA

tests [11]. Each diagnostic test has its own test characteristics. Therefore, there is no true "gold standard" for the detection of infected animals, as no diagnostic test available is either 100% sensitive or specific. Tests relying on detection of bacteria are often highly specific, but lack sensitivity as bacteria may not be present in the sample, or at time of sampling [42, 46]. Alternatively, detection of immune responses are highly reliant on age, the length of time an animal has been infected, and they have the potential to cross-react with other types of mycobacterial infections [21, 46, 47]. Due to disagreements between diagnostic methods, the long incubation of the bacteria and imperceptible changes between stages of disease, it is impossible to detect all MAP-infected animals in a herd with a single test at any point in time [25, 37, 41, 44]. Regardless of the method used to detect infection, the difficulty to accurately determine the number of MAP-infected animals in the herd, and the inability to quantify the amount of MAP bacteria being shed into the environment, are other limitations for controlling this disease.

1.2.1 MAP culture from feces and tissue

Fecal and tissue culture both rely on detection of MAP bacteria from samples acquired, and are therefore considered ideal for the detection of infected animals. Tissue culture is considered the "gold standard" method for detection of MAP-infected animals [10]; however, samples for tissue culture are collected post-mortem which is not always feasible or desired. Fecal culture is an alternative method of detection commonly used for identification of shedding animals; however, false-negative outcomes and false-positives are possible due to intermittent shedding and passive shedding, respectively [69]. Because both fecal and tissue culture methods detect MAP *per se*, they are considered to be nearly 100% specific if a positive test is reported when combined with detection of

MAP-specific genetic material (F57) by conventional Polymerase Chain Reaction (PCR), or quantitative PCR (qPCR) [47, 56, 71]. Regardless, fecal shedding does not always indicate infection, and a negative fecal culture results does not indicate a MAP-negative animal [71]. Infectious individuals can shed MAP intermittently, and it has been reported that MAP can pass-through the animal without causing infection (passive shedding), making confirmation of an infected animal inconsistent when using fecal tests [29, 45, 71]. Additionally, stage of infection (i.e., newly infected, subclinical infection, clinical infection etc.), level of shedding and antibody response impact on the sensitivity of fecal culture [70, 79].

Culture protocols for both tissue and fecal samples are typically conducted in three phases: 1) disinfection of sample, 2) culture and growth of bacteria either in liquid broth or on a plate, and 3) detection of MAP-specific DNA [21]. Due to the slow growing nature of MAP, culture procedures can take up to 4 mo [20, 72]; however, this enables detection of MAP when present in low quantities. Samples are often frozen prior to processing, and survival of MAP after the freezing of fecal samples is well documented [27, 51]. However, despite tissue samples being commonly stored at -80°C before culture, there are apparently no reports regarding the effects of detecting positive samples in frozen-thawed tissues.

1.2.2 Direct detection of MAP-specific DNA

Detection of MAP bacteria is possible without culture, as DNA can be directly extracted from either fecal or tissue samples and detected using MAP-specific primers and PCR [54]. One advantage of using this method is the ability to use an internal amplification control (IAC) which allows for the distinction between true and false negative results.

Additionally, because growth is not allowed by culture, quantification is possible when using qPCR and single copy genes, as higher CT values are directly associated with the amount of DNA being replicated [30]. F57 is a genetic sequence that is MAP-specific, and only present as a single copy; therefore, it is ideal both for identification of positive samples and quantification of the amount of MAP present [54]. Detection of positive samples with direct DNA extraction depends on the quantity of MAP present in feces or tissues being at detectable levels [54]; however, the method does not rely on live bacteria, which allows for handling and storage (i.e., freeze-thaw cycles, prolonged transportation etc.) that may alter the viability of MAP [27, 62, 73].

1.2.3 Detection of an immune response

Detection of MAP-positive animals can also be achieved through the detection of either a cellular or humoral response [59]. The INF-γ assay or the Johnin skin test detects the initial cellular response due to infection, and is often used for early diagnosis of infection with a reasonably high sensitivity of 57-100% [26, 58]. However, interpretation of the test is highly variable and difficult to interpret, and most literature recommends it be used as a supportive diagnostic tool to detect exposure within a herd [23, 24].

Antibodies produced as part of the type-2 humoral response can be detected with ELISA in either blood, or milk samples; however, reported sensitivity range from 7 to 94% depending on antigen used, gold standard to make test characteristic estimates, and individual progression of infection [47]. For both INF-γ assay and antibody ELISA's, there is a large amount of individual variation in the host immune response to an infection that affects the ability to accurately identify positive animals [16, 43]. It is widely regarded that serological studies greatly underestimate the prevalence of MAP

infection in a herd [63], as the humoral response tested by an ELISA may not always be detected due to delayed antibody response [16].

1.2.4 Environmental sampling

Environmental sampling on a farm is a cost-effective and reliable method for detection of MAP-positive herds [7], especially for the detection of low-prevalence herds [32]. The most common sampling technique requires six samples to be collected from various locations on a dairy farm, typically from areas of high manure concentration, e.g. alleyways, gutters, or manure storage sites. These samples are then cultured the same way as fecal culture, and presence of MAP is confirmed by detection of MAP-specific DNA sequences using conventional PCR or qPCR. Although it is not possible to identify individual positive animals with environmental sampling, there is evidence that the proportion of environmental samples on farm is correlated with the within-herd prevalence of infection [7, 17]. Additionally, environmental samples are estimated to have a sensitivity of 87% for detection of an infected herd [18], and due to the survivability of MAP in the environment, detection is not as affected by intermittent shedding as are fecal samples [72]. Due to the convenience, reliable, and cost-effective characteristics of environmental sampling, control programs have started to use it as a sampling method for detection and surveillance across the US and Canada [68, 76].

1.3 Control programs

In the absence of an effective treatment or vaccine for JD, control of the disease is currently based on preventing the transmission of MAP, and control programs have been developed and implemented worldwide [5]. Control programs can vary and include testing and culling positive animals, preventing spread and protecting negative herds, and

decreasing prevalence in bulk tank milk. The type of control program implemented is largely based on the overall aims of control of MAP infection in the region or country such as certification, eradication of clinical disease, protecting the exportation of milk, or decreasing transmission and prevalence [5]. Canadian Johne's disease control initiatives rely on detection of MAP-positive herds and subsequent risk assessments and corresponding recommendation by trained veterinarians, resulting in changes to management practices that will lead to a decrease of new infections on the farm [77]. One underlying assumption for current management protocols is that only cows can be infectious and only calves are susceptible; this has led to JD prevention and control programs encouraging separation of calves from their dams immediately after birth and placing them in calf pens [38]. An effective JD control program, includes two main objectives: 1) decrease calf exposure to manure (decrease incidence of new infections); and 2) reduce the number of infected animals that shed the bacteria in their manure (decrease prevalence) [38]. Although control programs have been implemented worldwide, the evaluation of the effectiveness of these programs have been underreported, largely due to the difficulties associated with measuring cow-level, within-herd, and herd-level prevalence [5].

1.4 Prevalence

Prevalence of MAP infection can be measured at either the cow-level (proportion of cows infected in total population), within-herd (proportion of cows infected within a herd), or at the herd-level (proportion of herds infected). The level at which prevalence can be estimated is based on the diagnostic test(s) being used, as "pooled" tests such as bulk milk tank ELISA or environmental samples can only estimate herd-level prevalence,

compared to individual tests such as fecal culture, serum ELISA, or INF-γ assay. Accurate prevalence estimates are essential for control, surveillance, and monitoring the effectiveness of a control program over time [5]; however, these estimates can be extremely difficult to make or compare due to the complexity of infection progression as well as the variability of diagnostic tests [5, 67].

In the United States, herd prevalence estimates based on environmental samples have been estimated to be 70%, and could be as high as 91% when adjusted for sensitivity and specificity of the test [33]. Across regions in Canada, prevalence has been estimated using environmental samples, bulk tank ELISA, tissue culture from slaughtered animals and serum ELISA; however, the same method has not been used across the whole country, making comparisons between regions difficult [36, 49, 76]. Herd-prevalence estimates in Canada have been estimated based on seroprevalence of antibodies as detected by ELISA and ranged from 8 to 50%; however, these are likely underestimating true prevalence as confirmation of prevalence based culture from fecal pooling has resulted in estimated ranging from 28 to 57% of farms being infected [63]. Most recently, environmental samples have been used to estimate the true prevalence in Alberta and Saskatchewan and have resulted in prevalence estimates of 68 and 76%, respectively [76]. The large variation and range of herd-level prevalence estimates are difficult to interpret and compare, which can cause complications in monitoring and surveillance of the success of JD control programs across regions and countries [5].

Due to the nature of the infection, it can take 4 to 8 y to detect any effect of the control program may have on prevalence or economic consequences [48]. However, based on long-term studies, herd prevalence estimates decrease over time when control programs

are in place [12, 57]. Because of the large amount of resources required for a long term study of changing prevalence, research investigating effectiveness of control programs can be modelled based on transmission both within, and between herds [13, 35]. These models are an opportunity to investigate what factors for control are currently being missed in control programs, and estimate changes in cow-level, within-herd, and herd-level prevalence as a results of various management changes [6].

1.5 Modelling transmission, and infection in young stock

Researching long-term effects of management practices changes and transmission dynamics are notoriously difficult due to the prolonged incubation of MAP, latently infected animals, variability of test characteristics, and a lack of long-term randomized control studies [35]. Therefore, mathematical modelling techniques have been implemented to study transmission parameters [40, 50], assess impact of varying infectious animals, as well as varying levels of environmental contamination [22], economic impacts of disease [55], and to determine effectiveness of control programs and interventions on the spread and control of JD [13, 31]. These modelling techniques rely on educated guesses regarding the dynamics of an infectious agent, in order to estimate impacts of various intervention strategies [65]. These transmission dynamics, commonly the reproduction ratio (R_0), can be estimated based on analysis of transmission experiments in which individuals are either susceptible (S), infected (I), or removed (R). The rate and probability distribution at which individuals move between these categories allow for transmission parameters to be estimated [65].

The association between JD control programs, management practices, and within-herd prevalence has been well established both through direct observations and mathematical

modelling [2, 31, 38, 57]. However, despite implementation of best management practices, decreasing within-herd prevalence has proven to be difficult and takes 6-10 y for changes to be detected [12, 48, 57], with eradication unlikely after 25 y [34]. Although most control programs focus on decreasing transmission to young stock, the potential risk of calf-to-calf transmission is largely overlooked and may contribute to new infection on farm. Calves with confirmed infections can begin shedding MAP following experimental infection as early as 2-6 mo following inoculation challenge, and can be susceptible to infection up to 12 mo of age [42-44]. Additionally, a relatively high proportion of young stock on confirmed MAP-positive farms are shedding MAP in their feces, indicating that a potential route of transmission is currently being overlooked [67, 78]. Therefore, the separation of calves from cows, and subsequent group-housing, may not be an effect practice to prevent new infections and eliminate the spread of MAP within a herd. Factors that may contribute to calf-to-calf transmission are the frequency with which calves shed MAP and quantity of MAP being shed, are currently not known; however, there is evidence that calf-to-calf transmission can occur [14, 64]. Mathematical modelling studies inconsistently include calf-to-calf transmission, with one literature review concluding that only one of eight MAP modelling studies included this transmission route [35]. One of the difficulties with including calf-to-calf transmission is the inconsistent findings regarding transmission parameter estimates, and the implications or impact of this route of transmission on control within herds [34, 39, 53, 64, 66]. Further investigation is needed to determine the extent to which calf-to-calf transmission occurs, in order to determine management changes that would be most beneficial for the improvement of current JD control programs.

1.6 Outline of thesis

The overall aims of this thesis were first to determine prevalence of MAP in Canada based on a single diagnostic method, and secondly to determine the extent to which calf-to-calf transmission occurs in group-housed calves. Environmental samples have been used to estimate herd prevalence, and the association between the sample characteristics and odds of being positive at a given time point have been elucidated [75, 76]. However, the extent to which these environmental sample characteristics are associated with prevalence estimates over time is not known. Chapter 2 describes changes in herd prevalence of MAP infection in Saskatchewan dairy herds at two time periods, 3 y apart. Furthermore, the association between the odds of a sample testing positive and sample characteristics (cow type contributing to the sample, pen-type from where sample was collected, and location in the barn) or herd size were examined.

Herd prevalence has been estimated across Canada for several provinces and regions, using a number of diagnostic techniques including environmental sampling, milk bulk tank ELISA, seroprevalence, tissue culture and fecal pooling [36, 49, 76]. However, no study has used the same method across each of these regions within Canada, making comparisons prevalence and any changes associated with control programs difficult [5]. Chapter 3 describes the sensitivity and specificity of estimating the true herd prevalence when using two environmental samples collected at a single time point, and the subsequent prevalence estimates for Western Canada, Ontario, Quebec, and Atlantic Canada.

Despite implementation of control programs and management changes on farm within Canada, JD remains prevalent with no evidence of eradication [5], indicating continued

transmission of infection to susceptible animals. Current programs focus on the transmission route from cow-to-calf, but other routes of transmission may be missed, leading to persistence of infection within a herd. Based on previous research, calves have been shown to be infectious as early as 1 mo following an inoculation challenge, and susceptible up to 12 mo of age [42, 43]; however, the extent to which these calve can infect pen mates is not known.

The following chapters describe the results of an experimental transmission trial involving 32 newborn calves group-housed in pens for 3 mo to study calf-to-calf transmission. Chapter 4 describes the diagnostic results (fecal shedding, INF-γ response, antibody response, tissue infection and culture of environmental samples) of the experimental transmission trial. Chapter 5 implements mathematical modelling techniques to estimate the transmission parameter R_0 for calves that were group housed together, based on fecal shedding, immune responses, or tissue infection. Very little is known about mechanisms of shedding MAP bacteria during infection, leading to a gap in knowledge regarding frequency of shedding, as well as quantity of MAP shed at any given time [28, 39, 45]. Chapter 6 compares the frequency with which shedding occurred between calves inoculated or exposed in the experimental transmission trial. Additionally, the quantity of MAP in fecal samples from three different inoculation doses were compared.

Freezing of samples prior to diagnostic testing is common, however, there is limited information regarding the effects it has on the ability to culture MAP from tissue samples. Chapter 7 compares culture results of MAP infected tissue that are processed immediately following necropsy and 18 mo following freezing at -80°C.

In Chapter 8, results of all studies presented in this thesis are discussed.

1.7 References

1. Arsenault RJ, Maattanen P, Daigle J, Potter A, Griebel P, Napper S (2014) From mouth to macrophage: mechanisms of innate immune subversion by *Mycobacterium avium* subsp. *paratuberculosis*. Vet Res 45:54.

2. Bakker D (2010) Paratuberculosis control measures in Europe. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. CABI, Wallingford, UK.

3. Bannantine JP, Bayles DO, Waters WR, Palmer MV, Stabel JR, Paustian ML (2008) Early antibody response against *Mycobacterium avium* subspecies *paratuberculosis* antigens in subclinical cattle. Proteome Sci 6:5.

4. Barkema HW, Hendrick S, De Buck J, Kaplan GG, Rioux K: Crohn's disease in humans and Johne's disease in catted-linked diseases? In: *Zoonotic pathogens in the food chain*. Edited by Krause D, Hendrick S: CAB International; 2010: 197-213.

5. Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten V, Bannantine JP, Keefe GP, Kelton DF, Wells SJ, Whittington RJ *et al* (2018) Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection. Transbound Emerg Dis., in press.

6. Beaunee G, Vergu E, Ezanno P (2015) Modelling of paratuberculosis spread between dairy cattle farms at a regional scale. Vet Res 46:111.

7. Berghaus RD, Farver TB, Anderson RJ, Jaravata CC, Gardner IA (2006) Environmental sampling for detection of *Mycobacterium avium* spp. *paratuberculosis* on large California dairies. J Dairy Sci 89:963-970.

8. Chi J, VanLeeuwen JA, Weersink A, Keefe GP (2002) Direct production losses and treatment costs from bovine viral diarrhoea virus, bovine leukosis virus, *Mycobacterium avium* subspecies *paratuberculosis*, and *Neospora caninum*. Prev Vet Med 55:137-153.

9. Cho J, Tauer LW, Schukken YH, Smith RL, Lu Z, Gröhn YT (2013) Cost-effective control strategies for Johne's disease in dairy herds. Can J Agr Econ 61:583-608.

10. Cocito C, Gilot P, Coene M, de Kesel M, Poupart P, Vannuffel P (1994) Paratuberculosis. Clin Microbiol Rev 7:328-345.

11. Collins MT (2011) Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract 27:581-591.

12. Collins MT, Eggleston V, Manning EJ (2010) Successful control of Johne's disease in nine dairy herds: results of a six-year field trial. J Dairy Sci 93:1638-1643.

13. Collins MT, Morgan IR (1991) Epidemiological model of paratuberculosis in dairy cattle. Prev Vet Med 11:131-146.

14. Corbett CS, De Buck J, Orsel K, Barkema HW (2017) Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48:27.

15. Coussens PM (2004) Model for immune responses to *Mycobacterium avium* subspecies *paratuberculosis* in cattle. Infect Immun 72:3089-3096.

16. Dargatz DA, Beverly AB, Barber LK, Sweeney RW, Whitlock RH, Shulaw WP, Jacobson RH, Stabel JR (2001) Evaluation of a commercial ELISA for diagnosis of paratuberculosis in cattle. JAVMA 218:1163-1166.

17. Donat K, Kube J, Dressel J, Einax E, Pfeffer M, Failing K (2015) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture. Epidemiol Infect 143:975-985.

18. Donat K, Schau U, Soschinka A (2011) Identfication of *Mycobacteriaum avium* ssp. *paratuberculosis* infected dairy herds by environmental sampling. Berl Munch Tierarztl 124:360-367.

19. Eisenberg SW, Koets AP, Nielen M, Heederik D, Mortier R, De Buck J, Orsel K (2011) Intestinal infection following aerosol challenge of calves with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 42:117.

20. Grant IR (2005) Zoonotic potential of Mycobacterium avium ssp. paratuberculosis: the current position. J Appl Microbiol 98:1282-1293.

21. Harris NB, Barletta RG (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin Microbiol Rev 14:489-512.

22. Humphry RW, Stott AW, Adams C, Gunn GJ (2006) A model of the relationship between the epidemiology of Johne's disease and the environment in suckler-beef herds. Vet J 172:432-445.

23. Jungersen G, Huda A, Hansen JJ, Lind P (2002) Interpretation of the gamma interferon test for diagnosis of subclinical paratuberculosis in cattle. Clin Vaccine Immunol 9:453-460.

24. Jungersen G, Mikkelsen H, Grell SN (2012) Use of the johnin PPD interferon-gamma assay in control of bovine paratuberculosis. Vet Immunol Immunopathol 148:48-54.

25. Kalis CH, Collins MT, Barkema HW, Hesselink JW (2004) Certification of herds as free of *Mycobacterium paratuberculosis* infection: actual pooled faecal results versus certification model predictions. Prev Vet Med 65:189-204.

26. Kalis CHJ, Collins MT, Hesselink JW, Barkema HW (2003) Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. Vet Microbiol 97:73-86.

27. Khare S, Adams LG, Osterstock J, Roussel A, David L (2008) Effects of shipping and storage conditions of fecal samples on viability of *Mycobacterium paratuberculosis*. J Clin Microbiol 46:1561-1562.

28. Koets AP, Eda S, Sreevatsan S (2015) The within host dynamics of *Mycobacterium avium* ssp. *paratuberculosis* infection in cattle: where time and place matter. Vet Res 46:61.

29. Kralik P, Pribylova-Dziedzinska R, Kralova A, Kovarcik K, Slana I (2014) Evidence of passive faecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in a Limousin cattle herd. Vet J 201:91-94.

30. Kralik P, Slana I, Kralova A, Babak V, Whitlock RH, Pavlik I (2011) Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR. Vet Microbiol 149:133-138.

31. Kudahl AB, Ostergaard S, Sorensen JT, Nielsen SS (2007) A stochastic model simulating paratuberculosis in a dairy herd. Prev Vet Med 78:97-117.

32. Lavers CJ, McKenna SL, Dohoo IR, Barkema HW, Keefe GP (2013) Evaluation of environmental fecal culture for *Mycobacterium avium* subspecies *paratuberculosis* detection in dairy herds and association with apparent within-herd prevalence. Can Vet J 54:1053-1060.

33. Lombard JE, Gardner IA, Jafarzadeh SR, Fossler CP, Harris B, Capsel RT, Wagner BA, Johnson WO (2013) Herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in United States dairy herds in 2007. Prev Vet Med 108:234-238.

34. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C (2011) Predicting fadeout versus persistence of paratuberculosis in a dairy cattle herd for management and control purposes: a modelling study. Vet Res 42:36.

35. Marcé C, Ezanno P, Weber MF, Seegers H, Pfeiffer DU, Fourichon C (2010) Invited review: modeling within-herd transmission of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle: a review. J Dairy Sci 93:4455-4470.

36. McKenna SL, Keefe GP, Barkema HW, McClure J, VanLeeuwen JA, Hanna P, Sockett DC (2004) Cow-level prevalence of paratuberculosis in culled dairy cows in Atlantic Canada and Maine. J Dairy Sci 87:3770-3777.

37. McKenna SL, Keefe GP, Barkema HW, Sockett DC (2005) Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. Vet Microbiol 110:105-111.

38. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

39. Mitchell RM, Medley GF, Collins MT, Schukken YH (2012) A meta-analysis of the effect of dose and age at exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows. Epidemiol Infect 140:231-246.

40. Mitchell RM, Whitlock RH, Stehman SM, Benedictus A, Chapagain PP, Grohn YT, Schukken YH (2008) Simulation modeling to evaluate the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on commercial dairy farms in the United States. Prev Vet Med 83:360-380.

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

42. Mortier R, Barkema HW, Orsel K, Wolf R, De Buck J (2014) Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 45:71.

43. Mortier RA, Barkema HW, De Buck J (2015) Susceptibility to and diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy calves: A review. Prev Vet Med 121:189-198.

44. Mortier RA, Barkema HW, Negron ME, Orsel K, Wolf R, De Buck J (2014) Antibody response early after experimental infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy calves. J Dairy Sci 97:5558-5565.

45. Munster P, Volkel I, Wemheuer W, Schwarz D, Doring S, Czerny CP (2013) A longitudinal study to characterize the distribution patterns of *Mycobacterium*

avium ssp. *paratuberculosis* in semen, blood and faeces of a naturally infected bull by IS 900 semi-nested and quantitative real-time PCR. Transbound Emerg Dis 60:175-187.

46. Nielsen SS, Toft N (2006) Age-specific characteristics of ELISA and fecal culture for purpose-specific testing for paratuberculosis. J Dairy Sci 89:569-579.

47. Nielsen SS, Toft N (2008) Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. Vet Microbiol 129:217-235.

48. Nielsen SS, Toft N (2011) Effect of management practices on paratuberculosis prevalence in Danish dairy herds. J Dairy Sci 94:1849-1857.

49. Pieper L, Sorge US, DeVries T, Godkin A, Lissemore K, Kelton D (2015) Comparing ELISA test-positive prevalence, risk factors and management recommendations for Johne's disease prevention between organic and conventional dairy farms in Ontario, Canada. Prev Vet Med 122:83-91.

50. Pouillot R, Dufour B, Durand B (2004) A deterministic and stochastic simulation model for intra-herd paratuberculosis transmission. Vet Res 35:53-68.

51. Raizman EA, Espejo LA, Wells SJ (2011) Long-term survival of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples obtained from naturally infected cows and stored at -18 degrees C and -70 degrees C. Vet Med Int 2011:1-5.

52. Sanderson JD, Moss MT, Tizard MLV, Hermon-Taylor J (1992) *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. Gut 33:890-896.

53. Santema WJ, Poot J, Segers RP, Van den Hoff DJ, Rutten VP, Koets AP (2012) Early infection dynamics after experimental challenge with *Mycobacterium avium* subspecies *paratuberculosis* in calves reveal limited calf-to-calf transmission and no impact of Hsp70 vaccination. Vaccine 30:7032-7039.

54. Slana I, Kralik P, Kralova A, Pavlik I (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. Int J Food Microbiol 128:250-257.

55. Smith RL, Al-Mamun MA, Gröhn YT (2017) Economic consequences of paratuberculosis control in dairy cattle: a stochastic modeling study. Prev Vet Med 138:17-27.

56. Sockett DC, Conrad TA, Thomas CB, Collins MT (1992) Evaluation of four serological tests for bovine paratuberculosis. J Clin Microbiol 30:1134-1139.

57. Sorge US, Lissemore K, Godkin A, Jansen J, Hendrick S, Wells S, Kelton DF (2011) Changes in management practices and apparent prevalence on Canadian dairy farms participating in a voluntary risk assessment-based Johne's disease control program. J Dairy Sci 94:5227-5237.

58. Stabel J (2000) Transitions in immune responses to *Mycobacterium paratuberculosis*. Vet Microbiol 77:465-473.

59. Stabel JR (2006) Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal. Anim Health Res Rev 7:61-70.

60. Sweeney RW (2011) Pathogenesis of paratuberculosis. Vet Clin North Am Food Anim Pract 27:537-546, v.

61. Sweeney RW, Uzonna J, Whitlock RH, Habecker PL, Chilton P, Scott P (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. Res Vet Sci 80:253-259.

62. Timms VJ, Mitchell HM, Neilan BA (2015) Optimisation of DNA extraction and validation of PCR assays to detect *Mycobacterium avium* subsp. *paratuberculosis*. J Microbiol Methods 112:99-103.

63. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW (2006) Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47:874-882.

64. van Roermund HJ, Bakker D, Willemsen PT, de Jong MC (2007) Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: calves can transmit the infection to other calves. Vet Microbiol 122:270-279.

65. Velthuis AG, De Jong MC, De Bree J (2007) Comparing methods to quantify experimental transmission of infectious agents. Math Biosci 210:157-176.

66. Weber MF, Groenendaal H (2012) Effects of infectious young stock on results of certification, surveillance and control programmes for paratuberculosis in dairy herds. Vet Microbiol 154:272-281.

67. Weber MF, Kogut J, de Bree J, van Schaik G, Nielen M (2010) Age at which dairy cattle become *Mycobacterium avium* subsp. *paratuberculosis* faecal culture positive. Prev Vet Med 97:29-36.

68. Whitlock RH (2010) Paratuberculosis control measure in the USA. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. Wallingford, UK.

69. Whitlock RH, Fyock T, Schukken Y, Van Kessel J, Kams J, Hovingh E, Smith J (2009) Passive MAP fecal shedding in dairy cattle. In: *International Colloquium on Paratuberculosis: 2009; Minneapolis, Minnesota*.

70. Whitlock RH, Rosenberger AE, Sweeney RW, Spencer PA (1996) Distribution of *M. paratuberculosis* in tissues of cattle from herds infected with Johne's disease. In: *Fifth International Colloquium on Paratuberculosis: 1996; Madison, Wisconsin, USA*; 168-173.

71. Whitlock RH, Wells S, Sweeney RW, Van Tiem J (2000) ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. Vet Microbiol 77:387-398.

72. Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA (2004) Survival and Dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the Environment. Appl Environ Microbiol 70:2989-3004.

73. Whittington RJ, Reddacliff L, Marsh I, Saunders V (1999) Detection of *Mycobacterium avium* subsp *paratuberculosis* in formalin-fixed paraffin-embedded intestinal tissue by IS*900* polymerase chain reaction. Aust Vet J 77:392-397.

74. Windsor PA, Whittington RJ (2010) Evidence for age susceptibility of cattle to Johne's disease. Vet J 184:37-44.

75. Wolf R, Barkema HW, De Buck J, Orsel K (2015) Sampling location, herd size, and season influence *Mycobacterium avium* ssp. *paratuberculosis* environmental culture results. J Dairy Sci 98:275-287.

76. Wolf R, Barkema HW, De Buck J, Slomp M, Flaig J, Haupstein D, Pickel C, Orsel K (2014) High herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Western Canadian dairy farms, based on environmental sampling. J Dairy Sci 97:6250-6259.

77. Wolf R, Clement F, Barkema HW, Orsel K (2014) Economic evaluation of participation in a voluntary Johne's disease prevention and control program from a farmer's perspective-The Alberta Johne's Disease Initiative. J Dairy Sci 97:2822-2834.

78. Wolf R, Orsel K, De Buck J, Barkema HW (2015) Calves shedding *Mycobacterium avium* subspecies *paratuberculosis* are common on infected dairy farms. Vet Res 46:1-8.

79. Yamamoto T, Murai K, Hayama Y, Kobayashi S, Nagata R, Kawaji S, Osaki M, Sakakibara SI, Tsutsui T (2018) Evaluation of fecal shedding and antibody

response in dairy cattle infected with paratuberculosis using national surveillance data in Japan. Prev Vet Med 149:38-46.

CHAPTER 2. ENVIRONMENTAL SAMPLE CHARACTERISTICS AND HERD SIZE ASSOCIATED WITH DECREASE IN HERD-LEVEL PREVALENCE OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS

2.1 Abstract

Environmental sampling is an effective method for estimating regional dairy herd-level prevalence of infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). However, factors affecting prevalence estimates based on environmental samples are not known. The objective was to determine whether odds of environmental samples collected on farm changed culture status over 2 sampling times and if changes were specific for location and type of housing (free-stall, tie-stall or loose housing), the sample collected (i.e. manure of lactating, dry or sick cows; namely, cow group), and effects of herd size. In 2012-2013 (sampling 1 (S1)) and 2015-2017 (sampling 2 (S2)), 6 environmental samples were collected and cultured for MAP from all 167 (99%) and 160 (95%) farms, respectively, in the province of Saskatchewan, Canada. Only the 148 dairy farms sampled at both sampling periods were included in the analysis. A mixed effects logistic regression was used to determine whether differences between sampling periods were associated with herd size and sample characteristics (cow group contributing to environmental sample, type of housing and location). In S1 and S2, 55 and 34%, respectively, of farms had at least 1 MAP-positive environmental sample. Correcting for sensitivity of environmental sampling, the estimated true prevalence in S1 and S2 was 79 and 48%, respectively. Herds with > 200 cows were more often MAP-positive than herds with < 51 cows in both S1 and S2. Percentage of positive samples was lower in S2 compared to S1, for all sampled areas, cow groups contributing to samples, types of housing where samples were collected, and herd size categories. However, samples collected from the dry cow areas had the largest decrease in MAP-positive samples in S2 compared to all other cow group samples. Herds that were MAP-negative in S1 with a

herd size 51-100 or 101-150 were more likely stay MAP negative, whereas MAP-positive herds with > 200 cows stayed more frequently MAP-positive. There was no difference in the odds of a sample being MAP-positive among housing types or location of sample collection in both sample periods. Of all farms sampled, 104 (70%) did not change status type from S1 to S2. In conclusion, when herd-level MAP prevalence decreased over the 3-year interval, the change in prevalence differed among herd size categories and was larger in samples from dry cow areas. It was, however, not specific to other characteristics of environmental samples collected.

Keywords: paratuberculosis, environmental samples, Johne's disease, herd size, Saskatchewan

2.2 Introduction

Johne's disease (JD), a chronic enteritis caused by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP), has an adverse economic impact on the dairy industry worldwide due to decreased milk production, increased risk of culling and decreased slaughter value [9, 18, 20]. There is no effective vaccine for prevention of MAP infection and no cure; therefore, control programs are primarily based on decreasing the risk of new infections within a dairy herd [8, 11]. Canadian JD control initiatives rely on detection of MAP-positive herds and subsequent risk assessments resulting in changes to management practices to decrease new infections within a herd [27]. Following detection of a positive herd, producers can opt for individual cow testing to remove infectious animals; however, detection of these animals is difficult due to the prolonged incubation period, unreliable diagnostics, and variability of immune and symptomatic responses [1, 10]. Due to high variability among diagnostic tests in test characteristics, prevalence estimates can vary drastically; therefore, test sensitivities and specificities must be considered when estimating true prevalence [1].

True cow-level prevalence estimates are difficult to estimate, as diagnostic tests rely on detecting latent and varying immune responses of animals, or detection of the pathogen, which is intermittently shed in milk and feces. Infected animals can be identified as negative if sampled at a time of no shedding or before immune responses develop, and this may result in low-prevalence herds being categorized as negative, despite MAP-infected animals being present [7, 17]. Fecal shedding can be intermittent and the extent of shedding is highly variable [12], which has large consequences for transmission of MAP as the primary route of infection is fecal oral. However, due to the survivability of

MAP in the environment for prolonged intervals [24], environmental samples are a cost-effective and reliable method for detection of MAP-positive herds [3], and are currently used in control programs in the United States and Canada [23, 27].

The most common sampling method protocol for environmental samples requires 6 samples to be collected from various locations on a dairy farm [3]. The type of sample collected is important, as sample characteristics impact the likelihood of a MAP-positive result [25]. For example, environmental samples from the lactating cow area are more likely to be positive than those from sick/calving pens or dry cow pens; furthermore, samples collected from locations where manure from several cows accumulate (e.g. alley ways or lagoons) are more likely to be positive than bedded packs or manure piles [25]. These sample-type specific characteristics of environmental samples can be grouped based on cows contributing to the sample (cow group, i.e. lactating, dry, sick, etc.), type of pen that cows are housed in (housing type, i.e. free-stall, tie-stall, loose housing, etc.) and location collected (location type, i.e. alley, gutter, bedding pack etc.). Additionally, larger herds are more likely to have MAP-positive samples and have higher within-herd prevalence than smaller herds [22]; however, there is no evidence that herd size affects sample-type specific results [25].

Accurate prevalence estimates are essential for control, surveillance, and monitoring the effectiveness a control program over time [1]. In long-term studies, herd MAP prevalence estimates decrease over time when control programs are in place [5, 19]. Most programs used milk or serum antibody ELISA to estimate herd prevalence, but it is unknown how or if herd MAP prevalence estimates based on environmental samples are associated with characteristics of environmental samples (cow group, housing, and location) and interact

to skew apparent changes in prevalence at various time points, or if they change in association with prevalence estimates. The prevalence of MAP-infected herds based on environmental samples in Saskatchewan, Canada, has been reported [26]. However, stability of herd infection status and associations with herd size following implementation of a control program has not been documented. The objective was to determine if odds of environmental samples collected on-farm changing MAP culture status over the 2 sampling times, were specific for location and type of housing (free-stall, tie-stall or loose housing) the sample has been collected, whether it included manure of lactating, dry or sick cows (cow group), and whether it was associated with herd size.

2.3 Materials and methods

2.3.1 Herds

Environmental samples were collected from all 167 and 160 dairy farms in the province of Saskatchewan in the first and second samplings, respectively, as part of the Saskatchewan JD surveillance program of the Saskatchewan Ministry of Agriculture (Regina, SK, Canada) and Sask Milk (Regina, SK, Canada), the Saskatchewan dairy producer marketing board. Only farms sampled at both sampling periods were included in the analysis (n=148). Farms were visited once by a herd veterinarian or employee of the producer organization SaskMilk (Saskatoon, SK, Canada) between August 2012 and October 2013 (sampling period 1; S1), and a second time between September 2015 and February 2017 (sampling period 2; S2). Mean interval between sampling periods was 3.5 years. Farms were recruited as part of a JD control initiative by SaskMilk and the Saskatchewan Ministry of Agriculture (Regina, SK, Canada). Herd size was categorized into 5 size categories: < 51, 51-100, 101-150, 151-200, and > 200 cows. Herd size

information was collected at S1 and applied to farms at S2. Following the first environmental sample collection in S1, MAP-positive farms were offered an option to enroll in whole-herd testing (individual serum ELISA and/or fecal testing), along with completion of a risk assessment by their herd veterinarian for the improvement of on farm management practices. Any changes to improve JD control were individualized and specific to the risk assessment performed by the veterinarian, and no information on these actions or results of individual whole herd testing were available for analysis due to strict privacy rules associated with the JD control program.

2.3.2 Sample collection

Sample collection was done as described [26]. Briefly, samples collectors were instructed to collect a total of 6 environmental samples, 2 samples in each of 3 pre-determined locations. The latter included: 1) manure accumulation sites from lactating cow alleys or scraper lines; 2) manure storage areas such as lagoon, pits or piles; and 3) if 2 or more cows were present, accumulated or concentrated manure from dry, calving, close-up, or sick pens. For each sample, the type of cows (lactating, dry, sick, combination) contributing to the sample, housing type (tie-stall, free-stall, loose housing, other) and location (manure storage, exercise and/or bedding pack, gutter or alley) were recorded using a standardized description sheet. Any sample without a label was categorized as "not specified" and excluded from sample-type analysis. Each sample contained a subset of 4 samples that were thoroughly mixed before storing on ice and mailing to the University of Calgary using Express Mail. All samples were processed within 7 days after collection using the TREK ESP II (TREK para-JEM® ; TREK Diagnostic Systems,

Cleveland, OH, USA) culture protocol with subsequent F57-specific qPCR [13]. Samples with a CT value < 40 were categorized as MAP-positive.

2.3.3 Statistical analysis

Analyses were conducted using R version 3.4.2 (R Core Team, Vienna, Austria, 2017) using the statistical R package "lme4" [2] and $P < 0.05$ was considered significant.

The apparent herd-level MAP prevalence was estimated based on at least 1 environmental sample from a farm being MAP-positive. The true herd-level prevalence for each sampling period was calculated by adjusting the apparent prevalence for imperfect sensitivity and specificity of the diagnostic test [6]. Sensitivity and specificity of a MAP test, based on 6 environmental samples, were previously estimated and were 0.69 and 0.99, respectively [26].

The number of positive samples in: S1 for farms that changed from positive to negative; S1 for farms that stayed positive; S2 for farms that changed from negative to positive; and S2 for farms that stayed positive, were compared using a Poisson regression model with a log link. Each of these 4 categories was included as a nominal predictor, with the outcome being number of MAP-positive environmental samples on the farm. Because this model assessed only records for farms classified as positive, the number of MAP-positive samples ranged from 1 to 6. The model was estimated using the Iteratively Reweighted Least Squares algorithm.

To assess association of within-farm changes with herd size, 2 logistic regression models were used on subsets of the data. The first subset used only farms that tested positive in S1, with herd size as a predictor and S2 status as the outcome, whereas the second subset used only farms that tested negative in S1, with herd size as a predictor and S2 status as

the outcome. This regression modeled the ratio of the odds of changing status in S2 to the odds of maintaining the same status in S2, conditional on herd status in S1. To determine the association between the number of MAP-positive samples per farm in S1 and test status in S2, a logistic regression was conducted on a subset of the data that only included farms positive in S1. The outcome for this model was farm status in S2, with the only predictor being number of positive samples per farm in S1. This regression modeled the change in log odds of a herd testing MAP-positive in S2 per additional positive sample in S1.

The association between an environmental sample being MAP-positive based over the sampling periods and other predictor variables was assessed in 2 steps using mixed effect logistic regression models. First, regression models were independently fit for each of 4 predictor variables: 1) type of cows (lactating, dry, sick or combinations) from which the environmental sample was collected; 2) housing (tie-stall, free-stall or loose housing); 3) location of sample collection (alley, gutter, or manure storage); and 4) number of adult cattle. Sampling period and an interaction of the predictor variable with sampling period were then forced into the model to allow for differences in temporal patterns within categories of other predictors. Coefficients involving sampling period can be interpreted as a measure of change between the 2 sampling periods, averaged across all herds in Saskatchewan. Variables significant at $P \leq 0.05$ were included in the final model. Models were estimated by maximum likelihood where the likelihood function was estimated using 50 quadrature points and optimized using the limited-memory algorithm. In case of divergent models, they were updated for a further $2x10^8$ iterations using Nelder-Mead

optimization until convergence was reached. Following convergence, Wald 95% confidence intervals were calculated.

2.4 Results

2.4.1 Prevalence

True herd prevalence for all 167 farms sampled during S1, and 160 farms sampled during S2 was 77% (95% CI: 56-97%) and 49% (95% CI: 29-68%), respectively. When only using the 148 farms that were sampled in both S1 and S2, in S1, 55% of the Saskatchewan dairy farms had at least 1 MAP-positive environmental sample, resulting in a true herd prevalence estimate of 79% (95% CI: 59-99%). In S2, proportion of herds with at least 1 MAP-positive environmental sample was 34%, resulting in the true herd prevalence estimate of 48% (95% CI: 26-69%), a decrease compared to S1 (P = 0.03). A total of 104 (70%) of farms sampled both in S1 and S2 did not change status (Table 2-1). Overall, MAP-negative farms in S1 were more likely to stay negative in S2, whereas S1-positive farms stay test positive more frequently in S2 than test negative (Table 2-1). Of farms that were MAP-negative in S1, those with a herd size of 51-100 or 101-150 cows were more likely to stay MAP-negative than change to positive (Table 2-1). Of farms that were MAP-positive in S2, herds of sizes 101-150 cows and > 200 cows were more likely to remain positive (Table 2-1).

Thirty-one (21%) farms that were MAP-positive in S1 but negative in S2 had, on average, 2.3 MAP-positive environmental samples of the 6 collected in S1, which was less than the average 3.0 samples on farms that stayed positive in S2 (P = 0.01). The 13 (9%) farms that were MAP-negative in S1 and positive in S2, had on average 1.8 positive samples in S2, which was not different from those positive only in S1 (P = 0.33). Among

farms that had at least 1 MAP-positive environmental sample in S1, each additional MAP-positive sample reduced the odds of testing negative in S2 (OR = -0.41; 95% CI= 0.16- -2.53; P = 0.01). For farms with at least 1 MAP-positive environmental sample in either sampling period, mean number of positive samples per farm did not change between S1 (2.9) and S2 (2.8) (P = 0.63).

2.4.2 Characteristics of environmental samples

Environmental samples in S1 and S2 were most frequently collected from locations containing lactating cows, from free-stall pens, and collected from alleys (Table 2-2). When analysing MAP-positive samples within characteristic type (cow group, housing, location or herd size) independently, there were differences in proportion of positive samples among lactating, dry, sick and combination cows in S1 (Table 2-2). Additionally, a smaller proportion of samples collected from loose housing were MAP-positive than samples from free-stall housing in S1 and S2, and tie-stalls had a fewer proportion of positive samples in S2 (Table 2-2). In both S1 and S2, the proportion of bedding packs samples were less often positive than samples collected from the alley (Table 2-2.) In both sampling periods, herds > 200 lactating cows had a higher odds of testing positive compared to herds < 51 cows (Table 2-2 and Table 2-3).

All environmental samples, regardless of characteristics (cow group, housing or location) had decreased odds in culturing MAP-positive in S2 compared to S1; however, the largest decrease in odds of testing MAP-positive was from samples collected from dry cow areas (Tables 2-2 and 2-3). When analysing all sample characteristics together, the decrease in the odds of being MAP-positive from S1 to S2 occurred across all sample characteristics; there were no differences among housing types, or locations within the

herd. However, herds with > 200 cows had increased odds of remaining positive than herds with < 51 cows (Table 2-3). The within-herd variance in the final mixed effects logistics regression was 3.75 (Table 2-3).

2.5 Discussion

The prevalence of MAP-infected Saskatchewan dairy herds based on environmental sampling decreased in the 3.5 y between the 2 sampling periods. All sample types were less frequently MAP-positive in S2 compared to S1, regardless of cow group contributing to the sample, housing type where the sample was collected, or location of collection. The largest decrease in the odds of being positive in S2 samples was among dry cows. Large herds (> 200 cows) were more often MAP-positive than the smallest herds. Overall, prevalence of positive environmental samples decreased across all areas sampled within the farms and was not specific to environmental sample characteristics. Following the first sampling period, MAP-positive farms were offered whole-herd testing, and a risk assessment to be completed on farm, a protocol consistent with several control programs [1, 27]. It has been reported that participation in control programs will only lead to an observable affect after 4-5 years [14]. However, based on milk ELISA, there were decreases in MAP prevalence at both herd and animal levels after only 2 years of participation in a control program [21]. It is important to note that the decrease in herd prevalence for the current study was based on environmental samples, which have been reported as identifying a higher proportion of farms as MAP-positive than pooled fecal testing, or ELISA testing, despite the high correlation to prevalence estimates based on these methods [3].

Infection status of a herd was based on at least 1 MAP-positive environmental sample; however, the number of positive samples detected can provide a tool for estimating within-herd prevalence and monitoring how it changes from S1 to S2 [7, 15]. Herds that changed from MAP-negative to positive had an average of 1.8 positive samples, whereas those that went from positive to negative had an average 2.3 positive samples. Additionally, each added positive sample reduced the odds that a farm would test negative in S2. Therefore, it is likely that farms that became MAP-negative in S2 had a lower within-herd prevalence than the farms that remained positive [7, 16, 21]. Differences of being MAP-positive in S1 or S2 were present within cow-group when looking at sample characteristics independently; however, these differences disappeared when all characteristics were considered together in 1 model. This was likely due to confounding, as certain characteristics are likely highly associated with each other (e.g., tie-stalls herds are smaller than free-stall or bedded pack herds), in addition to the wide range of number of samples that are included in each group. All sample types were less often positive in S2 than in S1; however, it should be noted that the decrease in the proportions of positive samples was present among the groups that had the largest number of samples collected. The only characteristic that was different in S2 than S1 when considering characteristics independently, or together, was the larger decrease in the odds of MAP-positive samples in dry cow samples than in other groups. Most control programs have 2 main aims for controlling within-herd spread of infection: 1) preventing new infections of newborn calves, and 2) reducing number of infected animals shedding MAP [11]. Therefore, because whole herd individual testing was offered to environmental sample-positive farms, it is likely that MAP-positive cows were identified

in many herds, and that most of these cows were removed from the herd based on current recommendations in Canadian control programs [4, 11], resulting in the change observed in S2. In Canada, calving is not seasonal, and dry cow groups are therefore always smaller than lactating cow groups. When within-herd prevalence decreases, the odds that at least 1 cow sheds MAP in the smaller group of dry cows will therefore decrease more than in the larger group of lactating cows.

Largest herds were more likely to test positive in both S1 and S2 when compared to smaller herds. This was consistent with findings that environmental samples collected from larger herds are more likely positive, and it is more difficult to decrease within-herd MAP prevalence in large herds over time [15, 22]. Although prevalence decreased in herds of all sizes, the majority of herds with > 200 cows tested positive at both sampling periods. Alternatively, more medium-sized herds, categories 51-100 and 100-151, changed status from S1 to S2, with the majority of the smallest herds testing negative both times. This may support previous findings that herd size impacts the ability to control MAP infection, and it should be taken into consideration for future control programs implementation and surveillance.

One of the limitations of the current study is the lack of information (due to privacy reasons) regarding which farms participated in the whole herd sampling, and whether changes were made based on suggestions outlined by the risk assessment. Currently, the authors can only speculate as to possible explanations for the decreased prevalence that was observed, based on control programs at the time of the study.

Additionally, based on the level of within-herd variance, there were unaccounted variables between farms contributing to differences (Table 3). Although this variance was

accounted for in the model, this variance indicated there were other influences, aside from environmental sample characteristics, not accounted for but affecting prevalence estimates.

2.6 Conclusions

Seventy percent of the farms did not change in status between S1 and S2; therefore, the large decrease in the estimated herd-level MAP prevalence was due to a few farms testing positive in S1 and changing to negative status in S2, which was not specific to herd size. For farms with at least 1 positive sample in S1, each additional positive sample decreased the odds of testing negative in S2. All environmental samples regardless of type, had decreased odds of testing positive in S2, with the largest decrease in environmental samples collected from areas containing dry cows. Herds > 200 cows had an increased odds of testing positive in at least 1 environmental sample, when compared to the smallest herds.

2.7 References

1. Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten V, Bannantine JP, Keefe GP, Kelton DF, Wells SJ, Whittington RJ *et al* (2018) Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection. Transbound Emerg Dis., in press.

2. Bates D, Mächler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67.

3. Berghaus RD, Farver TB, Anderson RJ, Jaravata CC, Gardner IA (2006) Environmental sampling for detection of *Mycobacterium avium* spp. *paratuberculosis* on large California dairies. J Dairy Sci 89:963-970.

4. Cho J, Tauer LW, Schukken YH, Smith RL, Lu Z, Gröhn YT (2013) Cost-effective control strategies for Johne's disease in dairy herds. Can J Agr Econ 61:583-608.

5. Collins MT, Eggleston V, Manning EJ (2010) Successful control of Johne's disease in nine dairy herds: results of a six-year field trial. J Dairy Sci 93:1638-1643.

6. Dohoo IR, Wayne MW, Henrik WM (2003) Screening and diagnostic tests. In: McPike MS (eds) Veterinary epidemiologici research. Atlantic Veterinary College, Charlottetown, PE, Canada.

7. Donat K, Kube J, Dressel J, Einax E, Pfeffer M, Failing K (2015) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture. Epidemiol Infect 143:975-985.

8. Kalis CH, Hesselink JW, Barkema HW, Collins MT (2001) Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp *paratuberculosis* in dairy herds. Am J Vet Res 62:270-274.

9. Lombard JE (2011) Epidemiology and economics of paratuberculosis. Vet Clin North Am Food Anim Pract 27:525-535.

10. Marcé C, Ezanno P, Weber MF, Seegers H, Pfeiffer DU, Fourichon C (2010) Invited review: modeling within-herd transmission of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle: a review. J Dairy Sci 93:4455-4470.

11. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

12. Mitchell RM, Schukken Y, Koets A, Weber M, Bakker D, Stabel J, Whitlock RH, Louzoun Y (2015) Differences in intermittent and continuous fecal shedding patterns between natural and experimental *Mycobacterium avium* subspecies *paratuberculosis* infections in cattle. Vet Res 46:66.

13. Mortier R, Barkema HW, Orsel K, Wolf R, De Buck J (2014) Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 45:71.

14. Nielsen SS, Toft N (2011) Effect of management practices on paratuberculosis prevalence in Danish dairy herds. J Dairy Sci 94:1849-1857.

15. Pillars R, Grooms D, Kaneene JB (2009) Longitudinal study of the distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project. Can Vet J 50:1039-1046.

16. Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE (2004) The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J Dairy Sci 87:2959-2966.

17. Raizman EA, Wells SJ, Godden SM, Fetrow J, Oakes JM (2007) The associations between culling due to clinical Johne's disease or the detection of *Mycobacterium avium* subsp. *paratuberculosis* fecal shedding and the diagnosis of clinical or subclinical diseases in two dairy herds in Minnesota, USA. Prev Vet Med 80:166-178.

18. Smith RL, Al-Mamun MA, Gröhn YT (2017) Economic consequences of paratuberculosis control in dairy cattle: a stochastic modeling study. Prev Vet Med 138:17-27.

19. Sorge US, Lissemore K, Godkin A, Jansen J, Hendrick S, Wells S, Kelton DF (2011) Changes in management practices and apparent prevalence on Canadian dairy farms participating in a voluntary risk assessment-based Johne's disease control program. J Dairy Sci 94:5227-5237.

20. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW (2006) Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47:874-882.

21. Verdugo C, Toft N, Nielsen SS (2015) Within- and between-herd prevalence variation of *Mycobacterium avium* subsp. *paratuberculosis* infection among control programme herds in Denmark (2011-2013). Prev Vet Med 121:282-287.

22. Wells S, Wagner BA (2000) Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between

familiarity of the herd manager with the disease or prior diagnosis of the disease in the herd and use of preventive measures. J Am Vet Med A 216:1450-1497.

23. Whitlock RH (2010) Paratuberculosis control measure in the USA. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. Wallingford, UK.

24. Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA (2004) Survival and Dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the Environment. Appl Environ Microbiol 70:2989-3004.

25. Wolf R, Barkema HW, De Buck J, Orsel K (2015) Sampling location, herd size, and season influence *Mycobacterium avium* ssp. *paratuberculosis* environmental culture results. J Dairy Sci 98:275-287.

26. Wolf R, Barkema HW, De Buck J, Slomp M, Flaig J, Haupstein D, Pickel C, Orsel K (2014) High herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Western Canadian dairy farms, based on environmental sampling. J Dairy Sci 97:6250-6259.

27. Wolf R, Clement F, Barkema HW, Orsel K (2014) Economic evaluation of participation in a voluntary Johne's disease prevention and control program from a farmer's perspective-The Alberta Johne's Disease Initiative. J Dairy Sci 97:2822-2834.

Table 2-1. *Mycobacterium avium* subsp. *paratuberculosis* status of 148 Saskatchewan dairy herds at the 2 sampling periods (categorized for herd size).

MAP status		Adult cow herd size					
		< 51	51-100	101-150	151-200	> 200	All
S1[1]	S2[1]	No. (%)[2]	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Negative	Negative	6 (100)	23 (79)	17 (77)	9 (100)	2 (50)	57 (81)
	Positive	0 (0)	6 (21)[3]	5 (23)[3]	0 (0)	2 (50)	13 (19)[4]
Positive	Negative	5 (83)	11 (50)	4 (29)	6 (43)	5 (22)[3]	31 (40)[5]
	Positive	1 (17)	11 (50)	10[6] (71)	8 (57)	17[6] (78)	47 (60)
Total		12	51	36	23	26	148

[1]Sampling period 1 (S1); Sampling period 2 (S2).

[2]Percentage of farms with this MAP status within MAP-status group S1.

[3]Different ($P < 0.05$) from the number of farms that did not change status from S1 to S2 within the same herd size and S1 status.

[4]Different ($P < 0.05$) from the number of farms that did not change status from S1 to S2 across herds of all sizes.

[5]Tendency for difference ($0.05 > P > 0.10$) from the number of farms that did not change status from S1 to S2 across herds of all sizes.

[6]Odds of changing status from S1 to S2 different ($P < 0.05$) from odds of changing status in herds of size 1-50.

Table 2-2. Percentage of *Mycobacterium avium* subsp. *paratuberculosis*-positive environmental samples collected from different locations on farm and herd sizes, during 2 sampling periods 3.5 y apart.

	August 2012 - Oct. 2013 (S1)		Sept. 2015 - Feb. 2017 (S2)	
	No. samples[1]	% MAP-pos.	No. samples	% MAP-pos.
Cow group				
Lactating cows[2]	632	28	552	25[4]
Dry cows	128	20[3]	177	6[3,4,5]
Sick cows	18	11[3]	7	14
Combination	46	17[3]	62	6[3]
Not specified	64	28	90	14
Housing				
Free-stall[2]	479	30	451	25[4]
Tie-stall	64	11	68	4[3]
Loose housing	279	23[3]	313	14[3]
Other	0	0	1	1
Not specified	66	21	55	9
Location				
Alley[2]	455	30	449	26[4]
Exercise and/or bedding pack	255	21[3]	293	13[3]
Gutter	62	15	50	6
Manure storage	95	24	86	12[3]
Not specified	21	33	10	0
Herd size in S1 (no. lactating cows)				
< 51[2]	72	13	72	6
51-100	306	20	306	15
101-150	216	18	216	14
151-200	138	30	138	21
> 200	156	51[3]	156	37[3]

[1]Number of samples collected in total each sampling period. For herd size, number of farms.

[2]Reference category used as baseline in logistic regression model for that variable.

[3]Different ($P < 0.05$) from reference within sampling period.

[4]Different ($P < 0.05$) from S1 within the same category.

[5]Magnitude of change between S1 and S2 is different ($P < 0.05$) from reference.

Table 2-3. Final mixed effects logistic regression model on the association between *Mycobacterium avium* subsp. *paratuberculosis* environmental sample culture results and sample type in 148 Saskatchewan dairy herds samples in 2 sample periods.

Predictor/parameter	Coefficient[1]	SE	95% CI[2]	*P*-value	OR[3]
Cow group					
Lactating cows (intercept)[4]	-2.21	0.75	-3.68 to -0.74	0.003	-
Dry cows	-0.43	0.37	-1.17 to 0.30	0.247	0.65
Sick cows	-1.67	0.91	-3.46 to 0.12	0.067	0.19
Combination	-1.01	0.57	-2.13 to 0.10	0.074	0.36
Interaction					
Sample period 2 x lactating cows	-0.43	0.18	-0.78 to -0.09	0.014	-
Sample period 2 x dry cows	-1.10	0.50	-2.08 to -0.13	0.027	-
Sample period 2 x sick cows	1.40	1.57	-1.67 to 4.48	0.371	-
Sample period 2 x combination	-0.40	0.84	-2.05 to 1.25	0.634	-
Housing					
Free-stall		Reference[4]			-
Tie-stall	-1.22	0.71	-2.62 to 0.18	0.088	0.30
Loose housing	0.19	0.37	-0.54 to 0.92	0.611	1.21
Other[5]	-14.38	-	-	-	5.68×10^{-7}
Location sampled					
Alley		Reference[4]			-
Exercise and/or bedding pack	-0.58	0.37	-1.31 to 0.14	0.116	1.79
Gutter	0.11	0.62	-1.11 to 1.32	0.862	1.12
Manure storage	-0.49	0.35	-1.18 to 0.2	0.165	0.61
Herd size					
< 51		Reference[4]			-
51-100	0.48	0.80	-1.1 to 2.05	0.553	1.62
101-150	0.02	0.84	-1.63 to 1.66	0.985	1.02
151-200	1.03	0.87	-0.68 to 2.73	0.239	2.80
> 200	2.50	0.85	0.84 to 4.16	0.003	12.18
Within-herd variance	3.75	1.94			

[1]Untransformed coefficients estimated by the model (log odds)

[2]95% Wald confidence interval

[3]Transformed odds ratios from coefficients: OR < 1 indicates a sample from the reference category is more likely to test MAP-positive than the comparator; OR > 1 indicates a

sample from the comparator is more likely to test MAP-positive than the reference category. Missing values represent coefficients that cannot be interpreted as ORs.

[4]Baseline group against which others were compared was lactating cows in sampling period 1, in free-stall herds of size < 51 cows where the sample was collected from an alley. Between sampling period differences are assumed common to all variables except cow group based on models 1-4.

[5]Not enough herds used "Other" housing to allow for accurate estimation of SE.

CHAPTER 3: PREVALENCE OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTIONS IN CANADIAN DAIRY HERDS

3.1 Abstract

Johne's disease (JD) is a progressive, chronic infection and inflammation of the small intestine of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Accurately estimating prevalence of MAP infections is important when controlling spread of infection or monitoring effectiveness of control programs. In the absence of a consistent test method used in prevalence studies across Canada, prevalence estimates among regions and programs cannot be compared. The aim of the current study was to estimate and compare prevalence of MAP infection in Western Canada, Ontario, Québec, and the Atlantic provinces, as well as among varying herd sizes and housing types. On 362 dairy farms located in all 10 provinces of Canada, environmental samples were collected from the lactating cow area and manure storage and cultured for detection of MAP. An additional sample was collected from breeding age heifers (BAH). Diagnostic sensitivity (Se) and specificity (Sp) were calculated for the ability to detect MAP-positive farms using only 2 environmental samples from the lactating cow area and manure storage, based on previous research, resulting in a Se and Sp of 0.40 and 0.99, respectively. There was no difference in Se and Sp when including BAH environmental samples. Test characteristics were applied to environmental culture results from the 362 participating farms in all 4 regions, resulting in true prevalence estimates of 66% for farms in Western Canada, 54% in Ontario, 24% in Québec, and 47% in Atlantic Canada. Herds housed in tie-stalls had lower prevalence than free-stall housed herds, and herds with 101-150 and >151 cows had higher prevalence than herds with ≤ 100 cows. This was the first time MAP prevalence was determined using 1 detection method, 1

laboratory, and within a single year across Canada, enabling direct comparisons of prevalence among regions, housing types, and herd sizes.

Keywords: Johne's disease, prevalence, Canada, environmental samples, herd size, housing

3.2 Introduction

Johne's disease (JD) is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and results in progressive chronic enteritis in most ruminant species [8]. Infection is widespread worldwide among dairy cattle, leading to economic losses due to reduced productivity and increased risk of culling [22]. In the absence of a vaccine to prevent MAP infection and treatment for infected animals, control is primarily based on preventing new infections within a herd. Control programs for JD have been implemented in countries and regions around the world and vary based on their aims of either eradication, surveillance, certification, or decreasing transmission [2, 4, 9, 14]. Accurate and reliable prevalence estimates can be used to monitor success of implemented control programs, as well as to estimate economic impacts of the disease [3]. Prevalence estimates, either at animal- or herd-levels, are calculated based on results of screening tests for detection of MAP infection. These tests rely on detection of the bacteria or the immune response produced by an infected animal in response to infection. However, accurate diagnosis can be difficult, due to variable disease progression leading to an immune response, intermittent shedding of bacteria in feces and milk, and varying sensitivities (Se) and specificities (Sp) of diagnostic tests [4, 18, 33]. Regardless of method used, apparent prevalence will depend on test characteristics and must be adjusted for test Se and Sp [13, 34]. However, there is little consensus regarding appropriate adjustments for Se and Sp for MAP infection, as there is no "gold standard" or reference for detection, making comparisons of true estimates difficult [31]. Prevalence of MAP infection has been estimated among regions in Canada, using environmental samples, bulk tank ELISA, tissues cultured from slaughtered animals, and

serum or milk ELISA [20, 25, 38]. Because choice of test used for detection of MAP infection impacts ability to identify infection status at both animal and herd levels, and estimates of test characteristics are not always accurate, comparing prevalence across regions or time frames is often difficult and unreliable [4, 18].

Herd-level prevalence within a region has been estimated using environmental sampling of herds and without the need for individual cow sampling [38]. When comparing prevalence of MAP infection across regions, it is important to not only use the same screening test method [13], but to also account for differences in herd size and housing type of the cows, as these have an effect on detecting positive herds [6, 37]. As herd size increases, the probability of a positive test increases, as does the ability to detect MAP in the environment, as more cattle are likely to be shedding in feces [32, 37]. Also, odds of herds testing MAP-positive is lower in tie-stall compared to free-stall herds, likely due to differences in contact structure of animals, less mixing of feces, and management practices [38]. Additionally, there is increasing evidence that young stock shed MAP into their environment [7, 39]. It is, however, not known whether including environmental samples from young stock housing affects identification of MAP-positive farms or resulting herd-prevalence estimates. Typically, 6 environmental samples are collected from areas of manure accumulation, with no samples collected from the environment of young stock [6, 38]. Sensitivities and Sp for detection of herds when collecting 6 environmental samples have been estimated and used for estimation of true herd-level prevalence [38]; however, Se and Sp have not been estimated when collecting fewer samples for detecting MAP-infection status at the herd-level, or when including environmental samples from young stock. Decreasing the number of environmental

samples used for herd detection would decrease cost, and potentially improve surveillance across large regions.

Objectives of this study were to: 1) determine Se and Sp for detecting MAP-positive farms based on 2 environmental samples, and 3 environmental samples when including an additional sample from breeding age heifers (BAH); 2) estimate true prevalence of MAP infection across 4 regions of Canada; and 3) compare estimated apparent and true MAP prevalence across herd size categories and housing types.

3.3 Materials and methods

3.3.1 Herd selection and surveys

Farms included in the study were used as a convenience sample for our present study, selected based on the criteria for the Canadian National Dairy Study (NDS) [5]. Briefly, producers were recruited to participate in the NDS, based on information provided by the 10 provincial milk marketing boards across Canada. All licensed Canadian dairy producers were invited to participate in a national survey. From the 1,062 respondents that completed the national survey, a subset of herds was randomly selected within each province to participate in an on-farm visit for an additional survey and collection of biological samples. The number of herds selected for a farm visit was based on a power calculation for the ability to detect common pathogens for mastitis in bulk milk tank samples, and resulted in the need to include 368 farms. These farms were selected to equally represent each region in Canada, as well as to be proportional to the number of producers located in each province: Western Canada (60; British Columbia (20), Alberta (20), Saskatchewan (10), Manitoba (10)), Ontario (120), Québec (120), and Atlantic Canada (65; New Brunswick (20), Nova Scotia (20), Prince Edward Island (20), and

Newfoundland (5)) [5]. These numbers were slightly modified due to limited numbers of sampling staff in some regions, and costs of travel to remote locations. Herd size and housing type of cows were recorded at each farm visit.

3.3.2 Environmental sample collection and MAP culture

At each farm visit, 1 environmental sample was collected from the area of the farm where lactating cows were housed (LAC), and 1 environmental sample from the manure storage (MS) or area of manure accumulation. An additional environmental sample was collected from the area where BAH were housed. Each environmental sample consisted of 4 subsamples collected by a technician wearing a latex glove who placed each of these ~150 g samples in a Ziploc bag, thoroughly mixed them and then filled a 50 mL screw-top plastic container. In tie-stall barns, these 4 subsamples were collected from the lactating herd in the manure gutter corners and high-traffic areas, whereas free-stall samples were collected from crossover alleys and near waterers. Manure storage sub-samples were collected from a manure pile using a gloved hand that reached 10 to 15 cm below the external surface or from a manure pit using a 1.2 m long golf ball retriever (extendable to 4.5 m) with a 50-mL plastic container taped to the end. Sub-samples from the BAH pens consisted of a handful from the manure pack of every pen of heifers or 4 handfuls from well-traveled areas if they were all kept in the same area and mixed as per the lactating cow herd. All samples collected from across the country were stored on ice packs, and express mailed (guaranteed 48 h delivery) to the University of Calgary (Calgary, AB, Canada) for processing. All samples were processed within 7 d after collection, using the TREK ESP II (TREK para-JEM®; TREK Diagnostic Systems,

Cleveland, OH, USA) culture protocol and media, and detection was based on subsequent F57-specific qPCR as described [23].

3.3.3 Statistical analyses

As the present method of environmental sampling had not been implemented in previous studies, first test characteristics (Se and Sp) for this type of environmental sampling (one LAC and one MS on the same day) needed to be estimated using the Bayesian models described below. Prior Se and Sp estimate inputs were calculated based on a complete dataset available from a previous study [38], in order to more accurately determine true prevalence across Canada (NDS). Sensitivity, Sp, and true prevalence were estimated using R v3.4.2 (R Core Team, 2017), the 'BetaBuster' function in the 'epiR' package [29], the 'rjags' package [27], the 'runjags' package [11], and utility programs included with *Doing Bayesian Data Analysis: a Tutorial with R, JAGS and Stan* [15].

Prior test characteristics. Test characteristics (Se and Sp) were estimated based on MAP environmental sample results collected by the Alberta Johne's Disease Initiative (AJDI) between November 2010 and May 2014 [38], during which time 6 environmental samples were collected from the same farms, 3 years in a row (6S3Y). Briefly, samples were collected in duplicate from 3 areas on 430 Alberta dairy farms from: 1) MS, 2) LAC, and 3) dry, sick, or calving pens; resulting in 6 samples per farm. As not all sampling records specified the location at which each environmental sample was collected, a subset of farms (n=62) was identified which were sampled in 3 consecutive years, and each year had at least 1 MS and 1 LAC area sample identified.

The Se and Sp estimates for 6S3Y were used to further determine the Se and Sp for these 62 farms when only 2 samples (MS + LAC) were collected once (2S1Y) to simulate the

NDS sampling scheme. On these 62 farms, 2 samples were randomly selected from 1 MS and 1 LAC area sample from a randomly selected year within the 3 y available for each herd. Herd-level MAP infection status was based on environmental samples from the same areas within the same herds; therefore, Se and Sp were estimated using a Bayesian model that accounted for conditional dependence between diagnostic tests [10]. Prior distributions for Se and Sp of 6S3Y, as well as MAP prevalence, were defined as following a beta distribution whose parameters were determined by the estimates by Wolf et al. (2014), using an implementation of the BetaBuster program (http://www.epi.ucdavis.edu/diagnostictests/betabuster.html) in R. Beta parameters were determined by specifying the range where the true parameter lies, with 95% confidence. The beta parameters α and β can also be interpreted as scaled versions of number of true positives and false-negatives, respectively. Prior distributions for Se and Sp of 2S1Y were chosen to represent minimal prior knowledge. Specifications for prior distribution are shown (Table 3-1). Sensitivity and Sp of both 6S3Y and 2S1Y were simultaneously estimated using a Gibbs sampler, and modes of the resulting posterior distributions were presented with their respective probability intervals. Gibbs samplers were run using 6 chains in parallel, for a combined total of 25,000 iterations after a burn-in of 5000 iterations. Convergence was verified by ensuring that posterior distributions were unimodal and that the Gelman and Rubin R statistic (Gelman and Rubin 1992) reached 1 by the end of the chains.

Estimating MAP prevalence. True herd-level MAP prevalence in dairy herds across Canada was estimated using a Bayesian model using prior distributions of the Se and Sp as described above, and based on the 362 herds sampled in 2015 (NDS). The model also

compared MAP status based on 2 samples (LAC and MS samples), with MAP-status based on 3 samples (LAC, MS and BAH) that also estimated Se, Sp of the third sampling scheme (3S1Y). The model was run for the same number of iterations as the model determining test characteristics, and convergence was also monitored using the Gelman and Rubin R statistic (Gelman and Rubin, 1992).

***Region, housing type and herd size*.** The 362 NDS herds were used to estimate region-, housing type-, and herd size specific prevalence in Canada. Stratified prevalence estimates were based on the 2 samples, LAC and MS (2S1Y). Regional, herd size specific and barn type-specific prevalence of MAP infection were estimated using a logistic regression model applied in a Bayesian framework to correct for the imperfect Se and Sp of the sampling scheme. A Se analysis was conducted to determine appropriate prior distributions for coefficients in the logistic regression model specifying region, barn type, and herd size. Distributions included in the Se analysis were centered on a prevalence of 50% (log odds of 0, used as a mean for a normal distribution) based on an estimate for prevalence estimated in model 2 comparing 2S1Y and 3S1Y. Values of precision varied between 0.0001 and 10,000 to represent very vague, weakly informative priors (low precision) and narrow, strongly informative priors (high precision); respectively. An intermediate level of precision was chosen that optimized the trade-off between high variability in the posterior distribution (results not interpretable or useful) and proximity of estimates to the prior (priors that are too strong may result in posterior estimates almost independent of observed data). Consequently, a weakly informative prior was chosen for all coefficients, centered on a prevalence of 50%, with 95% probability of estimating a prevalence between 12.3 and 87.6% (Table 3-1). These

parameters were estimated using a Gibbs sampler, with modes of resulting posterior distributions being used as the parameter estimates with their respective probability intervals. The model was run for the same number of iterations as the test accuracy model, and convergence was also monitored using the Gelman and Rubin R statistic (Gelman and Rubin, 1992).

Statistical significance of differences across characteristics (region, housing type and herd size) was assessed using the apparent prevalence and maximum-likelihood estimation in a logistic regression model using the "glm" function in base R (R Core Team 2017). A *P*-value of < 0.05 was considered significant. The models included either region, herd size, or housing type as predictors, with odds of testing MAP-positive as the outcome. Only farms categorized as free-stall or tie-stall were included in the housing-specific prevalence estimate models, as bedding pack, other farms or not specified, were removed due to low sample size (n=9, 1 and 5; respectively). Models with herd size and housing type as predictors were modelled using a mixed effects logistic regression with region as a random effect using the package "lme4" (Bates et al., 2015). A random-effects model adjusts estimates to account for unmeasured correlation between herds within a region.

3.4 Results

3.4.1 Herd characteristics

Of the 362 dairy farms across Canada participating in the NDS on farm assessments, 55 (15%) were from Western Canada (20 in British Columbia, 16 in Alberta, 9 in Manitoba, and 10 in Saskatchewan), 131 (36%) were located in Ontario, 117 (32%) in Québec and

59 (16%) in the Atlantic provinces (18 in Nova Scotia, 17 in New Brunswick, 20 in Prince Edward Island, and 4 in Newfoundland; Table 3-2).

In Western Canada, Ontario, and Atlantic Canada, the majority of participating farms 45 (82%) housed their cows in free-stalls (82, 54 and 59%; respectively), whereas in Québec the majority of farms housed the cows in tie-stalls (73%; Table 3-2). Across all 4 regions, free-stall farms had a larger average herd size than tie-stall farms (Table 3-2).

3.4.2 Environmental sample results

In all 4 regions across Canada, a higher number of MAP-positive environmental samples was collected from MS than either the LAC or BAH areas (Table 3-3). When including BAH environmental samples for identification of MAP-positive farms, 4 additional farms from Ontario and Québec were MAP-positive. These farms included both tie-stall and free-stall farms, and all herd size categories except for > 150 cows (Table 3-3).

3.4.3 Prior test characteristics

Of the 62 farms with 6 samples collected in 3 consecutive years, 45 (73%) had at least 1 positive environmental sample during at least 1 of the 3 sampling events (6S3Y). The estimated Se and Sp for these herds were 93 and 99%, respectively (Table 3-4). When collecting only 2 environmental samples from a herd at a single time point, the Se of detecting positive herds was estimated at 40% (2S1Y; Table 3-4).

3.4.4 Estimating MAP prevalence

On the 362 dairy farms sampled for the NDS, Se and Sp were estimated at 38 and 100%, respectively, when collecting 2 environmental samples (LAC and MS; 2S1Y). These estimates did not change when including the third environmental sample from the BAH (3S1Y; Table 3-5).

3.4.5 Region, housing type and herd size

True herd-level MAP prevalence for all of Canada was 46%, with true regional prevalence ranging from 24% in Québec to 66% in Western Canada (Table 3-6). The apparent prevalence varied for herd sizes, with herds having 101-150, and > 151 cows having a higher apparent prevalence than herds with ≤ 50 cows (Table 3-6). Additionally, across Canada, tie-stalls had a lower apparent MAP prevalence than free-stalls (Table 3-6).

3.5 Discussion

In the current study, environmental sampling was used to determine the herd-level prevalence of MAP infection in Canadian dairy herds. Sensitivity and Sp for collecting 2 environmental samples for detection of a positive farm were estimated at 38 and 100%, respectively. Based on these test characteristics, true prevalence across Canada was 46%, with Québec having a lower prevalence than the other 3 regions. This was the first time that the prevalence of MAP infection was determined using the same sampling technique across all provinces of Canada, enabling accurate comparisons, not only of regional affects, but of housing and herd size effects across the country.

Prevalence in Canada, the Netherlands, and the United States has primarily been estimated based on diagnostic outcome of serum ELISA testing, ranging from 9.8% in Ontario to 40% in Alberta, 17-28% in the US, and 54% in the Netherlands [24, 31]. However, ability to compare regions is extremely difficult, due to the variability introduced by types of kits used, cut-off points, laboratory environments, sampling technique and processing, and number of positive animals to define a MAP-positive herd

[21, 31]. Although Se and SP of various diagnostic tests have been investigated [1, 16, 17, 21], these test characteristics are often not applied to prevalence studies, and only apparent prevalence is reported. Additionally, test characteristics are often not estimated in a population that is representative for the population of study [3]. True prevalence estimates in the current study using environmental samples were higher than previous research using serum ELISA across Canada [31]; however, this was likely due to an underestimation of prevalence by overestimating Se of serum and milk ELISA.

Distribution of free-stall and tie-stall farms in Canada collected in this study corresponded to the situation in Canada (available through the Canadian Dairy Information Centre (CDIC) (dairyinfo.gc.ca.)). In Western and Atlantic Canada, 88 and 50% of DHI-participating farms house adult cows in a free-stall, respectively (CDIC), whereas 93% of Quebec farms have a tie-stall, similar to distributions of housing types collected in the current study (82, 59 and 73%, respectively). In the province of Ontario, however, the proportion of free-stall farms is higher according to the CDIC (69%) than in this study (42%). These differences between CDIC housing distributions and those acquired in this study were likely an artifact due to random sampling, and a smaller sample size, and should be considered for future investigations regarding housing and regional differences.

Québec has in number of lactating cows and farms the largest dairy industry next to Ontario in Canada, the lowest apparent prevalence and true prevalence (9.4, 23.6; respectively), and the highest proportion of tie-stall farms (69%; Bauman et al., 2018). Herd size is highly associated with housing type, as tie-stall farms usually have <100 cows [38]. The lower prevalence estimates in the tie-stall herds and Québec may be

explained by different manure and management practices and contact structure of the herd, as environmental samples may not accurately represent a “pooled” sample from the herd. Environmental samples collected from MS of free-stalls have high Se for detection of MAP-positive farms due to accumulation of fecal material and long-term survival of MAP in the environment [6, 19, 35]; however, larger and free-stall herds also tend to have liquid manure handling and storage. Most tie-stall barns have gutter scrapers that move manure to form a pile where there is much less mixing of manure, and this may lead to decreased detection among smaller herds, and tie-stall herds among MS samples. However, this decreased detection among tie-stall herds may not be due to manure storage, as MS sites alone detected 11 of the 14 MAP-positive tie-stall farms in Canada in the current study (Table 3-3). Although the majority of literature regarding Se and Sp of environmental sampling has been conducted on free-stall herds [6, 12, 28], there is evidence that it can be just as effective on tie-stall herds requiring inclusion of a manure storage site [1].

The MAP-prevalence was highest in larger herds, 33% in herds with 101-150 cows, and 49% identified as positive with ≤ 150 cows, a finding consistent with research indicating that environmental samples collected from larger herds are more likely to be positive [37]. The higher prevalence of MAP infected farms among larger herds may be due to a higher within-herd prevalence [26], or the higher likelihood that at least 1 cow sheds MAP; however, the cause of the association between herd size and within-herd prevalence is not known, and hypothesized to be due to difference in management practices for young stock, and purchasing of replacement heifers increasing risk of transmission [36].

The addition of BAH samples to the standard LAC and MS samples identified 4 additional farms as being MAP-positive. These farms were only positive based on the BAH sample, and therefore would be incorrectly identified as negative had this sample not been included; however, MAP-positive BAH samples more often occurred when either LAC or storage site samples were positive. Additionally, these BAH MAP-positive samples were not specific to regions, housing, or herd size categories. Identification of positive herds should not rely only on BAH environmental samples; however, their detection is an important indicator of potential transmission to, and within young stock. Positive environmental samples have been identified in all ages of calves [26, 28, 39], and should be considered an important tool to identify calf-to-calf transmission as a risk factor when examining within-herd prevalence and transmission.

Sensitivity estimates for 2 environmental samples were lower than previous estimates for 6 samples, which was to be expected as fewer samples being taken on the farm increase the odds that a truly positive farm is missed. However, the estimated prevalence in Alberta based on 2 environmental samples (70%) in the subset of 62 herds was nearly the same as those previous estimated based on 6 environmental samples (68%) in all 360 participating Alberta dairy herds indicating the accuracy of the test characteristic estimates [38]. The addition of the BAH samples did not affect the Se estimates, likely because the majority of farms only had MAP-positive BAH samples when also testing positive to either LAC or MS sample. Therefore, collecting 2 environmental samples, LAC and MS, enabled reliable estimation of true herd-level MAP prevalence in a region when accounting for adjusted test characteristics; however, inclusion of young stock

environmental samples should be considered for future control programs, as it may be an indicator of transmission among young stock.

The number farms recruited for on-farm sampling were based on ability to detect common mastitis pathogens in bulk milk tank samples (Bauman et al., 2018). Because prevalence of herd-level MAP infection is similar to the bulk tank prevalence of the most common udder pathogen *Staphylococcus aureus* in Canadian dairy herds (46%; Bauman et al., 2018), sample size calculation used was also appropriate for MAP infection. However, because detection of MAP in the environment is sensitive to within-herd prevalence, low MAP prevalence herds may have remained undetected in the 6S3Y sampling of Wolf et al. (2014), as well as 2S1Y from NDS sampling, resulting in an overestimation of the Se and an underestimation of true herd prevalence [17, 28].

A limitation of the study was an inability to account for ways that region, herd size, and housing were linked. The variables could not be stratified by more than 1 variable, as the number of herds that would be categorized in each stratum was too small to make a meaningful difference. For example, Western Canada only had 4 (7%) tie-stall farms that were sampled, and herd sizes among these farms ranged from 47-130 and in Quebec, 85 (73%) of farms were tie-stalls with herd size ranging from 7-130.

3.6 Conclusions

The prevalence of MAP infection across Canada was estimated based on the culture of 2 environmental samples from a proportional number of farms in each of the 4 regions within the country. Prevalence estimates were compared among regions, herd sizes, and housing types, indicating a lower prevalence in Québec, among tie-stall herds, and in

smaller herds. The inclusion of BAH samples did not significantly change the Se of the test or prevalence estimates.

3.7 References

1. Arango-Sabogal JC, Cote G, Pare J, Labrecque O, Roy JP, Buczinski S, Dore E, Fairbrother JH, Bissonnette N, Wellemans V *et al* (2016) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in tie-stall dairy herds using a standardized environmental sampling technique and targeted pooled samples. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire 80:175-182.

2. Bakker D (2010) Paratuberculosis control measures in Europe. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. CABI, Wallingford, UK.

3. Barkema HW, Hesselink JW, McKenna SL, Benedictus G, Groenendaal H (2010) Global prevalence and economics of infection with *Mycobacterium avium* susbsp. *paratuberculosis* in ruminants. In: Behr MA, Collins D (eds) Paratuberculosis: organism, disease, control. CAB International.

4. Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten V, Bannantine JP, Keefe GP, Kelton DF, Wells SJ, Whittington RJ *et al* (2018) Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection. Transbound Emerg Dis., in press.

5. Bauman CA, Barkema HW, Dubuc J, Keefe GP, Kelton DF (2018) Canadian National Dairy Study: Herd-level milk quality. J Dairy Sci 101:2679-2691.

6. Berghaus RD, Farver TB, Anderson RJ, Jaravata CC, Gardner IA (2006) Environmental sampling for detection of *Mycobacterium avium* spp. *paratuberculosis* on large California dairies. J Dairy Sci 89:963-970.

7. Bolton MW, Pillars RB, Kaneene JB, Mauer WA, Grooms DL (2011) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in naturally exposed dairy heifers and associated risk factors. J Dairy Sci 94:4669-4675.

8. Cocito C, Gilot P, Coene M, de Kesel M, Poupart P, Vannuffel P (1994) Paratuberculosis. Clin Microbiol Rev 7:328-345.

9. Collins MT, Eggleston V, Manning EJ (2010) Successful control of Johne's disease in nine dairy herds: results of a six-year field trial. J Dairy Sci 93:1638-1643.

10. Dendukuri N, Joseph L (2001) Bayesian approaches to modeling the conditional dependence between multiple diagnostic tests. Biometrics 57:158-167.

11. Denwood MJ (2016) runjags: AnRPackage Providing Interface Utilities, Model Templates, Parallel Computing Methods and Additional Distributions for MCMC Models inJAGS. J of Stat Softw 71.

12. Donat K, Kube J, Dressel J, Einax E, Pfeffer M, Failing K (2015) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture. Epidemiol Infect 143:975-985.

13. Garcia AB, Shalloo L (2015) Invited review: The economic impact and control of paratuberculosis in cattle. J Dairy Sci 98:5019-5039.

14. Kalis CH, Collins MT, Barkema HW, Hesselink JW (2004) Certification of herds as free of *Mycobacterium paratuberculosis* infection: actual pooled faecal results versus certification model predictions. Prev Vet Med 65:189-204.

15. Kruschke JK: Doing bayesian data analysis, Second Edition: a tutorial with R, JAGS, and Stan. In: *Academic Press / Elsevier*. 2015.

16. Lavers CJ, Barkema HW, Dohoo IR, McKenna SL, Keefe GP (2014) Evaluation of milk ELISA for detection of *Mycobacterium avium* subspecies *paratuberculosis* in dairy herds and association with within-herd prevalence. J Dairy Sci 97:299-309.

17. Lavers CJ, McKenna SL, Dohoo IR, Barkema HW, Keefe GP (2013) Evaluation of environmental fecal culture for *Mycobacterium avium* subspecies *paratuberculosis* detection in dairy herds and association with apparent within-herd prevalence. Can Vet J 54:1053-1060.

18. Lombard JE (2011) Epidemiology and economics of paratuberculosis. Vet Clin North Am Food Anim Pract 27:525-535.

19. Lombard JE, Wagner BA, Smith RL, McCluskey BJ, Harris B, Payeur JB, Garry F, Salman MD (2006) Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subspecies *paratuberculosis* distribution and herd infection status on US dairy operations. J Dairy Sci 89:4163-4171.

20. McKenna SL, Keefe GP, Barkema HW, McClure J, VanLeeuwen JA, Hanna P, Sockett DC (2004) Cow-level prevalence of paratuberculosis in culled dairy cows in Atlantic Canada and Maine. J Dairy Sci 87:3770-3777.

21. McKenna SL, Keefe GP, Barkema HW, Sockett DC (2005) Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. Vet Microbiol 110:105-111.

22. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

23. Mortier R, Barkema HW, Orsel K, Wolf R, De Buck J (2014) Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 45:71.

24. Muskens J, Barkema HW, Russchen E, van Maanen K, Schukken Y, Bakker D (2000) Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. Vet Microbiol 77:253-261.

25. Pieper L, Sorge US, DeVries T, Godkin A, Lissemore K, Kelton D (2015) Comparing ELISA test-positive prevalence, risk factors and management recommendations for Johne's disease prevention between organic and conventional dairy farms in Ontario, Canada. Prev Vet Med 122:83-91.

26. Pillars R, Grooms D, Kaneene JB (2009) Longitudinal study of the distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project. Can Vet J 50:1039-1046.

27. Plummer M (2016) Bayesian graphical models using MCMC. R package version 4-6. https://cran.r-project.org/package=rjags.

28. Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE (2004) The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J Dairy Sci 87:2959-2966.

29. Stevenson M, Nunes T, Heuer C, Marshall J, Sanchez J, Thornton R, Reiczigel J, Robinson-Cox J, Sebastiani P, Johann P *et al*: epiR: Tools for the analysis of epidemiological data. R package version 0.9-93. https://cran.r-project.org/package=epiR In.; 2017.

30. R Core Team: R: A language and environment for statistical computing. In. Edited by Computing RFfS. Vienna, Austria; 2017.

31. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW (2006) Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47:874-882.

32. van Schaik G, Stehman SM, Schukken YH, Rossiter CR, Shin SJ (2003) Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence. J Vet Diagn Invest 15:233-241.

33. Weber M (2006) Risk management of paratubersulosis in dairy herds. Ir Vet J 59:555-561.

34. Whitlock RH, Wells S, Sweeney RW, Van Tiem J (2000) ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. Vet Microbiol 77:387-398.

35. Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA (2004) Survival and Dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the Environment. Appl Environ Microbiol 70:2989-3004.

36. Wolf R, Barkema HW, De Buck J, Orsel K (2015) Factors affecting management changes on farms participating in a Johne's disease control program. J Dairy Sci 98:7784-7796.

37. Wolf R, Barkema HW, De Buck J, Orsel K (2015) Sampling location, herd size, and season influence *Mycobacterium avium* ssp. *paratuberculosis* environmental culture results. J Dairy Sci 98:275-287.

38. Wolf R, Barkema HW, De Buck J, Slomp M, Flaig J, Haupstein D, Pickel C, Orsel K (2014) High herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Western Canadian dairy farms, based on environmental sampling. J Dairy Sci 97:6250-6259.

39. Wolf R, Orsel K, De Buck J, Barkema HW (2015) Calves shedding *Mycobacterium avium* subspecies *paratuberculosis* are common on infected dairy farms. Vet Res 46:1-8.

Table 3-1. Parameters used for prior distributions to estimate test accuracy of the environmental sampling scheme used and herd-level prevalence stratified by region, herd size or barn-type of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection on Canadian dairy farms.

Parameter	Distribution type	Distribution parameter	Value
Prior test characteristics[1]			
6S3Y sensitivity	Beta	α[4] β[4]	7.55 2.64
6S3Y specificity	Beta	α β	34.17 1.34
2S1Y sensitivity	Uniform	a[5] b[5]	0.00 1.00
2S1Y specificity	Uniform	a b	0.00 1.00
Alberta MAP prevalence	Beta	α β	13.18 7.85
Estimating MAP prevalence[2]			
2S1Y Sensitivity	Beta	α β	9.50 13.65
2S1Y Specificity	Beta	α β	14.40 1.12
3S1Y Sensitivity	Uniform	a b	0.00 1.00
3S1Y Specificity	Uniform	a b	0.00 1.00
Canada MAP prevalence	Uniform	a b	0.00 1.00
Region, housing type and herd size[3]			
Region/barn-type/herd-size specific prevalence	Gaussian	μ (mean) τ (precision)	0.00 1.00
2S1Y Sensitivity	Beta	α β	9.50 13.65
2S1Y Specificity	Beta	α β	14.40 1.12

[1]Model comparing herd-level MAP-infection status based on 1 positive in 6 samples collected for 3 y (6S3Y) to estimate based on 1 MAP-positive sample out of 2 collected in 1 y (2S1Y), consisting of a manure storage (MS) and a lactating cow area sample

(LAC). Data were collected in Alberta between 2010 and 2014 (Wolf et al., 2014), so a prior distribution for prevalence also needed to be specified.

[2]Model estimating prevalence based on 2 samples (MS and LAC) collected in a single year (2S1Y), compared to sensitivity and specificity of collecting 1 additional sample from breeding-age heifers area (3S1Y).

[3]Model estimating regional, barn-type specific and herd-size specific herd-level MAP-infection prevalence in Canada in 2015, based on 2 samples taken from each herd (2S1Y).

[4]Shape parameters for a beta distribution. In the context of sensitivity/specificity estimation α is proportional to the number of true positives in the sample, and β is proportional to number of false-negatives.

[5]Lower (a) and upper (b) bounds for uniform distribution. All values in that range have equal probability.

Table 3-2. Housing and herd size of the 362 farms sampled across Canada for the Canadian National Dairy Study.

Region[1]	Housing type	No. herds (%)	Average adult herd size (range)
Western Canada		55	166 (40-802)
	Free-stall	45 (83)	170 (40-802)
	Tie-stall	4 (7)	69 (47-130)
	Bedded pack	1 (2)	234 (NA)
	Not specified	5 (9)	188 (91-319)
Ontario		131	90 (16-600)
	Free-stall	71 (54)	129 (33-600)
	Tie-stall	55 (42)	43 (16-106)
	Bedded pack	4 (3)	56 (35-72)
	Not specified	1 (1)	69 (NA)
Québec		117	62 (7-261)
	Free-stall	32 (27)	96 (37-261)
	Tie-stall	85 (73)	49 (7-130)
Atlantic Canada		59	84 (13 – 429)
	Free-stall	35 (59)	110 (22-429)
	Tie-stall	20 (34)	47 (22-150)
	Bedded pack	4 (7)	42 (13-70)

[1]Western Canada: British Columbia (20 herds), Alberta (16), Saskatchewan (10), and Manitoba (9); Atlantic Canada: New Brunswick (17 herds), Nova Scotia (18), Prince Edward Island (20), and Newfoundland (4).

Table 3-3. Herds with *Mycobacterium avium* subsp. *paratuberculosis* (MAP)-positive environmental samples collected from either lactating cows (LAC), manure storage (MS), or breeding age heifers (BAH).

Item	No. Farms	No. (%) LAC-positive	No. (%) MS-positive	No. (%) BAH-positive	No. (%) farms positive based on LAC and MS	No. (%) farms positive based on LAC, MS and BAH	No. additio farms due MAP-positi BAH samp
Region[1]							
Western Canada	55	11 (20)	13 (24)	1 (1)	16 (29)	16 (29)	0
Ontario	131	21 (16)	24 (18)	8 (6)	30 (23)	33 (25)	3
Québec	117	4 (3)	7 (6)	2 (2)	11 (9)	12 (10)	1
Atlantic Canada	59	7 (12)	8 (13)	1 (1)	11 (19)	11 (19)	0
Lactating cow housing							
Free-stall	183	33 (18)	37 (20)	9 (5)	49 (27)	51 (28)	2
Tie-stall	164	5 (3)	11 (7)	3 (2)	14 (9)	16 (10)	2
Bedded pack	9	1 (11)	1 (11)	0	1 (11)	1 (11)	0
Other	1	0	0	0	0	0	0
Not specified	5	4 (80)	3 (60)	0	4 (80)	4 (80)	0
Number of cows							
< 51	141	7 (5)	11 (8)	4 (3)	15 (9)	17 (12)	2
51-100	125	10 (8)	9 (7)	2 (1)	13 (10)	14 (11)	1
101-150	45	9 (20)	10 (22)	1 (2)	15 (33)	16 (36)	1
> 150	51	17 (33)	22 (43)	5 (10)	25 (49)	25 (49)	0

[1]Western Canada: British Columbia (20 herds), Alberta (16), Saskatchewan (10), and Manitoba (9); Atlantic Canada: New Brunswick (17 herds), Nova Scotia (18), Prince Edward Island (20), and Newfoundland (4).

Table 3-4. Prior sensitivity and specificity estimates of herd-level *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection status based on at least 1 positive sample out of 6 collected every year for 3 y (6S3Y) and MAP infection status based on minimally 1 positive sample of 2 collected in 1 y (2S1Y).

Parameter	Estimate[1]	95% CI[2]
6S3Y		
Sensitivity	0.93	0.79 – 0.99
Specificity	0.98	0.88 – 1.00
2S1Y[3]		
Sensitivity	0.40	0.28 – 0.55
Specificity	0.99	0.81 – 1.00

[1]Estimate of the mode of the posterior distribution defined using a Bayesian model.

[2]95% credibility interval from a posterior distribution using a Bayesian model. Represents the range of the central 95% probability of the distribution.

[3]Consisting of a manure storage and a lactating cow area sample.

Table 3-5. Sensitivity and specificity of herd-level *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection status for estimation of prevalence in National Canadian Dairy Study. Based on at least 1 MAP-positive environmental sample out of 2 collected in 1 y (2S1Y), compared to 1 positive environmental sample out of 3 collected in 1 y (3S1Y).

Parameter	Estimate[1]	95% PI[2]
2S1Y		
Sensitivity	0.38	0.26 – 0.49
Specificity	1.00	0.92 – 1.00
3S1Y[3]		
Sensitivity	0.39	0.27 – 0.52
Specificity	0.98	0.90 – 1.00
True Canada herd-level prevalence	0.46	0.29 – 0.75

[1]Estimate of the mode of the posterior distribution defined using a Bayesian model.

[2]Refers to 95% probability interval from a posterior distribution using a Bayesian model. Represents range of the central 95% probability of the distribution.

[3]Consisting of a manure storage and a lactating cow area, and breeding age heifer area sample.

Table 3-6. Herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection in Canadian dairy herds in 2015 stratified by region, herd size and housing type.

Predictor Level	Apparent Prevalence (%)[1]	True Prevalence (%)[2]	95% PI[3]
Canada	18.8	46.4	28.5 - 74.6
Region			
Western Canada[Ref.]	29.1	65.8	36.8 – 89.6
Ontario	22.9	54.1	0.3 – 83.7
Québec	9.4[4]	23.6	9.6 – 48.5
Atlantic Canada	18.6	47.3	21.6 – 79.2
Herd size (no. of lactating cows)			
≤ 50 [Ref.]	10.5	21.1	8.8 – 36.4
51 – 100	10.3	20.7	8.5 – 37.0
101 – 150	33.3[4]	61.2	35.9 – 86.7
> 150	48.4[4]	79.9	56.9 – 93.9
Housing-type			
Free-stall[Ref.]	26.5	63.7	40.2 – 88.9
Tie-stall	8.5[4]	20.8	7.5 – 39.8
Bedded pack	10.9	-[5]	-[5]
Other	0	-[5]	-[5]

[1]Proportion of farms with at least 1 of 2 environmental samples (manure storage and lactating cow area) being MAP-culture positive.

[2]Calculated by taking the logit transformation of the model coefficients.

[3]95% probability interval from a posterior distribution using a Bayesian model. Represents the range of the central 95% probability of the distribution.

[4]Different ($P < 0.05$) from apparent prevalence in reference group within predictor.

[5]Number of farms was too small (9 and 1 for bedded pack and other, respectively) to reliably estimate true prevalence.

CHAPTER 4: FECAL SHEDDING AND TISSUE INFECTIONS DEMONSTRATE TRANSMISSION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN GROUP-HOUSED DAIRY CALVES

4.1 Abstract

Current Johne's disease control programs primarily focus on decreasing transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from infectious adult cows to susceptible calves. However, potential transmission between calves is largely overlooked. The objective was to determine the extent of MAP infection in calves contact-exposed to infectious penmates. Thirty-two newborn Holstein-Friesian calves were grouped into 7 experimental groups of 4, consisting of 2 inoculated (IN) calves, and 2 contact-exposed (CE) calves, and 1 control pen with 4 non-exposed calves. Calves were group housed for 3 months, with fecal samples were collected 3 times per week, blood and environmental samples weekly, and tissue samples at the end of the trial. The IN calves exited the trial after 3 months of group housing, whereas CE calves were individually housed for an additional 3 months before euthanasia. Control calves group were housed for the entire trial. All CE and IN calves had MAP-positive fecal samples during the period of group housing; however, fecal shedding had ceased at time of individual housing. All IN calves had MAP-positive tissue samples at necropsy, and 7 (50%) of the CE had positive tissue samples. None of the calves had a humoral immune response, whereas INF-γ responses were detected in all IN calves and 5 (36%) CE calves. In conclusion, new MAP infections occurred due to exposure of infectious penmates to contact calves. Therefore, calf-to-calf transmission is a potential route of uncontrolled transmission on cattle farms.

Keywords: Calf, transmission, fecal shedding, immune response, tissue, group-house

4.2 Introduction

Johne's disease (JD) is a chronic, progressive, inflammatory disease in the small intestine of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is well established that MAP infection is widespread in cattle and causes substantial economic losses to dairy producers worldwide [6, 23, 50]. Clinical stages of disease cause severe diarrhea and shedding of bacteria into the environment; however, subclinical animals also contribute to the infectious load in the environment and economic losses incurred by the producer, due to reduced milk yield, increased risk of culling and decreased slaughter value [37, 42].

Although vaccines for use in cattle have been developed [15, 18], these vaccines only prevent clinical signs of JD, and there is currently no vaccine available for cattle to prevent infection or shedding of MAP. Therefore, control programs are based on decreasing both the number of new MAP introductions in negative herds and within-herd transmission [6, 8, 23]. The primary route of infection is fecal-oral through ingestion of milk, feed, or water contaminated by infectious animals shedding MAP bacteria in their feces [13, 36, 42]. The assumptions that cows are infectious, calves are susceptible, and calves do not shed until later in life has led to the focus of most control programs interrupting direct and indirect contact of fecal material from infectious adult cows to susceptible young stock [12, 46]. Although the association between JD control programs, management practices, and MAP infections on farms has been well established [2, 23, 38, 46], the potential risk of calf-calf transmission is largely overlooked. However, calves can begin shedding as early as 1 month after inoculation [27], calves up to at least 12 months of age are susceptible to MAP infection [26, 43], and a relatively high proportion

of young stock on infected farms are shedding MAP in their feces [45, 51]. Although most calves are separated from their dams shortly after birth to prevent transmission, fecal-oral transmission may still be possible during those first hours to days in the calving pen, or even prenatally via intra-uterine transmission [32, 48]. Therefore, group-housing of calves (even though they are isolated from adult cows) may not be an effective practice to eliminate the spread of MAP or prevent new infections.

Recent infection trials have yielded new knowledge that calves inoculated with MAP at an earlier age had more severe tissue lesions [26], and increased fecal shedding was associated with increased numbers of MAP-positive tissue samples [27]. The ability of calves to both infect and become infected has led to several transmission and modelling studies to determine the role of calf-to-calf transmission in causing new infections on farm. However, there have been inconsistent findings regarding the role of calf transmission and its importance for control [21, 25, 34, 43]. Furthermore, the extent of infection, subclinical infections, or the ability to suppress an infection, and detecting the signs of infection, all vary depending on several factors, including inoculation dose, immune capabilities, frequency of sampling and individual variability [24, 25, 41]. Therefore, there is a need for an experimental study to examine the extent of infection due to calf-to-calf transmission.

The objective was to determine extent and magnitude of MAP infection in contact-exposed calves resulting from transmission of MAP from inoculated pen-mates, based on fecal shedding and positive tissue samples due to 3 months of group housing.

4.3 Materials and methods

4.3.1 Calves

Thirty-two newborn Holstein-Friesian bull calves were purchased from 13 Alberta (Canada) dairy farms selected based on annual testing as part of the Alberta Johne's Disease Initiative [49] and participation in the JD herd health status program in Alberta. All farms had tested negative for at least 4 years using 6 environmental samples and 1 of the following: bacteriological culture of 60 individual fecal samples tested as pooled samples into groups of 5, individual milk ELISA of the whole milking herd, or serum ELISA of the entire herd.

4.3.2 Nutrition, health and husbandry

All calves were collected immediately after birth (to prevent contamination from fecal material on farm or ingesting colostrum), and transported to the research facility. Nutrition was similar to that described by Mortier et al. [26]. In short, calves were fed 6 L (in 2-L portions) of high-quality colostrum within the first 8 h after birth. Colostrum was collected from 4 of the 13 farms that had tested negative consistently for ≥ 4 years. Starting the 2nd day of their life, calves were fed milk replacer, followed by calf starter (without antimicrobials) and high-quality hay. Calves were gradually weaned by 8 week of age, and had *ad libitum* access to water and hay (supplemented with concentrates). Calves were housed in a biosecurity Level 2 facility. The facility included 15 custom-built housing units with waterproof liners to contain all bedding and fecal material. Group-housing pens were 10 × 10 feet and 6 feet tall (3.05 × 3.05 × 1.82 m). Each housing unit consisted of a marked-off area containing the pen, 2 pairs of boots, 2 pairs of coveralls and gloves dedicated specifically for use in the pen within the unit. All

personnel were trained to monitor health daily, and to observe strict biosafety and isolation protocols to prevent transmission of MAP between pens by any vectors, e.g. buckets, scoops for feed, personnel, etc. All protocols and the experimental design were approved by the University of Calgary Veterinary Sciences Animal Care Committee (protocol AC14-0168).

4.3.3 Study design

Calves were assigned to pens based on time of birth and entry into the research facility. The first 14 calves were designated to be inoculated animals (IN), with 2 calves in each of the 7 experimental pens. The next 14 calves to enter the barn were assigned as contact-exposed (CE) and individually housed temporarily in separate pens from the IN calves. The last 4 calves to enter the barn were designated as the control group, and placed together in the control pen. At 2 week of age, the IN calves in each pen were inoculated over 2 consecutive days. After 2 week (to allow the inoculum to pass through the calves), pens were relined with new liners and bedded with fresh shavings and straw. Calves designated as CE had to reach a minimum of 1 week of age with no health complications to ensure that they could drink from a bucket without assistance, and that only healthy calves were added to the study. When both CE calves entering the same pen reached a minimum 1 week of age, they were placed into the clean, re-lined experimental pen with the IN calves. Four calves (2 IN and 2 CE) were then group-housed for 3 months following the first day of group housing. The IN calves were euthanized and necropsied after 3 months of group housing. The CE calves were then individually housed in relined and clean pens for an additional 3 months. All 4 control calves were group housed (1 pen) for the entirety of the study.

4.3.4 Inoculum

The inoculum was a virulent MAP cattle type strain from a clinical JD case in Alberta (Cow 69) [26]. In short, a culture was prepared in 7H9/mycobactin/OADC liquid broth, from a first passage frozen stock and quantified using a combination of optical density (OD) at 600 nm, the wet weight method, and qPCR, as described [10]. Once culture grew to a concentration of 5×10^8 CFU/mL, 1 mL aliquots were frozen at −80 °C until 1 week prior to inoculation. Before each inoculation, 1 tube was thawed and suspended in 50 mL 7H9 broth for 1 week, during which time inoculum was tested for contamination. 2.5×10^8 CFU's was quantified using the wet weight method, diluted in 20 mL of broth, placed in a 20-mL syringe and transported to the research facility. Calves were allowed to suckle the syringe containing the inoculum and it was expelled at the root of the tongue (on 2 consecutive days).

4.3.5 Fecal sampling and culture

Fecal samples were collected daily for 14 days following inoculation of IN calves to ensure viability of the inoculum, and monitor passive shedding. As of 14 days after inoculation, shedding was attributed to active MAP infection. For the remainder of the trial, fecal samples from each calf were collected 3 times/week during group housing for all calves. Following group housing, when calves were housed individually, fecal samples were collected weekly from CE calves for the remainder of the trial. Samples were stored at 4 °C until processing, which occurred within 7 days after collection.

All samples were processed using a modified TREK ESP II culture media (TREK para-JEM®; TREK Diagnostic Systems, Cleveland, OH, USA) with subsequent F57-specific qPCR, as described [27]. Briefly, 2 g of fecal sample was thoroughly mixed with 30 mL

of distilled water and left to settle for 30 min. Then, 5 mL of supernatant was transferred to 25 mL of a 0.9% hexadecylpyridinium chloride (HPC) half-strength brain heart infusion (BHI) solution for decontamination. Samples were then incubated for 24 h at 37 °C, followed by centrifugation at 3000 × *g* for 20 min, and the pellet re-suspended in a mixture of antibiotic solution (AS; para-Jem®), water, and full strength BHI. Tubes were incubated again for 24 h at 37 °C and then 1 mL was added to liquid culture medium in TREK para-JEM® culture bottles (TREK Diagnostic Systems, Cleveland, OH, USA) and incubated at 37 °C for 49 days.

4.3.6 Environmental sampling and culture

Environmental samples were collected once per week from each pen for the duration of the trial. Samples were collected from 5 locations within the pen, and mixed together, resulting in 1 composite sample from each pen. Samples were collected from the surface of the bed pack (individual piles of feces were avoided). Samples were stored at 4 °C until processing, and were subjected to the same protocol (described above) as fecal samples.

4.3.7 Necropsies and tissue cultures

The IN calves were euthanized after 3 months of group housing at 4 months of age by intravenous injection of barbiturate (Euthanyl Forte®, DIN 00241326, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada), whereas CE were euthanized at 6 months of age, after an additional 3 months of individual housing. Control calves were euthanized last, after all other animals had exited the trial. Necropsies were performed immediately after euthanasia. No other ruminants were examined in the pathology room during necropsies, and the pathology room and tables were thoroughly cleaned and

disinfected before and after each necropsy. Thirteen tissue samples were collected from each calf, including 2 sections of the duodenum, the ileum (including ileal-cecal valve), 3 sections of jejunum, and spleen. All associated lymph nodes with each gastrointestinal tract section were also collected, as well as the inguinal lymph nodes. Sample locations were marked and isolated with zip ties prior to collection (to prevent movement of intestinal contents). A new set of disinfected instruments and a new pair of gloves was used for collection of each new sample to prevent cross contamination, and PBS was used to rinse fecal content from intestinal tissues.

Samples were transported to the laboratory, and processed immediately on the same day using a modified version of a previous protocol [26]. Briefly, 2.5 g of tissue was dissociated using gentleMACS M tubes (Miltenyi Biotech Inc, Auburn, CA, USA) in 10 mL 0.5% triton x-100 PBS solution. Samples were then transferred to a falcon tube and centrifuged at 4700 × *g* for 15 min and the pellet re-suspended in 25 mL of 0.75% HPC, ½ strength BHI, 4-mm sterile glass beads and vortexed vigorously for 1-2 min. Samples were then incubated at 37 °C for 3 h, before centrifugation at 4700 × *g* for 15 min. The pellet was then re-suspended in 3 mL of antibiotic brew (paraJEM®) and incubated overnight, and 1 mL added to paraJEM® culture bottles and incubated at 37 °C for 49 days.

4.3.8 qPCR procedure

Following liquid culture of fecal and tissue samples, DNA was extracted as described [11]. A duplex qPCR targeting the MAP-specific F57 region and an internal amplification control (IAC) was performed, with primers, probes, and IAC sequences identical to those described [35]. Amplification conditions for qPCR were as follows:

50 °C for 2 min, 95 °C for 20 s to allow for initial denaturation, then 42 cycles of 95 °C for 30 s and 59 °C for 30 s. Samples were considered positive when the cycle threshold (CT) value was < 40.

4.3.9 Blood sampling, IFN-γ release assay and ELISA

Blood samples were collected weekly from the jugular vein of all calves, alternating between sides. Whole blood was transported to the lab in heated coolers with hot water bottles (25-35 °C), and processed within 2 h for detection of IFN-γ release, as described [30]. Briefly, each sample of whole blood was treated with 100 μL avium Purified Protein Derivative (aPPD; 0.3 mg/mL; Canadian Food Inspection Agency, Ottawa, ON, Canada), 100 μL of pokeweed mitogen (positive stimulation control; 0.3 mg/mL; Sigma–Aldrich Canada Co., Oakville, ON, Canada), and 100 μL sterile PBS (negative stimulation control). Following overnight incubation at 37 °C, serum was collected after centrifugation and stored at −20 °C until all samples were collected and assayed using the sandwich ELISA BOVIGAM® (Prionics, La Vista, NE, USA). Inclusion criteria and interpretation of the IFN-γ release assay were as described [27] and [16]. Consequently, observations were excluded from analysis if negative assay controls were <0.25, the difference between the positive and negative assay controls was <0.45 or there was a difference of <0.20 between the negative stimulation and negative assay control. These criteria resulted in only 12 samples being excluded from the study. The %IFN-γ was calculated as follows: [(PPD Johnin-negative assay control)/(positive- negative assay control)] × 100 [16, 30].

Serum was collected for antibody testing following centrifugation and stored at −20 °C until antibody ELISA testing was performed, based on manufacturer's directions

(IDEXX Laboratories Inc.), with analysis as described [29]. Briefly, sample results were expressed as a proportion of the positive control corrected for the negative control (S/P ratio), and a ratio ≥ 60 was considered positive.

4.3.10 Data and statistical analyses

All statistical analyses were performed using STATA 11.2 (StataCorp LP, College Station, TX, USA). For all analyses, $P < 0.05$ was considered significant.

To define shedding events, isolated fecal culture-positive samples (sample collected week prior and subsequent week were negative), and groups of positive samples in which a positive sample was immediately followed by a subsequent positive fecal sample(s), were categorized as a single shedding event. Difference in mean number of fecal positive samples and shedding events, and length of shedding period between IN and CE calves was evaluated using a Student's *t*-test. The average length of events for IN and CE calves was calculated separately. Calves were also separated into fecal shedding categories based on the number of positive samples during group housing, where: 0 = calves with 0-4 positive fecal samples; 1 = calves with 5-9 positive fecal samples; 2 = calves 10-14 positive fecal samples; and 3 = calves with ≥ 15 positive fecal samples of all 38 samples collected during group housing.

The INF-γ results were dichotomized using a cutoff of 100% IFN-γ by calculating the average of presumed negative calves (control calves) + 1.96 the standard deviation [14]. All samples with a value of %IFN-γ exceeding 100, immediately followed by a sample below 100% IFN-γ, were considered false-positive spikes and removed from analysis (28 samples were excluded).

Differences in fecal shedding category, tissue culture and IFN-γ results between IN and CE calves, as well as the association between having at least 1 positive IFN-γ sample and having at least 1 tissue-positive sample, were evaluated using a Fisher's Exact test.

4.4 Results

4.4.1 Tissue culture

All IN calves had at least 3 MAP-positive tissue cultures (range, 3 to 11), whereas 7 (50%) of the CE calves had at least 1 MAP-positive tissue culture, but no more than 2 positive tissue samples (Table 4-1; Figure 4-1; $p < 0.001$). None of the control calves had positive tissue cultures.

All tissue locations were positive in at least 2 IN calves. No location was MAP culture-positive in all calves; however, lymph nodes associated with the jejunum were most frequently MAP-positive, especially among tissue-positive CE calves (Figure 4-1).

4.4.2 Immune responses

For all calves, all samples were antibody ELISA-negative, except for 2 pre-infection samples of Calves 15 and 16 that tested positive on 1 occasion before testing negative for the remainder of the study, and this may be due to the transfer of maternal antibodies absorbed from colostrum intake.

All IN calves had at least 5 positive IFN-γ responses, whereas 5 (36%) CE calves had at least 1 positive IFN-γ response (Figure 4-2). Additionally, 1 control calf (C29) had 2 positive INF-γ samples at 2 consecutive time points (35 and 42 days after beginning of group housing) during the experimental trial.

The IN calves started to have positive INF-γ samples on average 55 days after inoculation (41 days after the start of group housing), with the earliest and latest being 33 and 73

days after inoculation, respectively. Eight (64%) of the IN calves had their first INF-γ response on or after 56 days following inoculation and the average interval after the start of group housing for the CE calves to have an INF-γ response was 45 days, with the earliest being the day of exposure, and the latest day 89 of group housing ($p = 0.32$); however, 3 (60%) of these CE calves had positive INF-γ response on or before 33 days after the start of group housing.

Among CE calves, the tissue sample outcome was not associated with the INF-γ result ($p = 0.58$).

4.4.3 Fecal shedding of MAP

No calf fecally shed MAP prior to exposure (inoculation for IN, and group housing for CE). Positive fecal samples were detected consistently in all IN animals for at least 7 days after inoculation (14 days before group housing), and as many as 10 days. The first positive fecal sample collected from a CE calf occurred 5 days after the start of group housing, whereas the latest first shedding event was detected 31 days after the start of group housing (Figure 4-2). All IN and CE calves had at least 2 positive fecal samples during the 3 months of group housing (Figure 4-2); however, all fecal samples from CE calves were negative after group housing ended and they were housed individually (Figure 4-2). Fecal samples from control calves were culture-negative for all time points, except for day 21 after the start of group housing at which time all calves had a positive sample (Figure 4-2), whereas Calf 29 also had 1 additional positive sample on day 56. Mean number of shedding events was 5.6 (95% CI 4.6-6.7) and 4.1 (95% CI 3.4-4.9) in IN and CE calves, respectively ($p = 0.02$). Mean number of positive samples was 17.6 (95% CI 14.4-20.9) and 5.1 (95% CI 3.9-6.2) in IN and CE calves, respectively

($p < 0.001$). Thirteen (93%) of the IN calves were categorized into Groups 3 and 4, whereas 13 (93%) CE calves were categorized into Groups 1 and 2 (Table 4-1). Average length of a shedding event among the IN calves was 7.5 days, ranging from 2-54 days, whereas an average shedding event of CE calves lasted 2.9 days, ranging from 2-9 days ($p = 0.001$; Figure 4-2).

4.4.4 Environmental culture

All experimental pens had at least 2 MAP culture-positive environmental samples with a maximum of 7 of the weekly 12 samples collected during group housing (Table 4-2). The earliest environmental samples were culture-positive was 3 days after the start of group housing, and the latest first positive sample was collected 28 days after the start of group housing. The control pen had no environmental culture-positive samples. There were no significant correlations between positive environmental samples and shedding in all calves ($p = 0.47$), among CE shedding ($p = 0.30$), IN shedding ($p = 0.41$), or positive tissue samples ($p = 0.49$).

4.5 Discussion

In 5 of the 7 experimental pens, at least 1 CE calf had MAP-positive tissue samples, indicating infection caused by exposure to IN animals in the group pen. In total, 50% of CE calves had MAP-positive tissue samples, 5 (36%) had a positive INF-γ response, and all CE calves shed MAP during group housing. However, there was no association between INF-γ, or MAP-positive tissue results among CE calves. The majority of MAP-positive tissue samples from all calves were isolated from the ileum, jejunum, and adjacent lymph nodes, consistent with other studies [1, 26, 40, 44].

A low to moderate inoculation dose was chosen to be representative of natural exposure [4, 28, 40]; however, the inoculation protocol will likely have led to a difference in MAP dose between IN and CE calves, as IN calves were artificially infected. Because CE calves were infected by exposure to a contaminated environment and infectious animals, the dose and number of exposure events among CE calves cannot be directly determined. A higher inoculation dose results in a higher number of MAP-positive tissues, and was likely the origin of differences between IN and CE calves in number of positive tissue samples detected [4, 26, 27, 40]. Although there is little known regarding mechanisms of MAP shedding, it is generally agreed that shedding occurs as a result of MAP excretion towards the intestinal lumen [19]. As the majority of positive tissue samples in CE calves were located in the LN associated with the jejunum, jejunal and ileal tissue samples were mostly negative in CE calves, this may explain cessation of shedding following individual housing, as MAP was not detected where shedding is hypothesized to occur [19]. Additionally, CE calves had considerably fewer positive tissue samples than IN calves, and an increased number of culture-positive tissue samples has been associated with an increased frequency of MAP shedding [27]; therefore, the extent of infection among the CE calves may have been less than the IN calves, leading to less frequent fecal shedding.

All calves had MAP-positive fecal samples during group housing, indicating exposure and risk for infection to all CE calves. Although all CE and IN calves had positive fecal samples, it is possible that a proportion of these samples were not due to active shedding of MAP caused by an infection, but rather the result of passive shedding from exposure to the contaminated environment. It was reported that a higher prevalence of MAP caused

more passive shedding events, due to increased environmental contamination [20]. Shedding ceased when CE calves were individually housed in a clean environment; and this may indicate that the shedding detected in group housing was due to passive shedding caused by ingestion of contaminated feces in the environment [41]. However, the decrease in frequency of sampling at that time may have also accounted for this lack of positive fecal samples, due to the frequency of intermittent shedding detected during group housing. It is noteworthy that decreases in calf fecal shedding at 4 months, and as early as 2 months, were reported in experimental trials [25, 27, 34, 39, 44], making it impossible to resolve the nature of this shedding.

Both IN and CE calves shed intermittently in our study, which was detected due to frequent fecal sampling. Others have reported intermittent shedding; however, the interval between positive samples largely depended on the interval between samplings [27, 31]. In the current study, positive fecal samples were followed by negative fecal samples for anywhere from 2 days to 5 weeks before another positive sample was detected. These findings may have large implications for sampling calves on farm and/or incorporating calf sampling into control programs, as calves may shed MAP 1 day, yet cease to be positive on following days/weeks. This creates narrow intervals for detection of potentially infectious young stock that may introduce new infections to pen-mates.

In addition to fecal testing for diagnosis, immune responses are also used to diagnose infected animals [7]. All calves were ELISA-negative for the duration of the experimental trial. This was not surprising, as the main limitation of the antibody ELISA is the ability to detect early stages of infection due to the humoral response being related to the severity of infection [5, 9, 13]. Additionally, the earliest that infection trials with

similar doses detected positive antibody responses was 4 months after exposure [3, 10, 17, 29, 44]. The INF-γ immune response is generally a more sensitive indication of early infection or indication that an animal has been exposed to MAP [9, 13]; however, concerns regarding interpretation of the test [16, 47], as well as high individual variability [30] indicate the need for guarded interpretation and further optimization. Among IN calves, consecutive INF-γ positive samples began as early as 43 days after inoculation, and consecutive positive samples continued for all IN calves until euthanasia. Interestingly, of those CE calves with an INF-γ response, it was first detectable at 33 days of group housing (first exposure) or sooner (Calf 17), which indicates that they had a quicker cellular immune response than in IN calves (55 days). It has been reported that a lower dose of antigen may lead to a faster, more effective response [33]. Furthermore, a lower dose of MAP given over a longer interval (trickle dose) may lead to an earlier cellular immune response [10]; however, further research is needed.

All 4 control calves had a positive fecal sample 21 d after the start of group housing, and 1 calf had an additional positive fecal sample on day 56, as well as 2 positive INF-γ samples. It is not unusual to detect positive INF-γ samples among non-infected control calves [22]. All control calves were MAP tissue culture-negative. Despite the 5 positive fecal samples collected from calves in the control pen, all environmental samples collected for the duration of the study were negative. Although the CT threshold is high, resulting in a high specificity of fecal culture to identify true negative samples, control fecal samples collected on day 21 all had CT values well below the cut-off. Perhaps passive shedding of MAP on d 21 resulted from transmission (via an object or air) from an experimental pen in the barn, or samples were contaminated on the day of sampling.

However, this was unlikely due to the stringent protocols and strict biosecurity measures in place. It is unlikely that any control calves became infected, based on negative results for tissues, fecal and environmental samples during the trial, and it is possible these samples became positive during processing in the laboratory, as all control animals tested positive on the same day.

In conclusion, this study provided strong evidence that CE calves can become infected with MAP, and are at risk for transmission from infectious calves in group pens. It was noteworthy that 50% of CE calves had MAP-positive tissue results after 3 months of group housing, 5 (36%) had evidence of a cellular immune response, and all had MAP-positive fecal samples (indicating shedding of bacteria). Transmission among group-housed calves is currently largely overlooked in current control programs, but based on evidence from the current study, calf-to-calf transmission may be a source of new infections within a herd. Although there are still important knowledge gaps in the field regarding pathogenesis, progression, and recovery among infected animals, potential transmission among group-housed calves should be considered in JD control and prevention programs.

4.6 References

1. Arsenault RJ, Maattanen P, Daigle J, Potter A, Griebel P, Napper S (2014) From mouth to macrophage: mechanisms of innate immune subversion by *Mycobacterium avium* subsp. *paratuberculosis*. Vet Res 45:54.

2. Bakker D (2010) Paratuberculosis control measures in Europe. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. CABI, Wallingford, UK.

3. Bannantine JP, Bayles DO, Waters WR, Palmer MV, Stabel JR, Paustian ML (2008) Early antibody response against *Mycobacterium avium* subspecies *paratuberculosis* antigens in subclinical cattle. Proteome Sci 6:5.

4. Begg DJ, Whittington RJ (2008) Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. Vet J 176:129-145.

5. Billman-Jacobe H, Carrigane M, Cockram F, Corner LA, Gill IJ, HIll JF, Milner AR, Wood PR (1992) A comparison of the interferon gamma assay with the absorbed ELISA for diagnosis of Johne's disease in cattle. Aus Vet J 69:25-28.

6. Cho J, Tauer LW, Schukken YH, Smith RL, Lu Z, Gröhn YT (2013) Cost-effective control strategies for Johne's disease in dairy herds. Can J Agr Econ 61:583-608.

7. Collins MT (2011) Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract 27:581-591.

8. Collins MT, Eggleston V, Manning EJ (2010) Successful control of Johne's disease in nine dairy herds: results of a six-year field trial. J Dairy Sci 93:1638-1643.

9. Dargatz DA, Beverly AB, Barber LK, Sweeney RW, Whitlock RH, Shulaw WP, Jacobson RH, Stabel JR (2001) Evaluation of a commercial ELISA for diagnosis of paratuberculosis in cattle. JAVMA 218:1163-1166.

10. Eisenberg SW, Koets AP, Nielen M, Heederik D, Mortier R, De Buck J, Orsel K (2011) Intestinal infection following aerosol challenge of calves with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 42:117.

11. Forde T, Kutz S, De Buck J, Warren A, Ruckstuhl K, Pybus M, Orsel K (2012) Occurence, diagnosis, and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* infection in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) in southwest Alberta. J Wildlife Dis 48:1-11.

12. Garry F (2011) Control of paratuberculosis in dairy herds. Vet Clin North Am Food Anim Pract 27:599-607.

13. Harris NB, Barletta RG (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin Microbiol Rev 14:489-512.

14. Jungersen G, Huda A, Hansen JJ, Lind P (2002) Interpretation of the gamma interferon test for diagnosis of subclinical paratuberculosis in cattle. Clin Vaccine Immunol 9:453-460.

15. Kalis CH, Hesselink JW, Barkema HW, Collins MT (2001) Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp *paratuberculosis* in dairy herds. Am J Vet Res 62:270-274.

16. Kalis CHJ, Collins MT, Hesselink JW, Barkema HW (2003) Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. Vet Microbiol 97:73-86.

17. Kawaji S, Nagata R, Whittington RJ, Mori Y (2012) Detection of antibody responses against *Mycobacterium avium* subsp. *paratuberculosis* stress-associated proteins within 30 weeks after infection in cattle. Vet Immunol Immunopathol 150:101-111.

18. Koets A, Hoek A, Langelaar M, Overdijk M, Santema W, Franken P, Eden W, Rutten V (2006) Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. Vaccine 24:2550-2559.

19. Koets AP, Eda S, Sreevatsan S (2015) The within host dynamics of *Mycobacterium avium* ssp. *paratuberculosis* infection in cattle: where time and place matter. Vet Res 46:61.

20. Kralik P, Pribylova-Dziedzinska R, Kralova A, Kovarcik K, Slana I (2014) Evidence of passive faecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in a Limousin cattle herd. Vet J 201:91-94.

21. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C (2011) Predicting fadeout versus persistence of paratuberculosis in a dairy cattle herd for management and control purposes: a modelling study. Vet Res 42:36.

22. McDonald WL, Ridge SE, Hope AF, Condron RJ (1999) Evaluation of diagnostic tests for Johne's disease in young cattle. Aust Vet J 77:113-119.

23. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

24. McNab B, Meek AH, Duncan RJ, Brooks BW, Van Dreumel AA, Martin SW, Nielson KH, Sugden EA, Turcotte C (1991) An evaluation of selected screening tests for bovine paratuberculosis. Can J Vet Res 55:252-259.

25. Mitchell RM, Medley GF, Collins MT, Schukken YH (2012) A meta-analysis of the effect of dose and age at exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows. Epidemiol Infect 140:231-246.

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

27. Mortier R, Barkema HW, Orsel K, Wolf R, De Buck J (2014) Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 45:71.

28. Mortier RA, Barkema HW, De Buck J (2015) Susceptibility to and diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy calves: A review. Prev Vet Med 121:189-198.

29. Mortier RA, Barkema HW, Negron ME, Orsel K, Wolf R, De Buck J (2014) Antibody response early after experimental infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy calves. J Dairy Sci 97:5558-5565.

30. Mortier RA, Barkema HW, Wilson TA, Sajobi TT, Wolf R, De Buck J (2014) Dose-dependent interferon-gamma release in dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Immunol Immunopathol 161:205-210.

31. Munster P, Volkel I, Wemheuer W, Schwarz D, Doring S, Czerny CP (2013) A longitudinal study to characterize the distribution patterns of *Mycobacterium avium* ssp. *paratuberculosis* in semen, blood and faeces of a naturally infected bull by IS 900 semi-nested and quantitative real-time PCR. Transbound Emerg Dis 60:175-187.

32. Pithua P, Espejo LA, Godden SM, Wells SJ (2013) Is an individual calving pen better than a group calving pen for preventing transmission of *Mycobacterium avium* subsp *paratuberculosis* in calves? Results from a field trial. Res Vet Sci 95:398-404.

33. Rogers PR, Dubey C, Swain SL (2000) Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. J Immunol 164:2338-2346.

34. Santema WJ, Poot J, Segers RP, Van den Hoff DJ, Rutten VP, Koets AP (2012) Early infection dynamics after experimental challenge with *Mycobacterium avium* subspecies *paratuberculosis* in calves reveal limited calf-to-calf transmission and no impact of Hsp70 vaccination. Vaccine 30:7032-7039.

35. Slana I, Kralik P, Kralova A, Pavlik I (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. Int J Food Microbiol 128:250-257.

36. Slater N, Mitchell RM, Whitlock RH, Fyock T, Pradhan AK, Knupfer E, Schukken YH, Louzoun Y (2016) Impact of the shedding level on transmission of persistent infections in *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Vet Res 47:38.

37. Smith RL, Grohn YT, Pradhan AK, Whitlock RH, Van Kessel JS, Smith JM, Wolfgang DR, Schukken YH (2016) The effects of progressing and nonprogressing *Mycobacterium avium* ssp. *paratuberculosis* infection on milk production in dairy cows. J Dairy Sci 99:1383-1390.

38. Sorge US, Lissemore K, Godkin A, Jansen J, Hendrick S, Wells S, Kelton DF (2011) Changes in management practices and apparent prevalence on Canadian dairy farms participating in a voluntary risk assessment-based Johne's disease control program. J Dairy Sci 94:5227-5237.

39. Subharat S, Shu D, Wedlock DN, Price-Carter M, de Lisle GW, Luo D, Collins DM, Buddle BM (2012) Immune responses associated with progression and control of infection in calves experimentally challenged with *Mycobacterium avium* subsp. *paratuberculosis*. Vet Immunol Immunopathol 149:225-236.

40. Sweeney RW, Uzonna J, Whitlock RH, Habecker PL, Chilton P, Scott P (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. Res Vet Sci 80:253-259.

41. Sweeney RW, Whitlock RH, Hamir AN, Rosenberger AE, Herr SA (1992) Isolation of *Mycobacterium paratuberculosis* after oral inoculation in uninfected cattle. Am J Vet Res 53:1312-1314.

42. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW (2006) Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47:874-882.

43. van Roermund HJ, Bakker D, Willemsen PT, de Jong MC (2007) Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an

experimental setting: calves can transmit the infection to other calves. Vet Microbiol 122:270-279.

44. Waters WR, Miller JM, Palmer MV, Stabel JR, Jones DE, Koistinen KA, Steadham EM, Hamilton MJ, Davis WC, Bannantine JP (2003) Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. Infect Immun 71:5130-5138.

45. Weber MF, Kogut J, de Bree J, van Schaik G, Nielen M (2010) Age at which dairy cattle become *Mycobacterium avium* subsp. *paratuberculosis* faecal culture positive. Prev Vet Med 97:29-36.

46. Whitlock RH (2010) Paratuberculosis control measure in the USA. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. Wallingford, UK.

47. Whitlock RH, Wells S, Sweeney RW, Van Tiem J (2000) ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. Vet Microbiol 77:387-398.

48. Whittington RJ, Windsor PA (2009) In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. Vet J 179:60-69.

49. Wolf R, Barkema HW, De Buck J, Slomp M, Flaig J, Haupstein D, Pickel C, Orsel K (2014) High herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Western Canadian dairy farms, based on environmental sampling. J Dairy Sci 97:6250-6259.

50. Wolf R, Clement F, Barkema HW, Orsel K (2014) Economic evaluation of participation in a voluntary Johne's disease prevention and control program from a farmer's perspective-The Alberta Johne's Disease Initiative. J Dairy Sci 97:2822-2834.

51. Wolf R, Orsel K, De Buck J, Barkema HW (2015) Calves shedding *Mycobacterium avium* subspecies *paratuberculosis* are common on infected dairy farms. Vet Res 46:1-8.

Table 4-1. Number of *Mycobacterium avium* subspecies *paratuberculosis* fecal culture and tissue culture-positive calves in the 3 experimental groups.

	Fecal culture[a]				Tissue culture[b]			
Calf status	1	2	3	4	0	1	2	3
Inoculated (*n* = 14)	0	1	3	10	0	1	4	9
Contact-exposed (*n* = 14)	3	10	1	0	7	7	0	0
Control (n=4)	4	0	0	0	4	0	0	0

[a.] 1 = calves with 0-4 fecal culture positive samples; 2 = calves with 5-9 fecal culture-positive samples; 3 = calves with 10-14 culture-positive samples; and 4 = calves with ≥15 fecal culture-positive samples.

[b.] 0 = calves with 0 tissue culture-positive samples; 1 = calves with 1-3 tissue culture-positive samples; 2 = calves with 4-6 tissue culture-positive samples; and 3 = calves with >6 tissue culture-positive samples.

Table 4-2. *Mycobacterium avium* subspecies *paratuberculosis* environmental sampling results for all pens during group housing.

Group housing (wk)	Pen ID								
	1	2	3	4	5	6	7	Controls	Total
1	-	+	-	+	+	+	-	-	4
2	-	-	+	-	-	-	.	-	1
3	-	-	-	+	+	-	+	-	3
4	+	+	-	-	-	-	+	-	3
5	+	+	+	+	-	+	+	-	6
6	+	+	+	-	-	-	+	-	4
7	+	+	+	-	-	+	+	-	5
8	+	-	+	-	-	-	+	-	3
9	-	-	+	-	-	+	+	-	3
10	-	-	-	-	-	-	-	-	0
11	-	-	-	-	-	-	-	-	0
12	-	-	-	-	-	-	-	-	0
Total	5	5	6	3	2	4	7	0	32

Figure 4-1. Proportion of calves with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) culture-positive tissue samples per location. The y-axis displays the proportion that 1 particular tissue was positive over all calves exposed to MAP, whereas the x-axis indicates tissue location. Numbers indicate number of calves with a culture-positive tissue in the particular location. LN = lymph node.

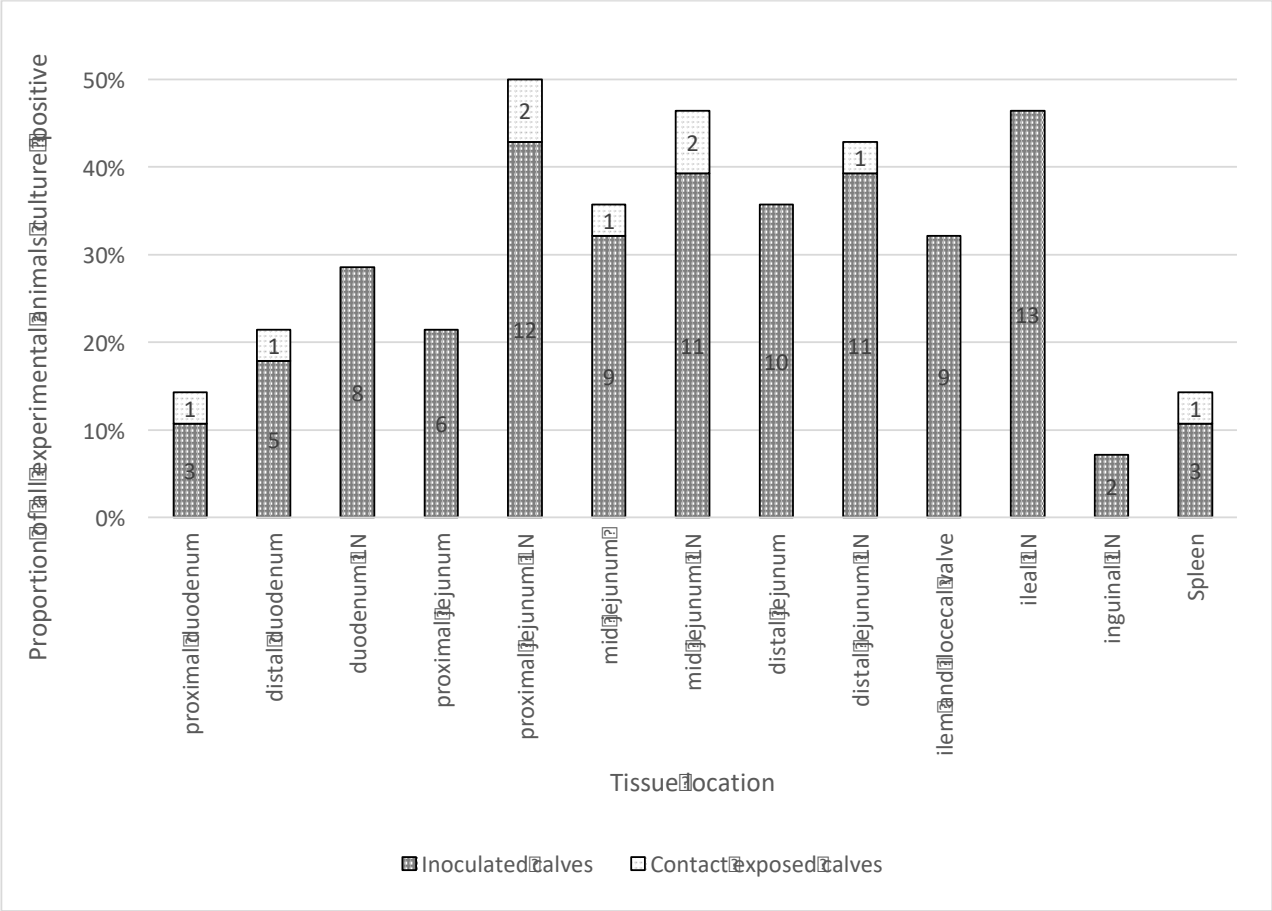
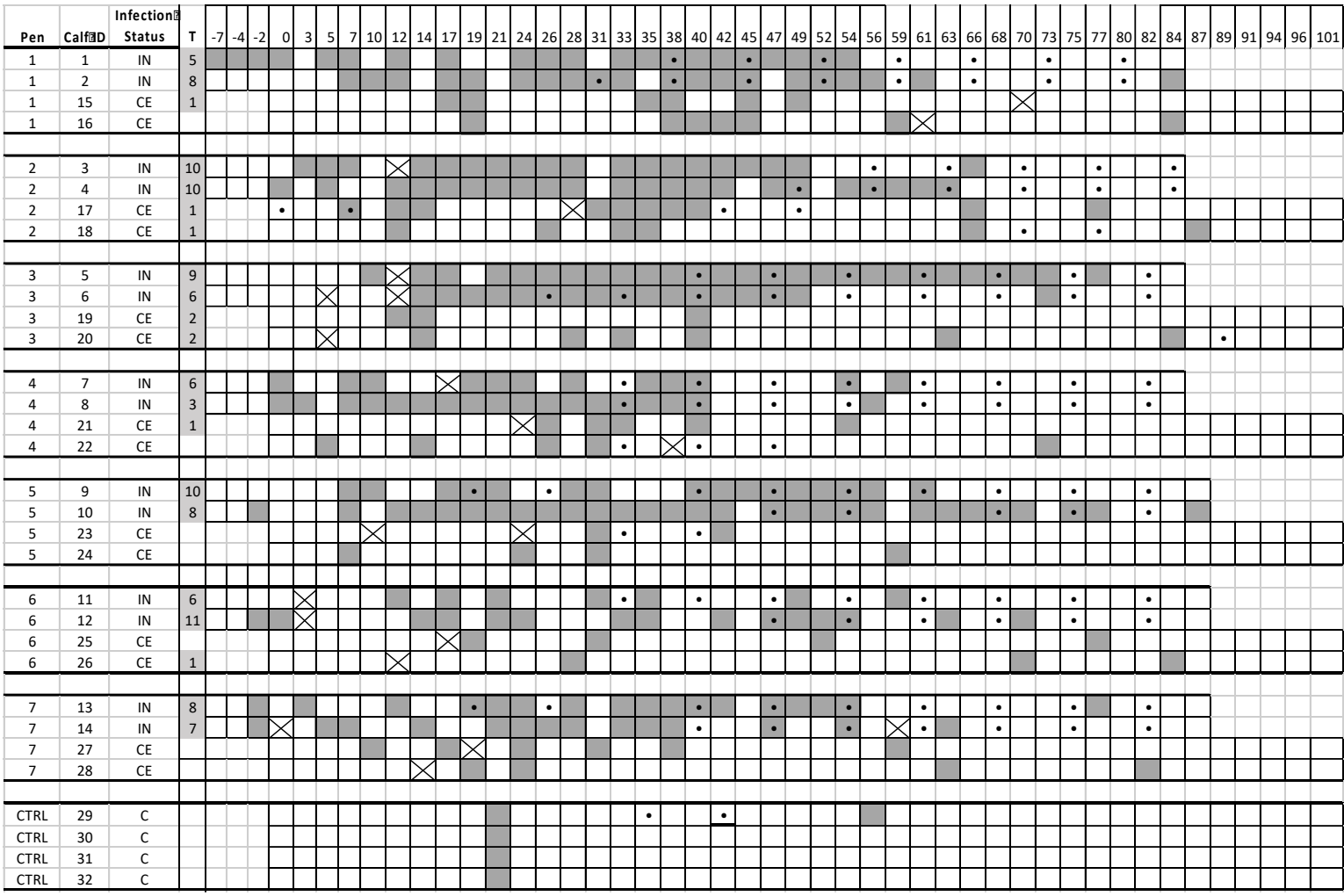


Figure 4-2. *Mycobacterium avium* subspecies *paratuberculosis* fecal culture, tissue culture and INF-γ results for individual calves per pen**.** A solid dark grey box indicates a positive fecal culture by F57-specific qPCR, a white box indicates a negative culture sample and box with a cross indicates a missing sample. "T" indicates the culture results for tissue samples, boxes shaded light grey indicate positive samples, and number of samples out of 13 that tested positive. Dots indicate blood samples that tested positive for



IFN-γ (based on 100% INF-γ cut-off).

CHAPTER 5: QUANTIFYING TRANSMISSION OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* AMONG GROUP-HOUSED DAIRY CALVES

5.1 Abstract

Johne's disease (JD), a chronic enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and control is primarily aimed at preventing infection among calves. The aim of the current study was to quantify calf-to-calf transmission of MAP among penmates in an experimental trial. Newborn Holstein bull calves (n=32) were allocated into pens of 4, with 2 inoculated (IN) calves and 2 calves that were contact exposed (CE). Calves were group-housed for 3 months, with frequent collection of fecal and blood samples, and tissue collection following euthanasia. The basic reproduction ratio (R_0) was estimated using a FS model with a susceptible-infected-recovered (SIR) model, based on ELISA and tissue culture followed by qPCR. In addition, transmission rate parameter (β) was estimated using a GLM with a susceptible-infected-susceptible (SIS) model based on culture, followed by qPCR, of fecal samples collected during group housing. The R_0 was derived for IN and CE calves separately, due to a difference in susceptibility, as well as differences in duration of shedding events. Based on the GLM model, R_0 for CE calves (R_0^{CE}) was 3.24 (1.14, 7.41), whereas R_0 for IN calves (R_0^{IN}) was 24.7 (4.57, 133.3). Based on the FS model, interferon-γ results from blood samples resulted in a R_0^{I} of 0.90 (0.24, 2.59), and tissue results a R_0^{T} of 1.36 (0.45, 3.94). We concluded that transmission of MAP infection between penmates occurred, and that transmission at this level may be an important cause of persistent MAP infection on dairy farms, despite implementation of JD control programs.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, transmission rate, reproduction ratio, calf, group-housing

5.2 Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic enteritis primarily affecting ruminants and causing substantial losses to dairy industries worldwide [8, 34, 56]. There is currently no treatment, cure, or vaccine for prevention of MAP infection; therefore, control is primarily based on preventing transmission of MAP and reducing new infections within the herd [10, 23, 25, 34].

The primary route of MAP transmission is fecal-oral through contaminated feed, milk, water and the environment caused by infectious animals intermittingly shedding MAP in their feces [19, 44, 46]. To decrease transmission from cows to calves and limit exposure of young stock to MAP, calves are removed from adult cows as soon as possible after birth and placed in calf barns or pens [18, 53]; however, calves up to 1 year of age have demonstrated susceptibility to MAP infection, and calves can begin shedding MAP bacteria in the feces as early as 2 weeks after exposure [12, 37, 38, 57]. Therefore, separating calves from cows, and subsequent group housing may not be an effective method for prevention of new infections in young stock. There is evidence that calf-to-calf transmission can occur [12, 47]; however, findings regarding implications and impact of this route of transmission on control within herds were inconsistent [30, 35, 42, 47, 52]. Additionally, earlier statistical analysis of data from 21 MAP infected farms had suggested that transmission among calves was necessary to explain the observed patterns of transmission within a herd [48].

Susceptible-infected-susceptible (SIS) models are modified from susceptible-infected-recovered (SIR) models, and both have been used to model transmission of pathogens in

a population [4, 5, 20, 49]. Transmission of pathogens is quantified using the basic reproduction ratio (R_0), the average number of new cases caused by one typically infectious individual introduced into a completely susceptible population [15, 50]. The threshold at which an outbreak can occur is when the R_0 value is > 1, whereas an infection is certain to fade out in a population if R_0 is < 1 [15]. Transmission dynamics for MAP infection are notoriously difficult to determine, due to long incubation, latently infected animals, variability of diagnostic tests, and lack of long-term randomized control studies [32]. Deterministic mathematical modelling techniques have been used to investigate MAP spread and transmission parameters [36, 41], assess impact of varying infectious animals, as well as varying levels of environmental contamination [21], economic impacts of disease [45], and to determine effectiveness of control programs and interventions on the spread and control of disease [11, 29]. Due to complications regarding MAP infection, these models all rely on educated guesses regarding infectivity and susceptibility of animals in a herd, as well as impacts of various transmission routes. Increasing knowledge regarding transmission within a herd will enhance understanding of disease maintenance and spread within a herd, and enable control programs to be optimised to better manage spread of infection [2]. Although transmission among calves is possible, sufficient quantitative information regarding the amount of transmission is lacking. The aim of the current study was therefore to fill the current knowledge gap regarding quantification of calf-to-calf transmission of MAP infection among penmates.

5.3 Materials and methods

5.3.1 Experimental design

Study design, calf collection, and sample collection were as described [12]. Briefly, 32 newborn Holstein-Friesian bull calves were purchased from 13 Alberta (Canada) dairy farms that had tested negative for MAP for at least 4 years by culture of environmental samples and milk ELISA or individual fecal sampling of the herd. Calves were assigned to infection status and pen based on birth order and entry into the Biosecurity Level-2 facility, with 7 experimental group pens each consisting of 2 inoculated calves (IN), and 2 recipient contact-exposed calves (CE). The last 4 calves to enter the barn were designated controls. Calves were group-housed for 3 months after inoculation. After 3 months of group housing, IN calves were euthanized, and remaining calves in the experimental pens were individually housed for an additional 3 mo. At the end of the trial, all remaining calves were euthanized for tissue sampling. Control calves were group-housed for the entirety of the study. All protocols and the experimental design were approved by the University of Calgary Veterinary Sciences Animal Care Committee (protocol AC14-0168).

5.3.2 Inoculum

Inoculum preparation was as described [12]. A virulent strain from a clinical case of JD (cow 69) in Alberta was used for inoculation. Two calves in each experimental pen were inoculated with an oral dose of 2.5×10^8 CFU on 2 consecutive days at 2 weeks of age. Following inoculation, calves remained individually housed for an additional 2 weeks to allow for passive shedding of the inoculum to cease before being group-housed with recipient calves.

5.3.3 Sampling

Fecal, blood, and tissue samples were all collected as described [12]. Briefly, individual fecal samples were collected 3 times/week for 3 months, starting 1 d after the onset of group housing. Samples were stored at 4°C and processed within 7 days after collection. All fecal samples were processed using modified TREK ESP II culture media (TREK para-JEM®; TREK Diagnostic Systems, Cleveland, OH, USA) as described [12, 38]. Following processing, fecal samples were incubated at 37°C for 49 d.

Blood samples were collected weekly from the jugular vein, alternating between sides. Within 2 hours after collection, samples were transported to the laboratory (in an insulated box with hot water bottles at 25-35°C).

After 3 months of group housing (i.e. 4 months of age), IN calves were euthanized by intravenous injection of barbiturates (Euthanyl Forte®, DIN 00241326, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada), necropsied, and tissue samples collected. After an additional 3 months of being housed individually, CE calves were euthanized, necropsied and tissues collected. Thirteen tissue samples were collected from each calf, including sections of the ileum, jejunum, duodenum, all associated lymph nodes and spleen. After transportation to the laboratory, samples were processed immediately for culture, as described [12]. Briefly, 2.5 g of tissue was dissociated and disinfected prior to incubation within paraJEM® culture bottles and incubated at 37°C for 49 days.

5.3.4 Detection of MAP

Following liquid culture of fecal and tissue samples for 49 days, DNA was extracted as described [12, 16]. A duplex qPCR was performed targeting the MAP-specific F57 region and an internal amplification control (IAC) with primers, probes and IAC

sequences identical to those described [43]. Samples were considered positive if the cycle threshold (CT) value was < 40.

5.3.5 Detection of an immune response

Following transportation to the laboratory, blood samples were processed for detection of IFN-γ release, as described [12, 39]. Each whole-blood sample was treated with 100 μL avium Purified Protein Derivative (aPPD; 0.3 mg/ml; Canadian Food Inspection Agency, Ottawa, ON, Canada), 100 μL of pokeweed mitogen (positive stimulation control; 0.3 mg/ml; Sigma–Aldrich Canada Co., Oakville, ON, Canada), and 100 μL sterile PBS (negative stimulation control). Following overnight incubation at 37°C and centrifugation serum was collected and assayed using the BOVIGAM® sandwich ELISA (Prionics, La Vista, NE, USA). The %IFN-γ was calculated as follows: [(PPD Johnin-negative assay control)/(positive- negative assay control)] × 100 [24, 39].

5.4 Quantification of MAP transmission

5.4.1 GLM and FS model

The transmission rate parameter β is the average number of new infections in a fully susceptible population caused by one typically infectious animal per unit of time [50]. To quantify transmission parameters, we used for the transmission rate parameter β a generalized linear model (GLM), and for the basic reproduction ratio R_0 we used final size (FS) models. Models were based on an SIS model for the GLM (infection status identified during experiment) and a SIR model for the FS model (infection status determined at end point); [49], in which infectious dynamics were based on number of recipient animals, i.e. susceptible (S), infectious (I) and total number of animals (N). Thus, the probability of a single susceptible calf becoming infected during a period Δt is:

$$p = 1 - e^{-\beta_T \times \frac{I_t}{N_t} \times \Delta t}$$

where β_T is the total transmission rate parameter [50].

The GLM used the binomial distribution, with the dependent variable being the number of new cases (C) and the total number of susceptible (S) calves as the binomial total. The analysis was done with a complementary log-log (cloglog) link function, a binomial error term, and an offset explained below [50, 51].

The expression for the GLM was:

$$\text{cloglog } E\left(\frac{C}{S}\right) = \log\beta_{T_t} + \log\left(\frac{I_{T_t}}{N_t} \cdot \Delta t\right),$$

where $\log\beta_{T_t}$ is the intercept and thus the logarithm of the transmission parameter is the (intercept) regression coefficient and $\log\left(\frac{I_{T_t}}{N_t} \cdot \Delta t\right)$ is the offset variable; $E\left(\frac{C}{S}\right)$ = expected number of cases (C) during the infectious interval (t, t+ Δt) divided by the number of susceptible individuals (S) at the start of the time interval (i.e. at t); β_T = total transmission rate parameter; I_T = total amount of infectious material (animals and environment) measured in units equivalent to what one infectious animal excretes per day also at the start of the time interval (t); Δt = duration of the time interval; and N_T = total number of animals at the start of the time interval (t) as this measures the size of the area given that density is constant [13].

Note that the β_T is the total transmission rate parameter. Due to several potential routes of transmission, different transmission effects may contribute to β_T, and hence different transmission rate parameters can be estimated depending on the population composition, as discussed below.

Data were analyzed using STATA 11 (StataCorp LP, College Station, TX). All time intervals where a pen had 0 susceptible (S) calves were removed from the analysis (n=9). The 95% confidence interval (CI) of the estimated β parameters was calculated using the standard error of the mean of log β.

5.4.2 Estimation of MAP concealment rate in the environment: β_E

Environmental contamination caused by fecal shedding calves was included in the model as a route of transmission for infection (expressed as parameter β_E). We assumed that environmental contamination on a specific day (E_t) depended on the shedding and excretion of MAP by infectious individuals (either IN or CE) on the previous days ($E_{(t-1)}$), as well as the remaining exposed MAP in the environment from previous days, discounted by the concealment rate (γ). Concealment and resulting exposure rate of MAP were estimated as described [4] accounting for potential difference in contamination caused by IN or CE calves. Briefly, E_T was calculated as the sum of environmental contamination caused by IN (E_I) and CE (E_C) calves. The same concealment rate (γ) was applied to both E_I and E_C, and was calculated based on the assumption that fecal-oral transmission from the environment after correction for concealment will be the same as fecal-oral transmission from an infectious pen mate on the same day [4]. This is the case, as oral transmission to other calves within the same period as the infectious material is shed, is basically the same as will occur from the equivalent but diminished amount of material in subsequent time periods, due to the resilient nature of MAP. However, over time, MAP will be concealed (diminished) in the environment and exposure of the other animals decreased, resulting in lower transmission [4]. Using new cases (C) as the result of environmental contamination (new cases that occurred following shedding in the pen),

we estimated γ to be equal 0.1422 day^{-1}. A sensitivity analysis in which the concealment rate was increased to 0.5 and decreased to 0.001 was performed to ensure the best estimate was made for the model, and was in agreement with current literature [14]. Time intervals in the current study were either 2 or 3 days; therefore, the exposure rate (σ) was calculated based on the following equation: $\sigma = e^{-\gamma * \Delta t}$. E_T for period t was then calculated as follows:

$$E_{T_t} = (\sigma_{(t-1)}\left(I_{IN_{(t-1)}} + E_{IN_{(t-1)}}\right)) + (\sigma_{(t-1)}\left(I_{CE_{(t-1)}} + E_{CE_{(t-1)}}\right)),$$

where $(\sigma_{(t-1)}\left(I_{IN_{(t-1)}} + E_{IN_{(t-1)}}\right))$ is the environmental contamination caused by the IN calves in the time interval prior to t; and $(\sigma_{(t-1)}\left(I_{CE_{(t-1)}} + E_{CE_{(t-1)}}\right))$ is the environmental contamination caused by CE calves in the time interval period to time t.

5.4.3 Transmission rate parameters

The transmission rate parameter β_T is dependent on the population composition in a pen and represents all the transmission that could occur between the pen mates in a group housing pen. Susceptibility and infectivity among calves can be variable and part of that variation may be explained by observable differences, e.g. inoculation status. These differences influence the transmission rate parameter β as not all calves can be assumed to have the same susceptibility and infectivity. All calves were either inoculated (IN) or contact exposed (CE) and this dichotomy can have an effect on susceptibility to begin shedding or the infectivity i.e. the propensity to cause shedding in other calves by the infected calf. For susceptibility status we used an explanatory dummy variable (INO) 0 or 1 for the recipient animal to be either inoculated (INO=1) or contact infected (INO=0). The dummy regression variable associated with infectivity is the fraction of infected

calves plus environment that are caused by inoculated calves, both from direct fecal shedding and contamination of the environment. The expression for infectivity is:

$$f_{IN} = \frac{I_{IN} + E_{IN}}{I_{IN} + E_{IN} + I_{CE} + E_{CE}}$$

where $I_{IN} + E_{IN}$ is the total infectivity caused by IN calves (I_{IN}) and $I_{IN} + E_{IN} + I_{CE} + E_{CE}$ is total infectivity caused by all calves (I_T).

Additionally, we can test whether transmission within the same period or in subsequent periods via the environment were indeed the same based on the fraction of the total environmental infectivity where $f_E = \frac{E_{IN}+E_{CE}}{I_T}$ and $E_{IN} + E_{CE}$ is equal to total infectivity in the environment (E_T).

Yet another explanatory variable we added was the day since the start of the experiment (startday). This variable has the same value for all animals in the pen for each observed interval and thus the corresponding regression coefficient contains both the susceptibility and infectivity effects. When main effects were significant we also looked at possible interactions.

Thus, based on the above, the following transmission route parameters were quantified using GLM: direct contact from IN calves (β_{IND}), environmental contamination caused by IN calves (β_{INE}), direct contact from CE calves shedding (β_{CED}), and environmental contamination caused by CE calves (β_{CEE}), all based on the regression coefficients in the following equation:

$$\text{cloglog } E\left(\frac{C}{S}\right) = C0 + C1 * ino + C2 * startday + C3 * \frac{I_{IN} + E_{IN}}{I_T} + C4\ \frac{E_{IN} + E_{CE}}{I_T} + C5 \text{ ino} * \text{startday} + \log\left(\frac{I_T}{N_t} \cdot \Delta t\right)$$

From this, the transmission rate parameters can be derived as follows (assuming that all regression coefficients were significantly different from zero):

$\beta_{IN\_IN_D} = e^{C0+C1+C2+C5}$ is the transmission rate parameter for infectious IN calf to transmit to a susceptible IN calf through direct contact;

$\beta_{IN\_IN_E} = e^{C0+C1+C2+C3+C4+C5}$ is the transmission rate parameter for infectious IN calf to transmit to a susceptible IN calf through the environment;

$\beta_{CE\_CE_D} = e^{C0+C2}$ is the transmission rate parameter for infectious CE calf to transmit to a susceptible CE calf through direct contact;

$\beta_{CE\_CE_E} = e^{C0+C2+C4}$ is the transmission rate parameter for infectious CE calf to transmit to a susceptible CE calf through the environment;

$\beta_{IN\_CE_D} = e^{C0+C2+C3}$ is the transmission rate for infectious IN calf to transmit to a susceptible CE calf through direct contact;

$\beta_{IN\_CE_E} = e^{C0+C2+C3+C4}$ is the transmission rate parameter for infectious IN to transmit to a susceptible CE calf through the environment;

$\beta_{CE\_IN_D} = e^{C0+C1+C2+C5}$ is the transmission rate parameter for infectious CE to transmit to a susceptible IN calf through direct transmission; and

$\beta_{CE\_IN_E} = e^{C0+C1++C2+C4+C5}$ is the transmission rate parameter for infectious CE to transmit to a susceptible CE calf through the environment.

Regression coefficients were tested for being different from zero. For regression coefficients that were not significant (P>0.05), the corresponding explanatory variable was dropped from the model unless it led to confounding (>25% change in the regression

coefficient of the other explanatory variables). Interaction terms were only calculated when both main effects were statistically significant.

5.4.4 Infectious periods: T_{IN} and T_{CE}

The infectious periods were calculated based on duration of shedding events for IN and CE calves as described [12]. The first time a calf tested MAP-positive in fecal samples was considered day 1 of its infectious period. The infectious period then would end if the next sample was negative, or continue until a negative sample was detected. A shedding event was defined as continuous streak of positive fecal samples, and ranged from 2 days (one positive fecal sample) up to 54 days (23 consecutive positive fecal samples) during the 90+ d of group housing.

Mean duration of infectious period for IN calves (T_{IN}) and CE calves (T_{CE}) was calculated as the average length of shedding events for IN calves or CE calves, respectively. The 95% confidence intervals (CI) were calculated using the standard variation of the mean of log T_{IN} and log T_{CE} for IN calves and CE calves, respectively.

5.5 Reproduction ratio R_0

The basic reproduction ratio (R_0) represents the average number of new infections in a totally susceptible population caused by one typical infectious calf during its infectious period, and was estimated using 2 approaches.

5.5.1 Using the GLM: R_0^{CE}

The GLM analysis is based on interval data, and was therefore based on fecal samples collected over the course of the trial using the SIS model. Infectious (I) individuals were those with MAP-positive fecal results, susceptible individuals (S) had MAP-negative fecal results, and cases (C) were calves who had negative fecal culture results at the

previous observation point, and became infectious (I) in the current time interval. The R_0 can be estimated by multiplying the rate of transmission (β) by the infectious period (T). The concealment rate by the environment due to new bedding being added and infectious material being covered was also taken into consideration using the variable σ, thus $(1-\sigma)^{-1}$ represents the total exposure rate of MAP in the environment for each day of the infectious period T. The equation for estimating R_0 can be seen below:

$$R_0 = \beta * T * (1-\sigma)^{-1}$$

The 95% CI of R_0 was calculated using the variance and regression constant of the GLM results (log β) and the variance and the average of the logarithm of the infectious period T.

5.5.2 Using the final size model: R_0^T, R_0^I

The FS model was based on the SIR model, which uses the total number of susceptible animals remaining at the end of the experiment [49]. Two FS models were used to account for varying definitions of MAP infection. In the first FS model, infectious (I) calves were counted as those with at least one culture-positive tissue sample at the end of the experiment, and susceptible (S) calves as those with no positive tissue sample. R_0^T was the estimate of the number of new tissue-positive infections that would result from the introduction of one tissue-positive calf to the susceptible population. In the second FS model, infectious (I) calves were those with at least 1 positive INF-γ sample by the end of the experiment, whereas susceptible (S) calves had no positive INF-γ samples. R_0^I was the estimate of the number of new INF-γ positive infections that would result from the introduction of 1 INF-γ positive calf to the susceptible population. For both definitions, IN calves started the trial with infectious status (I), and CE calves were considered

susceptible (S). By the end of the trial, outcomes for the number of cases in a pen could be 0, 1 or 2 where the number of susceptible calves will be 2, 1, or 0 (respectively) depending on the extent of transmission, and the formula for the probability of each outcome is as follows:

$$p[\mathrm{s}=2]=\mathrm{p}[\mathrm{c}=0]=\left(\frac{4}{2R_0+4}\right)^2$$

$$p[\mathrm{s}=1]=\mathrm{p}[\mathrm{c}=1]=\left(\frac{4}{2R_0+4}+\frac{4}{R_0+4}\right)\left(\frac{2R_0}{2R_0+4}+\frac{4}{R_0+4}\right)^2$$

$$p[\mathrm{s}=0]=\mathrm{p}[\mathrm{c}=2]=1-((p[\mathrm{s}=2])+(p[\mathrm{s}=1]))$$

thus, R_0 can be derived analytically by the maximum likelihood estimate (MLE) given these probabilities for the definition of tissue infection (R_0^T) or INF-γ infection (R_0^I) [28, 50].

5.6 Results

5.6.1 Detection of infection

Descriptive results were presented more extensively in the previous report [12]. All IN and CE calves had MAP-culture positive fecal samples at various times during group housing; however, CE calves ceased shedding after individual housing (Table 5-1). All IN calves and 5/14 CE calves had positive INF-γ samples, whereas all IN calves and 7/14 CE calves had at least one MAP culture-positive tissue sample (Table 5-1).

5.6.2 GLM

Susceptibility for MAP infection (identified through fecal shedding) was different for IN and CE calves; therefore, the contrast was included as an explanatory variable in the final GLM model ($p<0.001$). Explanatory variables also included in the final model were time passage following group housing (startday), the interaction between housing date and

inoculation status, and the intercept. All estimates present are calculated for the beginning of the experiment at startday=0, therefore the interaction between startday and susceptibility status was not included in the final model (Table 5-2). When the interaction is included in the model, there is no significant difference in how the model fits the data, and the difference in the coefficients can be seen in table 5-2. No coefficients of the explanatory variables changed more than 11%; it was therefore concluded that no confounding was present.

The IN and CE calves had different infectious intervals; therefore, R_0 had to be estimated separately for these 2 groups of calves. The transmission rate parameter β_{CE_CE} was estimated as 0.158 per d (0.109, 0.230). The average infectious period for CE calves was 2.91 (0.98, 6.08) d. Based on the GLM, the estimated basic reproduction value for CE calves (R_0^{CE}) was 3.24 (1.41, 7.41).

For the IN calves, the transmission rate parameter β_{IN_IN} was 0.649 per day (0.437, 0.965) and the average infectious period was 7.49 (0, 25.3) days. Therefore, the estimated reproduction value for IN calves R_0^{IN} was 24.6 (4.57, 133.3).

5.6.3 FS model

When quantifying transmission based on the INF-γ definition of MAP infection, in 3 pens no susceptible (S) calves became infected (I), in 2 pens only one of the 2 susceptible (S) calves became infected (I), and in 1 pen, all susceptible (S) calves became infected (I). Therefore, the estimated reproduction number for INF-γ (R_0^I) was 0.90 (0.24, 2.59).

When quantifying transmission based on being tissue culture-positive as a definition of MAP infection, in 2 pens none of the susceptible (S) calves became infected (I), in 3 pens, 1 susceptible calf (S) became infected, and in 2 pens all susceptible (S) calves

became infected (I). Therefore, the estimated reproduction value for tissue (R_0^T) was 1.36 (0.45, 3.94).

5.7 Discussion

Quantification of transmission of MAP infection based on fecal shedding and accumulation of environmental contamination indicated that one CE calf that has started shedding and is introduced to a completely susceptible population of calves in a clean environment could cause approximately 3 calves to begin shedding (R_0^{CE}=3.24; 1.41, 7.41). This estimate represents the MAP transmission for calves in a herd entering a clean environment as it starts from a "naturally" infected calf. Using MAP-positive tissue culture, or cellular immune response to MAP, one infectious calf will cause approximately one CE calf to become infected in group housing (R_0^T= 1.36; and R_0^I= 0.90, respectively). Thus, the R_0 value for MAP transmission among naturally infected calves lies between 0.9 and 3.24 depending on the definition of infection (fecal shedding, INF-γ response, or tissue infection). Our study indicated that transmission of MAP between group-housed dairy calves occurred (R>1) and that potential shedding outbreaks may occur. When including the interaction between startday and susceptibility status, the transmission estimate for CE calves decreases, indicating that over time infectiousness changes; however, because this change may be due to several extraneous variables specific to the trial (bed pack, addition of clean material, cleaning of environment etc.), the model without the interaction provides a better estimate of the transmission estimate for group housed calves entering a clean environment.

The R_0^{CE} value for transmission of fecal shedding among calves was higher than previously described [47] which may be due to the consideration of environmental

contamination in the current study. All calves shed MAP into the group pen, and due to the resilience of MAP, the presence of MAP in this environment remained a source of infection for a prolonged interval. Presence of MAP in the environment, especially resulting from a high herd prevalence of MAP, has a large impact on fecal-oral transmission that occurs from cows to calves [27, 54, 55]. In the analysis of our experiments, only when environmental contamination with MAP was taken into consideration did the model better capture the transmission of fecal shedding occurring between calves. Both IN and CE calves shed MAP into the environment at equal infectivities; however, there was a difference in the IN or CE calves' susceptibilities to begin shedding, and length of shedding events. The IN calves were more likely to begin shedding, and had longer shedding events, leading to a higher point estimate R_0 value (31.7) than CE calves (2.1). The indirect transmission of infection occurring through the environment is likely to have a large impact on calf-to-calf transmission, especially considering the large amount of intermittent shedding that occurs within these animals and opportunities to miss detection of shedding events.

One of the difficulties associated with MAP is that an animal can be "infectious", and/or "infected"; therefore a GLM model was used to identify "infectious" calves, whereas the FS model was used to identify "infected" calves and the transmission of infected status [40]. Although fecal shedding may not be a true indication of MAP infection and could be the result of either passive or active shedding, the evidence that 1 CE calf will cause 3 more calves in the population to start shedding has huge implications for JD control programs. Of all the CE calves that were fecal shedding, half (7/14) had culture-positive tissue samples, and the estimated R_0^T value for the "transmission" of MAP-positive tissue

samples from 1 CE calf to a susceptible calf was 1.36, however this was not significantly larger than 1, due to confidence intervals encompassing 1. Calves that are tissue-positive, may cause 1 other calf in the group pen to become tissue-positive through fecal shedding, potentially leading to infection and symptoms later in life; however, this is only part of the whole MAP transmission dynamics that occur on farm. Additionally, it should be taken into consideration that testing of tissue samples in non-clinical calves may lead to a high number of false-negative samples, due to low MAP concentrations in the tissue and few samples sites that are positive, which would lead to an underestimation of the number of infected calves [7, 26, 33, 37]. The tendency for calves to begin shedding, but only some with detectable positive tissue samples, may be one explanation for maintenance of MAP infection and a low, consistent prevalence on farm.

Epidemiological models are a useful tool when testing the hypothesis in the field is difficult or improbable, as is the case with MAP, as infections are slow progressing with a long latent period, susceptibilities and infectivities may vary between age groups, there are low sensitivities and specificities of diagnostic tests, and not all transmission routes (including the environmental impact) are clearly understood [32]. These models are simplified representations based on current knowledge designed to investigate specific hypotheses such as: MAP transmission dynamics in a herd [1, 31], impact of control programs [29, 45], predicting fadeout and persistence of disease [30, 36], and effects of infectious young stock on disease control [52]. The outcomes of each of these models rely on the availability of knowledge regarding the underlying mechanisms of the disease, as well as assumptions made to fill in knowledge gaps and the modelling objective to answer the question at hand [32]. There are currently inconsistent values

used to model the impact of infection and transmission of young stock, and herd transmission dynamics, due to the contradictory findings regarding the impact of calf-to-calf transmission [30, 31, 36, 47, 52]. The current study provides R_0 values for calf-to-calf transmission that can be used to better model the transmission dynamics on farm, with potential to examine different infectious abilities. The fecal shedding R_0 value demonstrates infectiousness of calves and the contaminated environment in group-housed pens; however, this value did not necessarily represent infections that will persist over time. Based on INF-γ or tissue results, a more conservative estimate for persistent infections in the calves could be obtained. Taken together, results from the current study allow for better and more accurate modelling of infection and transmission of MAP within dairy farms, leading to more effective control and prevention programs. Currently, control programs focus on decreasing transmission to susceptible calves from infectious cows by separating young stock soon after birth, based on the assumption that calves will not transmit infection to each other [18]. However, results from this study indicate that shedding of MAP may be more easily transmitted to other pen mates than previously assumed, not only from direct contact, but potentially more importantly, due to environmental contamination caused by shedding animals. Individual calf housing may be one solution to the risk of calf-to-calf transmission; however, this method of calf housing may not be possible for all herds, due to increased labour costs [6], transition to automated feeding systems [3], decreased calf welfare [9, 17, 22], and the need for careful cleaning between successive calves. A second solution would be regular and vigorous maintenance of a clean group-housed environment, in which contaminated material is removed and replaced with new bedding and base, and sides of the enclosure

are disinfected. Additionally, young stock should be considered for inclusion of MAP testing in a herd testing program and positive animals should be immediately removed from group-housing and monitored/tested in the future to determine infection status. Results from this research indicate a high potential for group-housed calves to cause shedding among penmates, leading sometimes to more extensive infection as evidenced by tissue and immune responses; however, consideration should be made for parameters of the experimental situation when applying results to the field. Future research is required regarding calf-transmission that occurs on farms, and effects of a contaminated environment, as positive young stock have been detected in positive herds [57]. Ideally, a longitudinal study on positive farms should be conducted in which fecal samples, environmental samples, and blood samples are collected regularly from all penmates that are group-housed, beginning at birth, for all months of group housing. This will lead to a better understanding of the transmission and infection status that may occur due to direct contact and environmental contamination that occurs in a commercial dairy herd.

In conclusion, in this study, transmission of MAP among group-housed calves was quantified using with GLM and FS modelling and using fecal shedding, tissue, or immune responses as a definition of infectiousness. Based on the definition of infection, the R_0 value for lies between 0.9 and 3.24. Using the GLM model of fecal shedding over time and an SIS model, the R_0^{CE} for an infectious contact-exposed animal was estimated to be 3.24. Although this model only considered fecal shedding, and therefore was not a perfect indication of infection among calves, the FS models with tissue and immune responses had R_0 values equal to 1.36 and 0.85, respectively. Together, this study provided strong evidence that transmission of MAP infection among group housed calves

is not only present, but may be a contributing factor to maintenance of infections within a herd, and therefore, should be seriously considered in future Johne's disease prevention and control programs.

5.8 References

1. Al-Mamun MA, Smith RL, Schukken YH, Grohn YT (2016) Modeling of *Mycobacterium avium* subsp. *paratuberculosis* dynamics in a dairy herd: An individual based approach. J Theor Biol 408:105-117.

2. Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten V, Bannantine JP, Keefe GP, Kelton DF, Wells SJ, Whittington RJ *et al* (2017) Knowledge gaps that hamper prevention and control of Mycobacterium avium subspecies paratuberculosis infection. Transbound Emerg Dis.

3. Barkema HW, von Keyserlingk MA, Kastelic JP, Lam TJ, Luby C, Roy JP, LeBlanc SJ, Keefe GP, Kelton DF (2015) Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J Dairy Sci 98:7426-7445.

4. Bravo de Rueda C, de Jong MC, Eble PL, Dekker A (2015) Quantification of transmission of foot-and-mouth disease virus caused by an environment contaminated with secretions and excretions from infected calves. Vet Res 46:43.

5. Broens EM, Espinosa-Gongora C, Graat EAM, Vendrig N, Van Der Wolf PJ, Guarabassi L, Butaye P, Neilsen JP, De Jong MCM, Van De Giessen AW (2012) Longitudinal study on transmission of MRSA CC398 within pig herds. Vet Res 8:58-68.

6. Broom DM, Leaver JD (1978) Effects of group-rearing or partial isolation on later social behaviour of calves. Anim Behav 26:1255-1263.

7. Buergelt CD, Hall C, McEntee K, Duncan JR (1978) Pathological evaluation of paratuberculosis in naturally infected cattle. Vet Pathol 15:196-207.

8. Cho J, Tauer LW, Schukken YH, Smith RL, Lu Z, Gröhn YT (2013) Cost-effective control strategies for Johne's disease in dairy herds. Can J Agr Econ 61:583-608.

9. Chua B, Coenen E, van Delen J, Weary DM (2002) Effects of pair versus individual housing on the behavior and perfomance of dairy calves. J Dairy Sci 85:360-364.

10. Collins MT, Eggleston V, Manning EJ (2010) Successful control of Johne's disease in nine dairy herds: results of a six-year field trial. J Dairy Sci 93:1638-1643.

11. Collins MT, Morgan IR (1991) Epidemiological model of paratuberculosis in dairy cattle. Prev Vet Med 11:131-146.

12. Corbett CS, De Buck J, Orsel K, Barkema HW (2017) Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48:27.

13. de Jong MCM, Diekmann O, Heesterbeek H (1995) How does transmission of infection depend on population size? In: Mollison D (eds) Epidemic models: Their structure and relation to data. Cambridge: Cambridge University Press.

14. De Silva KR, Eda S, Lenhart S (2017) Modeling environmental transmission of MAP infection in dairy cows. Math Biosci Eng 14:1001-1017.

15. Diekmann O, Heesterbeek JAP, Metz JAJ (1990) On the definition and the computation of the basic reproduction ratio R_0 in models for infectious diseases in heterogeneous populations. J Math Biol 28:365-382.

16. Forde T, Kutz S, De Buck J, Warren A, Ruckstuhl K, Pybus M, Orsel K (2012) Occurence, diagnosis, and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* infection in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) in southwest Alberta. J Wildlife Dis 48:1-11.

17. Gaillard C, Meagher RK, von Keyserlingk MA, Weary DM (2014) Social housing improves dairy calves' performance in two cognitive tests. PLoS One 9:e90205.

18. Garry F (2011) Control of paratuberculosis in dairy herds. Vet Clin North Am Food Anim Pract 27:599-607.

19. Harris NB, Barletta RG (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin Microbiol Rev 14:489-512.

20. Huijbers PM, Graat EA, van Hoek AH, Veenman C, de Jong MC, van Duijkeren E (2016) Transmission dynamics of extended-spectrum beta-lactamase and AmpC beta-lactamase-producing *Escherichia coli* in a broiler flock without antibiotic use. Prev Vet Med 131:12-19.

21. Humphry RW, Stott AW, Adams C, Gunn GJ (2006) A model of the relationship between the epidemiology of Johne's disease and the environment in suckler-beef herds. Vet J 172:432-445.

22. Jensen MB, Larsen LE (2014) Effects of level of social contact on dairy calf behavior and health. J Dairy Sci 97:5035-5044.

23. Kalis CH, Hesselink JW, Barkema HW, Collins MT (2001) Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp *paratuberculosis* in dairy herds. Am J Vet Res 62:270-274.

24. Kalis CHJ, Collins MT, Hesselink JW, Barkema HW (2003) Specificity of two tests for the early diagnosis of bovine paratuberculosis based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. Vet Microbiol 97:73-86.

25. Koets A, Hoek A, Langelaar M, Overdijk M, Santema W, Franken P, Eden W, Rutten V (2006) Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. Vaccine 24:2550-2559.

26. Koets AP, Eda S, Sreevatsan S (2015) The within host dynamics of *Mycobacterium avium* ssp. *paratuberculosis* infection in cattle: where time and place matter. Vet Res 46:61.

27. Kralik P, Pribylova-Dziedzinska R, Kralova A, Kovarcik K, Slana I (2014) Evidence of passive faecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in a Limousin cattle herd. Vet J 201:91-94.

28. Kroese AH, De Jong MCM: Design and analysis of transmission experiments In: *Meeting for the Society for Veterinary Epidemiology and Preventative Medicine*. Society for Veterinary Epidemiology and Preventative Medicine; 2001: xxi-xxxvii.

29. Kudahl AB, Ostergaard S, Sorensen JT, Nielsen SS (2007) A stochastic model simulating paratuberculosis in a dairy herd. Prev Vet Med 78:97-117.

30. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C (2011) Predicting fadeout versus persistence of paratuberculosis in a dairy cattle herd for management and control purposes: a modelling study. Vet Res 42:36.

31. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C (2011) Within-herd contact structure and transmission of *Mycobacterium avium* subspecies *paratuberculosis* in a persistently infected dairy cattle herd. Prev Vet Med 100:116-125.

32. Marcé C, Ezanno P, Weber MF, Seegers H, Pfeiffer DU, Fourichon C (2010) Invited review: modeling within-herd transmission of *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle: a review. J Dairy Sci 93:4455-4470.

33. McDonald WL, Ridge SE, Hope AF, Condron RJ (1999) Evaluation of diagnostic tests for Johne's disease in young cattle. Aust Vet J 77:113-119.

34. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

35. Mitchell RM, Medley GF, Collins MT, Schukken YH (2012) A meta-analysis of the effect of dose and age at exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows. Epidemiol Infect 140:231-246.

36. Mitchell RM, Whitlock RH, Stehman SM, Benedictus A, Chapagain PP, Grohn YT, Schukken YH (2008) Simulation modeling to evaluate the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on commercial dairy farms in the United States. Prev Vet Med 83:360-380.

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

38. Mortier R, Barkema HW, Orsel K, Wolf R, De Buck J (2014) Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 45:71.

39. Mortier RA, Barkema HW, Wilson TA, Sajobi TT, Wolf R, De Buck J (2014) Dose-dependent interferon-gamma release in dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Immunol Immunopathol 161:205-210.

40. Nielsen SS, Toft N (2006) Age-specific characteristics of ELISA and fecal culture for purpose-specific testing for paratuberculosis. J Dairy Sci 89:569-579.

41. Pouillot R, Dufour B, Durand B (2004) A deterministic and stochastic simulation model for intra-herd paratuberculosis transmission. Vet Res 35:53-68.

42. Santema WJ, Poot J, Segers RP, Van den Hoff DJ, Rutten VP, Koets AP (2012) Early infection dynamics after experimental challenge with *Mycobacterium avium* subspecies *paratuberculosis* in calves reveal limited calf-to-calf transmission and no impact of Hsp70 vaccination. Vaccine 30:7032-7039.

43. Slana I, Kralik P, Kralova A, Pavlik I (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. Int J Food Microbiol 128:250-257.

44. Slater N, Mitchell RM, Whitlock RH, Fyock T, Pradhan AK, Knupfer E, Schukken YH, Louzoun Y (2016) Impact of the shedding level on transmission of persistent infections in *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Vet Res 47:38.

45. Smith RL, Al-Mamun MA, Gröhn YT (2017) Economic consequences of paratuberculosis control in dairy cattle: a stochastic modeling study. Prev Vet Med 138:17-27.

46. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW (2006) Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47:874-882.

47. van Roermund HJ, Bakker D, Willemsen PT, de Jong MC (2007) Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: calves can transmit the infection to other calves. Vet Microbiol 122:270-279.

48. van Roermund HJ, Vos AM, De Jong MC (2002) Within herd transmission of paratuberculosis and the possible role of infectious calves. In: *Proc 7th Intern Colloq Paratuberculosis: 2002; Bilbao, Spain*; 368-370.

49. Velthuis AG, De Jong MC, De Bree J (2007) Comparing methods to quantify experimental transmission of infectious agents. Math Biosci 210:157-176.

50. Velthuis AGJ, De Jong MCM, De Bree J, Nodelijk G, Van Boven M (2002) Quantification of transmission in one-to-one experiments. Epidemiol Infect 128:193-204.

51. Velthuis AGJ, De Jong MCM, Kamp EM, Stockhofe N, Verheijden JHM (2003) Design and analysis of an *Actinobacillus pleuropneumoniae* transmission experiment. Prev Vet Med 60:53-68.

52. Weber MF, Groenendaal H (2012) Effects of infectious young stock on results of certification, surveillance and control programmes for paratuberculosis in dairy herds. Vet Microbiol 154:272-281.

53. Whitlock RH (2010) Paratuberculosis control measure in the USA. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. Wallingford, UK.

54. Windsor PA, Whittington RJ (2010) Evidence for age susceptibility of cattle to Johne's disease. Vet J 184:37-44.

55. Wolf R, Barkema HW, De Buck J, Orsel K (2016) Dairy farms testing positive for *Mycobacterium avium* ssp. *paratuberculosis* have poorer hygiene practices and are less cautious when purchasing cattle than test-negative herds. J Dairy Sci 99:4526-4536.

56. Wolf R, Barkema HW, De Buck J, Slomp M, Flaig J, Haupstein D, Pickel C, Orsel K (2014) High herd-level prevalence of *Mycobacterium avium* subspecies

paratuberculosis in Western Canadian dairy farms, based on environmental sampling. J Dairy Sci 97:6250-6259.

57. Wolf R, Orsel K, De Buck J, Barkema HW (2015) Calves shedding *Mycobacterium avium* subspecies *paratuberculosis* are common on infected dairy farms. Vet Res 46:1-8.

Table 5-1. *Mycobacterium avium* subsp. *paratuberculosis* culture results detected by F57-specific qPCR for fecal and tissue samples, and INF-γ detection from whole blood samples. "I" indicate an infectious animal and "C" indicates a new shedding event.

Pen	Calf ID	IN:CE	MAP detection with F57 qPCR following culture of individual fecal samples																																									INF-γ	Tissue culture
			Days following group housing																																										
			0	3	5	7	10	12	14	17	19	21	24	26	28	31	33	35	38	40	42	45	47	49	52	54	56	59	61	63	66	68	70	73	75	77	80	82	84	87	89	91	94	96	101		
1	1	IN	I		C	I		C		C			C	I	I		C	I	I	I	I	I	I	I	I	I																			+	+
1	2	IN				C	I	I		C	I		C	I	I	I	I		C	I	I	I		C	I	I	I		C																+	+
1	15	CE								C	I							C	I			C		C																					-	+
1	16	CE									C								C	I	I	I							C											C						-	-
2	3	IN		C	I	I			C	I	I	I	I	I	I		C	I	I	I	I	I	I	I							C															+	+
2	4	IN	I		C			C	I	I	I	I	I	I	I		C	I	I	I	I		C	I		C	I	I	I	I																+	+
2	17	CE				C		C	I							C	I	I	I	I											C					C										+	+
2	18	CE						C						C			C	I													C									C						+	+
3	5	IN					C		C	I		C	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I		C										+	+
3	6	IN							C	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I										C											+	+
3	19	CE						C	I											C																									-	+
3	20	CE							C						C		C			C											C									C						+	+
4	7	IN	I			C	I				C	I	I		C			C	I	I							C		C																	+	+
4	8	IN	I	I		C	I	I	I	I	I	I	I	I	I	I	I	I	I	I								C																	+	+
4	21	CE												C		C	I			C							C																		-	+
4	22	CE			C				C					C		C																			C										+	-
5	9	IN				C	I			C	I	I			C	I	I			C	I	I	I	I	I	I	I		C																+	+
5	10	IN				C		C	I	I	I	I	I	I	I	I	I	I	I	I	I		C	I	I	I	I		C	I	I	I	I		C	I				I					+	+
5	23	CE														C					C																								+	-
5	24	CE				C							C			C													C																-	-
6	11	IN						C		C		C				C		C						C				C																	+	+
6	12	IN	I						C	C		C	I				C	I			C		C	I	I	I				C			C												+	+
6	25	CE									C					C									C											C									-	-
6	26	CE													C																		C						C						-	+
7	13	IN		C				C			C	I	I		C		C	I	I	I	I		C	I	I	I										C									+	+
7	14	IN			C	I			C			C	I	I	I		C	I	I				C			C				C															+	+
7	27	CE					C			C			C			C			C										C																-	-
7	28	CE									C		C																	C								C							-	-

Table 5-2. Estimates for coefficients and their 95% confidence for fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in final General Linearized Model (GLM) with and without the interaction term for start day*susceptibility status.

Model	Model Coefficient (code)	Estimate (day^{-1})	p-value	95% Confidence interval	AIC value for model fit
Final model with no interaction between susceptibility and start day					530.67
	Susceptibility status (ino)	1.41	<0.001	1.05, 1.77	
	Time since group housing (start day)	-0.027	<0.001	-0.035, -0.019	
	Infectiousness of contact exposed calves (_cons)	-1.84	<0.001	-2.21, -1.47	
Final model including interaction between susceptibility and start day					527.86
	Susceptibility status (ino)	2.08	<0.001	1.37,2.79	
	Time since group housing (start day)	-0.014	0.033	-0.028, -0.001	
	Interaction (ino*start day)	-0.019	0.028	-0.036, -0.002	
	Infectiousness of contact exposed calves (_cons)	-2.26	<0.001	-2.82, -1.71	

CHAPTER 6: QUANTIFYING FECAL SHEDDING OF *MYCOBACTERIUM AVIUM* SUPSP. *PARATUBERCULOSIS* FROM CALVES AFTER EXPERIMENTAL INFECTION AND EXPOSURE

6.1 Abstract

Johne's disease (JD), a chronic enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), causes large economic losses to the dairy industry worldwide. Fecal shedding of MAP contaminates the environment, feed and water and contributes to new infections on farm, yet, there is limited knowledge regarding mechanisms of shedding, extent of intermittent shedding, and numbers of MAP bacteria shed. The objectives were to: 1) compare (in an experimental setting) the frequency at which intermittent shedding occurred and the quantity of MAP shed among penmates that were inoculated or contact-exposed (CE) calves, and; 2) determine whether there was an association between inoculation dose and quantity of MAP shed. In Experiment A, 32 newborn Holstein-Friesian bull calves were allocated to pens in groups of 4, whereby 2 calves were inoculated with a moderate dose (MD) of 5 x 10^8 CFUs of MAP and 2 calves acted as CE. Calves were group-housed for 3 months and fecal samples were collected, cultured, and culture-positive samples quantified. In Experiment B, 6 calves were inoculated with either a low (LD) or high (HD) dose of MAP (1 x 10^8 and 1 x 10^{10} CFUs, respectively), and fecal samples were collected for 3 months, cultured for detection of MAP, and amount of MAP quantified using direct DNA extraction (DE) and F57-specific qPCR. In Experiment A, the average amount of MAP in all culture-positive samples did not differ between MD and CE calves. When comparing inoculation doses, LD had the lowest proportion of MAP-positive culture samples and HD had the highest, but there was no difference between average quantity of MAP shed. This study provided new information in regards to JD research and control regarding shedding from various

inoculation doses and from CE animals; these data should inform future experimental trials and control programs.

Key-words: Paratuberculosis, fecal, shedding, quantity, calf

6.2 Introduction

Johne's disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic enteritis primarily affecting ruminants, resulting in substantial losses to dairy industries worldwide due to decreased milk production, increased risk of culling and decreased slaughter value [16, 27]. Although most MAP-infected animals are culled before reaching the clinical stage of the disease, subclinically infected animals contribute to the infectious load in the herd through fecal shedding [15, 31, 33]. Johne's disease prevention and control programs are largely based on decreasing MAP transmission within herds, as there is currently no effective vaccine for the prevention of infection, or treatment for infected animals [8, 16, 28]. The primary route of MAP transmission is fecal-oral through the ingestion of contaminated milk, water, feed, and contact with the contaminated environment due to fecal shedding [9, 26, 31]. Although ingestion of shed bacteria is the main cause of new infections, very little is known about the mechanisms of shedding, the amount of MAP shed, and the frequency of intermittent shedding, all which has contributed to animals traditionally being classified as strictly shedders or non-shedders [11, 13, 14, 17, 20, 22]. However, as more research adds to our current base of knowledge regarding shedding, further insights can be made. Recently, it has been accepted that MAP shedding can be intermittent, due to passive (pass-through) or active infection, and shedding quantities can be roughly estimated based on number of colony forming units (CFUs) following culture on solid media or time to detection; however, these quantification methods have not been standardized and do not result in absolute quantification, as 1 CFU may not be formed by a single bacterium [23]. Additionally, shedding was originally believed to only occur in adult cows, but calves can begin

shedding MAP as early as 2 weeks after exposure [20, 32, 36]. Due to the increasing knowledge base regarding shedding, new insights can be gained from researching further into the specific patterns of intermittent shedding, and quantification of these fecal shedding events.

Dose and method of infection affect frequency of MAP shedding; however, knowledge gaps still remain regarding fecal shedding quantities and its relation to shedding on farm [17, 18, 20]. Consensus recommendations on doses and methods of inoculation for consistent experimental infection are available, but there is uncertainty regarding to what extent these doses relate to natural exposure, leading to varying recommendations, based on the outcome of interest [2, 10]. Consequently, doses for infection in transmission trials have ranged from 4×10^4 to 10^{10} CFUs, potentially affecting shedding intervals, immune responses, and degree of tissue infections [5, 17, 20, 24, 32].

Although a recent meta-analysis examined shedding patterns in experimental studies [17], knowledge is lacking regarding the amount of MAP and the consistency of the quantity shed by inoculated and naturally exposed animals, as well as the impact of shedding animals on fecal shedding (passive or active) of pen mates [1]. Therefore, the aims of the current study were to: 1) compare the frequency at which intermittent shedding occurred and the quantity of MAP shed over the course of group housing between inoculated and CE calves, and 2) determine the effects of inoculation dose on the quantity of MAP shed by calves over the course of 3 months.

6.3 Materials and methods

The study was conducted using fecal samples collected in 2 independent calf MAP challenge experiments. Fecal samples in Experiment A were used to compare frequency

of shedding (based on culture) and quantities of MAP shed among inoculated and exposed (naturally infected) calves. All samples collected in Experiment A were cultured, and only culture-positive samples were quantified. Selected fecal samples from 5 calves in Experiment A, and all fecal samples from calves in Experiment B were used to quantify fecal shedding and quantity related to dose of inoculation. Fecal samples were categorized as either "pass-through shedding" of the inoculum (samples collected within the first 7 days after inoculation), and those remaining were categorized as "active shedding"; therefore, there were 5 samples per calf in each category.

6.3.1 Experiment A

Study design, calf collection and care, and sample collection were as described [4]. Briefly, 32 newborn Holstein-Friesian bull calves were collected from 13 Alberta (Canada) dairy farms that had tested negative for MAP for over 4 years using 6 environmental samples and 1 of the following: bacteriological culture of 60 individual fecal samples tested as pooled samples into groups of 5, individual milk ELISA of the whole milking herd, or serum ELISA of the entire herd. Calves were group-housed in 7 experimental pens in a biosecurity level 2 facility, based on birth order. Each pen contained 4 calves, 2 of which were inoculated with a moderate dose (MD) and 2 that acted as contact-exposed (CE). An additional 4 calves acted as non-inoculated control calves. Inoculum was prepared as described [4]. Briefly, a strain obtained from a clinical case (Cow 69) in Alberta (Canada) was used for inoculation. Following culture in 7H9/mycobactin/OADC broth from a frozen first-passage stock, 2 calves (2 wk of age) in each pen were inoculated with an moderate dose (MD) of 2.5 x 10^8 CFU on 2 consecutive days orally. At least 2 wk after inoculation (range: 14 d – 21d following

inoculation), CE calves were added to the group pen (to allow for pass through of the inoculum), with MD and CE calves group housed for 3 mo. The MD calves were euthanized after 3 mo of group housing, whereas CE calves were euthanized after an additional 3 mo of individual housing. Control calves were group-housed throughout the study, and were the last to be euthanized.

All samples were collected as described [4]. Briefly, individual fecal samples were collected 3 times per wk, starting on the first day of group housing. Additional fecal samples were collected from 5 MD calves on days 1-4, 7 and 14 after inoculation. Fecal samples were aliquoted into 3 separate containers; 1 was cultured as described below within 1 wk after collection, and the rest were frozen at -80°C pending further use.

6.3.2 Experiment B

Study design and sample collection were as described by [20]; however, only fecal samples collected from calves that were inoculated at 2 weeks of age with additional samples on days 1-4 were used (3 out of 5 calves in each dose group). Briefly, newborn Friesian-Holstein bull calves, originating from dairy farms that were negative for MAP based on similar herd infection testing described for Experiment A, were inoculated (2 wks of age) with either a low dose (LD, 5 x 10^7 CFUs) or a high dose (HD, 5 x 10^9 CFUs) on 2 consecutive days, with the same MAP strain as in Experiment A. Calves were housed individually in strictly separated pens. Fecal samples were collected on days 1-4 after inoculation, then weekly on days 7, 14, 21, 28, and finally monthly on days 57 and 91. All samples were cultured, and then frozen at -80°C until direct extraction (DE) and PCR detection of MAP as described below. All DE-positive samples were used for quantification, regardless of culture status.

6.3.3 Culture and direct extraction of MAP

Fecal samples collected on days 1-4, 7, 14, 21, 28, 57 and 91 following inoculation from 5 MD calves of experiment A, and all samples from 3 LD and 3 HD calves from Experiment B were used. All fecal samples were cultured as described [4, 20]. Briefly, 2 g of fecal sample were disinfected in 0.9% hexadecylpyridinium chloride (HPC), in ½ strength brain heart infusion (BHI) broth at 37°C overnight following centrifugation of each sample. The pellet was re-suspended in a mixture of antibiotic solution (AS; para-Jem®, TREK Diagnostic Systems, Cleveland, OH, USA), water, and full strength BHI and incubated at 37°C overnight, and 1 mL was added to paraJEM® culture bottles and incubated at 37°C for 49 d. Following incubation, DNA was extracted as described [7], and a duplex qPCR targeting the single copy, MAP-specific F57 region with an internal amplification control (IAC) was done [25]. Based on fecal culture and qPCR results, MAP culture-positive fecal samples from the MD calves were selected to undergo DE of DNA from samples. All fecal samples from LD and HD calves underwent both culture and DE. Fecal samples were thawed, and DE performed using a MagMAX total nucleic acid isolation kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions, with modifications as described [6]. Briefly, 0.3 g of fecal material was used, and a bead beater was used to release DNA. The final lysate product was then used for F57-specific duplex qPCR with an IAC, using the same protocol as for PCR confirmation after culture.

6.3.4 qPCR, quantification and verification

Duplex qPCR targeting the MAP-specific F57 region and an IAC was performed with primers and probes identical to those described [25] after DE of fecal samples or after

MAP culture. Amplification conditions for qPCR were as follows: 50°C for 2 min, 95°C for 20 s to allow for initial denaturation, then 42 cycles of 95°C for 30 s and 59°C for 30 s. Eluate from DE was run in duplicate, with the lowest CT value from both runs retained for quantification.

Cultured fecal samples were considered MAP-positive when the cycle threshold (CT) value was < 40. All culture-positive samples from MD and CE calves, and all samples collected from LD and HD calves, were DE for quantification. For DE fecal samples, CT values from the qPCR were used to estimate relative amount of MAP present in each sample using a plasmid standard containing the F57 region cloned into pCR 2.1 cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations and as described [25]. Plasmid concentration was estimated using Qubit Fluorometric Quantification ® (Invitrogen, Life Techonologies), and 10-fold serially diluted 9 times. The F57 copy numbers from the DE fecal samples were extrapolated from CT values, based on a plasmid standard curve generated from the plasmid standard dilutions to estimate the relative number of MAP bacteria in each g of feces per sample [14].

A spiking experiment was conducted in triplicate, as described [14], to verify DE and quantification method. Briefly, a MAP culture suspension was prepared as described above for inoculation and quantified after a 10-fold serial dilution in broth, by counting cells in a Neubauer chamber. Only the first 4 serial dilutions could be quantified using cell counting; therefore, dilutions were extrapolated based on average of the first 3 serial dilutions. Seven serial dilutions of MAP cells were used to spike negative cattle fecal samples, with the number of cells ranging from approximately 5 x 10^{8}-$10^{1.}$ Following

spiking, fecal samples underwent DE as described above, and quantification following F57-specific qPCR. The efficiency of DE and quantification was determined as described [14]; briefly, the estimated recovered MAP per g feces was based on CT value and plasmid curve was divided by the theoretical input concentration (based on cell count) and multiplied by 100. Mean efficiency for each dilution was calculated.

6.3.5 Statistical analyses

All statistical analyses were performed using STATA 11.2 (StataCorp LP, College Station, TX, USA). For all analyses, $P < 0.05$ was considered significant. Normality was assessed using the Shapiro-Wilk test. The difference between quantities shed before and after group housing were compared using the Wilcoxon rank sum test. Differences between the mean MAP shedding quantity of MD and CE calves and differences between proportions of positive samples for each inoculation group were compared using a Kruskal-Wallis rank test. A regression was used to determine if there was a relationship between shedding quantities and days following group housing for MD and CE calves.

6.4 Results

6.4.1 Fecal culture results (Experiment A)

A total of 325 MAP culture-positive fecal samples of 1176 were collected during group housing; 254 (78%) of these were from MD calves and 71 (22%) from CE calves. All calves had at least 2 MAP culture-positive fecal samples during the 3 mo of group housing. Although shedding was detected intermittently throughout group housing, a peak with >50% calves with MAP culture-positive fecal samples occurred between 24 and 40 d (Figure 6-1). Following this peak, the proportion of MAP culture-positive fecal samples decreased among inoculated samples. The proportion of all culture-positive fecal samples varied daily, ranging from 0 to 57% (P<0.01)(Figure 6-1).

6.4.2 Spiking experiment: quantification verification

Mean efficiency for the DE and quantification of MAP from spiked fecal samples was 93.6% (95% CI: 40.5 - 146.7), with no difference between the first 3 serial dilutions and the last 3 (P=0.44) (Table 6-1). The MAP quantified from the CT values following F57-specific qPCR were within a half log of the spiked-in concentration and highly correlated (r=0.98); therefore, DE and quantification were further used to quantify fecal samples.

6.4.3 Quantification of MAP in culture-positive fecal samples in MD and CE

Of the 325 MAP culture-positive samples collected during group housing, 111 (34%) were positive for both culture and DE, 8 from CE and 103 from MD calves (Figure 6-2). Quantification using the standard plasmid curve was applied to all 111 fecal samples that were MAP-positive for both culture and DE. Average MAP shed in all positive samples tended to be lower for MD than for CE calves, 7.6 x 10^5 MAP per g (103 samples) and 3.4 x 10^6 MAP per g (8 samples), respectively (P = 0.08).

Mean MAP shed per g feces among MD calves was higher before group housing (2.8 x 10^6) than during group housing (1.3 x 10^5) (P = 0.003); however, there was no difference among calves for average MAP per g feces during group housing (P = 0.24), and there was no difference in shedding quantities over time during group housing for either MD or CE calves (P=0.32 and P=0.50, respectively) (Figure 6-3).

6.4.4 Quantification of MAP in LD, MD and HD

All fecal samples collected from the 3 calves in the LD group, 3 calves in the HD group, and 5 calves in the MD group were cultured, and DE with relative quantification (Figure 6-4). A lower proportion of samples of the LD calves were MAP culture-positive compared to samples from HD calves (P = 0.04); furthermore, the proportion of MD samples tended to be higher than the LD samples (P = 0.09), but was not different from HD samples (P = 0.16). Of the 136 MAP culture-positive samples, 74 (54%) were positive following DE (Figure 4). Of all fecal samples collected from all 3 doses (251 samples), 98 (40%) samples were MAP-positive after DE.

For all 3 doses, there was a higher average quantity of MAP shed during pass-through shedding (first 7 days after inoculation) than in the average of active shedding (all samples after the first week) (Table 6-2). Quantities shed during pass-through versus active shedding did not differ among doses (Table 6-2).

6.5 Discussion

In the first 7 days after inoculation, calves shed the highest quantity of MAP in fecal samples, regardless of dose, higher than the average of all remaining samples collected over the next 3 months, and shedding did not increase over time. The highest proportion of samples were MAP culture-positive 24-40 d of group housing of calves.

Proportion of MAP-culture positive samples increased with increasing inoculation dose, and the average quantity of MAP shed by CE calves was similar to calves inoculated with a MD. After the first week following inoculation, there was no differences between the 3 doses for quantity of MAP shed. Therefore, the primary difference between doses was in frequency of shedding, rather than quantity shed.

Frequency of fecal shedding detected by culture was highest among HD calves, followed by the MD, LD, and lastly the CE group, consistent with findings from previous trials where dose and type of exposure (inoculation or exposure) had a direct effect on frequency of shedding [17, 20]. Although MD and CE were group-housed, and HD and LD calves were individually housed, positive environmental samples among MD and CE were not associated with shedding frequency [4]; however, the accumulative effect of the environmental contamination in group housing on the amount of MAP shed among MD and CE cannot be elucidated, and should be taken into consideration when interpreting the results. Direct extraction was performed on all MAP culture-positive samples; however, fewer positive samples were detected by this method. Although DE allows for estimation of MAP present in a fecal sample, low amounts of MAP that may be detected via the culture method due to growth during incubation, may not be detected using the DE technique. Calves inoculated with a lower dose resulting in fewer culture-positive samples may be shedding less MAP, therefore resulting in more DE-negative samples (due to decreased detectability). Low shedding quantities among calves in all doses groups resulted in negative DE samples in 46% of culture-positive fecal samples, which may result in overestimation of quantities of MAP shed, particularly among LD or CE calves. Additionally, low concentrations of MAP in fecal samples that can be detected by

DE may be overestimated using the quantification method, as indicated by spiking validation in this experiment and others [14], potentially resulting in a false plateau of shedding for all doses. Regardless, the proportion of culture-positive samples detected in each dose group clearly indicated that a higher dose caused more frequent fecal shedding events than lower doses. It should be taken into consideration that the amount of MAP being shed may vary between calves of the same inoculation dose, and this was not accounted for in the analysis as only the average of positive samples was calculated.

Although culture may be a more sensitive technique to identify MAP-positive and negative fecal samples, DE allows for estimation of the relative quantity of MAP present in fecal samples [14]. The average amount of MAP shedding after the first week following inoculation did not differ among the 3 doses; however, these amounts were lower than those in the first week after inoculation. These differences between the amount of MAP detected in the first week following inoculation, and subsequent shedding events may be indicative of the difference between pass-through shedding of the inoculum, and active shedding following infection [10, 30]. Although a minimum infection dose for MAP is not known, it has been suggested that 1.5×10^6 CFUs is sufficient to reliably detect infection in the tissues, with doses as low as 10^3 being able to cause infection [2, 29]. Based on quantification results from the current study, shedding detected in calves could be sufficient to cause infection on farm, and may have huge implications considering shedding levels > 10^4 CFUs/g feces is extremely high, with "super shedders" being categorized as shedding 1×10^4 CFU's per g feces; however, it should be considered that the relative quantification method uses copy number based on the single copy gene F57, and therefore may be a more accurate estimation of the number

of MAP in a sample [35]. Additionally, the minimum threshold at which the DE and relative quantification can reliably detect F57 was reported to be 1.0 x 10^4 [14], and although results from the spiking experiment indicate that lower numbers can be detected with qPCR, it is possible that detection and quantification of MAP may be more difficult in low-shedding samples collected from the calves due low quantities and the unknown mechanisms of shedding [11].

Once pass-through of the inoculum had occurred (7 days following inoculation) in group-housed MD calves, the quantity of MAP shed was consistent; however, the frequency at which MAP was detected in fecal samples was highly variable. In agreement with previous studies, inoculated calves shed more frequently than CE calves, and a higher dose increased frequency of positive samples [17, 18, 20]. Although individual shedding patterns were intermittent, it is important to consider the accumulative impact on the environmental contamination due to MAP fecal shedding in calves, as any shedding calf will contribute to environmental contamination, potentially leading to new infections of susceptible calves.

The main objectives of current JD prevention and control programs are to decrease incidence of new MAP infections by decreasing transmission from infectious cows to susceptible calves and, to decrease the prevalence of infection by preventing entry of infectious animals into the herd and identifying and removing infectious animals from the herd [3, 8, 16]. Currently, identification and removal of infectious animals within the herd focuses on cows that have calved at least once; however, based on results from this study and others, it may be imperative to begin fecal testing of young stock [21, 34, 36]. Direct extraction and qPCR targeting F57 may facilitate rapid identification of

MAP-positive calves shedding on farm, as well as quantification of MAP being shed. However, repeated sampling may be needed, and culture-based methods appear to be more sensitive, especially if low quantities of MAP are shed [12, 14]. It is also important to take into consideration that the results from this experimental study may not entirely reflect fecal shedding occurring in naturally infected animals in a herd; however, important insights can still be made to improve control programs especially regarding the impact of shedding calves. Control programs should include testing of environmental samples collected from areas that house young stock, as due to the nature of intermittent shedding MAP-positive calves may not be detected, but the environment may act as a reservoir of accumulated infectious material previously shed by infectious calves [4, 19, 36].

In conclusion, inoculation dose and method were associated with the frequency of shedding MAP in feces as detected by culture, but not with quantity of MAP shed in months following the first week after inoculation. Frequency of shedding increased with dose, and was more frequent in inoculated compared to CE calves; however, it was not related to pen mate shedding, but was individually variable. Based on quantification of these samples, immediately after inoculation, MAP quantities were relative to the dose administered; however, later average amount of MAP shed per g feces were consistent at between 9×10^4 to 2×10^5 during the first 4 months, regardless of dose. This study provided important information regarding shedding quantities in calves inoculated with three different doses, patterns of shedding among pen mates, and quantities of MAP shed in CE calves, allowing future experimental trials to implement this information in the decision-making process regarding inoculation doses. Additionally, understanding

shedding patterns and quantities in naturally infected calves should improve JD control programs, allowing inclusion of effective calf sampling, and the addition of young stock environmental sampling to better prevent new infections from occurring in positive herds.

6.6 References

1. Barkema HW, Orsel K, Nielsen SS, Koets A, Rutten V, Bannantine JP, Keefe GP, Heuer C, Forde T, Ritter C *et al* (2017) Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection. Transb Emerg Dis.

2. Begg DJ, Whittington RJ (2008) Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. Vet J 176:129-145.

3. Cho J, Tauer LW, Schukken YH, Smith RL, Lu Z, Gröhn YT (2013) Cost-effective control strategies for Johne's disease in dairy herds. Can J Agr Econ 61:583-608.

4. Corbett CS, De Buck J, Orsel K, Barkema HW (2017) Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48:27.

5. Eisenberg SW, Koets AP, Nielen M, Heederik D, Mortier R, De Buck J, Orsel K (2011) Intestinal infection following aerosol challenge of calves with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 42:117.

6. Forde T, De Buck J, Elkin B, Kutz S, van der Meer F, Orsel K (2013) Contrasting results of culture-dependent and molecular analyses of *Mycobacterium avium* subsp. *paratuberculosis* from wood bison. Appl Environ Microbiol 79:4448-4454.

7. Forde T, Kutz S, De Buck J, Warren A, Ruckstuhl K, Pybus M, Orsel K (2012) Occurence, diagnosis, and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* infection in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) in southwest Alberta. J Wildlife Dis 48:1-11.

8. Garry F (2011) Control of paratuberculosis in dairy herds. Vet Clin North Am Food Anim Pract 27:599-607.

9. Harris NB, Barletta RG (2001) *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin Microbiol Rev 14:489-512.

10. Hines ME, 2nd, Stabel JR, Sweeney RW, Griffin F, Talaat AM, Bakker D, Benedictus G, Davis WC, de Lisle GW, Gardner IA *et al* (2007) Experimental challenge models for Johne's disease: a review and proposed international guidelines. Vet Microbiol 122:197-222.

11. Koets AP, Eda S, Sreevatsan S (2015) The within host dynamics of *Mycobacterium avium* ssp. *paratuberculosis* infection in cattle: where time and place matter. Vet Res 46:61.

12. Kralik P, Beran V, Pavlik I (2012) Enumeration of *Mycobacterium avium* subsp. *paratuberculosis* by quantitative real-time PCR, culture on solid media and optical densitometry. BMC Res Notes 5:114.

13. Kralik P, Pribylova-Dziedzinska R, Kralova A, Kovarcik K, Slana I (2014) Evidence of passive faecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in a Limousin cattle herd. Vet J 201:91-94.

14. Kralik P, Slana I, Kralova A, Babak V, Whitlock RH, Pavlik I (2011) Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR. Vet Microbiol 149:133-138.

15. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C (2011) Within-herd contact structure and transmission of *Mycobacterium avium* subspecies *paratuberculosis* in a persistently infected dairy cattle herd. Prev Vet Med 100:116-125.

16. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

17. Mitchell RM, Medley GF, Collins MT, Schukken YH (2012) A meta-analysis of the effect of dose and age at exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows. Epidemiol Infect 140:231-246.

18. Mitchell RM, Schukken Y, Koets A, Weber M, Bakker D, Stabel J, Whitlock RH, Louzoun Y (2015) Differences in intermittent and continuous fecal shedding patterns between natural and experimental *Mycobacterium avium* subspecies *paratuberculosis* infections in cattle. Vet Res 46:66.

19. Moravkova M, Babak V, Kralova A, Pavlik I, Slana I (2012) Culture- and quantitative IS900 real-time PCR-based analysis of the persistence of *Mycobacterium avium* subsp. *paratuberculosis* in a controlled dairy cow farm environment. Appl Environ Microbiol 78:6608-6614.

20. Mortier R, Barkema HW, Orsel K, Wolf R, De Buck J (2014) Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 45:71.

21. Mortier RA, Barkema HW, De Buck J (2015) Susceptibility to and diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy calves: A review. Prev Vet Med 121:189-198.

22. Munster P, Volkel I, Wemheuer W, Schwarz D, Doring S, Czerny CP (2013) A longitudinal study to characterize the distribution patterns of *Mycobacterium avium* ssp. *paratuberculosis* in semen, blood and faeces of a naturally infected bull by IS 900 semi-nested and quantitative real-time PCR. Transbound Emerg Dis 60:175-187.

23. Pradhan AK, Mitchell RM, Kramer AJ, Zurakowski MJ, Fyock TL, Whitlock RH, Smith JM, Hovingh E, Van Kessel JA, Karns JS *et al* (2011) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* in a longitudinal study of three dairy herds. J Clin Microbiol 49:893-901.

24. Santema WJ, Poot J, Segers RP, Van den Hoff DJ, Rutten VP, Koets AP (2012) Early infection dynamics after experimental challenge with *Mycobacterium avium* subspecies *paratuberculosis* in calves reveal limited calf-to-calf transmission and no impact of Hsp70 vaccination. Vaccine 30:7032-7039.

25. Slana I, Kralik P, Kralova A, Pavlik I (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. Int J Food Microbiol 128:250-257.

26. Slater N, Mitchell RM, Whitlock RH, Fyock T, Pradhan AK, Knupfer E, Schukken YH, Louzoun Y (2016) Impact of the shedding level on transmission of persistent infections in *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Vet Res 47:38.

27. Smith RL, Grohn YT, Pradhan AK, Whitlock RH, Van Kessel JS, Smith JM, Wolfgang DR, Schukken YH (2016) The effects of progressing and nonprogressing *Mycobacterium avium* ssp. *paratuberculosis* infection on milk production in dairy cows. J Dairy Sci 99:1383-1390.

28. Sorge US, Lissemore K, Godkin A, Jansen J, Hendrick S, Wells S, Kelton DF (2011) Changes in management practices and apparent prevalence on Canadian dairy farms participating in a voluntary risk assessment-based Johne's disease control program. J Dairy Sci 94:5227-5237.

29. Sweeney RW, Uzonna J, Whitlock RH, Habecker PL, Chilton P, Scott P (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. Res Vet Sci 80:253-259.

30. Sweeney RW, Whitlock RH, Hamir AN, Rosenberger AE, Herr SA (1992) Isolation of *Mycobacterium paratuberculosis* after oral inoculation in uninfected cattle. Am J Vet Res 53:1312-1314.

31. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW (2006) Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47:874-882.

32. van Roermund HJ, Bakker D, Willemsen PT, de Jong MC (2007) Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: calves can transmit the infection to other calves. Vet Microbiol 122:270-279.

33. Weber M (2006) Risk management of paratubersulosis in dairy herds. Ir Vet J 59:555-561.

34. Weber MF, Kogut J, de Bree J, van Schaik G, Nielen M (2010) Age at which dairy cattle become *Mycobacterium avium* subsp. *paratuberculosis* faecal culture positive. Prev Vet Med 97:29-36.

35. Whitlock RH, Sweeney RW, Fyock T, Smith J (2005) MAP super-shedders: Another factor in the control of Johne's disease. In: *Proc 8th Intern Colloq Paratuberculosis: 2005;* Madison, WI.

36. Wolf R, Orsel K, De Buck J, Barkema HW (2015) Calves shedding *Mycobacterium avium* subspecies *paratuberculosis* are common on infected dairy farms. Vet Res 46:1-8.

Table 6-1. Validation of the efficiency of direct extraction to quantify the total *Mycobacterium avium* subsp. *paratuberculosis* (MAP) DNA isolation from spiked cattle feces based on F57-specific qPCR.

Input of MAP cells [a]		Experimental output of MAP cells[b]		Mean MAP DNA extraction and quantification efficiency (%)[d]
Mean[c]	SE	Mean[c]	SE	
4.8×10^8	1.7×10^7	2.1×10^8	3.5×10^7	44.3
5.3×10^7	5.0×10^6	2.6×10^7	7.3×10^6	50.2
3.5×10^6	9.8×10^5	4.4×10^6	2.6×10^6	107.2
4.5×10^5	5.0×10^4	6.0×10^5	3.0×10^5	122.3
4.5×10^4	5.0×10^3	2.4×10^4	7.9×10^3	56.5
4.5×10^3	5.0×10^2	9.3×10^3	4.9×10^3	187.0
4.5×10^2	5.0×10^1	7.8×10^2	7.8×10^2	181.4
4.5×10^1	5.0×10^0	0	0	0

[a]Number of MAP cells (quantified using cell counting) used for spiking cattle feces.

[b]Number of MAP cells recovered following direct extraction of DNA and quantification following F57-specific qPCR.

[c]Mean values from triplicate serial dilutions corresponding to amount of MAP per gram of feces.

[d]Experimental output of MAP per g feces divided by the input concentration, and multiplied by 100.

Table 6-2. Average quantity of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) per gram of feces shed following inoculation with a low, moderate or high dose for direct extraction (DE) positive fecal samples based on F57 qPCR.

Inoculation status (CFUs)	Number of DE-positive samples	Mean MAP per g feces for the first 7 days after inoculation (Pass-through)	Mean MAP per g feces for the remaining days following 1 week after inoculation (Active)
Low dose (1×10^8)	14	$8.2 \times 10^{6\ a}$	$1.3 \times 10^{5\ b}$
Moderate dose (5×10^8)	61	$1.3 \times 10^{6\ a}$	$9.4 \times 10^{4\ b}$
High dose (1×10^{10})	23	$1.6 \times 10^{8\ a}$	$1.7 \times 10^{5\ b}$

[a,b]Means without a common superscript differed ($P < 0.05$).

Figure 6-1. Proportion of all fecal samples collected that were *Mycobacterium avium* subsp. *paratuberculosis* (MAP) culture-positive based on F57-specific qPCR. Culture-positive fecal samples from moderate dose (MD; 5 x 10^8 CFUs) calves are represented by the dark grey bars, and contact-exposed (CE) by light bars.

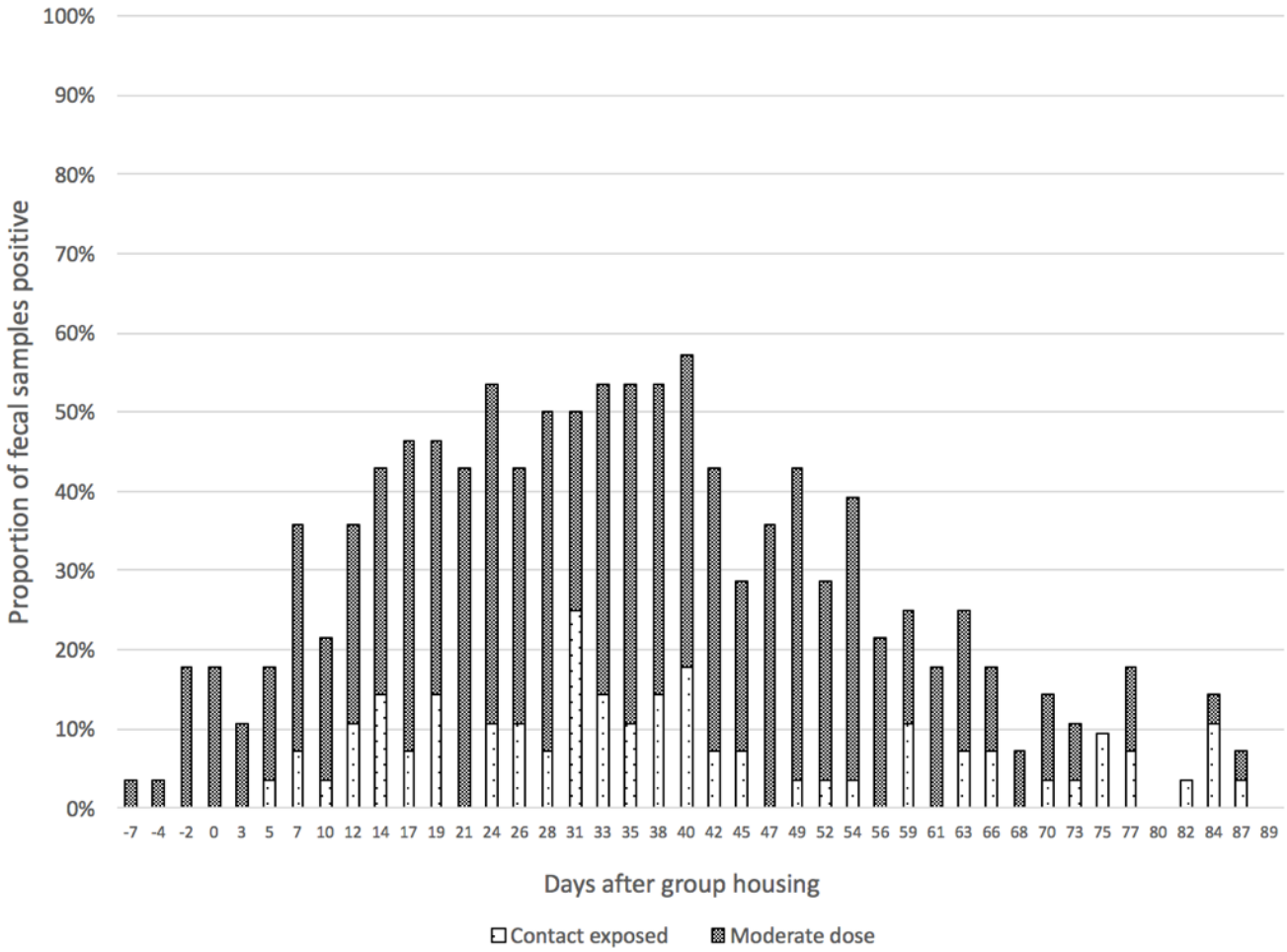


Figure 6-2. Fecal samples collected from group-housed calves that were included in quantification study for shedding of *Mycobacterium avium* subsp. *paratuberculosis*. Fecal culture-positive samples were direct extracted (DE) from calves inoculated at a moderate dose (MD; 5 x 10^8 CFUs) and contact-exposed (CE) calves.

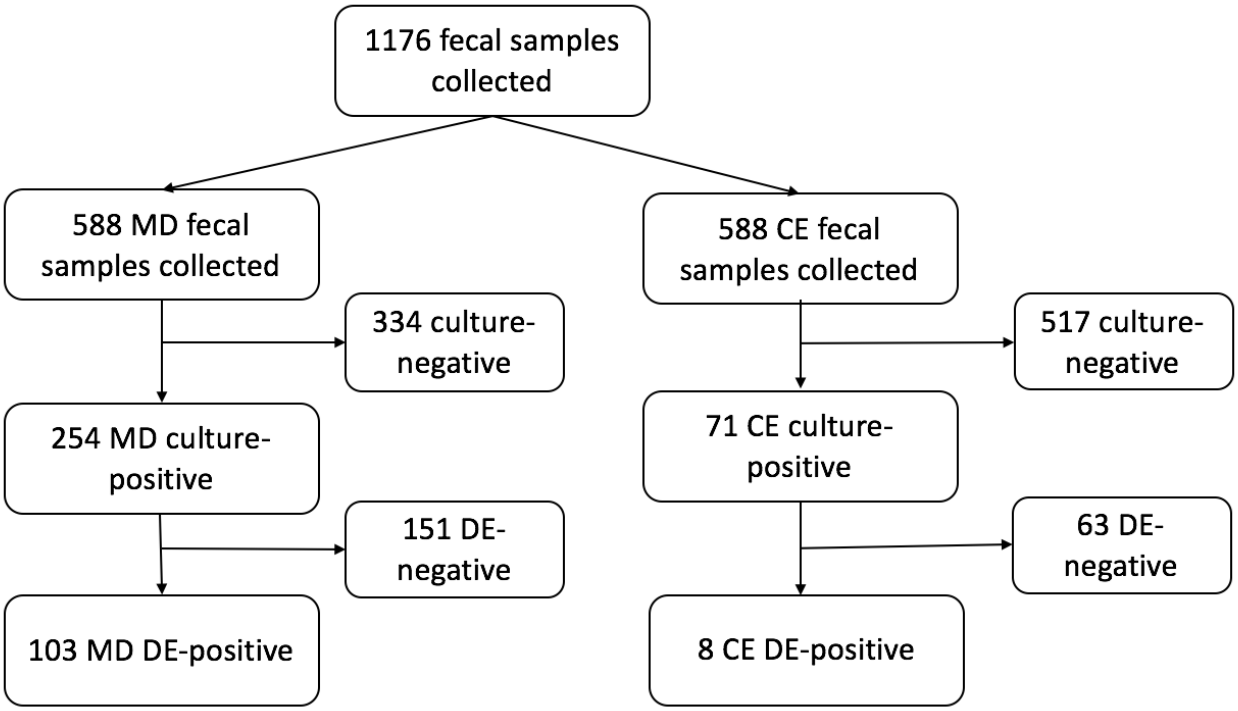


Figure 6-3. Fecal shedding quantities of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) based on F57 copies for calves during 3 months of group housing (starting on Day 0). Dark bars indicate log MAP copies per gram of feces quantified for calves inoculated at a moderate dose (MD; 5 x 10^8 CFUs). Multiple calf samples on the same day are indicated by white horizontal lines across dark bars. Light bars indicate log MAP copies per g feces quantified for contacted-exposed (CE) calves.

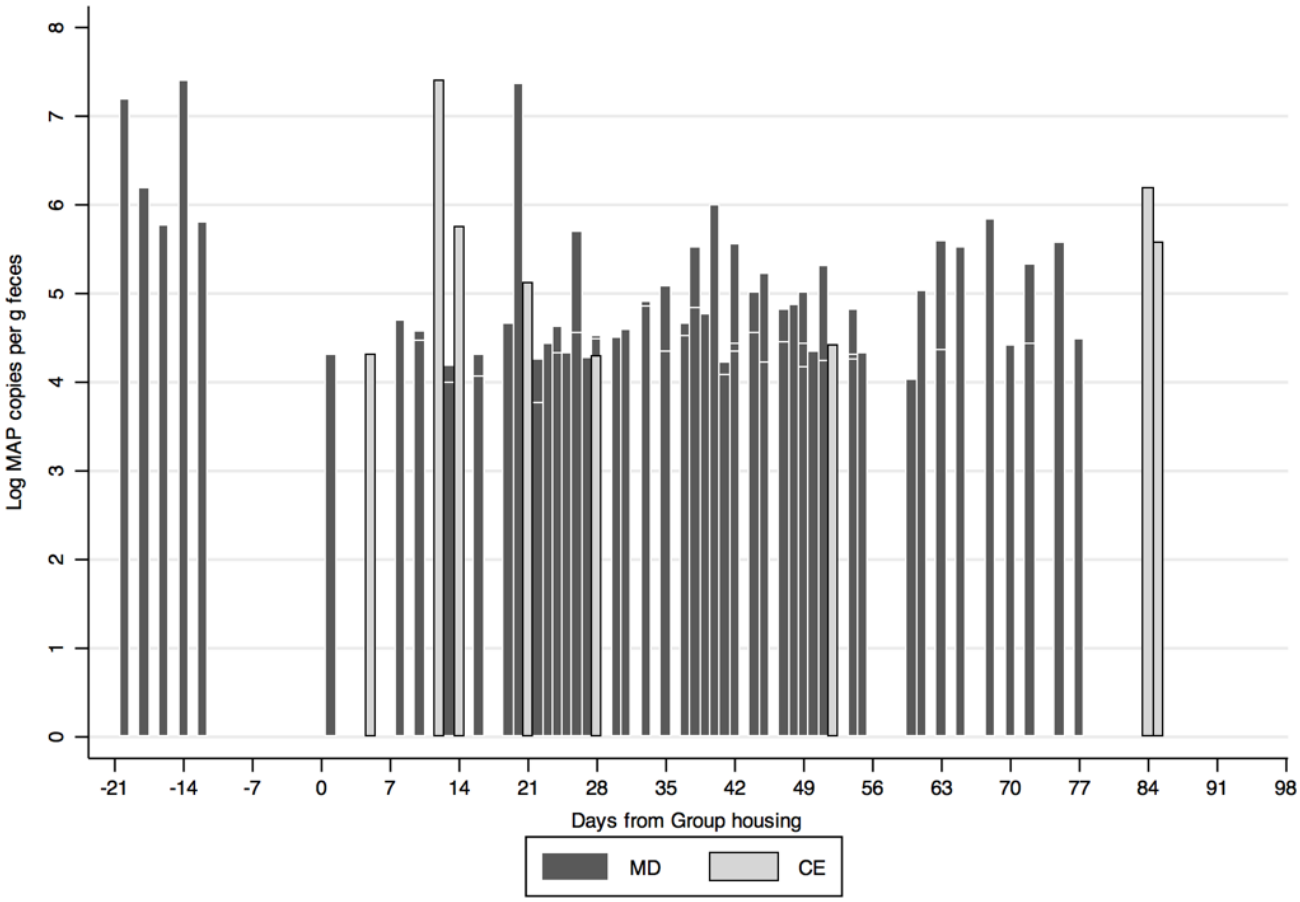
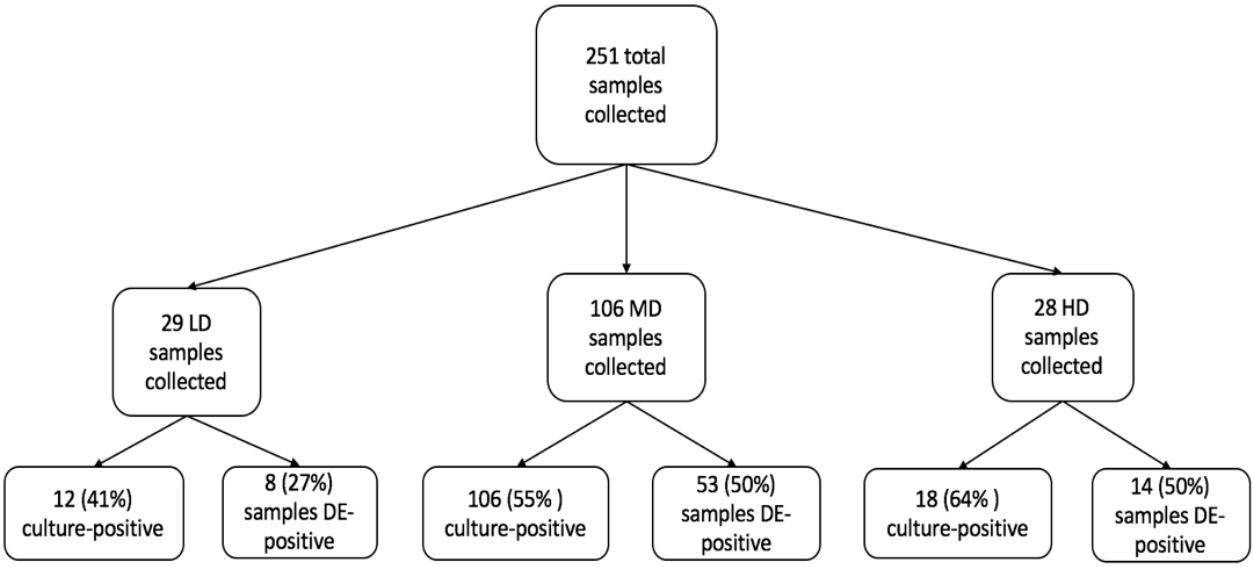


Figure 6-4. Fecal samples collected from calves inoculated with a low dose (LD; 1 x 10^8; n=3), moderate dose (MD; 5 x 10^8 CFUs; n=5) and high dose (HD; 1 x 10^{10}; n=3) of *Mycobacterium avium* subsp. *paratuberculosis*. Breakdown of culture results for each dose, and direct extraction results.



CHAPTER 7: EFFECTS OF FREEZING ON ABILITY TO DETECT *MYCOBACTERIUM AVIUM* SUSBSP. *PARATUBERCULOSIS* FROM BOVINE TISSUE FOLLOWING CULTURE

7.1 Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the bacterium that causes Johne's disease in cattle. Although infected cattle can be identified by examining fecal, blood, or milk samples, the gold standard is identification of MAP in tissue samples postmortem. Although tissue samples are commonly frozen, the ability to detect MAP in frozen-thawed tissue samples has apparently not been reported. We therefore determined the ability to detect MAP in tissue samples following freezing. Tissue samples were collected from calves that were either inoculated (IN) 3 mo prior, or contact-exposed (CE) for 3 mo. Following autopsy, tissues were immediately processed for culture, followed by DNA extraction and detection by qPCR. Samples were categorized as positive or negative based on the cycle threshold (Ct) value. The remaining unprocessed tissue samples were frozen at -80°C. After 18 mo, 50 tissue samples designated MAP-positive were thawed and processed for detection of MAP. Four (8%) samples were qPCR-negative, and Ct values of the remaining 46 samples were higher after freezing. Given the small numerical change in Ct values for MAP-positive samples after 18 mo of frozen storage, freezing and thawing may have had some deleterious effects on MAP detection in tissues. Although the decrease in ability to detect MAP-positive samples was minor for IN calves, there may be a greater effect for CE calves that should be considered when freezing tissue samples.

Key words: Culture; detection; freezing; paratuberculosis; tissue.

7.2 Introduction

Johne's disease is a production-limiting, costly disease in dairy cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) [7, 14]. Although identification of MAP-infected cattle is an important factor in control of the disease, diagnosis can be difficult given the prolonged incubation period, variable infection progression and immune responses, and unreliable detection tests as a result of poor test sensitivity[1, 11]. Detection tests include individual fecal sampling and culture, interferon-γ test, antibody ELISA, and culture of tissue samples post mortem [3, 20]. However, it is common to keep samples frozen prior to laboratory analysis.

Although tissue samples are often frozen for convenience or preservation, the freeze-thaw process may impact the resulting categorization of a sample as MAP-positive, or –negative [8]. However, research has focused primarily on the effects of freezing on fecal samples prior to culture. Initial studies indicated that the quantity of MAP recovered from fecal samples decreased after freezing at -80°C [17], although recent results indicated that this temperature had little or no effect on viability [9, 16]. However, despite tissue samples being commonly stored at -80°C before culture, there are apparently no reports regarding the effects of detecting positive samples in frozen-thawed tissues.

Deleterious effects of freezing on bacteria were attributed to formation of intracellular ice [12], although more recently it was reported that damage may be the result of intra- and extra-cellular osmotic imbalances during warming [5]. Additionally, viability of MAP may vary depending on the sample matrix from which the bacteria are isolated (tissue versus fecal; natural infection versus artificially spiked); it was reported that fewer MAP were lost from the feces of naturally infected cattle after freezing compared to artificially

spiked specimens [17]. Culture followed by PCR is considered optimal for detection of MAP, because it allows for low numbers of bacteria to grow to a point at which genetic markers can be detected via PCR or quantitative PCR (qPCR) [13]. Although direct DNA extraction from tissue samples is documented, there is a risk that positive samples may be missed given the low numbers of bacteria in the tissue, especially among young stock soon after infection [19]. Tissue culture is considered the gold standard for the detection of MAP infection [2]. However, following freezing, positive samples may be incorrectly identified as negative because of a loss of viability and decreased growth. Our objective was to determine the effects of freezing tissues at -80°C for 18 mo on culture-based detection of MAP.

7.3 Materials and Methods

Tissue samples were collected during an experimental infection study [4]. All protocols and the experimental design were approved by the University of Calgary Veterinary Sciences Animal Care Committee (Protocol AC14-0168). Briefly, 32 newborn Holstein-Friesian bull calves were collected from 13 dairy farms in Alberta (Canada) that had tested negative for MAP for at least 4 y. Calves were group-housed in 7 experimental pens consisting of 2 inoculated (IN) calves and 2 contact-exposed (CE) calves for 3 mo. Inoculum was prepared from a strain obtained from a clinical case (cow 69) in Alberta (Canada), and 2 calves in each experimental pen were inoculated with 2.5 x 10^8 CFU on 2 consecutive days at 2 wk of age. Following 3 mo of group housing, IN calves were euthanized for tissue sampling, and CE calves were individually housed for an additional 3 mo before euthanasia. Full details regarding tissue collection, culture, and results of this conventional culture have been published [4]. At the time of the initial study, only 2.5 g

of the total sample of collected tissue was processed. The remaining tissue sample was stored frozen and then opportunistically used as a convenience sample for our present study.

Of the tissue samples categorized in the initial study as culture-positive following fresh processing [4], 50 were systematically selected to be processed 18 mo after the initial autopsy and processing dates. The number of samples selected was based on a power calculation to detect a large or medium effect for categorization of samples or cycle threshold (Ct) values, respectively. Based on the power calculation, 32 samples were required to detect an effect at a *p*-value of 0.05; however, given the lack of literature regarding the size of a potential freezing effect, 50 samples were selected. Given the variability of MAP-positive tissue cultures (intestinal tissue versus lymph node) [15], samples for processing after freezing were selected to include at least 1 intestinal tissue and 1 associated lymph node. For IN calves, samples from the ileum were selected first. However, if no positive ileal or ileal LN sample was available, the next closest jejunal samples were selected. All 4 MAP-positive spleen samples were selected for additional processing. Additionally, 6 of the 50 samples were selected from CE calves, which represented 86% of all positive CE tissues. These tissues were thawed overnight in a 4°C refrigerator before processing. All sample processing procedures, culture, DNA extraction and detection were identical to those used in our initial study [4]. In brief, lymph nodes were cut into 2-cm cubes, mucosa was scraped from small intestinal samples and samples placed into pre-labelled Whirl-Pak bags. Thereafter, a 2.5 g sample of tissue was removed from bags, weighed, and dissociated (gentleMACS dissociator, M tubes, Miltenyi Biotech, Auburn, CA, USA) before undergoing a 24 h disinfection

procedure specialized for retention of MAP [4]. Briefly, the disinfection process involved incubation at 37°C for 3 h with 0.75% hexadecylpyridinium chloride (HPC) in half-strength brain heart infusion (BHI), followed by centrifugation (4,700 × g, 15 min) and re-suspension in a mixture of antibiotic solution (AS; para-JEM, TREK Diagnostic Systems, Cleveland, OH, USA), water, and full-strength BHI and incubated at 37°C overnight [4]. Then, 1 mL of solution from the disinfected sample was added to culture bottles (TREK para-JEM) and incubated at 37°C for 49 d. Following culture, DNA was extracted from the culture bottles [6]. A duplex qPCR assay targeting the MAP-specific F57 region and an internal amplification control was performed with primers and probes identical to those described [18]. Samples were categorized as positive if the Ct value was <40.

All statistical analyses were performed using STATA 11.2 (StataCorp LP, College Station, TX, USA). For all analyses, $p < 0.05$ was considered significant. A Wilcoxon signed rank test was used to compare Ct values obtained before and after freezing of tissue samples, and a McNemar chi-squared test to compare number of positives before and after freezing.

7.4 Results and discussion

We collected 91 culture-positive tissues from calves in the initial study [4], with 50 samples selected for processing after being frozen for 18 mo (Table 7-1). Of the 50 positive tissue samples that were processed and cultured after freezing, 4 samples were culture-negative based on qPCR. These samples included 2 spleen samples (1 IN, 1 CE), and 2 LN samples (both CE). Lymph nodes, and to some extent the spleen, have been shown to be fairly reliable tissues for identifying infected animals [15]. Of the 4 spleen

samples that were positive at initial culture, only 2 had to be re-classified after a freeze-thaw cycle. Additionally, 3 of 4 negative samples following freezing originated from CE calves. Given the nature of transmission in the trial design, CE animals were not directly inoculated, rather they became infected through direct and indirect contact with IN calves, which may have resulted in a lower infectious dose. A lower challenge dose leads to fewer tissue-positive samples and fewer MAP bacteria being present in infected tissues [15, 19]. Although culture allows for a few bacteria to multiply to a detectable number, freezing may have reduced the viability of MAP present, causing a decrease in the ability to detect true positive samples. Fewer MAP, in combination with a decrease in viability caused by freezing, may explain why more CE samples changed status after freezing compared to IN samples. This apparent difference between IN and CE tissue samples to remain positive following freezing should be considered when culturing frozen samples from naturally infected animals, because the number of bacteria in tissue may differ. A negative culture result following freezing of a sample that would have been identified as positive if cultured before storing could have large implications for testing and surveillance purposes. Future studies would benefit from culturing field and abattoir-derived tissue samples to determine effects of freezing collected from naturally infected animals.

Positive tissue samples that were processed fresh had a mean Ct value of 24.8 (SD: 3.9, range: 20.4-37.1), whereas post-freezing samples had a mean Ct value of 26.6 (SD: 2.5, range: 24.2-37.0) following removal of negative samples (n = 4). Samples that were processed fresh had lower Ct values than those processed after freezing (p = 0.005). Although samples were frozen for an extended interval, which may have affected MAP

viability, it is the number of freeze-thaw cycles that may negatively impact bacteria viability rather than freeze duration [16, 17]. Quantification of MAP is possible based on DNA and resulting Ct values from qPCR [10, 18], but there is no way to differentiate between living and dead bacteria. Therefore, comparison of viable bacteria between fresh and frozen samples was not possible in our study. The lower Ct values would normally indicate a greater quantity of MAP in the samples; however, other deleterious effects from freezing may have caused slower growth, which would increase Ct values, and it cannot be determined how many MAP bacteria were killed in the freeze/thaw process. Despite an inability to quantify viable bacteria, it was noteworthy that the Ct values increased after a freeze and thaw cycle, and this should be considered when quantifying or detecting MAP from tissues in the future.

Although there was no difference between samples categorized as positive or negative ($p = 0.13$) given the change in Ct values, it is important to note that 4 positive samples were re-categorized as negative after freeze-thawing. Therefore, it is important to not only take multiple samples from a single animal, but if possible, process samples immediately. Additionally, naturally exposed animals may be more difficult to detect; therefore, freezing effects may be more important in this population. It should be taken into consideration that tissue samples were only cultured once before freezing; perhaps the change in Ct values following freezing may be the result of variability in sample homogeneity of MAP. However, samples processed after freezing had consistently higher CT values, consistent with an effect of freezing, rather than with within-sample variation. Additionally, only positive tissue samples were selected for re-processing following

freezing; therefore, no conclusions regarding specificity were made, because no negative samples were re-processed after freezing.

Further research investigating MAP viability after a freeze-thaw cycle of tissues would provide further insights and would be of great importance for studies that culture MAP from tissues. Impacts on MAP viability could be investigated by plating on solid media or time-to-positive culturing techniques, rather than exclusively DNA-based measures of detection. Additionally, a study of intervals between freezing and thawing and various thaw methods may provide insight regarding optimal storage and thawing procedures for tissues suspected of being infected with MAP. Ultimately, further understanding regarding the loss of MAP in tissue samples following freezing will lead to more credible research findings regarding MAP infection, and identification of infected animals.

7.5 References

1. Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten V, Bannantine JP, Keefe GP, Kelton DF, Wells SJ, Whittington RJ *et al* (2018) Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection. Transbound Emerg Dis., in press.

2. Cocito C, Gilot P, Coene M, de Kesel M, Poupart P, Vannuffel P (1994) Paratuberculosis. Clin Microbiol Rev 7:328-345.

3. Collins MT (2011) Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract 27:581-591.

4. Corbett CS, De Buck J, Orsel K, Barkema HW (2017) Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48:27.

5. Fonseca F, Marin M, Morris GJ (2006) Stabilization of frozen *Lactobacillus delbrueckii* subsp. *bulgaricus* in glycerol suspensions: Freezing kinetics and storage temperature effects. Appl Environ Microbiol 72:6474-6482.

6. Forde T, Kutz S, De Buck J, Warren A, Ruckstuhl K, Pybus M, Orsel K (2012) Occurence, diagnosis, and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* infection in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) in southwest Alberta. J Wildlife Dis 48:1-11.

7. Garcia AB, Shalloo L (2015) Invited review: The economic impact and control of paratuberculosis in cattle. J Dairy Sci 98:5019-5039.

8. Gutierrez CB, Rodriguez Barbosa JI, Gonzalez OR, Tascon RI, Rodrguez Ferri EF (1992) Viability of *Actinobacillus pleuropneumoniae* in frozen pig lung samples and comparison of different methods of direct diagnosis in fresh samples. Comp Immunol Microbiol Infect Dis 15:89-95.

9. Khare S, Adams LG, Osterstock J, Roussel A, David L (2008) Effects of shipping and storage conditions of fecal samples on viability of *Mycobacterium paratuberculosis*. J Clin Microbiol 46:1561-1562.

10. Kralik P, Beran V, Pavlik I (2012) Enumeration of *Mycobacterium avium* subsp. *paratuberculosis* by quantitative real-time PCR, culture on solid media and optical densitometry. BMC Res Notes 5:114.

11. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C (2011) Within-herd contact structure and transmission of *Mycobacterium avium* subspecies *paratuberculosis* in a persistently infected dairy cattle herd. Prev Vet Med 100:116-125.

12. Mazur P (1977) Stabilization of frozen *Lactobacillus delbrueckii* subsp. *bulgaricus* in glycerol suspensions: Freezing kinetics and storage temperature effects. Appl Environ Microbiol 72:6473-6482.

13. McKenna SL, Keefe GP, Barkema HW, Sockett DC (2005) Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. Vet Microbiol 110:105-111.

14. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

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

16. Raizman EA, Espejo LA, Wells SJ (2011) Long-term survival of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples obtained from naturally infected cows and stored at -18 degrees C and -70 degrees C. Vet Med Int 2011:1-5.

17. Richards WD, Thoen CO (1977) Effect of freezing on the viability of *Mycobacterium paratuberculosis* in bovine feces. J Clin Microbiol 6:392-395.

18. Slana I, Kralik P, Kralova A, Pavlik I (2008) On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. Int J Food Microbiol 128:250-257.

19. Sweeney RW, Uzonna J, Whitlock RH, Habecker PL, Chilton P, Scott P (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. Res Vet Sci 80:253-259.

20. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW (2006) Johne's disease in Canada Part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J 47:874-882.

Table 7-1. Results of testing of tissue samples processed for *Mycobacterium avium* subsp. *paratuberculosis* following storage at -80°C for 18 mo. All samples selected for processing were initially positive based by qPCR (Ct < 40) detection following culture of the fresh sample.[4]

Calf status	Location (total no. samples collected in initial study)*	No. of culture-positive fresh samples	No. samples selected for processing after freezing (no. positive after freeze thaw)
Inoculated			
	Ileum (14)	9	9 (9)
	Jejunum (42)	25	13 (13)
	Ileum LN (14)	13	13 (13)
	Jejunum LN (42)	34	6 (6)
	Spleen (14)	3	3 (2)
Contact-exposed			
	Ileum (14)	0	0 (0)
	Jejunum (42)	1	1 (1)
	Ileum LN (14)	0	0
	Jejunum LN (42)	5	4 (2)
	Spleen (14)	1	1 (0)

* LN= Lymph nodes.

CHAPTER 8: SUMMARIZING DISCUSSION

8.1 Concluding Remarks

The overall aims of this thesis were to: elucidate influences of environmental sample characteristics on infection status outcome of a herd; estimate prevalence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP)-infected herds based on 2 environmental samples commonly collected for detection of positive herds, or addition of a novel sample from breeding age heifers (BAH) on estimates (Chapters 2 and 3), in Canada; and quantify extent of calf-to-calf transmission among pen mates (Chapters 4-6). All environmental samples regardless of type, had decreased odds of testing positive in the second sampling, and the largest herds had increased odds of testing positive than smaller herds at both sample periods (Chapter 2). The Canadian national prevalence study was the first to use a single detection method (environmental samples) across all 10 provinces to estimate prevalence, including environmental samples collected from BAH (Chapter 3). Although the latter samples did not affect prevalence estimates (Chapter 3), they provided additional evidence that young stock are shedding MAP on farm [20, 33]. Detection of positive environmental samples among young stock is not a novel finding; however, that there was extreme intermittent shedding among calves (Chapter 6), substantial amounts of MAP shed by calves (Chapter 6), and calf-to-calf transmission that occurred and caused infection (Chapters 4 and 5), provided new evidence that may help to explain persistent infections on farms despite implementation of Johne's disease (JD) control programs. Additionally, freezing had only a minor impact on MAP detection in tissues of inoculated calves, although some tissue samples did change infection status, primarily among contact exposed (CE) calves (Chapter 7). Effects of freezing tissue should be considered when detecting MAP-infected cattle, as tissue samples are

considered the "gold standard" for detection (Chapter 7). The impact of these findings, and future directions for JD control programs as well as research, will be discussed below.

8.1.1. Environmental sampling and prevalence

Environmental sampling has been used to estimate herd prevalence; however, herd size, seasonality, housing type, and location of sample on farm affected odds of a sample being MAP culture-positive when collecting 6 samples, which affects herd status categorization [30, 32]. Similarly, when only collecting 2 environmental samples for detection of an infected herd, the apparent prevalence was higher in larger herds, as well herds with free-stall housing across the country at 1 sampling time point (Chapter 3). Although prevalence of MAP was associated with herd size and housing type (Chapter 3), herd prevalence over time decreased uniformly across all environmental sample types, herd sizes, and housing types (Chapter 2). Based on these findings, at a single time point of sampling, certain herd/sample characteristics (housing type, herd size, location of sample) may have affected odds of detecting a MAP-positive farm [32] (Chapter 3). However, changes in prevalence of positive environmental samples over time occurred across the entire herd, regardless of specific environmental/herd characteristics (Chapter 2). Therefore, sample/herd characteristics may impact odds of a sample being positive or a farm being identified as positive at a single point in time, although longitudinally, changes in prevalence occurred across all herds and sample types. It should be taken into consideration, that the cow population in a single dairy herd changes over time as the average lifespan of a dairy cow is about 4 years; longitudinal dairy studies are, therefore, likely to have variability due to the changing population. However, due to the chronic,

slow growing nature of MAP, lack of evidence regarding eradication despite control programs being in place, and reported effects of control programs taking at least 4-5 years to observe [19], it is likely that the turn-over of the cow population does not have a large effect on prevalence estimates.

The association between number of positive environmental samples from a herd and within-herd prevalence of MAP has been reported [5]. In Chapter 2, each additional positive environmental sample detected in the first sampling period decreased the odds of testing negative 3 years later. Additionally, farms that changed status had fewer MAP-positive environmental samples than those that were negative in both years (Chapter 2). Therefore, we inferred that herds that changed status had lower within-herd prevalence and highlighted the importance of repeated screening to reliably detect low-prevalence herds. Low within-herd prevalence farms are more likely to have fewer positive environmental samples, and therefore may be more difficult to detect, especially when only collecting 2 samples rather than 6. Although 6 samples were more sensitive to determine MAP-infection status and within-herd prevalence estimates for a specific herd, if test characteristics such as Se and Sp are calculated and applied appropriately (Chapter 3), collecting fewer samples may provide a more cost-effective alternative for large-scale herd prevalence studies. It is important to have repeated prevalence estimates over time, as low-prevalence herds are likely to have fewer positive samples (Chapter 2), as well as adjust apparent prevalence estimates to true prevalence estimates, based on test characteristics (Chapter 3).

Inclusion of environmental samples collected from young stock did not appear to affect ability to accurately identify MAP-positive farms (Chapter 3). However, positive

environmental samples from young stock housing may be an important indicator of infections among young stock, and could be used to identify a gap in control methods currently implemented on farm. These samples also highlighted the potential of calf-to-calf transmission. A MAP-positive environmental sample among young stock not only indicates that at least 1 animal has been shedding, potentially leading to new infections among susceptible pen mates, but also that calves may be becoming infected from cows, despite preventative management practices. Positive environmental samples have been identified in all ages of young stock [20, 22, 33] (Chapter 3), and should be considered standard in future control programs for better control and understanding of MAP transmission.

8.1.2 Importance of calf-to-calf transmission

It has been well established that calves up to 12 mo of age can become infected [17] and young stock are shedding MAP on farm [33]; however, findings regarding the role of calf transmission and its importance for control have been inconsistent [10, 12, 23, 27]. Based on research in this thesis, calf-to-calf transmission is possible among infectious (shedding) and infected calves (immune response detection and tissue culture), and this route of transmission results in tissue infection in at least half of CE animals (Chapters 4 and 5). It should be considered that CE calves may have had a lower infectious dose due to the nature of exposure, resulting in fewer tissue-positive samples, and fewer MAP bacteria being present in infected tissues that could lead to positive identification of the sample (Chapter 7) [15, 26]; therefore, the true number of tissue-infected CE calves may have been greater. Identification of MAP-positive tissue samples in CE calves has important implications for control programs, as it indicates that calves are not only

passively shedding MAP, but becoming infected, which may result in active shedding later in life, and transmission to other cows, calves, and offspring throughout their lifetime.

The distinction between infectious (shedding) and infected (immune responses and tissue infection) calves in quantitative modelling of transmission in Chapter 5 was important, due to variability in disease progression [3, 17]. Depending on the definition of MAP infection, the R_0 value varied from 0.9-3.24, with implications for on-farm transmission (Chapter 5). Shedding detected among calves may be due to passive shedding rather than an active infection [21]; however, the distinction between types is difficult or nearly impossible to make, due to difficulties in detection based on immune responses [3, 17], or requires post-mortem identification of MAP-positive tissue samples. Although R_0 values estimated are based on fecal culture, immune responses, or tissue infection (Chapter 5), it is important to consider that half of all CE calves in the transmission trial had positive tissue samples indicative of MAP infection (Chapter 4). Overall, it was concluded that 1 infectious (shedding) calf may cause 3 more calves to begin shedding (Chapter 5), but only half (1 or 2 calves) may develop a tissue infection (Chapter 4), which was also supported by the R_0 values when a positive immune response or tissue sample was considered the definition of infectious (R_0^I=0.90, R_0^T=1.36; respectively). These results may explain positive environmental samples or fecal cultures collected from young stock [29, 33], and the persistence of low-level prevalence in dairy herds [2, 10, 14].

Although the minimum infectious dose of MAP for calves, young stock, and cows is not definitively known, a dose most commonly used for experimental infections "consistently results in infection of experimental animals, but is not so high as to overwhelm

interventions" [7]. When comparing 3 inoculation doses for frequency of intermittent shedding and quantity of MAP shed, the largest difference among doses was the frequency with which MAP was detected (Chapter 6). Additionally, "pass-through" of the inoculum for the 7 days immediately after inoculation resulted in higher quantities of MAP detected for all 3 doses when compared to "active shedding" (shedding events after the first 7 days), but differences among doses were not observed at either pass-through or active shedding periods (Chapter 6). It is important to take into consideration that the sample size to detect the difference between shedding quantities may not provide enough power to detect differences between the doses of inoculation, as the effect of dose on shedding quantity has not been previously elucidated. Additionally, the high amount on individual variability of disease progression, and the difference in detection between culture and DE methods, may affect the quantitative accuracy of fecal shedding and further research is required with larger number of animals to confirm these findings. However, these findings provide new information regarding fecal shedding and will aid future experimental infection trials to better select inoculation doses, as dose appeared to affect frequency with which MAP was shed, rather than the quantity. Additionally, intermittent shedding among IN and CE was detected at intervals previously not described (2-3 days) (Chapters 4 and 6), likely due to the frequency of fecal sampling being thrice weekly compared to monthly, or biannual sampling in previous research [13, 16, 18]. The unprecedented frequency with which intermittent shedding among calves both IN and CE provided evidence that repeated testing of young stock and calves may be necessary to identify infectious animals and decrease new transmission events due to calf-to-calf transmission.

8.2 Control programs with young stock

In general, the aim of dairy JD control programs in Canada were to decrease MAP prevalence at farm, herd and national levels to decrease economic losses due to MAP-infected cattle [1, 11]. The assumption that calves are susceptible and at risk of transmission from infectious cows led most control programs to focus on stopping the transmission route from cow-to-calf, largely overlooking potential impacts of calf-to-calf transmission [6, 31]. Although it has been well established that calves can be infectious, whether transmission among calves has an impact on the effectiveness of control programs is uncertain [12, 23, 28]. However, persistence of MAP infection world-wide, despite implementation of control programs, highlights the need to improve management recommendations and aims. One of these changes should include monitoring and testing young stock.

The decision to include young stock in JD monitoring is not straightforward. Benefits to a program that incorporates young stock would include early detection of infectious and potentially infected animals (to prevent new transmissions), environmental sampling (to detect young stock housing as a potential transmission site) and identification of MAP-positive young stock (potential indicator of cow-calf transmission). However, challenges arise when a decision needs to be made regarding the diagnostic test used to identify MAP-positive calves, and what is done with the calves identified as MAP-positive. Fecal shedding among calves can be intermittent, changing day-to-day, making detection of infectious calves difficult and highly variable (Chapters 4 and 5). Furthermore, fecal shedding does not necessarily indicate that a calf has a tissue infection (Chapter 4). Therefore, repeated testing, separating positive calves from potentially susceptible pen

mates, and continued monitoring would be advised as an alternative option to immediate culling. Methods for surveillance of potentially infectious calves will have an immediate economic impact to producer, due to repeated testing, as well implementing separate designated housing areas with designated equipment for these fecal-positive animals. Additionally, supplementary testing of young stock may impact certificate-based programs as well as herd status-based programs, as MAP-positive environmental samples from young stock are possible when all other samples are negative (Chapter 3). A decision would have to be made as to whether testing (fecal, environmental sampling) of young stock would be used for monitoring of prevalence estimates, herd status categorization, or as a tool to help identify and control within-herd transmission. Environmental sample testing is currently a cost-effective tool to monitor prevalence over time and among regions (Chapters 2 and 3), and inclusion of BAH samples did not affect prevalence estimates, although there were farms identified as being infected based solely on positive BAH environmental (Chapter 3). Improvement of current JD control programs to include young stock and calves as a transmission route is highly recommended. However, careful consideration needs to be made regarding how to include young stock sampling in such a program, and the recommendations to producers after identifying positive calves or environmental samples. Further research is required to better understand susceptibility/resistance on farm, progression of infection in naturally infected calves, and modelling economic/prevalence impacts of various detection and control programs for young stock.

8.3 Future directions for research

A wealth of information regarding MAP infection and JD control has been generated in the last 60 years of MAP research. However, important knowledge gaps have been identified regarding effective control programs, producer participation, improvement of diagnostic tests and vaccines, transmission routes and their impact for control, MAP bacteria and progression of disease, etc. [1]. Results and information generated by this thesis have increased understanding of prevalence and use of environmental samples, as well as the importance and potential impact of calf-to-calf transmission (Chapters 2-7). Regardless, future research is required to improve current JD control programs leading to fewer new infections on farm, which will result in a decrease in MAP-prevalence. Future research should focus on the following:

1) Calf-to-calf transmission on commercial dairy farms

Although calf-to-calf transmission based on fecal culture, immune responses, and tissue infection occurred in studies reported in this thesis, (Chapters 3-6), these findings were based on experimental parameters in a controlled setting. Although CE calves were assumed to be most representative of natural infections, it is noteworthy that they were exposed to inoculated calves in a controlled environment. Therefore, to better understand the extent that calf-to-calf transmission occurs in the field, on-farm observational and experimental studies are needed. Ideally, these would be longitudinal studies, based on identification of shedding calves at an early age, and monitoring extent of transmission to susceptible penmates, detection of positive environmental samples, and repeated testing of specific animals. Although it is recommended to cull MAP-positive cows, it is likely that a fecal positive calf would remain on farm for surveillance until active shedding

(identification with an immune response) is confirmed, during which time it could be monitored for disease progression, transmission (based on study design), and further follow-up studies. These studies could be conducted on commercial dairy farms, or a research/education farm, enabling long-term surveillance of positive and infectious cattle. The aims of the research should focus on infectious rate of calves, progression of disease among naturally infected calves and differences between susceptible calves that acquire infection and those that do not. This research would elucidate the role of infectious calves on the transmission routes in the dairy herd. Following on-farm transmission studies with naturally infected cattle, impacts of intervention strategies focusing on calf housing (group vs individual, hygiene practices) and resulting transmission among young stock will generate new knowledge to improve control programs, and recommendations that can be implemented on farm.

2) Control programs including young stock

Inclusion of young stock in future control programs is not trivial. Research needs to be conducted to improve early detection diagnostics, as well as elucidate causes of individual variability for resistance and disease progression. Research regarding host/pathogen interactions [8, 9], progression of disease and impact on farm transmission dynamics [24, 25], heritability of resistance/susceptibility [34, 35], and early detection of MAP infection with biomarkers [4] is currently underway. However, an economic analysis of various diagnostic tests for calves and effects that various definitions of “infectious” and “infected” calves will have on transmission dynamics, prevalence and economic consequences on farm, will facilitate informed decision making regarding surveillance. The decision to use 1) environmental samples for surveillance and

monitoring, 2) fecal samples for identification of infectious calves to separate/remove from pen mates, or 3) blood samples to detect immune responses and infection, will affect economic impacts to the producer, management practices, as well as long-term changes in farm-level prevalence. Additionally, samples undergo various stressors before processing, including interval from collection to assessment, transportation, freezing, long- and short-term storage. These pre-processing stressors likely differ among farms, but also among regions and across programs, making comparisons difficult. Freezing tissue samples impacted ability to detect MAP (Chapter 7); however, further investigation regarding variability of MAP in tissue and fecal samples should be conducted, as well as determining viability of immune cells (to detect cellular immunity) following transportation and storage. This will improve consistency, not only between the same diagnostic tests within a region, but improve comparisons of results among labs, studies, regions, and control programs.

3) Longitudinal prevalence studies using environmental samples

Environmental samples allow for estimation of prevalence and prevalence changes that occur over time (Chapters 2, 3). Using 2 environmental samples to estimate and monitor prevalence is a cost-effective method to compare prevalence estimates among countries and control programs. Because changes in prevalence over time were not dependent on herd or environmental characteristics (Chapter 2), 2 environmental samples could be effective to estimate true prevalence when test characteristics are applied (Chapter 3), as well effective for monitoring changes over time. Environmental sampling world-wide could provide new opportunities not only for prevalence comparisons, but would also

enable new analyses regarding risk factors and management programs regarding effectiveness of JD control and MAP infection.

8.4 References

1. Barkema HW, Orsel K, Nielsen SS, Koets AP, Rutten V, Bannantine JP, Keefe GP, Kelton DF, Wells SJ, Whittington RJ *et al* (2018) Knowledge gaps that hamper prevention and control of *Mycobacterium avium* subspecies *paratuberculosis* infection. Transbound Emerg Dis., in press.

2. Beaunee G, Vergu E, Ezanno P (2015) Modelling of paratuberculosis spread between dairy cattle farms at a regional scale. Vet Res 46:111.

3. Dargatz DA, Beverly AB, Barber LK, Sweeney RW, Whitlock RH, Shulaw WP, Jacobson RH, Stabel JR (2001) Evaluation of a commercial ELISA for diagnosis of paratuberculosis in cattle. JAVMA 218:1163-1166.

4. De Buck J, Shaykhutdinov R, Barkema HW, Vogel HJ (2014) Metabolomic profiling in cattle experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*. PLoS One 9.

5. Donat K, Kube J, Dressel J, Einax E, Pfeffer M, Failing K (2015) Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples by faecal culture and real-time PCR in relation to apparent within-herd prevalence as determined by individual faecal culture. Epidemiol Infect 143:975-985.

6. Garry F (2011) Control of paratuberculosis in dairy herds. Vet Clin North Am Food Anim Pract 27:599-607.

7. Hines ME, 2nd, Stabel JR, Sweeney RW, Griffin F, Talaat AM, Bakker D, Benedictus G, Davis WC, de Lisle GW, Gardner IA *et al* (2007) Experimental challenge models for Johne's disease: a review and proposed international guidelines. Vet Microbiol 122:197-222.

8. Koets AP, Eda S, Sreevatsan S (2015) The within host dynamics of *Mycobacterium avium* ssp. *paratuberculosis* infection in cattle: where time and place matter. Vet Res 46:61.

9. Koets AP, Grohn YT (2015) Within- and between-host mathematical modeling of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infections as a tool to study the dynamics of host-pathogen interactions in bovine paratuberculosis. Vet Res 46:60.

10. Marcé C, Ezanno P, Seegers H, Pfeiffer DU, Fourichon C (2011) Predicting fadeout versus persistence of paratuberculosis in a dairy cattle herd for management and control purposes: a modelling study. Vet Res 42:36.

11. McKenna SL, Keefe GP, Tiwari A, VanLeeuwen JA, Barkema HW (2006) Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. Can Vet J 47:1089-1099.

12. Mitchell RM, Medley GF, Collins MT, Schukken YH (2012) A meta-analysis of the effect of dose and age at exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows. Epidemiol Infect 140:231-246.

13. Mitchell RM, Schukken Y, Koets A, Weber M, Bakker D, Stabel J, Whitlock RH, Louzoun Y (2015) Differences in intermittent and continuous fecal shedding patterns between natural and experimental *Mycobacterium avium* subspecies *paratuberculosis* infections in cattle. Vet Res 46:66.

14. Mitchell RM, Whitlock RH, Stehman SM, Benedictus A, Chapagain PP, Grohn YT, Schukken YH (2008) Simulation modeling to evaluate the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on commercial dairy farms in the United States. Prev Vet Med 83:360-380.

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

16. Mortier R, Barkema HW, Orsel K, Wolf R, De Buck J (2014) Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subspecies *paratuberculosis*. Vet Res 45:71.

17. Mortier RA, Barkema HW, De Buck J (2015) Susceptibility to and diagnosis of *Mycobacterium avium* subspecies *paratuberculosis* infection in dairy calves: A review. Prev Vet Med 121:189-198.

18. Munster P, Volkel I, Wemheuer W, Schwarz D, Doring S, Czerny CP (2013) A longitudinal study to characterize the distribution patterns of *Mycobacterium avium* ssp. *paratuberculosis* in semen, blood and faeces of a naturally infected bull by IS 900 semi-nested and quantitative real-time PCR. Transbound Emerg Dis 60:175-187.

19. Nielsen SS, Toft N (2011) Effect of management practices on paratuberculosis prevalence in Danish dairy herds. J Dairy Sci 94:1849-1857.

20. Pillars R, Grooms D, Kaneene JB (2009) Longitudinal study of the distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment of dairy herds in the Michigan Johne's disease control demonstration herd project. Can Vet J 50:1039-1046.

21. Pradhan AK, Mitchell RM, Kramer AJ, Zurakowski MJ, Fyock TL, Whitlock RH, Smith JM, Hovingh E, Van Kessel JA, Karns JS *et al* (2011) Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* in a longitudinal study of three dairy herds. J Clin Microbiol 49:893-901.

22. Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE (2004) The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J Dairy Sci 87:2959-2966.

23. Santema WJ, Poot J, Segers RP, Van den Hoff DJ, Rutten VP, Koets AP (2012) Early infection dynamics after experimental challenge with *Mycobacterium avium* subspecies *paratuberculosis* in calves reveal limited calf-to-calf transmission and no impact of Hsp70 vaccination. Vaccine 30:7032-7039.

24. Smith RL, Grohn YT, Pradhan AK, Whitlock RH, Van Kessel JS, Smith JM, Wolfgang DR, Schukken YH (2016) The effects of progressing and nonprogressing *Mycobacterium avium* ssp. *paratuberculosis* infection on milk production in dairy cows. J Dairy Sci 99:1383-1390.

25. Smith RL, Schukken YH, Grohn YT (2015) A new compartmental model of *Mycobacterium avium* subsp. *paratuberculosis* infection dynamics in cattle. Prev Vet Med 122:298-305.

26. Sweeney RW, Uzonna J, Whitlock RH, Habecker PL, Chilton P, Scott P (2006) Tissue predilection sites and effect of dose on *Mycobacterium avium* subs. *paratuberculosis* organism recovery in a short-term bovine experimental oral infection model. Res Vet Sci 80:253-259.

27. van Roermund HJ, Bakker D, Willemsen PT, de Jong MC (2007) Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: calves can transmit the infection to other calves. Vet Microbiol 122:270-279.

28. van Roermund HJ, Vos AM, De Jong MC (2002) Within herd transmission of paratuberculosis and the possible role of infectious calves. In: *Proc 7th Intern Colloq Paratuberculosis: 2002; Bilbao, Spain*; 368-370.

29. Weber MF, Kogut J, de Bree J, van Schaik G, Nielen M (2010) Age at which dairy cattle become *Mycobacterium avium* subsp. *paratuberculosis* faecal culture positive. Prev Vet Med 97:29-36.

30. Wells S, Wagner BA (2000) Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in the herd and use of preventive measures. J Am Vet Med A 216:1450-1497.

31. Whitlock RH (2010) Paratuberculosis control measure in the USA. In: Behr MA, Collins DM (eds) Paratuberculosis - Organism, disease and control. Wallingford, UK.

32. Wolf R, Barkema HW, De Buck J, Orsel K (2015) Sampling location, herd size, and season influence *Mycobacterium avium* ssp. *paratuberculosis* environmental culture results. J Dairy Sci 98:275-287.

33. Wolf R, Orsel K, De Buck J, Barkema HW (2015) Calves shedding *Mycobacterium avium* subspecies *paratuberculosis* are common on infected dairy farms. Vet Res 46:1-8.

34. Zare Y, Shook GE, Collins MT, Kirkpatrick BW (2014) Genome-wide association analysis and genomic prediction of *Mycobacterium avium* subspecies *paratuberculosis* infection in US Jersey cattle. PLoS One 9:e88380.

35. Zare Y, Shook GE, Collins MT, Kirkpatrick BW (2014) Short communication: Heritability estimates for susceptibility to *Mycobacterium avium* subspecies *paratuberculosis* infection defined by ELISA and fecal culture test results in Jersey cattle. J Dairy Sci 97:4562-4567.

APPENDIX

Copyright permissions

RE: Obtain Permission – Journal request - Caroline Susan Corbett 2018-05-10, 7:08 PM

RE: Obtain Permission – Journal request

Vethakkan, Anita Mercy M. (ELS-CHN) [redacted]

Wed 2018-05-09 6:45 AM

To:Caroline Susan Corbett [redacted]

ELSEVIER

Dear Dr. Caroline Corbett

Thank you for your email.

Please note that, as one of the Authors of this article, you retain the right to reuse it in your thesis/dissertation. You do not require formal permission to do so. You are permitted to post this Elsevier article online if it is embedded within your thesis. You are also permitted to post your Author Accepted Manuscript online

However posting of the final published article is prohibited.

*"As per our Sharing Policy, authors are permitted to post the Accepted version of their article on their institutional repository – as long as it is for **internal institutional use only.***

It can only be shared publicly on that site once the journal-specific embargo period has elapsed. For a list of embargo periods please see: https://www.elsevier.com/__data/assets/pdf_file/0005/78476/external-embargo-list.pdf

You are not permitted to post the Published Journal Article (PJA) on the repository."

Please feel free to contact me if you have any queries.
Kind regards
Anita

Anita Mercy
Senior Copyrights Coordinator, Global Rghts Department | ELSEVIER |
Ascendas International Tech Park, Crest - 12th Floor | Taramani, Chennai 600113 • India |
Tel: [redacted]

Join the Elsevier Connect Community
www.elsevier.com
Twitter | Facebook | LinkedIn | Google+

https://outlook.office.com/owa/?viewmodel=ReadMessageItem&ItemI...XLCVjgADaCIshAAAAA%3D%3D&IsPrintView=1&wid=25&ispopout=1&path= Page 1 of 2

00872439 RE: Including publication in Thesis

"Joel Lagmay" [redacted]

Thu 2018-05-10 6:00 AM

To:Caroline Susan Corbett < [redacted]

Dear Caroline,

Thank you for contacting Springer Nature.

The open access articles published in BioMed Central's journals are made available under the Creative Commons Attribution (CC-BY) license, which means they are accessible online without any restrictions and can be re-used in any way, subject only to proper attribution (which, in an academic context, usually means citation).

The re-use rights enshrined in our license agreement (http://www.biomedcentral.com/about/policies/license-agreement) include the right for anyone to produce printed copies themselves, without formal permission or payment of permission fees. As a courtesy, however, anyone wishing to reproduce large quantities of an open access article (250+) should inform the copyright holder and we suggest a contribution in support of open access publication (see suggested contributions at http://www.biomedcentral.com/about/policies/reprints-and-permissions/suggested-contributions).

Please note that the following journals have published a small number of articles that, while freely accessible, are not open access as outlined above: Alzheimer's Research & Therapy, Arthritis Research & Therapy, Breast Cancer Research, Critical Care, Genome Biology, Genome Medicine, Stem Cell Research & Therapy.
You will be able to find details about these articles at http://www.biomedcentral.com/about/policies/reprints-and-

Gmail

Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

Jeroen De Buck[redacted] Thu, Mar 22, 2018 at 10:57 AM
To: Caroline Corbett [redacted]

"I, Jeroen De Buck, co-author of at least one of the listed manuscripts, grant Caroline Corbett permission to include the listed manuscript(s) in her doctoral thesis."

Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, J.P., Barkema, H.W. (submitted 2018). Environmental sample characteristics and herd size associated with decrease in herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis*. J Dairy Sci, submitted.

Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci

Corbett, C.S., De Buck, J., Orsel, K, Barkema, H.W., 2017. Fecal shedding and tissue infections demonstrate transmission of*Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48, 27.

Corbett, C.S., de Jong, M., Orsel, K., De Buck, J., Barkema, H.W. (submitted 2017). Quantifying transmission of *Mycobacterium avium*subsp. *paratuberculosis* among group-housed dairy calves. Vet Res, submitted

Corbett, C.S., Barkema, H.W., De Buck., J. 2018. Quantifying fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* from calves after experimental infection and exposure. J Dairy Sci 101, 1-10.

Gmail Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

John Kastelic [redacted] Thu, Mar 22, 2018 at 11:00 AM
To: Caroline Corbett [redacted]

To whom it may concern:

As co-author of themanuscript described below, I grant Caroline Corbett permission to include the listed manuscript in her doctoral thesis.

Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, JP., Barkema, H.W. (submitted 2018). Environmental sample characteristics and herd size associated with decrease in herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis*. J Dairy Sci, submitted.

Thanks
John Kastelic

John P. Kastelic, DVM, PhD, Dipl. ACT
Professor, Cattle Reproductive Health - Theriogenology
Head, Department of Production Animal Health
3330 Hospital Drive NW
Room HRIC 2AC58
Calgary, AB, Canada T2N 4NT

[redacted]

From: Caroline Corbett [redacted]
Sent: Thursday, March 22, 2018 10:55:39 AM
To: [redacted]
Subject: Permission to include manuscripts in thesis

[Quoted text hidden]

Gmail Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

Herman Barkema [redacted] Thu, Mar 22, 2018 at 11:31 AM
To: Caroline Corbett [redacted]
Cc: Jeroen De Buck [redacted]

Hi Caroline,

I, Herman Wildrik Barkema, co-author of the below listed manuscripts, grant Caroline Corbett permission to include the listed manuscript(s) in her doctoral thesis.

- Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, J.P., Barkema, H.W. (submitted 2018). Environmental sample characteristics and herd size associated with decrease in herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis*. J Dairy Sci, submitted.
- Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci
- Corbett, C.S., De Buck, J., Orsel, K, Barkema, H.W., 2017. Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48, 27.
- Corbett, C.S., de Jong, M., Orsel, K., De Buck, J., Barkema, H.W. (submitted 2017). Quantifying transmission of *Mycobacterium avium* subsp. *paratuberculosis* among group-housed dairy calves. Vet Res, submitted
- Corbett, C.S., Barkema, H.W., De Buck., J. 2018. Quantifying fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* from calves after experimental infection and exposure. J Dairy Sci 101, 1-10.
- Corbett, C.S., De Buck, J., Barkema, H.W. 2018. Effects of freezing on ability to

Gmail

Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

David Kelton <[redacted] Thu, Mar 22, 2018 at 11:07 AM
To: Caroline Corbett [redacted]

Hello Caroline,

I, David Kelton, co-author of the manuscript identified below, grant Caroline Corbett permission to include the listed manuscript in her doctoral thesis.

Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci

David

David Kelton, DVM, PhD
DFO Research Chair in Dairy Cattle Health
Professor of Epidemiology
Department of Population Medicine
University of Guelph
Guelph, Ontario, Canada
N1G 2W1
[redacted]

Gmail

Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

Karin Orsel [redacted] Thu, Mar 22, 2018 at 11:51 AM
To: Caroline Corbett [redacted]

"I, Karin Orsel, co-author of the listed manuscripts, grant Caroline Corbett permission to include the listed manuscript(s) in her doctoral thesis."

Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci

Corbett, C.S., De Buck, J., Orsel, K, Barkema, H.W., 2017. Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48, 27.

Corbett, C.S., de Jong, M., Orsel, K., De Buck, J., Barkema, H.W. (submitted 2017). Quantifying transmission of *Mycobacterium avium* subsp. *paratuberculosis* among group-housed dairy calves. Vet Res, submitted

, C.S., De Buck, J., Barkema, H.W. 2018. Effects of freezing on ability to detect *Mycobacterium avium* subsp. *paratuberculosis* from bovine tissues following culture. J Vet Diagn Invest, accepted.

DVM, MSc, PhD, Dipl. ECBHM
Faculty of Veterinary Medicine: Dept of Production Animal Health
Cumming School of Medicine, Dept of Community Health Sciences
Associate Professor

Gmail Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

Uehlinger, Fabienne <[redacted] Thu, Mar 22, 2018 at 12:26 PM
To: Caroline Corbett [redacted]

I, Fabienne Uehlinger, co-author of at least one of the listed manuscripts, grant Caroline Corbett permission to include the listed manuscript(s) in her doctoral thesis.

Fabienne Uehlinger

From: Caroline Corbett [mailto[redacted]]
Sent: March-22-18 10:56 AM
To: [redacted]
Subject: Permission to include manuscripts in thesis

Dear all,

[Quoted text hidden]

Gmail Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

Uliana Kanevets <[redacted] Thu, Mar 22, 2018 at 1:44 PM
To: Caroline Corbett [redacted]

I, Uliana Kanevets, co-author of at least one of the listed manuscripts, grant Caroline Corbett permission to include the listed manuscript(s) in her doctoral thesis.

Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, J.P., Barkema, H.W. (submitted 2018). Environmental sample characteristics and herd size associated with decrease in herd-level prevalence of *Mycobacterium avium*subsp. *paratuberculosis*. J Dairy Sci, submitted.

Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci

Corbett, C.S., De Buck, J., Orsel, K, Barkema, H.W., 2017. Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48, 27.

Corbett, C.S., de Jong, M., Orsel, K., De Buck, J., Barkema, H.W. (submitted 2017). Quantifying transmission of *Mycobacterium avium* subsp. *paratuberculosis* among group-housed dairy calves. Vet Res, submitted

Gmail

Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

Cathy Bauman [redacted] Thu, Mar 22, 2018 at 2:00 PM

[redacted]

Hi Caroline,

Attached are my suggested revisions, you have done a lot of work, well done. I am still unclear about what equations, priors etc. were used to calculate what Se/Sp and prevalences and the Bayesian details, but perhaps with revisions it will be clearer...

Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci

"I, *Cathy Bauman*, co-author of at least one of the listed manuscripts, grant Caroline Corbett permission to include the listed manuscript(s) in her doctoral thesis."

From: Caroline Corbett [redacted]
Sent: Thursday, March 22, 2018 12:55 PM
To: Herman Barkema; Jeroen De Buck; Karin Orsel; John Kastelic; Ali Naqvi; David Kelton; Uliana; Cathy Bauman; Uehlinger, Fabienne; Jong, Mart de
Subject: Permission to include manuscripts in thesis

[Quoted text hidden]

CAROLINE CORBETT NDS Prev March 16 HB Kelton KO CB.docx
145K

Gmail Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

Ali Naqvi[redacted] Thu, Mar 22, 2018 at 4:17 PM
To: Caroline Corbett [redacted]

I, S. Ali Naqvi co-author of the following two manuscripts, grant Caroline Corbett permission to include them in her doctoral thesis:

Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, J.P., Barkema, H.W. (submitted 2018). Environmental sample characteristics and herd size associated with decrease in herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis*. J Dairy Sci, submitted.

Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci

[redacted]

Dear all,

[Quoted text hidden]

Gmail

Caroline Corbett <c.corbett01@gmail.com>

Permission to include manuscripts in thesis

[redacted]

"I, *Mart C.M. de Jong* , co-author of at least one of the listed manuscripts, grant Caroline Corbett permission to include the listed manuscript(s) in her doctoral thesis."

(As long as you write my name with all initials as my surname is rather common in NL.)

Corbett, C.S., Naqvi, S.A., De Buck, J., Kanevets, U., Kastelic, J.P., Barkema, H.W. (submitted 2018). Environmental sample characteristics and herd size associated with decrease in herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis*. J Dairy Sci, submitted.

Corbett, C.S., Naqvi, S.A., Bauman, C., De Buck, J., Orsel, Uehlinger, F., K., Kelton, D.F., Barkema H.W. (to be submitted 2018). Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* on Canadian dairy farms. J Dairy Sci

Corbett, C.S., De Buck, J., Orsel, K, Barkema, H.W., 2017. Fecal shedding and tissue infections demonstrate transmission of *Mycobacterium avium* subsp. *paratuberculosis* in group-housed dairy calves. Vet Res 48, 27.

Corbett, C.S., de Jong, M.C.M., Orsel, K., De Buck, J., Barkema, H.W. (submitted 2017). Quantifying transmission of *Mycobacterium avium* subsp. *paratuberculosis* among group-housed dairy calves. Vet Res, submitted